American Institute of Integrative Oncology
RESEARCH & EDUCATION

Foundations of Integrative Oncology
COURSE

GRAND ROUNDS CALL
With Dr. Nalini Chilkov
August 14th, 2019
5:30 PM Pacific / 6:30 PM Mountain / 7:30 PM Central / 8:30 PM Eastern

Agenda

- Clinical Pearl:
  - HERBAL COMPOUNDS THAT INHIBIT Tumor Promoting Oncogene mTOR (mammalian target of rapamycin)

- Research Highlights:
  - Role of tumor microenvironment in tumorigenesis
  - Nearly half of cancer patients don't feel involved in treatment decisions, survey finds Patient insights on cancer care: opportunities for improving efficiency
  - Study Finds Primary Care Providers Are Engaged in Cancer Survivorship Care but Report Barriers to Optimal Care Delivery
  - A study of coagulation profile in neoplastic conditions

Clinical Pearl: **HERBAL COMPOUNDS THAT INHIBIT Tumor Promoting Oncogene mTOR (mammalian target of rapamycin)**
See Slides Below

Case Study:
Submitted by: No Case Study Submitted

Questions & Answers:
No Questions Submitted

Research:

REVIEW

Role of tumor microenvironment in tumorigenesis

Journal of Cancer 2017, Vol. 8

Maonan Wang1,2, Jingzhou Zhao et al

Abstract

Tumorigenesis is a complex and dynamic process, consisting of three stages: initiation, progression,and metastasis. Tumors are encircled by extracellular matrix (ECM) and stromal cells, and the physiological state of the tumor microenvironment (TME) is closely connected to every step of tumorigenesis. Evidence suggests that the vital components of the TME are fibroblasts and myofibroblasts, neuroendocrine cells, adipose cells, immune and inflammatory cells, the blood and lymphatic vascular networks, and ECM. This manuscript, based on the current studies of the TME, offers a more comprehensive overview of the primary functions of each component of the TME in cancer initiation, progression, and invasion. The manuscript also includes primary therapeutic targeting markers for each player, which may be helpful in treating tumors.

Research:

Nearly half of cancer patients don't feel involved in treatment decisions, survey finds

Patient insights on cancer care: opportunities for improving efficiency

Findings from the international All.Can patient survey

https://www.all-can.org/what-we-do/research/patient-survey/

"It is so important that, as physicians, we listen to what patients are telling us in this survey,"

The patient perspective is too often forgotten in considerations of how cancer care can be improved. The All.Can patient survey gave us a unique opportunity to ask this question directly to those who have had personal experience of cancer.

Almost 4,000 cancer patients and caregivers from more than 10 countries across the world responded to the survey to share their experiences. While most reported that their needs were sufficiently addressed during their care, they highlighted some specific areas where they encountered inefficiency.

We have identified four key opportunities for improvement based on the responses to the survey:

- **Ensure swift, accurate and appropriately delivered diagnosis**
- **Improve information-sharing, support and shared decision-making**
- **Make integrated multidisciplinary care a reality for all patients**
- **Address the financial impact of cancer**
- *It's impossible to get a bank loan… One is punished for being ill, and it all comes down to luck.* **Respondent from Belgium**
- *I think the psychological involvement part is forgotten. It is true that the main thing is to survive, but it is also necessary to feel accompanied and understood.* **Respondent from Spain**
- *I don't want more information, but better information.* **Respondent from the United States**
- *I had delays in diagnosis, and I felt I was made a fool of about the symptoms I had – they were trivialised as an intestinal virus and anxiety.* **Respondent from Italy**

Research:

Study Finds Primary Care Providers Are Engaged in Cancer Survivorship Care but Report Barriers to Optimal Care Delivery

By The ASCO Post
https://www.ascopost.com/News/60182?email=f5131f75e03b4964ecb0429aa2a88b9c6990b3fc521c5402a84c497cb05136d0&utm_medium=Email&utm_campaign=TAP%20EN Posted: 6/24/2019 12:26:09 PM

A new study published by McDonough et al in the *Journal of Oncology Practice* found that while primary care physicians are often involved in cancer survivorship care, many do not feel adequately prepared for key components of it.

The study shared the results of a survey of 117 primary care physicians in practices affiliated with a large academic medical center to evaluate four key areas: (1) current practices in care of cancer survivors, (2) sense of preparedness to deliver such care, (3) barriers to care of cancer survivors, and (4) preferences for shared care and communication between primary care physicians and oncologists.

A majority (82%) of respondents had been practicing for more than 10 years and saw a median of 36 patients per week. Nearly all survey respondents reported that they commonly assume primary responsibility for at least some aspects of survivorship care.

Key Points

- **84% reported regularly assuming primary responsibility for the management of chronic physical complications of cancer therapy; but, only 10% felt very prepared to do so.**
- **73% of those surveyed were uncertain about delegation of responsibility and 72% lacked needed training.**
- **97% wanted access to a cancer treatment summary, and 78% reported that they generally had such access; but 98% reported that they wanted a survivorship care plan with explicit follow-up care recommendations, and only 11% reported regularly having such access.**

Practice, Preferences, and Practical Tips From Primary Care Physicians to Improve the Care of Cancer Survivors

Allison L. McDonough, Julia Rabin, Nora Horick, Yvonne Lei, Garrett Chinn, Eric G. Campbell, Elyse R. Park, and Jeffrey Peppercorn
Journal of Oncology Practice 2019 15:7, e600-e606

References:

Peter Attia MD Podcast THE DRIVE

#62 - Keith Flaherty, M.D.: Deep dive into cancer—History of oncology, novel approaches to treatment, and the exciting and hopeful future
This link takes you to an episode landing page where you can find the show notes, listen to or watch the episode, access references from the conversation, and more.

"We can't keep hitting the same pillar and expect that we're going to cure cancer. . .we need the activators of the immune system, we need the inhibitors of the activated oncogenes, we need the drugs that target epigenetic regulators, and we need the metabolic switch regulators." — Keith Flaherty

In this episode, Keith Flaherty, director of clinical research and targeted cancer therapy at Massachusetts General Hospital, shares his vast wealth of knowledge in cancer starting with the history of treatment from chemotherapy to radiation to surgical therapy and where those methodologies seemed to have leveled off. He also walks us through the timeline of advancements (and lack thereof) from when the War on Cancer was declared in the 1970s, through the sequencing of the entire human genome, and all the way to today. Keith dives into the topic of immunotherapy, probably the most exciting recent development in cancer therapy, and also provides us a rundown of his notion of a different approach to cancer that attacks all the essential pillars of cancer growth and survival. Finally, we talk a little bit about liquid biopsies, we discuss the roles of CRISPR and other potentially over-hyped therapies with respect to cancer. We also touch on stem cell therapy a bit, as well as some other common cancer-related questions such as the role of vitamin D and sun exposure in melanoma, and much more.

Research:

A study of coagulation profile in neoplastic conditions
ICF Intravascular Coagulation and Fibrinolysis

Patel SM, Gupta S, Patel MM, Mahadik JD, Patel KA, Patel AS
Int J Med Sci Public Health 2016;5:402-407

Malignancies show an increased susceptibility to thromboembolic events when compared with benign tumors and the general population.

Ovarian, pancreatic, prostatic, and lung cancers and mucin-producing carcinomas of gastrointestinal tract are among the malignancies often associated with thromboembolic episodes.[2]

Thrombosis occurs spontaneously, after surgery, radiation therapy, and anticancer treatment and might be the first manifestation of underlying cancer.[3]

Abnormal coagulation activation encourages endothelial adhesion, metastatic spread, tumor cell growth, and survival.[2

Our study implies a relation between **activation of hemostasis mirrored by elevated D-dimer** and malignancy. **D-dimer might be used as a universal surrogate indicator of the relation between cancer and the activation of hemostasis and fibrinolysis, with elevated D-dimer levels symbolizing the pathogenesis of a more aggressive malignant process associated with poor clinical results.**

Amin et al.[7] showed that the percentage of patients with abnormal coagulation was 88%. Mohammed et al.[2] showed that the coagulation abnormalities were in 80% patients.
In our study, 94.28% malignancies showed coagulation abnormalities comparable with the above studies

In our study, the mean platelet count in malignancies was 334.14 ± 104.56 Å~ 103/cm, which was higher when compared with apparently normal controls (273.73 ± 126.52 Å~ 103/cm)

Patients with malignancies predominantly showed D-dimer > 0.5µg/mL (88.57%). Such patients with elevated D-dimer are said to have ICF syndrome. Malignancies tended to show elevated D-dimer when compared with benign lesions

Plasma D-dimer correlates with tumor burden, no. of metastatic sites, progression kinetics, cytokines related to angiogenesis,[11] invasion depth, lymph node metastasis, peritoneal dissemination, distant metastasis, tumor size, and TNM stage[12]

CONCLUSION
Increased D-dimer and altered coagulation parameters significantly correlated with malignant behavior of tumors and their spread. They might be useful indicators of aggressive tumor biology and behavior.

References:

1. Alayev, A., Berger, S. M., & Holz, M. K. (2015). **Resveratrol as a novel treatment for diseases with mTOR pathway hyperactivation**. *Annals of the New York Academy of Sciences,1348*(1), 116-123. doi:10.1111/nyas.12829

2. Chia, S., Gandhi, S., Joy, A., Edwards, S., Gorr, M., Hopkins, S., . . . Dent, S. (2015). **Novel agents and associated toxicities of inhibitors of the pi3k/Akt/mtor pathway for the treatment of breast cancer**. *Current Oncology,22*(1), 33. doi:10.3747/co.22.2393

3. Crane, C., Panner, A., Pieper, R. O., Arbiser, J., & Parsa, A. T. (2009). **Honokiol-mediated Inhibition of PI3K/mTOR Pathway**. *Journal of Immunotherapy,32*(6), 585-592. doi:10.1097/cji.0b013e3181a8efe6

4. Geissler, E. K. (2013). **The influence of mTOR inhibitors on immunity and the relationship to post-transplant malignancy**. *Transplantation Research,2*(S1). doi:10.1186/2047-1440-2-s1-s2

5. Ghițu, A., Schwiebs, A., Radeke, H. H., Avram, S., Zupko, I., Bor, A., . . . Danciu, C. (2019). **A Comprehensive Assessment of Apigenin as an Antiproliferative, Proapoptotic, Antiangiogenic and Immunomodulatory Phytocompound**. *Nutrients,11*(4), 858. doi:10.3390/nu11040858

6. Guri, Y., Nordmann, T. M., & Roszik, J. (2018). **MTOR at the Transmitting and Receiving Ends in Tumor Immunity**. *Frontiers in Immunology,9*. doi:10.3389/fimmu.2018.00578

7. Lamming, D. W., Cummings, N. E., Rastelli, A. L., Gao, F., Cava, E., Bertozzi, B., . . . Fontana, L. (2015). **Restriction of dietary protein decreases mTORC1 in tumors and somatic tissues of a tumor-bearing mouse xenograft model**. *Oncotarget,6*(31). doi:10.18632/oncotarget.5180

8. Liu, H., Dong, Y., Gao, Y., Du, Z., Wang, Y., Cheng, P., . . . Huang, H. (2016). **The Fascinating Effects of Baicalein on Cancer: A Review.** *International Journal of Molecular Sciences,17*(10), 1681. doi:10.3390/ijms17101681

9. Maaty, M. A., & Wölfl, S. (2017). **Vitamin D as a Novel Regulator of Tumor Metabolism: Insights on Potential Mechanisms and Implications for Anti-Cancer Therapy**. *International Journal of Molecular Sciences,18*(10), 2184. doi:10.3390/ijms18102184

10. Mahase, E. (2019). **Nearly half of cancer patients don't feel involved in treatment decisions, survey finds**. *Bmj,*L4877. doi:10.1136/bmj.l4877

11. Mcdonough, A. L., Rabin, J., Horick, N., Lei, Y., Chinn, G., Campbell, E. G., . . . Peppercorn, J. (2019). **Practice, Preferences, and Practical Tips From Primary Care Physicians to Improve the Care of Cancer Survivors.** *Journal of Oncology Practice,15*(7). doi:10.1200/jop.18.00740

12. Mead, H., Zeremski, M., & Guba, M. (2009). **MTOR Signaling in Angiogenesis.** *MTOR Pathway and MTOR Inhibitors in Cancer Therapy,*49-74. doi:10.1007/978-1-60327-271-1_3

13. Patel, S., Gupta, S., Patel, M., Mahadik, J., Patel, K., & Patel, A. (2016). **A study of coagulation profile in neoplastic conditions.** *International Journal of Medical Science and Public Health,5*(3), 402. doi:10.5455/ijmsph.2016.1807201565

14. Rivera, A. R., Castillo-Pichardo, L., Gerena, Y., & Dharmawardhane, S. (2016). **Anti-Breast Cancer Potential of Quercetin via the Akt/AMPK/Mammalian Target of Rapamycin (mTOR) Signaling Cascade.** *Plos One,11*(6). doi:10.1371/journal.pone.0157251

15. Shankar, E., Goel, A., Gupta, K., & Gupta, S. (2017). **Plant Flavone Apigenin: An Emerging**

Anticancer Agent. *Current Pharmacology Reports,3*(6), 423-446. doi:10.1007/s40495-017-0113-2

16. Shin, S., Jing, K., Jeong, S., Kim, N., Song, K., Heo, J., . . . Lim, K. (2013). **The Omega-3 Polyunsaturated Fatty Acid DHA Induces Simultaneous Apoptosis and Autophagy via Mitochondrial ROS-Mediated Akt-mTOR Signaling in Prostate Cancer Cells Expressing Mutant p53.** *BioMed Research International,2013*, 1-11. doi:10.1155/2013/568671

17. Siddiqui, F. A., Prakasam, G., Chattopadhyay, S., Rehman, A. U., Padder, R. A., Ansari, M. A., . . . Iqbal, M. A. (2018). **Curcumin decreases Warburg effect in cancer cells by down-regulating pyruvate kinase M2 via mTOR-HIF1α inhibition.** *Scientific Reports,8*(1). doi:10.1038/s41598-018-25524-3

18. Tan, H. K., Moad, A. I., & Tan, M. L. (2014). **The mTOR Signalling Pathway in Cancer and the Potential mTOR Inhibitory Activities of Natural Phytochemicals.** *Asian Pacific Journal of Cancer Prevention,15*(16), 6463-6475. doi:10.7314/apjcp.2014.15.16.6463

19. Tong, X., & Pelling, J. (2013). **Targeting the PI3K/Akt/mTOR Axis by Apigenin for Cancer Prevention.** *Anti-Cancer Agents in Medicinal Chemistry,13*(7), 971-978. doi:10.2174/18715206113139990119

20. Totiger, T. M., Srinivasan, S., Jala, V. R., Lamichhane, P., Dosch, A. R., Gaidarski, A. A., . . . Nagathihalli, N. S. (2019). **Urolithin A, a Novel Natural Compound to Target PI3K/AKT/mTOR Pathway in Pancreatic Cancer.** *Molecular Cancer Therapeutics,18*(2), 301-311. doi:10.1158/1535-7163.mct-18-0464

21. Wang, E., Fan, Q., Yue, Y., & Xu, L. (2018). **Ursolic Acid Attenuates High Glucose-Mediated Mesangial Cell Injury by Inhibiting the Phosphatidylinositol 3-Kinase/Akt/Mammalian Target of Rapamycin (PI3K/Akt/mTOR) Signaling Pathway.** *Medical Science Monitor,24*, 846-854. doi:10.12659/msm.907814

22. Wang, M., Zhao, J., Zhang, L., Wei, F., Lian, Y., Wu, Y., . . . Guo, C. (2017). **Role of tumor microenvironment in tumorigenesis.** *Journal of Cancer,8*(5), 761-773. doi:10.7150/jca.17648

23. Yang, J., Pi, C., & Wang, G. (2018). **Inhibition of PI3K/Akt/mTOR pathway by apigenin induces apoptosis and autophagy in hepatocellular carcinoma cells.** *Biomedicine & Pharmacotherapy,103*, 699-707. doi:10.1016/j.biopha.2018.04.072

Herbal Compounds that Inhibit Tumor Promoting Oncogene mTOR (mammalian Target Of Rapamycin)

Dr. Nalini Chilkov, L.Ac. OMD, Founder

American Institute of Integrative Oncology
RESEARCH & EDUCATION®

www.AIIORE.com

mTOR is an Atypical Protein Kinase

- phosphorylates tyrosine and serine/threonine
- mTOR is hyperactive in the majority of cancers
- mTOR is also important in normal physiology

Activates several oncogenic signaling pathways that promote cell survival

- Regulates growth factor signaling and proliferation
- Regulates nutrient metabolism
- Promotes protein, lipid and nucleotide synthesis
- Promotes angiogenesis and metastasis
- Promotes cell survival/Inhibits autophagy
- Promotes immunity
- Activates IGF-1 Receptor and Insulin Receptor

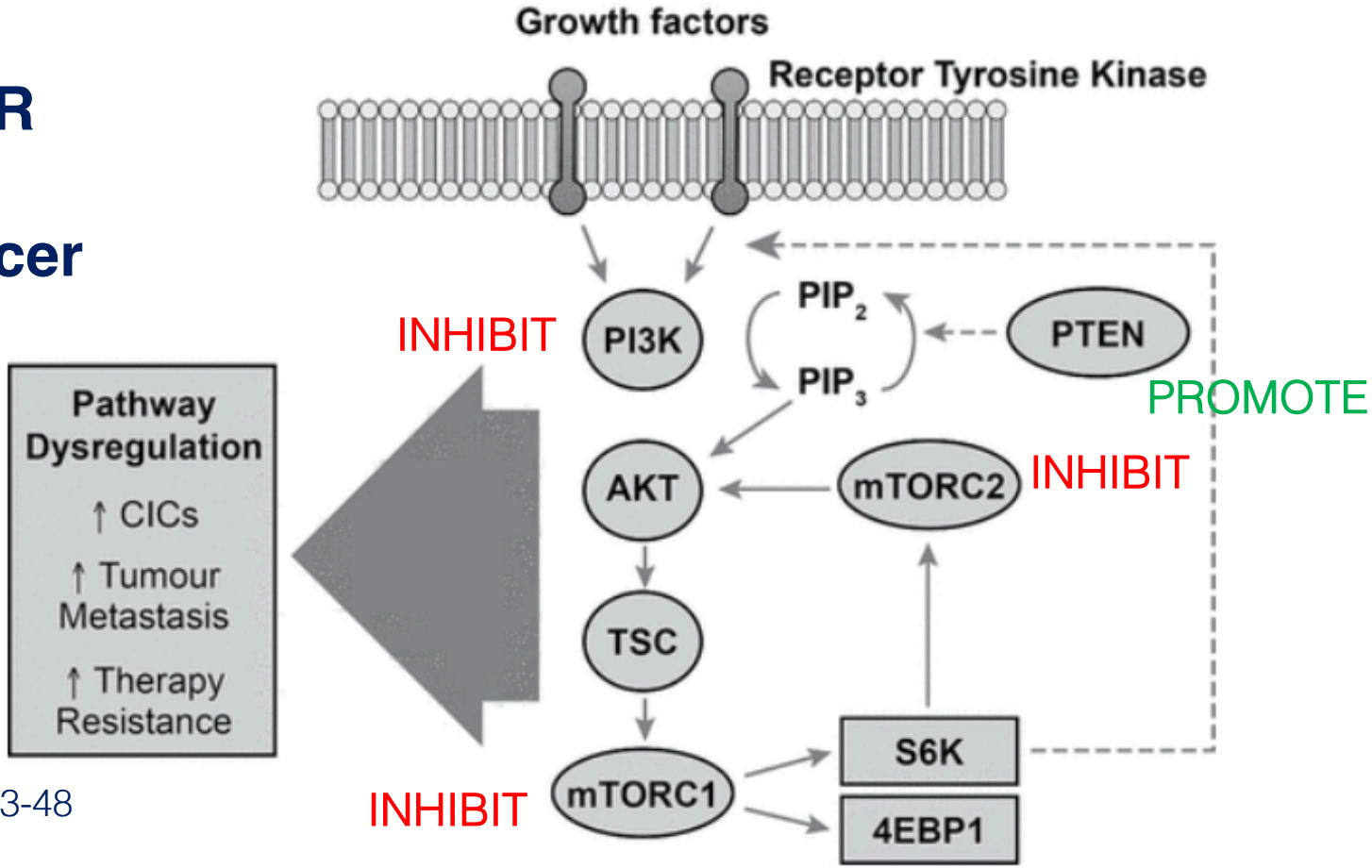
Activated by several oncogenic upstream pathways

- Ras/Raf/MEK/ERK. (MAPK) and PI3K/AKT/PTEN

PI3K/Akt/mTOR signaling pathway in cancer

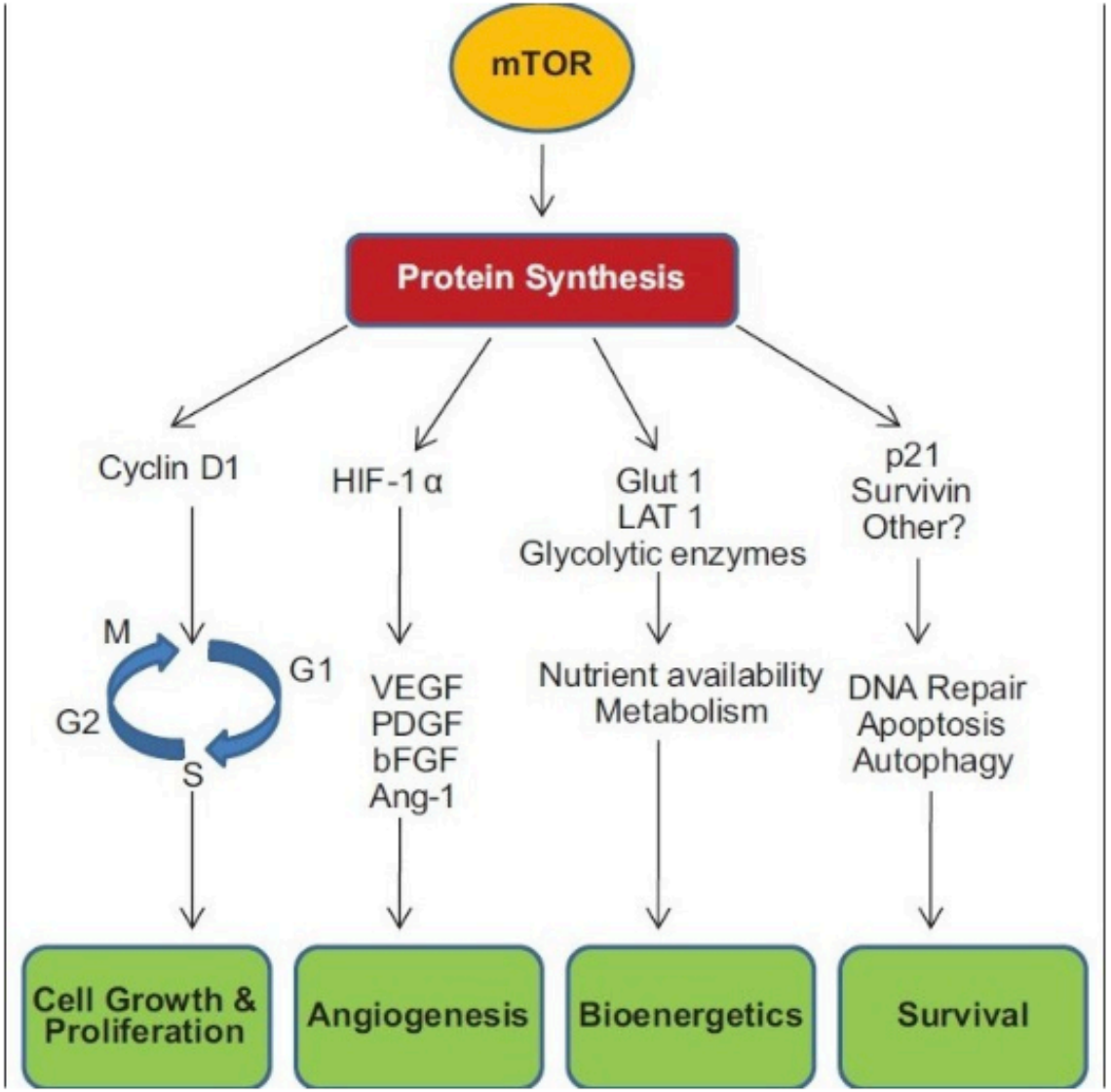
mTOR
Tumor Promoter

PTEN
Tumor Suppressor



Curr Oncol. 2015 Feb;22(1):33-48
doi: 10.3747/co.22.2393.

Treating Breast Cancer by Targeting the mTOR Pathway



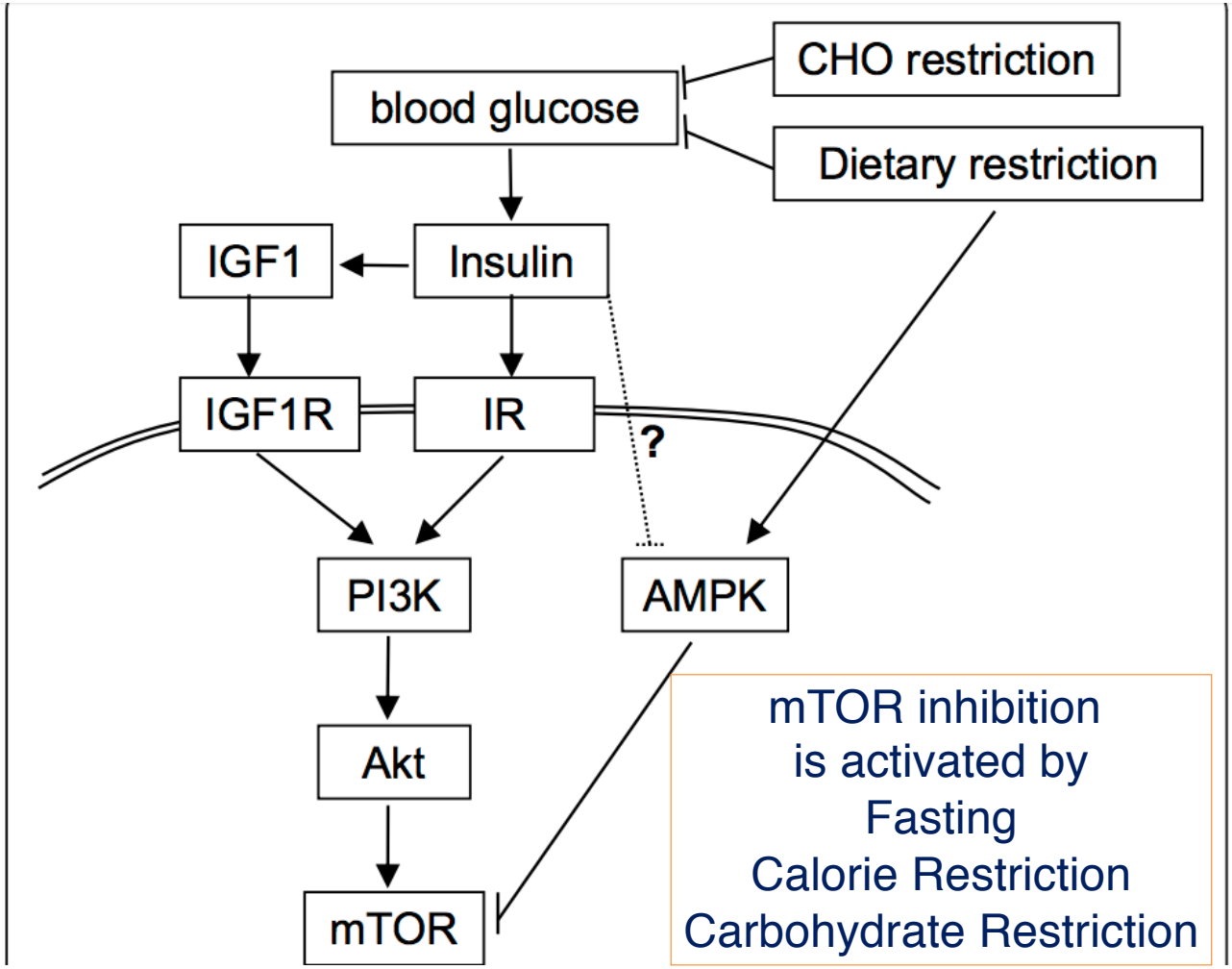
http://ubcf.org/treating-breast-cancer-targeting-mtor-pathway/

mTOR is frequently de-regulated in both solid tumors and hematologic cancers

Brain
Breast
Endometrial
Ovarian
Neuroendocrine
Pancreatic
Gastric
Colorectal
Thyroid
Prostate
Renal
Hepatic
Melanoma
Myelomas
Lymphomas
Leukemias

Effects of short-term fasting on cancer treatment

de Groot et al.
Journal of Experimental & Clinical Cancer Research (2019) 38:209
https://doi.org/10.1186/s13046-019-1189-9



mTOR Inhibition Effects are a function of the MicroEnvironment

mTor inhibition can either inhibit or promote immune response
depending on the antigenic stimulus and the tumor microenvironment signals

Inhibits Immune Response against transplant allografts
AND
Promotes Immune Response against micro-organisms and tumor cells

Transplant Res. 2013; 2(Suppl 1): S2.
Published online 2013 Nov 20. doi: 10.1186/2047-1440-2-S1-S2

Inhibition of mTOR and Beneficial Immune Modulation

ENHANCE ANTI TUMOR RESPONSE

- Induce memory cytotoxic CD8 + T lymphocytes (CTL) formation against tumor cells

REDUCE IMMUNOSUPPRESSIVE AND PRO GROWTH ACTIVITY OF

- Cancer Associated Fibroblasts
- Myeloid Derived Stem Cells
- Anti-Inflammatory Tumor Associated Macrophages
- Inhibition of PDL-1 Immune Suppression

Potential use WITH Cancer Immunotherapy

IMPROVE FOREIGN ANTIGEN RECOGNITION and Dendritic Cell Function

- Inhibition of infectious organisms and
- Reduced viral infections

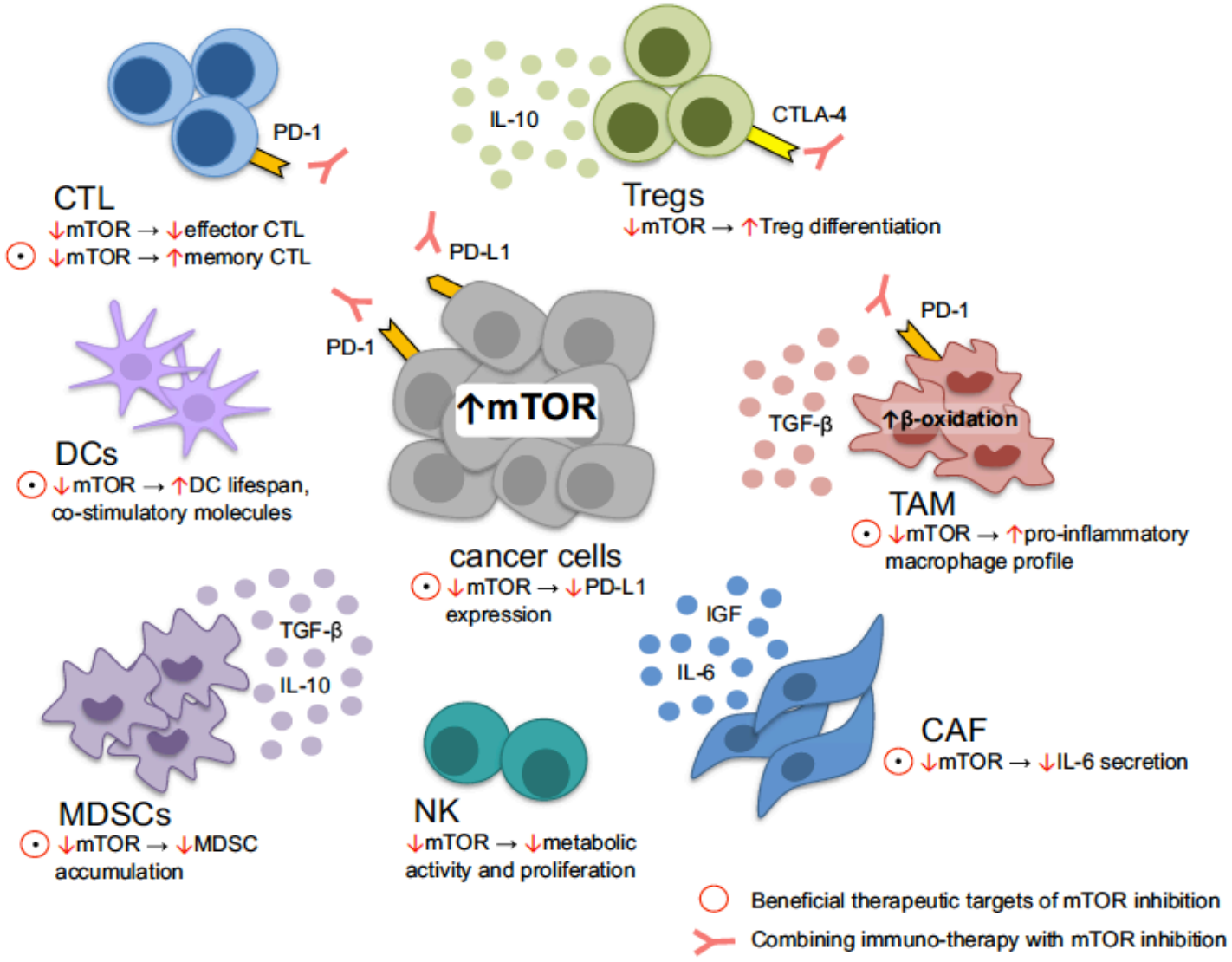
DOWNREGULATION of PI3K-mTOR-AKT pathway

Front Immunol. 2018; 9: 578.
Published online 2018 Mar
27. doi: 10.3389/fimmu.2018.00578

mTOR and Modulation of the Tumor Microenvironment

some of the anticancer effects of mTOR inhibition may be *via* **immune modulation**

Front Immunol. 2018; 9: 578.
Published online 2018 Mar
27. doi: 10.3389/fimmu.2018.00578



American Institute of Integrative Oncology
RESEARCH & EDUCATION®

Phytochemicals That Inhibit mTOR

Phytochemical	Type of Neoplasm	Reference
Curcumin Curcuma Longa	Colorectal	Anticancer Res. 2009 August; 29(8): 3185
Curcumin Curcuma Longa	Multifocal	Cancer Res 2009;69(3):1000–8
Curcumin Curcuma Longa	Prostate	Cancer Ther. 2008 Sep;7(9):2609-20.
Curcumin Curcuma Longa	Leiomyosarcoma	Gynecol Oncol. 2011 Jul;122(1):141-8. Epub 2011 Mar 29.
Curcumin +EGCG Curcuma longa+ Camelia sinensis	Leiomyosarcoma	Int J Clin Oncol. 2012 Feb 15.
EGCG Camelia sinensis	Breast	Br J Cancer. 2008 Oct 7;99(7):1056-63. Epub 2008 Sep 16.
Isoliquiritigenin Glycyrhhiza spp	Adenoid Cystic Carcinoma	Apoptosis. 2012 Jan;17(1):90-101.

Phytochemicals That Inhibit mTOR

Phytochemical	Type of Neoplasm	Reference
Salidroside Rhodiola rosea	Bladder	Mol Carcinog. 2011 Apr 22. doi: 10.1002/mc.20780.
Ursolic Acid Salvia spp, Heydotis/Oldenlandia	Breast	Nutr Cancer. 2010;62(8):1074-86.
Withaferin A Withania somnifera	Colorectal	Mol Cancer Ther. 2010 Jan ; 9(1): 202–210
Silibinin Silybium marianum	Breast, Prostate	Mol Cancer Ther. 2009 Jun;8(6):1606-12. Epub 2009 Jun 9.
Apigenin Matricaria, Petroselinum,	Breast, Prostate, Colon, Cervical, Lung, Ovary, Skin Liver, Pancreas, Hematologic,	Anticancer Agents Med Chem. 2013 Sep; 13(7): 971–978 Biomed Pharmacother. 2018 Jul;103:699-707. doi: 10.1016/j.biopha.2018.04.072. Epub 2018 Apr 24.
Genistein Soy	Breast	Genome Med. 2010; 2(12): 90.
Honokiol Magnolia spp	Breast, Prostate, Renal, Brain	J Immunother. 2009 Jul-Aug; 32(6): 585–592. doi: 10.1097/CJI.0b013e3181a8efe6

Inhibition of mTOR

Phytochemical	Type of Neoplasm	Reference
Resveratrol Polygonum cuspidatum	Multifocal	.Ann N Y Acad Sci. 2015 Aug;1348(1):116-23. doi: 10.1111/nyas.12829. Epub 2015 Jul 22. Anticancer Agents Med Chem. 2013 Sep;13(7):1032-8.
Baicailein Scutellaria baicelensis	Prostate, Breast, Hepatic, multi	Int J Mol Sci. 2016 Oct; 17(10): 1681. Pub online 2016 Oct 9. doi: 10.3390/ijms17101681 Cancer Lett. 2015 Mar 28;358(2):170-179. doi: 10.1016/j.canlet.2014.12.033. Mol Cell Biochem. 2015 Aug;406(1-2):111-9. doi: 10.1007/s11010-015-2429-8.
Quercetin-Isoquercitrin Camelia sinensis, Cruciferae spp, Allium spp, Rubus spp, +	Renal, Prostate, Colon, multi	PLoS One. 2016; 11(6): e0157251. Pub online 2016 Jun 10. doi: 10.1371/journal.pone.0157251 PLoS One. 2012; 7(10): e47516. Pub 2012 Oct 18. doi: 10.1371/journal.pone.0047516 Molecules. 2016 Jan; 21(1): 108. Publ online 2016 Jan 19. doi: 10.3390/molecules21010108
Urolithin Punica granatum	Pancreatic	Mol Cancer Ther DOI: 10.1158/1535-7163.MCT-18-0464 Vol 8 (2) February 2019. AACR
Vitamin D Cholecalciferol	Breast	Int J Mol Sci. 2017 Oct 19;18(10). pii: E2184. doi: 10.3390/ijms18102184. J Nutr Biochem. 2018 Mar;53:111-120. doi: 10.1016/j.jnutbio.2017.10.013.
Calorie Restriction **Carbohydrate restriction**	Multifocal	Oncotarget. 2015 Oct 13;6(31):31233-40. doi: 10.18632/oncotarget.5180. Cell. 2017 Feb 23; 168(5): 775–788.e12.doi: 10.1016/j.cell.2017.01.040
Omega 3 Fatty Acid DHA	Prostate	Biomed Res Int. 2013;2013:568671. doi: 10.1155/2013/568671

Pharmaceutical mTOR inhibitors

Afinitor	everolimus
Torisel	temsirolimus
Rapamune	sirolimus
Rapamycin	sirolimus

Adverse Effects

Stomatitis
Pneumonitis
Dermatitis-Rash
Hyperglycemia
Immunosuppression
Myelosuppression

Monitor

CBC + differential
Platelets
Liver Function
Glucose

THIS INFORMATION IS PROVIDED FOR THE USE OF PHYSICIANS AND OTHER LICENSED HEALTH CARE PRACTITIONERS ONLY. THIS INFORMATION IS INTENDED FOR PHYSICIANS AND OTHER LICENSED HEALTH CARE PROVIDERS TO USE AS A BASIS FOR DETERMINING WHETHER OR NOT TO RECOMMEND SUPPORT OR PRODUCTS TO THEIR PATIENTS. THIS MEDICAL AND SCIENTIFIC INFORMATION IS NOT FOR USE BY CONSUMERS. THE DIETARY SUPPLEMENTS ARE NOT INTENDED FOR USE BY CONSUMERS AS A MEANS TO CURE, TREAT, PREVENT, DIAGNOSE, OR MITIGATE ANY DISEASE OR OTHER MEDICAL CONDITION.

IVYSPRING INTERNATIONAL PUBLISHER

Journal of Cancer

2017; 8(5): 761-773. doi: 10.7150/jca.17648

Review

Role of tumor microenvironment in tumorigenesis

Maonan Wang[1,2], Jingzhou Zhao[2], Lishen Zhang[2], Fang Wei[2], Yu Lian[2], Yingfeng Wu[2], Zhaojian Gong[2], Shanshan Zhang[1], Jianda Zhou[3], Ke Cao[3], Xiayu Li[3], Wei Xiong[1,2,3], Guiyuan Li[1,2,3], Zhaoyang Zeng[1,2,3]✉, Can Guo[1,2,3] ✉

1. Key Laboratory of Carcinogenesis of Ministry of Health, Xiangya Hospital, Central South University, Changsha, Hunan 410078, China;
2. Key Laboratory of Carcinogenesis and Cancer Invasion of Ministry of Education, Cancer Research Institute, Central South University, Changsha, Hunan 410078, China;
3. Hunan Key Laboratory of Nonresolving Inflammation and Cancer, Disease Genome Research Center, The Third Xiangya Hospital, Central South University, Changsha, Hunan 410013, China.

✉ Corresponding authors: Zhaoyang Zeng, Email: zengzhaoyang@csu.edu.cn; or Can Guo, Email: guocde@csu.edu.cn

Received: 2016.09.20; Accepted: 2016.12.22; Published: 2017.02.25

Abstract

Tumorigenesis is a complex and dynamic process, consisting of three stages: initiation, progression, and metastasis. Tumors are encircled by extracellular matrix (ECM) and stromal cells, and the physiological state of the tumor microenvironment (TME) is closely connected to every step of tumorigenesis. Evidence suggests that the vital components of the TME are fibroblasts and myofibroblasts, neuroendocrine cells, adipose cells, immune and inflammatory cells, the blood and lymphatic vascular networks, and ECM. This manuscript, based on the current studies of the TME, offers a more comprehensive overview of the primary functions of each component of the TME in cancer initiation, progression, and invasion. The manuscript also includes primary therapeutic targeting markers for each player, which may be helpful in treating tumors.

Key words: cancer-associated fibroblasts (CAFs), neuroendocrine cells, adipose cells, immune-inflammatory cells, angiogenesis

Introduction

Currently, ten major characteristics of cancer have been universally recognized, including unlimited multiplication, evasion from growth suppressors, promoting invasion and metastasis, resisting apoptosis, stimulating angiogenesis, maintaining proliferative signaling, elimination of cell energy limitation, evading immune destruction, genome instability and mutation, and tumor enhanced inflammation (**Figure 1**) [1]. Although researchers now have an understanding of most characteristics of cancer [2-30], the characteristics regarding cancer formation, which is the focus of the current study, remains unknown. After the 'ecological therapy' strategy was widely employed [31], much effort has been devoted to determining how cellular and noncellular components of the tumoral niche help tumors to acquire these characters. These cellular and noncellular components of the tumoral niche comprise tumor the microenvironment (TME). The TME consists of extracellular matrix (ECM) as well as myofibroblasts and cellular players, such as fibroblasts, neuroendocrine (NE) cells, adipose cells, immune-inflammatory cells, and the blood and lymphatic vascular networks [32]. Furthermore, TME has increasingly been shown to dictate aberrant tissue function and play a critical role in the subsequent evolution of more stubborn and advanced malignancies [33]. Oncologists have also found that when the microenvironment in a healthy state, it can help protect against tumorigenesis and invasion. By contrast, if it is not in a healthy state, it will become an accomplice.

The intent of this paper was to summarize the existent knowledge on the potential role of each TME

component in tumorigenesis: initiation, progression, and metastasis, respectively. We have also summarized some of the main cellular players, such as cancer-associated fibroblasts, immune and inflammatory cells, blood and lymphatic vascular networks, adipose cells, neuroendocrine cells and ECM in the TME, as well as their corresponding targets in TME, in the hope of providing some clues for future TME research. We have also introduced the therapeutic target markers for various parts of TME based on the current research results.

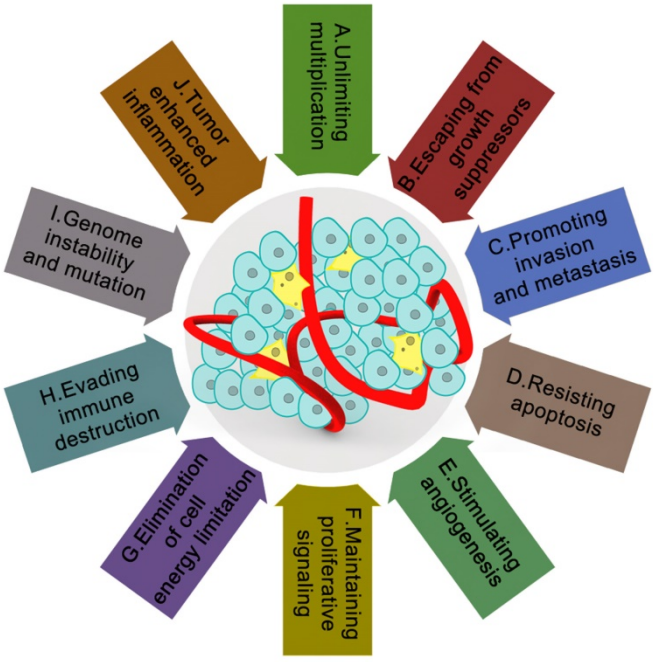


Figure 1. The tumor microenvironment and characteristics of cancer. It is currently widely recognized that tumor microenvironments are wildly influenced by the ten main characteristics of cancer: A. unlimited multiplication; B. escaping from growth suppressors; C. promoting invasion and metastasis; D. resisting apoptosis; E. stimulating angiogenesis; F. maintaining proliferative signaling; G. elimination of cell energy limitation; H. evading immune destruction; I. genome instability and mutation; J. tumor-enhanced inflammation. Lower cure rate and poor prognosis of cancer patients are closely related to these ten characteristics of cancer. These ten characteristics make cancer more mysterious within the complex tumor microenvironments.

Cancer-associated fibroblasts (CAFs)

A sub-population of fibroblasts with a myofibroblastic phenotype in cancerous wounds is distinguished as cancer-associated fibroblasts (CAFs). After activation, fibroblasts are known as CAFs or myofibroblasts [34-36]. During natural wound repair, myofibroblasts are transiently present [37]. Unlike the process of wound healing, CAFs at the site of a tumor remain perpetually activated, as in tissue fibrosis.

Several studies have demonstrated that only the activated fibroblasts are required to initiate and promote tumor growth [38-40]. Fibroblast activation may be induced through various impetuses when tissue lesions occur, including growth factors, direct cell-cell communication, adhesion molecules contacting with leukocytes, reactive oxygen species [41], and microRNA [42, 43]. When the fibroblasts remain activated after the initial insult has regressed, these activated fibroblasts may work with other molecular pathways to boost neoplasm initiation. These CAFs have a significant impact on cancer progression through remodeling ECM, inducing angiogenesis, recruiting inflammatory cells, and directly stimulating cancer cell proliferation via the secretion of growth factors, immune suppressive cytokines, and mesenchymal-epithelial cell interactions [41, 44]. For instance, Galectin-1 overexpression in CAFs advances the development of abutting cancer cells [45] and is correlated with poor prognosis in several types of cancer, including breast and prostate cancer and laryngeal carcinoma [46-49]. Chemokine (C–X–C motif) ligand 12 (CXCL12), violently uttered in CAFs, may induce epithelial-mesenchymal transition (EMT) of cancer cells to promote cancer progress in gastric and prostate cancers [50, 51]. Moreover, one team discovered that MMP-2, derived from senescent CAF-CMs, induced epithelial invasion and keratinocyte discohesion into collagen. Interleukin-22 (IL-22) is also expressed by CAFs to encourage gastric cancer cell invasion through STAT3 and ERK signaling [52]. Using a 3D invasion model, another study found that HCT116 cells manifested a substantially invasive phenotype, while media originated from human dermal fibroblasts (HDF) [53].

Since myofibroblasts can be distinguished by alpha-smooth muscle actin (α-SMA), laminin-1, transforming growth factor beta (TGF-β1), vascular endothelia growth factor A (VEGF-A), etc. [54, 55], CAFs have been recognized as playing an essential role in the metastasis and development of cancer [56]. Oncologists have found that through HGF, TGF-β, platelet-derived growth factor (PDGF) etc., CAFs may promote tumor growth and invasion (**Figure 2**). Through fibroblast growth factor 2 (FGF2), VEGF, etc., CAFs may promote tumor development by promoting angiogenesis [57]. Additionally, CAFs also interact with immune-inflammatory cells and neuroendocrine cells through different cell factors and cytokines to jointly promote the initiation, progression, and invasion of cancer [58-62]. However, many of the markers that have been gradually proven to be unable to identify all of the CAFs, are not unique to the CAFs [63]. The cardinal functions and the primary markers of CAFs are illustrated in **Table 1**. For instance, α–SMA, one of the previous major markers of CAFs, was found to be expressed in normal fibroblasts [64], pericytes, and smooth muscle

cells [65]. The cell-surface serine protease fibroblast activation protein α (FAPα), which is highly expressed in quiescent mesodermal cells in multiple tissue types [66], is also not specific to CAFs. Additional markers fibroblast-specific protein 1 (FSP-1) [67], vimentin, and certain proteins, including NG2 (Neuroglial Antigen-2), platelet-derived growth factor receptor-β (PDGFR-β), fibroblast-associated antigen, and prolyl 4-hydroxylase have been shown to be expressed in cells other than CAFs [63].

Table 1. The function of cell players in the tumor microenvironment.

Cell players	Main markers	Primary functions
Cancer-associated fibroblasts (CAFs)	PDGF*; FAP*; FGFR*; VDR*	Regulating inflammation; Participating in wound healing; Integrating collagen and protein to form the ECM fiber network; Escaping damage;
Immune & Inflammatory cell	TNF-α; IL-10; IL-12; TGF-β; Foxp3+*; HMGB1*; CD163+*; KIR*; PD-1+*	Treatment of wound healing and infection; Clearing dead cells and cellular debris; Having a double effect on tumor formation
The blood & lymphatic vascular networks	VEGRF3; LYVE-1; CD31; CD34; VEGF*; PlGF*; VEGF-B*; VEGF-C*; VEGF-D*	Require nutrients and oxygen; Evacuating metabolic wastes and carbon dioxide; Helping to escape immune surveillance.
Adipose cell	AIs*; MBD6*	Producing circulating blood estrogen; A major energy source; Relating with inflammation; Recruiting immune cells; Support vasculogenesis.
Neuroendocrine cell	NSE; CgA; K18&K8 cytokeratins; PGP9.5; Ki-67; IL-2; KE108*; DLL3*; EGF*	Extending lumina and adjacent epithelial cells; Regulating secretion and motility; Controlling lung branching morphogenesis; Providing a protective niche for a subset of lung stem cells.

Note: *, the targeting markers.

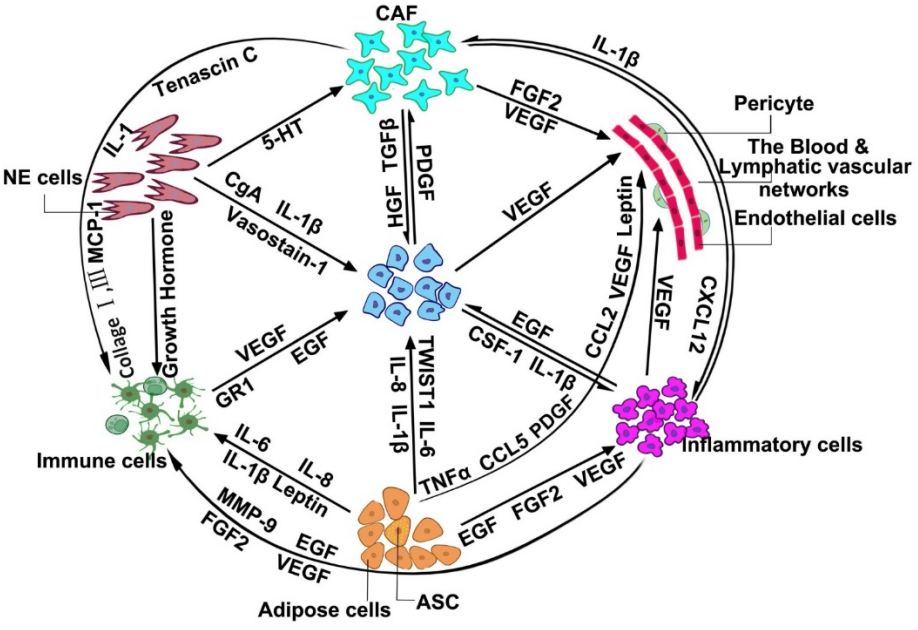


Figure 2. The inactive network of cancer cells and the tumor microenvironment.

Similarly, although there is no unique marker, there are still some targets for significant help in cancer treatment. CAFs work in two main ways in cancer treatment. One method is by directly reversing CAFs into the normal fibroblasts or inhibiting their growth. This method highlighted that efforts such as reconstituting miRNA expression had been proven to deactivate CAFs [68-70] and inhibit PDGF signaling in the mouse model of cervical carcinogenesis; it can also reduce tumor proliferation [71]. Additionally, the fibroblast growth factor receptor (FGFR) signaling pathway may be one of the therapeutic objectives in gastric cancer [72].The other objective is dedifferentiating CAF into a quiescent state. One data set showed that Vitamin D receptor (VDR) ligands promoted the dedifferentiation of satellite cells and abrogated fibrosis [73]. Using a murine xenograft model of colon carcinoma, another recent study found that when targeting fibroblast activation protein (FAP), the accumulation of CAFs was markedly reduced [74]. FAP is expected to become another marker of CAFs targeted therapy.

Immune and inflammatory cell

The main function of the mammalian immune system is to monitor tissue homeostasis, to protect against invading or infectious pathogens and to eradicate damaged cells [75]. The primary theory advises that immune surveillance has significant roles in recognizing and eradicating a large part of nascent tumor cells [1]. However, unlike normal functions, immune-inflammatory cells would persist in sites of chronic inflammation, linked to diverse tissue pathologies, including fibrosis, aberrant angiogenesis, and neoplasia [76]. In light of recent discoveries in immune system research, it is difficult to ignore the crucial issue that immune-inflammatory cells may be the early cradle of cancer [77-83].

Several studies have revealed the contribution of adaptive and innate immunity in cancer immunoediting, including the unmanipulated innate immune system without adaptive immunity [84]. Dunn et al. divided the dynamic process of cancer immunoediting into three steps: elimination,

equilibrium, and escape [85]. These three stages can also be used to express the role of the immune system in cancer initiation, progression, and invasion. He pointed out that in the elimination phase, the immunologic system can defeat nascent tumors. This is accomplished by different inflammatory cells [86-88] and signaling molecules [88, 89]. Once cancer cells have been completely eliminated, these active factors and immune cells may have an additional role in producing "immunologic sculpting" or "immunoediting" [85]. This means that in the equilibrium stage, tumor cells cannot be completely cleared, but the tumor growth can controlled. In order to escape the immune surveillance, cancer cells tend to evolve a number of phenotypic changes in this stage, such as EMT [90]. These cells, with survival advantage, would eventually develop into the primary solid tumor. In other words, the immune microenvironment helps cancer cells to select the dominant cells so that the tumor can progress at the fastest rate in a limited environment. Several studies in mice have revealed that the depletion of macrophages during tumor induction restrained tumor growth [91, 92]. Regarding the escape stage, the immune system may help tumor cells to format the clinical characterization of tumor immune escape mechanisms, while many experiments have proven that immune cells can reduce anti-cancer proteins or cytokines to promote cancer invasion [93, 94]. In addition, one experiment also found that immune cells may be related to the regulation of apoptosis [95]. However, the greatest divergence between these transitional immune cells and the other stroma cells is that these immune cells can be redesigned toward the tumor destruction in therapies. How to activate the normal function of immune cells will be the focus of a future study.

As depicted in Figure 2, CAFs [96], NE cells [97], adipose cells [98-100], and inflammatory cells [101-103] in the TME can affect the role of immune cells through the secretion of different cytokines, cell factors, or interacting proteins, which adds several difficulties to the search for markers and targets for cancer therapy [104, 105]. In different cell and tumor types, the complexity and heterogeneity of immune factors also provides a further complication to finding more specific markers of the immune cells. The immune system is divided into adaptive immunity and innate immunity. Adaptive immune cells include thymus-dependent lymphocytes (T cells), and bursa-dependent lymphocytes (B cells). Innate immune cells consist of dendritic cells (DC), killer lymphocytes, natural killer (NK) cells, hyaline leukocyte/macrophage, granulocytes, and mast cells [106]. According to the different clusters of differentiation, T cells are divided into $CD4^+$ T (helper T cells, Th) and $CD8^+$ T (cytotoxic T cells, Tc) cells. These secrete IFN-γ, TNF-α, and IL17, which have antitumor effects. B cells are mainly marked by different antigens in different physiological periods, such as mainly expressing CD19 and CD20 in pre-B cells, immature B cells, and plasma cells, mainly expressing IgM, IgD, and CR1 in mature B cells, and mainly expressing IgM, IgD, IgA, IgG in memory B cells. A key feature of human NK cells, which could efficiently recognize infected and malignant target cells, is the expression of HLA class I-specific receptors of the KIR and NKG2 gene families [107]. DCs express co-stimulatory molecules and innate inflammatory cytokines, such as IL-12, IL-23, and IL-1, that promote IFN-γ-secreting $CD4^+$ T cells and cytotoxic T lymphocyte responses [108]. DCs represent key targets for 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), which can directly induce T cells [109].

After the addition of immune-inflammatory cells into an emerging hallmark of cancer [1], studies have shown that immune cells were associated with immunosuppression. Some types of immune cells have an innate function of immune suppression, and some cytokines can also activate them through different signaling pathways. The main immunosuppressive cells are regulatory T (Treg) cells marked by $Foxp3^+$ [110], myeloid-derived suppressor cells (MDSC) marked by HMGB1 [111], and M2 macrophages marked by $CD163^+$ [108, 112], which have emerged as a leading method in the development of new immunotherapeutics. Studies have also found that some granulocytes can promote cancer development through the expression of cytokines, such as the hematopoietic growth factor (HGF), granulocyte colony-stimulating factor (G-CSF), or inducing changes in stromal cells [113-115]. $PD\text{-}1^+$ and cytotoxic T-lymphocyte antigen-4 (CTLA-4) expressed by "exhausted" $CD8^+$ T cells are also targeting markers in treating patients with breast and non-small cell lung (NSCL) cancer [116-118]. In addition to the mutual activation [119], the antitumor effects also can be suppressed by some co-inhibitory molecules expressed by antitumor immune cells, such as PD-1/PD-L1 [120, 121]. Some antitumor cytokines can also promote immunosuppression, such as IL-10 and TGF-β secreted by DCs, which may activate Treg cells that are recruited to the tumor under the influence of the chemokines, including CCL22 and CXCL12 [108]. As for these immunosuppressive cells, cell depletion strategies [122-125], tumor vaccines [126], intratumoral injection with an agonistic antibody [127], targeting the transcription factor, and suppression of activated receptors [128, 129] have

been widely used in improving immune surveillance and promoting antitumor immune responses. Agonist antibodies of CD40, 4-1BB, GITR, and OX-40 can also be used to enhance antigen-specific T cell responses [130], CD25 antibody can be used to reduce the number of Treg cells or inhibit Treg function [131], and promote the maturation of DC and rational use of cytokines and antibodies to break the immune tolerance [132]. The significant roles and the master markers of the immune and inflammatory cells are indicated in **Table 1**.

The blood and lymphatic vascular networks

Similar to normal tissues, the blood and lymphatic vascular networks supply oxygen and sustenance as well as removing carbon dioxide and metabolic wastes for sustaining the survival of neoplasm growth. These networks have two main features. First, the new vessels surrounding tumors are usually inefficient, tortuous, or leaky [133]. Second, the angiogenic switch is almost always activated and remains active during the process of the tumorigenesis, resulting in continued growth of new natural blood vessels [134]. In the tumor angiogenesis process, new blood vessels form from pre-existing vessels, making the blood and lymphatic vascular network more complex [135].

The blood and lymphatic vascular networks have different roles during the stages of tumorigenesis [136]. Tumor normal cells are confronted with the challenge of hypoxic surroundings [137]. To survive in hypoxic circumstances, primary tumor cells may adjust to the low oxygen setting, or migrate to and recruit blood vessels [138]. A selection shape can be chosen, which is more enterprising and metastatic, and is provided by a chronically hypoxic environment [139, 140]. In the process of tumor progression, one of the primary functions of the blood and lymphatic vascular networks is to help tumor cells escape immune surveillance. Escape measures are mainly divided into two categories. Directly, the lymphatic microenvironment will weaken or eliminate the normal function of immune cells. For instance, the myeloid-derived suppressor cells (MDSCs) and the immature DCs in the sentinel lymph nodes (SLNs) could restrict the normal operation of T cells [141-143]. When the metastatic tumor enters a novel environment, $CD4^+$and $CD8^+$ T cells may help them to evade the host immune system [144, 145]. The remodeling of unusual endothelial venules (HEVs) can indirectly influence immune cells to traffic into lymph nodes [146]. Though some immune cells traffic into the lymph nodes through the draining afferent lymphatic vessels, lymphocyte recruitment into the SLN via HEVs is fundamental [144].

Moreover, lymphatic vessels around the tumor also provide a physical link between the SLNs and primary tumor. When lymphatic vessels are obstructed, collateral lymphatic vessels can make up for the diminution in lymphatic capacity [144, 147]. This physical connection is like a highway through which tumor cells can reach other locations. Some phenomena showed that local tumor invasion correlated with excellent lymphatic vessel density in the tumor margin [148, 149]. Figure 2 shows that through different interacting factors, adipose cells, inflammatory cells, and CAFs can also closely connect with lymphatic vascular networks [150-153]. The physical and chemical connection makes the role of angiogenesis in tumor formation more of a mystery.

The important functions and the primary markers of the blood and lymphatic vascular networks are listed in Table 1. Banerji et al. found that the lymphatic vessel endothelial HA receptor (LYVE-1) was expressed predominantly in lymphatic vessels [154]. Evidence suggests that VEGF is among the most important factors for anti-angiogenic treatment [155, 156]. A number of studies have discovered that VEGF would inhibit the development of new vessels, block the VEGF or its signaling pathways, prune pre-existing vessels, and induce vessel normalization [133, 157, 158]. Furthermore, clinical trials that targeted VEGF and other markers showed prolonged survival [155, 159]. Members of the VEGF family include placental growth factor (PlGF), VEGF-B, VEGF-C, and VEGF-D, which were also good candidates for anti-angiogenic treatment [133, 160-164]. Another marker, PDGFβ, secreted by sprouting ECs, has two sides of the effects on tumors. It would recruit prostate cancer (PC) signaling through the presenting PDGF receptor-β (PDGFRβ) [165]. Studies have revealed that blockage of PDGFβ makes tumor vessels more sensitive to VEGF inhibitors [166, 167]. Consequently, the decrement of PDGFβ also enhances the risk of increased metastasis [158]. Generally, anti-PDGFβ drugs play an auxiliary role in anti-VEGF treatment. Some other markers, such as CRISP-3 [94], CCR7 [168], GATA2 [169], Prox1 [170], and Foxc2 [171] have also been found and are well used in the treatment. In conclusion, abnormal tumor vasculature exhibited remarkable spatiotemporal heterogeneousness, and not only damaged perfusion and drug delivery, but also made chemoradiotherapy less expeditious.

Adipose cells

Adipose tissue comprises two cell types, white adipose tissue (WAT) and adipocytes [172]. The

significant functions and major markers of adipose cells are shown in Table 1. Extensive evidence has proven that some features of adipose tissue are associated with cancer. First, obese adipose tissue hypoxia establishes a highly proinflammatory microenvironment, which is more likely to breed tumors [173-175]. Second, adipose cells also secrete more than 50 different cytokines, chemokines, and hormone-like factors [176, 177]. These factors, whose production may upregulate in obesity, may be accomplices in tumor initiation. Similar to interleukin (IL)-6, IL-8, IL-1β, tumor necrosis factor-α (TNF-α), VEGF, chemokine (C-C motif) ligand 2 (CCL2) and CCL5 [178, 179]. Third, in obese patients, adipose tissue accommodates a higher proportion of preadipocytes, in which figures of macrophages and monocytes synergistic increase [180]. These alterations may also contribute to cancer development locally. Fourthly, adipose tissue reprogramming and the associated systemic secretion may have an effect on cancer growth and progression [180]. Excess adiposity leads to high circulating blood estrogen [181] and chronic, low-grade inflammation, which is involved in cancer development [176, 182-184]. Cancer progression has been proven to be accompanied by recruiting progenitor mesenchymal stromal cells (MSCs) in reaction to setting factors free by TME with hypoxic and inflammatory conditions [185, 186].

Another type of cancer-associated adipose cell is the adipose stem cells (ASC), which have the capability to differentiate into multiple cell lineages [187-189]. ASC plays a chief role when it comes to the promotion of tumor progress. First, ASCs influence the tumor microenvironment (Figure 2). Eterno et al. [190] suggested that ASCs may worsen the tumorigenic behavior of c-Met-producing breast cancer cells by creating a TME characterized by inflammation. The TGFβ1 signaling pathway may also play a role in the interaction between ASCs and the TME [98]. Second, ASCs may promote angiogenesis [191]. Gehmert et al. [192] reported that ASCs might contribute to angiogenesis by migrating toward tumor-conditioned media through the platelet-derived growth factor BB/platelet-derived growth factor receptor-β (PDGF-BB/PDGFR-β) signaling pathway[193]. Third, ASCs may differentiate into carcinoma-associated cells. In breast-cancer tumor models, many studies have found that ASCs may differentiate into fibroblasts and promote tumor proliferation [194]. The same findings were also reflected in ovarian cancer and lung cancer progression [195-198]. Fourth, ASCs may promote EMT. Studies using pleural effusions or established breast cancer cell lines from breast cancer patients exemplified that either co-culture with ASCs or conditioned medium from could advance invasion and EMT of breast cancer cells [199-201].

Cancers are triggered by adipocytes to gain enterprising tumor phenotypes with some aggressive traits. In turn, cancer cells may express the corresponding markers. Although there is no unique marker for clinical identification, the increase or decrease in the number of a substance in adipose tissue may directly or indirectly reflect the initiation, progression, and invasion of the tumor, such as the EMT-like phenotypic alterations accompanied by the upregulation of matrix metallopeptidase 9 (MMP9) and TWIST1 [202, 203]. Higher aromatase activity and higher degrees of obesity may abate the efficacy of aromatase inhibitors [204]. Leptin, an adipocyte-derived cytokine, would arouse proliferation in some cell types, such as mammary epithelium, which has an effect on the neoplasm cell proliferation, apoptosis, and cell cycle [205-207]. Data has shown that high glucose levels can induce leptin signaling directly [208]. Additionally, adiponectin in given cells may inhibit proliferation promoting apoptosis through the adenosine monophosphate kinase (AMPK) and MAPK pathways [180].

Certain types of cancer have found adipose-related target markers. For instance, methyl-CpG-binding protein 6 (MBD6) is a direct target of octamer-binding transcription factor 4 (Oct4) and controls the stemness and differentiation of adipose tissue-derived mesenchymal stem cells (ASC)[209]. Aromatase is one of the newly discovered adipose-related markers for postmenopausal breast cancer. The majority of postmenopausal breast cancers are estrogen receptor (ER)-positive. Obesity results in the secretion of inflammatory factors, which stimulates the expression of the aromatase enzyme and converts androgens into estrogens in the adipose tissue [210]. Several clinical trials have revealed that many pathways could be targeted to specifically inhibit aromatase within the breast. For example, through targeting aromatase inhibitors (AIs), clinical treatment may inhibit the activity of aromatase enzymes and prevent estrogen production [211]. Although targeted therapy has achieved promising results, it also has certain side effects.

Neuroendocrine cell

In the late 1920s, Siegfried Oberndorfer found that the secretion of nerve cells in the hypothalamus enters the blood [212]. These cells are now called neuroendocrine (NE) cells, and a series of experiments proved that this type of cell exhibits a combination of neuronal and endocrine features [56]. In healthy organisms, normal NE cells play complex local

regulatory roles at the tissue level [213]. NE cells are part of the diffuse NE system, which is spread throughout the normal organism. They can also be found within endocrine glands or tissues, such as the hypothalamus, anterior pituitary gland, pineal gland, thyroid gland (calcitonin-secreting cells), thymus, breast, and the pancreatic islets of langerhans [212, 214, 215]. Under the electron microscope, two different morphologies were found: open-type cells and closed-type cells [212]. There is no difference in the location of these two kinds of cells, but the function is not the same, which increases the difficulty in studying NE cells.

NE cells are the accomplices of tumor formation [216]. Extensive evidence has proven that the NE system strongly influences the function of the immune system (Figure 2). The NE system can regulate the migration and cytotoxicity in NK cells through neurotransmitters [217]. Additionally, substance P showed the ability to block the β1-integrin-mediated adhesion of T lymphocytes [218] and increase their migratory activity [219]. Substance P also can induce the production of various cytokines in leukocytes [220]. Norepinephrine, another neurotransmitter, also showed a significant impact on T cells [219]. It could inhibit the generation of antitumor cytotoxic T-lymphocytes (CTLs) through the inhibition of TNF-α synthesis [221]. On the other hand, as a result of their secretory products, NE cells could stimulate the proliferation of prostate carcinoma cells and increase their aggressiveness [222], while in the development of NE-cell tumors, NE cells may play a leading role [223]. NE carcinomas are rare malignancies that originate from the hormone-producing cells of the body's NE system. While there is no conclusive evidence, several studies have reflected on this conclusion. For example, one result showed that androgen-dependent lymph node carcinomas of the prostate (LNCAP) could only develop in the presence of NE tumors in castrating mice, which proved that NE tumor cells may secrete specific factors [224]. NE tumors had a highly internal heterogeneity, and there were 13 different types of NE cells [225]. Different NEs produce different secretions, which leads to different or mixed symptoms. NE tumors are also highly aggressive. Approximately 50% of pancreatic NE tumors have hepatic metastases [226] due to the secretion of several peptide hormones. To improve the survival rate of NE tumors, it is becoming more and more urgent to find specific markers.

The significant functions and the major markers of NE are shown in Table 1. Many markers have gradually been found to be expressed in a large number of prostatic NE cancer cases[227], such as K18 and K8 cytokeratins [228], α-methylacyl-CoA-racemase (AMACR) [228], plasma anterior gradient 2 (AGR2) [229], and PGP9.5 [230]. Additionally, neuron-specific enolase (NSE) and chromogranin A (CgA) may be the most frequently expressed neuropeptides [231-233], but they also have limitations in sensitivity, specificity, reproducibility, etc. [234]. IL-2 was more specific than any marker other than Ki-67 in detecting gastroenteropancreatic NE tumors [235], even though some other targeting markers were promising for use in clinical treatment. Novel TDP-A-loaded and KE108-conjugated unimolecular micelles exhibited the best potential in suppressing NE cancer cell growth both in vitro and in vivo [236]. There also have also been some achievements in the fields of prostate, lung, pancreas, and gastrointestinal tracts. Delta-like canonical notch ligand 3 (DLL3) proteins are expressed on the surface of pulmonary NE tumor cells but not in normal adult tissues. One multiple model in vivo showed that a DLL3-targeted antibody-drug induced durable tumor regression [237]. In addition, more than 85% of lung cancer patients are diagnosed with NSCLC, while the other 15% are classified as small cell lung cancer (SCLC) [238, 239]. All the SCLC patients show histological features of NE morphology. Synaptophysin (Syn), and neural cell adhesion molecule 1 (NCAM1) are known as NE diagnostic markers. One recently study result showed that the positive expression rates of these three markers were 112 (58.3%), 160 (83.3%), and 166 (86.5%), respectively, in a total of 192 SCLC patients [240]. In addition to targeted therapy, peptide receptor radionuclide therapy is a promising new treatment modality for inoperable or metastasized gastroenteropancreatic NE tumors patients [241]. Some studies have found that EGF receptor inhibition may disrupt some signaling cascades, which may inhibit the growth of foregut NE tumors/pancreatic NE tumors [242]. Another result also proved that PRCRT is an effective treatment in patients with FDG-avid NE tumors, even in patients for whom conventional therapies have failed[243]. Recent genomic profiling studies, which have demonstrated that prostate cancers with an NE phenotype are enriched for loss of RB, loss or mutation of TP53, loss of AR, and AR target gene expression, and overexpression of MYCN and AURKA, may also provide a new treatment method [244].

ECM within the microenvironment

ECM, a dynamic and complicated environment, is characterized using biophysical, mechanical and biochemical properties specific for each tissue. ECM contains all the cytokines, growth factors, and

hormones secreted by stromal and tumor cells. Many assays have respectively demonstrated ECM components, including collagens [245-247], laminins [248], fibronectins [249], proteoglycans [250], and hyaluronans [251, 252] in a specific organization [225]. In vitro, one three-dimensional (3D) model indicated that ECM heterogeneity is crucial for controlling collective cell invasive behaviors and determining metastasis efficiency [253-262]. Because of the complexity of ECM heterogeneity in vivo, its influence on collective cell behavior has been described but not quantified [253].

Depending on the different organization and locations, ECM has complementary effects on the development and metastasis of tumors in diverse ways. First, ECM may affect tumors through extracellular secretion. During embryogenesis, fibroblastic mesenchyme determines the sexual phenotype of the gland, while the adipocyte mesenchyme controls mammary-specific ductal morphogenesis [263-266]. One study illustrated that innate ECM scaffolds, derived from decellularized tissues, lead the cells derived from stem cell differentiation that reside in the tissue from which the ECM was derived [267]. Second, ECM may alter the phenotype type of stromal cells or tumor cells. Oncogenic mutations are broadly thought to increase cellular fitness and result in the clonal expansion of receivers. One experiment revealed that the ability to clean apoptotic colon cancer cells can be promoted by tumor-associated macrophages (TAMs) through the expression of sulfoglycolipids (SM4s). During this procedure, phenotypic change of TAM was accompanied by expression of TGF-β1 and secretion of IL-6, which may have an advantage in further activating the angiogenic process [268]. Third, ECM can help neoplasms to get away from immune surveillance. For instance, colon cancer cells always display an increased production of Fas ligand binding to its receptor on immune cells [269]. Fourth, the ECM tumor will provide a hypoxic or acidic environment in which the tumor cells have greater survival advantages than normal cells. ECM will select survival cells to aid in tumor growth and invasion at the fastest rate.

Conclusion

Tumor management strategies include surgery for a cure or for cytoreduction, radiological intervention, chemotherapy, and somatostatin analogs to control symptoms. However, tumor cells are extremely elastic and may adapt to treatments and environmental modifications speedily [270-277]. Once one component has been obstructed, other mechanisms will quickly follow. This may be one of the main factors that lead to poor prognosis. The major interaction between different stromal cells and active factors in the advanced TME is shown in Figure 2. Different impeding mechanisms at the same time might lead to the best results of tumor development. In other words, it is important to understand the role of different components of the TME in the treatment and prevention of tumors. Moreover, the study of predictive biomarkers, which may fully address the complexities of the biology, will promote the development of therapies tailored to individual patients. At present, the emerging targeted microenvironment therapy has been widely accepted. According to the characteristics of different tissues, the synergistic therapies targeting multiple microenvironment stromal cells and the continuous discovery of multiple target markers may be the direction of future research.

Acknowledgement

This work was supported in part by grants from the National Natural Science Foundation of China (81372907, 81301757, 81472531, 81402009, 81572787, 81672993 and 81672683), the Natural Science Foundation of Hunan Province (14JJ1010, 2015JJ1022 and 2016JC2035), the Hunan Provincial Development and Reform Commission (422030001), the Open-End Fund for the Valuable and Precision Instruments and the Faculty Research Fund of Central South University (CSUZC201739), the Mittal Innovation Foundation of Central South University (15MX46, MX2016442), and the Fundamental Research Funds for Central Universities of Central South University (2014zzts066, 2016zzts478)

Competing Interests

The authors have declared that no competing interest exists.

References

1 Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011; 144: 646-74.
2 Xiong W, Zeng ZY, Xia JH, et al. A susceptibility locus at chromosome 3p21 linked to familial nasopharyngeal carcinoma. Cancer Res. 2004; 64: 1972-4.
3 Zeng Z, Zhou Y, Zhang W, et al. Family-based association analysis validates chromosome 3p21 as a putative nasopharyngeal carcinoma susceptibility locus. Genet Med. 2006; 8: 156-60.
4 Zeng Z, Zhou Y, Xiong W, et al. Analysis of gene expression identifies candidate molecular markers in nasopharyngeal carcinoma using microdissection and cDNA microarray. J Cancer Res Clin Oncol. 2007; 133: 71-81.
5 Zeng ZY, Zhou YH, Zhang WL, et al. Gene expression profiling of nasopharyngeal carcinoma reveals the abnormally regulated Wnt signaling pathway. Hum Pathol. 2007; 38: 120-33.
6 Zhou Y, Zeng Z, Zhang W, et al. Identification of candidate molecular markers of nasopharyngeal carcinoma by microarray analysis of subtracted cDNA libraries constructed by suppression subtractive hybridization. Eur J Cancer Prev. 2008; 17: 561-71.
7 Zhou Y, Zeng Z, Zhang W, et al. Lactotransferrin: a candidate tumor suppressor-Deficient expression in human nasopharyngeal carcinoma and inhibition of NPC cell proliferation by modulating the mitogen-activated protein kinase pathway. Int J Cancer. 2008; 123: 2065-72.

8 Zeng Z, Huang H, Zhang W, et al. Nasopharyngeal carcinoma: advances in genomics and molecular genetics. Sci China Life Sci. 2011; 54: 966-75.
9 Gong Z, Zhang S, Zhang W, et al. Long non-coding RNAs in cancer. Sci China Life Sci. 2012; 55: 1120-4.
10 Zhang W, Zeng Z, Fan S, et al. Evaluation of the prognostic value of TGF-beta superfamily type I receptor and TGF-beta type II receptor expression in nasopharyngeal carcinoma using high-throughput tissue microarrays. J Mol Histol. 2012; 43: 297-306.
11 Liao Q, Guo X, Li X, et al. Prohibitin is an important biomarker for nasopharyngeal carcinoma progression and prognosis. Eur J Cancer Prev. 2013; 22: 68-76.
12 Zhang W, Huang C, Gong Z, et al. Expression of LINC00312, a long intergenic non-coding RNA, is negatively correlated with tumor size but positively correlated with lymph node metastasis in nasopharyngeal carcinoma. J Mol Histol. 2013; 44: 545-54.
13 Zeng Z, Huang H, Huang L, et al. Regulation network and expression profiles of Epstein-Barr virus-encoded microRNAs and their potential target host genes in nasopharyngeal carcinomas. Sci China Life Sci. 2014; 57: 315-26.
14 Bo H, Gong Z, Zhang W, et al. Upregulated long non-coding RNA AFAP1-AS1 expression is associated with progression and poor prognosis of nasopharyngeal carcinoma. Oncotarget. 2015; 6: 20404-18.
15 Zhang W, Fan S, Zou G, et al. Lactotransferrin could be a novel independent molecular prognosticator of nasopharyngeal carcinoma. Tumour Biol. 2015; 36: 675-83.
16 Li H, Li X, Ge X, et al. MiR-34b-3 and miR-449a inhibit malignant progression of nasopharyngeal carcinoma by targeting lactate dehydrogenase A. Oncotarget. 2016; 7: 54838-51.
17 Li Q, Chen P, Zeng Z, et al. Yeast two-hybrid screening identified WDR77 as a novel interacting partner of TSC22D2. Tumour Biol. 2016; 37: 12503-12.
18 Liang F, Li Q, Li X, et al. TSC22D2 interacts with PKM2 and inhibits cell growth in colorectal cancer. Int J Oncol. 2016; 49: 1046-56.
19 Wang H, Zhao R, Guo C, et al. Knockout of BRD7 results in impaired spermatogenesis and male infertility. Sci Rep. 2016; 6: 21776.
20 Wang Y, Xue D, Li Y, et al. The Long Noncoding RNA MALAT-1 is A Novel Biomarker in Various Cancers: A Meta-analysis Based on the GEO Database and Literature. J Cancer. 2016; 7: 991-1001.
21 Xu K, Xiong W, Zhou M, et al. Integrating ChIP-sequencing and digital gene expression profiling to identify BRD7 downstream genes and construct their regulating network. Mol Cell Biochem. 2016; 411: 57-71.
22 Yu J, Liu Y, Gong Z, et al. Overexpression long non-coding RNA LINC00673 is associated with poor prognosis and promotes invasion and metastasis in tongue squamous cell carcinoma. Oncotarget. 2017; DOI: 10.18632/oncotarget.14200.
23 Zeng Z, Fan S, Zhang X, et al. Epstein-Barr virus-encoded small RNA 1 (EBER-1) could predict good prognosis in nasopharyngeal carcinoma. Clin Transl Oncol. 2016; 18: 206-11.
24 Zhou Y, Liao Q, Li X, et al. HYOU1, Regulated by LPLUNC1, Is Up-Regulated in Nasopharyngeal Carcinoma and Associated with Poor Prognosis. J Cancer. 2016; 7: 367-76.
25 Liu Y, Zhao R, Wang H, et al. miR-141 is involved in BRD7-mediated cell proliferation and tumor formation through suppression of the PTEN/AKT pathway in nasopharyngeal carcinoma. Cell Death Dis. 2016; 7: e2156.
26 Wang W, Yi M, Chen S, et al. Significance of the NOR1-FOXA1/HDAC2-Slug regulatory network in epithelial-mesenchymal transition of tumor cells. Oncotarget. 2016; 7: 16745-59.
27 Wang W, Yi M, Chen S, et al. NOR1 Suppresses Cancer Stem-Like Cells Properties of Tumor Cells via the Inhibition of the AKT-GSK-3beta-Wnt/beta-Catenin-ALDH1A1 Signal Circuit. J Cell Physiol. 2017; DOI: 10.1002/jcp.25706.
28 Yang L, Tang Y, He Y, et al. High Expression of LINC01420 indicates an unfavorable prognosis and modulates cell migration and invasion in nasopharyngeal carcinoma. J Cancer. 2017; 8: 97-103.
29 Tang Y, He Y, Yang L, et al. Co-expression of AFAP1-AS1 and PD-1 predicts poor prognosis in nasopharyngeal carcinoma. Oncotarget. 2017;
30 Wang Y, Mo Y, Gong Z, et al. Circular RNAs in Human Cancer. Molecular Cancer. 2017; 16: 25.
31 Pienta KJ, McGregor N, Axelrod R, et al. Ecological Therapy for Cancer: Defining Tumors Using an Ecosystem Paradigm Suggests New Opportunities for Novel Cancer Treatments. Transl Oncol. 2008; 1: 158-64.
32 Chen F, Zhuang X, Lin L, et al. New horizons in tumor microenvironment biology: challenges and opportunities. BMC Med. 2015; 13: 45.
33 Mroue R, Bissell MJ. Three-Dimensional Cultures of Mouse Mammary Epithelial Cells. In: Randell HS, Fulcher LM, eds. Epithelial Cell Culture Protocols: Second Edition. Humana Press: Totowa, NJ. 2013:221-50.
34 Karvonen HM, Lehtonen ST, Sormunen RT, et al. Lung cancer-associated myofibroblasts reveal distinctive ultrastructure and function. J Thorac Oncol. 2014; 9: 664-74.
35 Karki S, Surolia R, Hock TD, et al. Wilms' tumor 1 (Wt1) regulates pleural mesothelial cell plasticity and transition into myofibroblasts in idiopathic pulmonary fibrosis. FASEB J. 2014; 28: 1122-31.
36 Shu H, Li HF. Prognostic effect of stromal myofibroblasts in lung adenocarcinoma. Neoplasma. 2012; 59: 658-61.
37 Grotendorst GR, Rahmanie H, Duncan MR. Combinatorial signaling pathways determine fibroblast proliferation and myofibroblast differentiation. FASEB J. 2004; 18: 469-79.
38 Orimo A, Gupta PB, Sgroi DC, et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell. 2005; 121: 335-48.
39 Olumi AF, Grossfeld GD, Hayward SW, et al. Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. Cancer Res. 1999; 59: 5002-11.
40 Busch S, Acar A, Magnusson Y, et al. TGF-beta receptor type-2 expression in cancer-associated fibroblasts regulates breast cancer cell growth and survival and is a prognostic marker in pre-menopausal breast cancer. Oncogene. 2015; 34: 27-38.
41 Kalluri R, Zeisberg M. Fibroblasts in cancer. Nat Rev Cancer. 2006; 6: 392-401.
42 Tanaka K, Miyata H, Sugimura K, et al. miR-27 is associated with chemoresistance in esophageal cancer through transformation of normal fibroblasts to cancer-associated fibroblasts. Carcinogenesis. 2015; 36: 894-903.
43 Min A, Zhu C, Peng S, et al. Downregulation of Microrna-148a in Cancer-Associated Fibroblasts from Oral Cancer Promotes Cancer Cell Migration and Invasion by Targeting Wnt10b. J Biochem Mol Toxicol. 2016; 30: 186-91.
44 Wiseman BS, Werb Z. Stromal effects on mammary gland development and breast cancer. Science. 2002; 296: 1046-9.
45 Xue X, Lu Z, Tang D, et al. Galectin-1 secreted by activated stellate cells in pancreatic ductal adenocarcinoma stroma promotes proliferation and invasion of pancreatic cancer cells: an in vitro study on the microenvironment of pancreatic ductal adenocarcinoma. Pancreas. 2011; 40: 832-9.
46 Saussez S, Decaestecker C, Cludts S, et al. Adhesion/growth-regulatory tissue lectin galectin-1 in relation to angiogenesis/lymphocyte infiltration and prognostic relevance of stromal up-regulation in laryngeal carcinomas. Anticancer Res. 2009; 29: 59-65.
47 van den Brule FA, Waltregny D, Castronovo V. Increased expression of galectin-1 in carcinoma-associated stroma predicts poor outcome in prostate carcinoma patients. J Pathol. 2001; 193: 80-7.
48 Jung EJ, Moon HG, Cho BI, et al. Galectin-1 expression in cancer-associated stromal cells correlates tumor invasiveness and tumor progression in breast cancer. Int J Cancer. 2007; 120: 2331-8.
49 He XJ, Tao HQ, Hu ZM, et al. Expression of galectin-1 in carcinoma-associated fibroblasts promotes gastric cancer cell invasion through upregulation of integrin beta1. Cancer Sci. 2014; 105: 1402-10.
50 Eberlein C, Rooney C, Ross SJ, et al. E-Cadherin and EpCAM expression by NSCLC tumour cells associate with normal fibroblast activation through a pathway initiated by integrin alphavbeta6 and maintained through TGFbeta signalling. Oncogene. 2015; 34: 704-16.
51 Jung Y, Kim JK, Shiozawa Y, et al. Recruitment of mesenchymal stem cells into prostate tumours promotes metastasis. Nat Commun. 2013; 4: 1795.
52 Fukui H, Zhang X, Sun C, et al. IL-22 produced by cancer-associated fibroblasts promotes gastric cancer cell invasion via STAT3 and ERK signaling. Br J Cancer. 2014; 111: 763-71.
53 Dowling CM, Herranz Ors C, Kiely PA. Using real-time impedance-based assays to monitor the effects of fibroblast-derived media on the adhesion, proliferation, migration and invasion of colon cancer cells. Biosci Rep. 2014; 34
54 Desmouliere A, Guyot C, Gabbiani G. The stroma reaction myofibroblast: a key player in the control of tumor cell behavior. Int J Dev Biol. 2004; 48: 509-17.
55 Huang J, Li Z, Ding Z, et al. Different roles of myofibroblasts in the tumorigenesis of nonsmall cell lung cancer. Tumour Biol. 2015: 1-10.
56 Gout S, Huot J. Role of cancer microenvironment in metastasis: focus on colon cancer. Cancer Microenviron. 2008; 1: 69-83.
57 Hlatky L, Tsionou C, Hahnfeldt P, et al. Mammary Fibroblasts May Influence Breast Tumor Angiogenesis via Hypoxia-induced Vascular Endothelial Growth Factor Up-Regulation and Protein Expression. Cancer Res. 1994; 54: 6083-6.
58 Zucali JR, Dinarello CA, Oblon DJ, et al. Interleukin 1 stimulates fibroblasts to produce granulocyte-macrophage colony-stimulating activity and prostaglandin E2. J Clin Invest. 1986; 77: 1857-63.
59 Huleihel M, Douvdevani A, Segal S, et al. Regulation of interleukin 1 generation in immune-activated fibroblasts. Eur J Immunol. 1990; 20: 731-8.
60 Yu X, Graves DT. Fibroblasts, mononuclear phagocytes, and endothelial cells express monocyte chemoattractant protein-1 (MCP-1) in inflamed human gingiva. J Periodontol. 1995; 66: 80-8.
61 Ghatnekar A, Trojanowska M. GATA-6 is a novel transcriptional repressor of the human Tenascin-C gene expression in fibroblasts. Biochim Biophys Acta. 2008; 1779: 145-51.
62 Feig C, Jones JO, Kraman M, et al. Targeting CXCL12 from FAP-expressing carcinoma-associated fibroblasts synergizes with anti-PD-L1 immunotherapy in pancreatic cancer. Proc Natl Acad Sci U S A. 2013; 110: 20212-7.
63 Ohlund D, Elyada E, Tuveson D. Fibroblast heterogeneity in the cancer wound. J Exp Med. 2014; 211: 1503-23.
64 Berdiel-Acer M, Sanz-Pamplona R, Calon A, et al. Differences between CAFs and their paired NCF from adjacent colonic mucosa reveal functional heterogeneity of CAFs, providing prognostic information. Mol Oncol. 2014; 8: 1290-305.
65 Wendling O, Bornert JM, Chambon P, et al. Efficient temporally-controlled targeted mutagenesis in smooth muscle cells of the adult mouse. Genesis. 2009; 47: 14-8.
66 Roberts EW, Deonarine A, Jones JO, et al. Depletion of stromal cells expressing fibroblast activation protein-alpha from skeletal muscle and bone marrow results in cachexia and anemia. J Exp Med. 2013; 210: 1137-51.

67 Okada H, Danoff TM, Kalluri R, et al. Early role of Fsp1 in epithelial-mesenchymal transformation. Am J Physiol. 1997; 273: F563-74.
68 Musumeci M, Coppola V, Addario A, et al. Control of tumor and microenvironment cross-talk by miR-15a and miR-16 in prostate cancer. Oncogene. 2011; 30: 4231-42.
69 Bronisz A, Godlewski J, Wallace JA, et al. Reprogramming of the tumour microenvironment by stromal PTEN-regulated miR-320. Nat Cell Biol. 2012; 14: 159-67.
70 Aprelikova O, Palla J, Hibler B, et al. Silencing of miR-148a in cancer-associated fibroblasts results in WNT10B-mediated stimulation of tumor cell motility. Oncogene. 2013; 32: 3246-53.
71 Haubeiss S, Schmid JO, Murdter TE, et al. Dasatinib reverses cancer-associated fibroblasts (CAFs) from primary lung carcinomas to a phenotype comparable to that of normal fibroblasts. Mol Cancer. 2010; 9: 168.
72 Yashiro M, Matsuoka T. Fibroblast growth factor receptor signaling as therapeutic targets in gastric cancer. World J Gastroenterol. 2016; 22: 2415-23.
73 Ding N, Yu RT, Subramaniam N, et al. A vitamin D receptor/SMAD genomic circuit gates hepatic fibrotic response. Cell. 2013; 153: 601-13.
74 Li M, Li M, Yin T, et al. Targeting of cancerassociated fibroblasts enhances the efficacy of cancer chemotherapy by regulating the tumor microenvironment. Mol Med Rep. 2016; 13: 2476-84.
75 Davis CD, Milner JA. Molecular targets for nutritional preemption of cancer. Curr Cancer Drug Targets. 2007; 7: 410-5.
76 Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell. 2010; 140: 883-99.
77 Duan Z, Zheng H, Xu S, et al. Activation of the Ig Ialpha1 promoter by the transcription factor Ets-1 triggers Ig Ialpha1-Calpha1 germline transcription in epithelial cancer cells. Cell Mol Immunol. 2014; 11: 197-205.
78 Hu D, Duan Z, Li M, et al. Heterogeneity of aberrant immunoglobulin expression in cancer cells. Cell Mol Immunol. 2011; 8: 479-85.
79 Hu D, Zheng H, Liu H, et al. Immunoglobulin expression and its biological significance in cancer cells. Cell Mol Immunol. 2008; 5: 319-24.
80 Li M, Zheng H, Duan Z, et al. Promotion of cell proliferation and inhibition of ADCC by cancerous immunoglobulin expressed in cancer cell lines. Cell Mol Immunol. 2012; 9: 54-61.
81 Yang Y, Zhou H, Yang Y, et al. Lipopolysaccharide (LPS) regulates TLR4 signal transduction in nasopharynx epithelial cell line 5-8F via NFkappaB and MAPKs signaling pathways. Mol Immunol. 2007; 44: 984-92.
82 Zheng H, Li M, Ren W, et al. Expression and secretion of immunoglobulin alpha heavy chain with diverse VDJ recombinations by human epithelial cancer cells. Mol Immunol. 2007; 44: 2221-7.
83 Zhao R, Liu Y, Wang H, et al. BRD7 plays an anti-inflammatory role during early acute inflammation by inhibiting activation of the NF-small ka, CyrillicB signaling pathway. Cell Mol Immunol. 2017; DOI:10.1038/cmi.2016.31.
84 O'Sullivan T, Saddawi-Konefka R, Vermi W, et al. Cancer immunoediting by the innate immune system in the absence of adaptive immunity. J Exp Med. 2012; 209: 1869-82.
85 Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. Annu Rev Immunol. 2004; 22: 329-60.
86 Egeblad M, Nakasone ES, Werb Z. Tumors as organs: complex tissues that interface with the entire organism. Dev Cell. 2010; 18: 884-901.
87 Mantovani A. Molecular pathways linking inflammation and cancer. Curr Mol Med. 2010; 10: 369-73.
88 Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. Cell. 2010; 141: 39-51.
89 Murdoch C, Muthana M, Coffelt SB, et al. The role of myeloid cells in the promotion of tumour angiogenesis. Nat Rev Cancer. 2008; 8: 618-31.
90 Ricciardi M, Zanotto M, Malpeli G, et al. Epithelial-to-mesenchymal transition (EMT) induced by inflammatory priming elicits mesenchymal stromal cell-like immune-modulatory properties in cancer cells. Br J Cancer. 2015; 112: 1067-75.
91 Bronkhorst IH, Ly LV, Jordanova ES, et al. Detection of M2-macrophages in uveal melanoma and relation with survival. Invest Ophthalmol Vis Sci. 2011; 52: 643-50.
92 Ly LV, Baghat A, Versluis M, et al. In aged mice, outgrowth of intraocular melanoma depends on proangiogenic M2-type macrophages. J Immunol. 2010; 185: 3481-8.
93 Tao M, Liu L, Shen M, et al. Inflammatory stimuli promote growth and invasion of pancreatic cancer cells through NF-kappaB pathway dependent repression of PP2Ac. Cell Cycle. 2016; 15: 381-93.
94 Pathak BR, Breed AA, Apte S, et al. Cysteine-rich secretory protein 3 plays a role in prostate cancer cell invasion and affects expression of PSA and ANXA1. Mol Cell Biochem. 2016; 411: 11-21.
95 Jiang MH, Lim JE, Chi GF, et al. Substance P reduces apoptotic cell death possibly by modulating the immune response at the early stage after spinal cord injury. Neuroreport. 2013; 24: 846-51.
96 Konig A, Vilsmaier T, Rack B, et al. Determination of Interleukin-4, -5, -6, -8 and -13 in Serum of Patients with Breast Cancer Before Treatment and its Correlation to Circulating Tumor Cells. Anticancer Res. 2016; 36: 3123-30.
97 Savino W. Neuroendocrine control of T cell development in mammals: role of growth hormone in modulating thymocyte migration. Exp Physiol. 2007; 92: 813-7.
98 Razmkhah M, Jaberipour M, Erfani N, et al. Adipose derived stem cells (ASCs) isolated from breast cancer tissue express IL-4, IL-10 and TGF-beta1 and upregulate expression of regulatory molecules on T cells: do they protect breast cancer cells from the immune response? Cell Immunol. 2011; 266: 116-22.
99 Caspar-Bauguil S, Cousin B, Andre M, et al. Weight-dependent changes of immune system in adipose tissue: importance of leptin. Exp Cell Res. 2006; 312: 2195-202.
100 Winer S, Winer DA. The adaptive immune system as a fundamental regulator of adipose tissue inflammation and insulin resistance. Immunol Cell Biol. 2012; 90: 755-62.
101 Wei N, Chen Z, Xue Z, et al. Polymorphism of VEGF gene in susceptibility to chronic immune-mediated inflammatory diseases: a meta-analysis. Rheumatol Int. 2015; 35: 1351-60.
102 Middleton E, Jr., Kandaswami C. Effects of flavonoids on immune and inflammatory cell functions. Biochem Pharmacol. 1992; 43: 1167-79.
103 Lolmede K, Campana L, Vezzoli M, et al. Inflammatory and alternatively activated human macrophages attract vessel-associated stem cells, relying on separate HMGB1- and MMP-9-dependent pathways. J Leukoc Biol. 2009; 85: 779-87.
104 Zhang J, Silva T, Yarovinsky T, et al. VEGF blockade inhibits lymphocyte recruitment and ameliorates immune-mediated vascular remodeling. Circ Res. 2010; 107: 408-17.
105 Abbott RJ, Knott V, Roversi P, et al. Crystallization and preliminary X-ray diffraction analysis of three EGF domains of EMR2, a 7TM immune-system molecule. Acta Crystallogr D Biol Crystallogr. 2004; 60: 936-8.
106 Angell H, Galon J. From the immune contexture to the Immunoscore: the role of prognostic and predictive immune markers in cancer. Curr Opin Immunol. 2013; 25: 261-7.
107 Manser AR, Uhrberg M. Age-related changes in natural killer cell repertoires: impact on NK cell function and immune surveillance. Cancer Immunol Immunother. 2016; 65: 417-26.
108 Butt AQ, Mills KH. Immunosuppressive networks and checkpoints controlling antitumor immunity and their blockade in the development of cancer immunotherapeutics and vaccines. Oncogene. 2014; 33: 4623-31.
109 Baeke F, Korf H, Overbergh L, et al. Human T lymphocytes are direct targets of 1,25-dihydroxyvitamin D3 in the immune system. J Steroid Biochem Mol Biol. 2010; 121: 221-7.
110 Baruch K, Rosenzweig N, Kertser A, et al. Breaking immune tolerance by targeting Foxp3(+) regulatory T cells mitigates Alzheimer's disease pathology. Nat Commun. 2015; 6: 7967.
111 Parker KH, Sinha P, Horn LA, et al. HMGB1 enhances immune suppression by facilitating the differentiation and suppressive activity of myeloid-derived suppressor cells. Cancer Res. 2014; 74: 5723-33.
112 Tiainen S, Tumelius R, Rilla K, et al. High numbers of macrophages, especially M2-like (CD163-positive), correlate with hyaluronan accumulation and poor outcome in breast cancer. Histopathology. 2015; 66: 873-83.
113 Dobrenis K, Gauthier LR, Barroca V, et al. Granulocyte colony-stimulating factor off-target effect on nerve outgrowth promotes prostate cancer development. Int J Cancer. 2015; 136: 982-8.
114 Hu P, Wang G, Shen M, et al. Intratumoral polymorphonuclear granulocyte is associated with poor prognosis in squamous esophageal cancer by promoting epithelial-mesenchymal transition. Future Oncol. 2015; 11: 771-83.
115 Yoshimura T, Imamichi T, Weiss JM, et al. Induction of Monocyte Chemoattractant Proteins in Macrophages via the Production of Granulocyte/Macrophage Colony-Stimulating Factor by Breast Cancer Cells. Front Immunol. 2016; 7: 2.
116 Rizvi NA, Mazieres J, Planchard D, et al. Activity and safety of nivolumab, an anti-PD-1 immune checkpoint inhibitor, for patients with advanced, refractory squamous non-small-cell lung cancer (CheckMate 063): a phase 2, single-arm trial. Lancet Oncol. 2015; 16: 257-65.
117 Sun S, Fei X, Mao Y, et al. PD-1(+) immune cell infiltration inversely correlates with survival of operable breast cancer patients. Cancer Immunol Immunother. 2014; 63: 395-406.
118 Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer. 2012; 12: 252-64.
119 Hoechst B, Gamrekelashvili J, Manns MP, et al. Plasticity of human Th17 cells and iTregs is orchestrated by different subsets of myeloid cells. Blood. 2011; 117: 6532.
120 Park JH, Han JH, Kang HY, et al. Expression of follicular helper T-cell markers in primary cutaneous T-cell lymphoma. Am J Dermatopathol. 2014; 36: 465-70.
121 Reiss KA, Forde PM, Brahmer JR. Harnessing the power of the immune system via blockade of PD-1 and PD-L1: a promising new anticancer strategy. Immunotherapy. 2014; 6: 459-75.
122 Byrne WL, Mills KH, Lederer JA, et al. Targeting regulatory T cells in cancer. Cancer Res. 2011; 71: 6915-20.
123 Sinha P, Clements VK, Bunt SK, et al. Cross-Talk between Myeloid-Derived Suppressor Cells and Macrophages Subverts Tumor Immunity toward a Type 2 Response. J Immunol. 2007; 179: 977-83.
124 Puig-Kröger A, Sierra-Filardi E, Domínguez-Soto A, et al. Folate Receptor β Is Expressed by Tumor-Associated Macrophages and Constitutes a Marker for M2 Anti-inflammatory/Regulatory Macrophages. Cancer Res. 2009; 69: 9395.
125 Rasku MA, Clem AL, Telang S, et al. Transient T cell depletion causes regression of melanoma metastases. J Transl Med. 2008; 6: 12.
126 Kim Y, Pardoll D, Fu J, et al. Abstract 2495: STING agonists formulated into cancer vaccines (STINGVAX) can cure established tumor resistant to immune checkpoint blockade by activating NK cells. Cancer Res. 2015; 75: 2495.

127 Coe D, Begom S, Addey C, et al. Depletion of regulatory T cells by anti-GITR mAb as a novel mechanism for cancer immunotherapy. Cancer Immunol Immunother. 2010; 59: 1367-77.
128 Pyonteck SM, Gadea BB, Wang HW, et al. Deficiency of the macrophage growth factor CSF-1 disrupts pancreatic neuroendocrine tumor development. Oncogene. 2012; 31: 1459-67.
129 Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med. 2004; 10: 942-9.
130 Boesteanu AC, Katsikis PD. Memory T cells need CD28 costimulation to remember. Semin Immunol. 2009; 21: 69-77.
131 Parietti V, Monneaux F, Decossas M, et al. Function of CD4+,CD25+ Treg cells in MRL/lpr mice is compromised by intrinsic defects in antigen-presenting cells and effector T cells. Arthritis Rheum. 2008; 58: 1751-61.
132 Miwa S, Nishida H, Tanzawa Y, et al. TNF-alpha and tumor lysate promote the maturation of dendritic cells for immunotherapy for advanced malignant bone and soft tissue tumors. PLoS One. 2012; 7: e52926.
133 De Bock K, Cauwenberghs S, Carmeliet P. Vessel abnormalization: another hallmark of cancer? Molecular mechanisms and therapeutic implications. Curr Opin Genet Dev. 2011; 21: 73-9.
134 Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell. 1996; 86: 353-64.
135 Birbrair A, Zhang T, Wang ZM, et al. Type-2 pericytes participate in normal and tumoral angiogenesis. Am J Physiol Cell Physiol. 2014; 307: C25-38.
136 Hos D, Schlereth SL, Bock F, et al. Antilymphangiogenic therapy to promote transplant survival and to reduce cancer metastasis: what can we learn from the eye? Semin Cell Dev Biol. 2015; 38: 117-30.
137 Barankay T, Baumgartl H, Lubbers DW, et al. Oxygen pressure in small lymphatics. Pflugers Arch. 1976; 366: 53-9.
138 Chovatiya R, Medzhitov R. Stress, inflammation, and defense of homeostasis. Mol Cell. 2014; 54: 281-8.
139 Guillaumond F, Leca J, Olivares O, et al. Strengthened glycolysis under hypoxia supports tumor symbiosis and hexosamine biosynthesis in pancreatic adenocarcinoma. Proc Natl Acad Sci U S A. 2013; 110: 3919-24.
140 Koshikawa N, Iyozumi A, Gassmann M, et al. Constitutive upregulation of hypoxia-inducible factor-1[alpha] mRNA occurring in highly metastatic lung carcinoma cells leads to vascular endothelial growth factor overexpression upon hypoxic exposure. Oncogene. 2003; 22: 6717-24.
141 Ito M, Minamiya Y, Kawai H, et al. Tumor-derived TGFbeta-1 induces dendritic cell apoptosis in the sentinel lymph node. J Immunol. 2006; 176: 5637-43.
142 Huang RR, Wen DR, Guo J, et al. Selective Modulation of Paracortical Dendritic Cells and T-Lymphocytes in Breast Cancer Sentinel Lymph Nodes. Breast J. 2000; 6: 225-32.
143 Watanabe S, Deguchi K, Zheng R, et al. Tumor-induced CD11b+Gr-1+ myeloid cells suppress T cell sensitization in tumor-draining lymph nodes. J Immunol. 2008; 181: 3291-300.
144 Pereira ER, Jones D, Jung K, et al. The lymph node microenvironment and its role in the progression of metastatic cancer. Semin Cell Dev Biol. 2015; 38: 98-105.
145 Topalian SL, Drake CG, Pardoll DM. Targeting the PD-1/B7-H1(PD-L1) pathway to activate anti-tumor immunity. Curr Opin Immunol. 2012; 24: 207-12.
146 Padera TP, Meijer EF, Munn LL. The Lymphatic System in Disease Processes and Cancer Progression. Annu Rev Biomed Eng. 2016; 18: 125-58.
147 Nathanson SD, Shah R, Chitale DA, et al. Intraoperative clinical assessment and pressure measurements of sentinel lymph nodes in breast cancer. Ann Surg Oncol. 2014; 21: 81-5.
148 Shields JD, Borsetti M, Rigby H, et al. Lymphatic density and metastatic spread in human malignant melanoma. Br J Cancer. 2004; 90: 693-700.
149 Farnsworth RH, Karnezis T, Shayan R, et al. A role for bone morphogenetic protein-4 in lymph node vascular remodeling and primary tumor growth. Cancer Res. 2011; 71: 6547-57.
150 Dunn IF, Heese O, Black PM. Growth factors in glioma angiogenesis: FGFs, PDGF, EGF, and TGFs. J Neurooncol. 2000; 50: 121-37.
151 Christiaens V, Lijnen HR. Angiogenesis and development of adipose tissue. Mol Cell Endocrinol. 2010; 318: 2-9.
152 Kesavan P, McCabe F, Millar H, et al. Anti-CCL-2 / MCP-1 (monocyte chemoattractant protein-1) monoclonal antibodies effectively inhibit tumor angiogenesis and growth of human breast carcinoma. Cancer Res. 2005; 65: 254-.
153 Argaw AT, Zhang Y, Snyder BJ, et al. IL-1beta regulates blood-brain barrier permeability via reactivation of the hypoxia-angiogenesis program. J Immunol. 2006; 177: 5574-84.
154 Banerji S, Ni J, Wang S-X, et al. LYVE-1, a New Homologue of the CD44 Glycoprotein, Is a Lymph-specific Receptor for Hyaluronan. J Cell Biol. 1999; 144: 789-801.
155 Ellis LM, Hicklin DJ. VEGF-targeted therapy: mechanisms of anti-tumour activity. Nat Rev Cancer. 2008; 8: 579-91.
156 Crawford Y, Ferrara N. VEGF inhibition: insights from preclinical and clinical studies. Cell Tissue Res. 2009; 335: 261-9.
157 Horowitz A, Simons M. Branching morphogenesis. Circ Res. 2008; 103: 784-95.
158 Jain RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science. 2005; 307: 58-62.
159 Heath VL, Bicknell R. Anticancer strategies involving the vasculature. Nat Rev Clin Oncol. 2009; 6: 395-404.
160 Fischer C, Mazzone M, Jonckx B, et al. FLT1 and its ligands VEGFB and PlGF: drug targets for anti-angiogenic therapy? Nat Rev Cancer. 2008; 8: 942-56.
161 Olofsson B, Pajusola K, Kaipainen A, et al. Vascular endothelial growth factor B, a novel growth factor for endothelial cells. Proc Natl Acad Sci U S A. 1996; 93: 2576-81.
162 Joukov V, Pajusola K, Kaipainen A, et al. A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. EMBO J. 1996; 15: 290-8.
163 Yamada Y, Nezu J, Shimane M, et al. Molecular cloning of a novel vascular endothelial growth factor, VEGF-D. Genomics. 1997; 42: 483-8.
164 Tang Y, Zhang D, Fallavollita L, et al. Vascular endothelial growth factor C expression and lymph node metastasis are regulated by the type I insulin-like growth factor receptor. Cancer Res. 2003; 63: 1166-71.
165 Gaengel K, Genove G, Armulik A, et al. Endothelial-mural cell signaling in vascular development and angiogenesis. Arterioscler Thromb Vasc Biol. 2009; 29: 630-8.
166 Bergers G, Song S, Meyer-Morse N, et al. Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. J Clin Invest. 2003; 111: 1287-95.
167 Nisancioglu MH, Betsholtz C, Genove G. The absence of pericytes does not increase the sensitivity of tumor vasculature to vascular endothelial growth factor-A blockade. Cancer Res. 2010; 70: 5109-15.
168 Tutunea-Fatan E, Majumder M, Xin X, et al. The role of CCL21/CCR7 chemokine axis in breast cancer-induced lymphangiogenesis. Mol Cancer. 2015; 14: 35.
169 Kazenwadel J, Secker GA, Liu YJ, et al. Loss-of-function germline GATA2 mutations in patients with MDS/AML or MonoMAC syndrome and primary lymphedema reveal a key role for GATA2 in the lymphatic vasculature. Blood. 2012; 119: 1283-91.
170 Ishii J, Yazawa T, Chiba T, et al. PROX1 Promotes Secretory Granule Formation in Medullary Thyroid Cancer Cells. Endocrinology. 2016; 157: 1289-98.
171 Cui L, Dang S, Qu J, et al. FOXC2 is up-regulated in pancreatic ductal adenocarcinoma and promotes the growth and migration of cancer cells. Tumour Biol. 2016; 37: 8579-85.
172 Rajala MW, Scherer PE. Minireview: The adipocyte--at the crossroads of energy homeostasis, inflammation, and atherosclerosis. Endocrinology. 2003; 144: 3765-73.
173 Gilbert CA, Slingerland JM. Cytokines, obesity, and cancer: new insights on mechanisms linking obesity to cancer risk and progression. Annu Rev Med. 2013; 64: 45-57.
174 Roberts DL, Dive C, Renehan AG. Biological mechanisms linking obesity and cancer risk: new perspectives. Annu Rev Med. 2010; 61: 301-16.
175 Naugler WE, Karin M. The wolf in sheep's clothing: the role of interleukin-6 in immunity, inflammation and cancer. Trends Mol Med. 2008; 14: 109-19.
176 Donohoe CL, O'Farrell NJ, Doyle SL, et al. The role of obesity in gastrointestinal cancer: evidence and opinion. Therap Adv Gastroenterol. 2014; 7: 38-50.
177 Lago F, Gomez R, Gomez-Reino JJ, et al. Adipokines as novel modulators of lipid metabolism. Trends Biochem Sci. 2009; 34: 500-10.
178 Gilbert CA, Slingerland JM. Cytokines, Obesity, and Cancer: New Insights on Mechanisms Linking Obesity to Cancer Risk and Progression. Annu Rev Med. 2013; 64: 45-57.
179 Mohammadpour H, Pourfathollah AA, Nikougoftar Zarif M, et al. Irradiation enhances susceptibility of tumor cells to the antitumor effects of TNF-alpha activated adipose derived mesenchymal stem cells in breast cancer model. Sci Rep. 2016; 6: 28433.
180 Goodwin PJ, Stambolic V. Impact of the obesity epidemic on cancer. Annu Rev Med. 2015; 66: 281-96.
181 Key TJ, Appleby PN, Reeves GK, et al. Body Mass Index, Serum Sex Hormones, and Breast Cancer Risk in Postmenopausal Women. J Natl Cancer Inst. 2003; 95: 1218-26.
182 van Kruijsdijk RC, van der Wall E, Visseren FL. Obesity and cancer: the role of dysfunctional adipose tissue. Cancer Epidemiol Biomarkers Prev. 2009; 18: 2569-78.
183 Joshi RK, Lee SA. Obesity related adipokines and colorectal cancer: a review and meta-analysis. Asian Pac J Cancer Prev. 2014; 15: 397-405.
184 Parekh N, Chandran U, Bandera EV. Obesity in cancer survival. Annu Rev Nutr. 2012; 32: 311-42.
185 Kolonin MG, Simmons PJ. Combinatorial stem cell mobilization. Nat Biotechnol. 2009; 27: 252-3.
186 Kolonin MG, Evans KW, Mani SA, et al. Alternative origins of stroma in normal organs and disease. Stem Cell Res. 2012; 8: 312-23.
187 Lee YH, Mottillo EP, Granneman JG. Adipose tissue plasticity from WAT to BAT and in between. Biochim Biophys Acta. 2014; 1842: 358-69.
188 Iwen KA, Priewe AC, Winnefeld M, et al. Gluteal and abdominal subcutaneous adipose tissue depots as stroma cell source: gluteal cells display increased adipogenic and osteogenic differentiation potentials. Exp Dermatol. 2014; 23: 395-400.
189 Noel D, Caton D, Roche S, et al. Cell specific differences between human adipose-derived and mesenchymal-stromal cells despite similar differentiation potentials. Exp Cell Res. 2008; 314: 1575-84.

190 Eterno V ZA, Pavesi L, Villani L, Zanini V, Petrolo G, et al. Adiposederived mesenchymal stem cells (ASCs) may favour breast cancer recurrence via HGF/c-Met signaling. Oncotarget. 2014; 5: 613–33.
191 Rowan BG, Gimble JM, Sheng M, et al. Human adipose tissue-derived stromal/stem cells promote migration and early metastasis of triple negative breast cancer xenografts. PLoS One. 2014; 9: e89595.
192 Gehmert S, Gehmert S, Prantl L, et al. Breast cancer cells attract the migration of adipose tissue-derived stem cells via the PDGF-BB/PDGFR-beta signaling pathway. Biochem Biophys Res Commun. 2010; 398: 601-5.
193 Amos PJ, Shang H, Bailey AM, et al. IFATS collection: The role of human adipose-derived stromal cells in inflammatory microvascular remodeling and evidence of a perivascular phenotype. Stem Cells. 2008; 26: 2682-90.
194 Jotzu C, Alt E, Welte G, et al. Adipose tissue derived stem cells differentiate into carcinoma-associated fibroblast-like cells under the influence of tumor derived factors. Cell Oncol (Dordr). 2011; 34: 55-67.
195 Cho JA, Park H, Lim EH, et al. Exosomes from ovarian cancer cells induce adipose tissue-derived mesenchymal stem cells to acquire the physical and functional characteristics of tumor-supporting myofibroblasts. Gynecol Oncol. 2011; 123: 379-86.
196 Jeon ES, Heo SC, Lee IH, et al. Ovarian cancer-derived lysophosphatidic acid stimulates secretion of VEGF and stromal cell-derived factor-1 alpha from human mesenchymal stem cells. Exp Mol Med. 2010; 42: 280-93.
197 Do EK, Kim YM, Heo SC, et al. Lysophosphatidic acid-induced ADAM12 expression mediates human adipose tissue-derived mesenchymal stem cell-stimulated tumor growth. Int J Biochem Cell Biol. 2012; 44: 2069-76.
198 Park YM, Yoo SH, Kim SH. Adipose-derived stem cells induced EMT-like changes in H358 lung cancer cells. Anticancer Res. 2013; 33: 4421-30.
199 Zimmerlin L, Donnenberg AD, Rubin JP, et al. Regenerative therapy and cancer: in vitro and in vivo studies of the interaction between adipose-derived stem cells and breast cancer cells from clinical isolates. Tissue Eng Part A. 2011; 17: 93-106.
200 Devarajan E, Song YH, Krishnappa S, et al. Epithelial-mesenchymal transition in breast cancer lines is mediated through PDGF-D released by tissue-resident stem cells. Int J Cancer. 2012; 131: 1023-31.
201 Pinilla S, Alt E, Abdul Khalek FJ, et al. Tissue resident stem cells produce CCL5 under the influence of cancer cells and thereby promote breast cancer cell invasion. Cancer Lett. 2009; 284: 80-5.
202 Lee Y, Jung WH, Koo JS. Adipocytes can induce epithelial-mesenchymal transition in breast cancer cells. Breast Cancer Res Treat. 2015; 153: 323-35.
203 Quarto N, Senarath-Yapa K, Renda A, et al. TWIST1 silencing enhances in vitro and in vivo osteogenic differentiation of human adipose-derived stem cells by triggering activation of BMP-ERK/FGF signaling and TAZ upregulation. Stem Cells. 2015; 33: 833-47.
204 Goodwin PJ. Obesity and endocrine therapy: host factors and breast cancer outcome. Breast. 2013; 22 Suppl 2: S44-7.
205 Maccio A, Madeddu C, Mantovani G. Adipose tissue as target organ in the treatment of hormone-dependent breast cancer: new therapeutic perspectives. Obes Rev. 2009; 10: 660-70.
206 Yin N, Wang D, Zhang H, et al. Molecular mechanisms involved in the growth stimulation of breast cancer cells by leptin. Cancer Res. 2004; 64: 5870-5.
207 Delort L, Rossary A, Farges MC, et al. Leptin, adipocytes and breast cancer: Focus on inflammation and anti-tumor immunity. Life Sci. 2015; 140: 37-48.
208 Lopez R, Arumugam A, Joseph R, et al. Hyperglycemia enhances the proliferation of non-tumorigenic and malignant mammary epithelial cells through increased leptin/IGF1R signaling and activation of AKT/mTOR. PLoS One. 2013; 8: e79708.
209 Jung JS, Jee MK, Cho HT, et al. MBD6 is a direct target of Oct4 and controls the stemness and differentiation of adipose tissue-derived stem cells. Cell Mol Life Sci. 2013; 70: 711-28.
210 Wang X, Simpson ER, Brown KA. Aromatase overexpression in dysfunctional adipose tissue links obesity to postmenopausal breast cancer. J Steroid Biochem Mol Biol. 2015; 153: 35-44.
211 Ioannides SJ, Barlow PL, Elwood JM, et al. Effect of obesity on aromatase inhibitor efficacy in postmenopausal, hormone receptor-positive breast cancer: a systematic review. Breast Cancer Res Treat. 2014; 147: 237-48.
212 Montuenga LM, Guembe L, Burrell MA, et al. The diffuse endocrine system: from embryogenesis to carcinogenesis. Prog Histochem Cytochem. 2003; 38: 155-272.
213 Gunawardene AR, Corfe BM, Staton CA. Classification and functions of enteroendocrine cells of the lower gastrointestinal tract. Int J Exp Pathol. 2011; 92: 219-31.
214 di Sant'Agnese PA. Neuroendocrine differentiation in carcinoma of the prostate. Diagnostic, prognostic, and therapeutic implications. Cancer. 1992; 70: 254-68.
215 Linnoila RI. Functional facets of the pulmonary neuroendocrine system. Lab Invest. 2006; 86: 425-44.
216 Ayala GE, Dai H, Powell M, et al. Cancer-related axonogenesis and neurogenesis in prostate cancer. Clin Cancer Res. 2008; 14: 7593-603.
217 Lala PK, Parhar RS, Singh P. Indomethacin therapy abrogates the prostaglandin-mediated suppression of natural killer activity in tumor-bearing mice and prevents tumor metastasis. Cell Immunol. 1986; 99: 108-18.
218 Levite M. Nerve-driven immunity. The direct effects of neurotransmitters on T-cell function. Ann N Y Acad Sci. 2000; 917: 307-21.
219 Lang K, Drell TL, Niggemann B, et al. Neurotransmitters regulate the migration and cytotoxicity in natural killer cells. Immunol Lett. 2003; 90: 165-72.
220 Delgado AV, McManus AT, Chambers JP. Production of tumor necrosis factor-alpha, interleukin 1-beta, interleukin 2, and interleukin 6 by rat leukocyte subpopulations after exposure to substance P. Neuropeptides. 2003; 37: 355-61.
221 Lang K, Entschladen F, Weidt C, et al. Tumor immune escape mechanisms: impact of the neuroendocrine system. Cancer Immunol Immunother. 2006; 55: 749-60.
222 Epstein JI, Amin MB, Beltran H, et al. Proposed morphologic classification of prostate cancer with neuroendocrine differentiation. Am J Surg Pathol. 2014; 38: 756-67.
223 Tanaka M, Suzuki Y, Takaoka K, et al. Progression of prostate cancer to neuroendocrine cell tumor. Int J Urol. 2001; 8: 431-6; discussion 7.
224 Jin RJ, Wang Y, Masumori N, et al. NE-10 neuroendocrine cancer promotes the LNCaP xenograft growth in castrated mice. Cancer Res. 2004; 64: 5489-95.
225 Gout S, Huot J. Role of Cancer Microenvironment in Metastasis: Focus on Colon Cancer. Cancer Microenviron. 2008; 1: 69-83.
226 Kaltsas GA, Besser GM, Grossman AB. The diagnosis and medical management of advanced neuroendocrine tumors. Endocr Rev. 2004; 25: 458-511.
227 Jiang N, Zhu S, Chen J, et al. A-methylacyl-CoA racemase (AMACR) and prostate-cancer risk: a meta-analysis of 4,385 participants. PLoS One. 2013; 8: e74386.
228 Olar A, He D, Florentin D, et al. Biologic correlates and significance of axonogenesis in prostate cancer. Hum Pathol. 2014; 45: 1358-64.
229 Vlachostergios PJ, Papandreou CN. Targeting neuroendocrine prostate cancer: molecular and clinical perspectives. Front Oncol. 2015; 5: 6.
230 Leiblich A, Cross SS, Catto JW, et al. Human prostate cancer cells express neuroendocrine cell markers PGP 9.5 and chromogranin A. Prostate. 2007; 67: 1761-9.
231 Grigore AD, Ben-Jacob E, Farach-Carson MC. Prostate cancer and neuroendocrine differentiation: more neuronal, less endocrine? Front Oncol. 2015; 5: 37.
232 Tsao KC, Wu JT. Development of an ELISA for the detection of serum chromogranin A (CgA) in prostate and non-neuroendocrine carcinomas. Clin Chim Acta. 2001; 313: 21-9.
233 Von Dorsche HH, Fält K, Hahn H-J, et al. Neuron-specific enolase (NSE) as a neuroendocrine cell marker in the human fetal pancreas. Acta Histochemica. 1989; 85: 227-8.
234 Modlin IM, Bodei L, Kidd M. Neuroendocrine tumor biomarkers: From monoanalytes to transcripts and algorithms. Best Pract Res Clin Endocrinol Metab. 2016; 30: 59-77.
235 Berkovic MC, Jokic M, Marout J, et al. IL-2 -330 T/G SNP and serum values-potential new tumor markers in neuroendocrine tumors of the gastrointestinal tract and pancreas (GEP-NETs). J Mol Med (Berl). 2010; 88: 423-9.
236 Chen G, Jaskula-Sztul R, Harrison A, et al. KE108-conjugated unimolecular micelles loaded with a novel HDAC inhibitor thailandepsin-A for targeted neuroendocrine cancer therapy. Biomaterials. 2016; 97: 22-33.
237 Saunders LR, Bankovich AJ, Anderson WC, et al. A DLL3-targeted antibody-drug conjugate eradicates high-grade pulmonary neuroendocrine tumor-initiating cells in vivo. Sci Transl Med. 2015; 7: 302ra136.
238 Nagashio R, Sato Y, Matsumoto T, et al. Significant high expression of cytokeratins 7, 8, 18, 19 in pulmonary large cell neuroendocrine carcinomas, compared to small cell lung carcinomas. Pathol Int. 2010; 60: 71-7.
239 Sorhaug S, Steinshamn S, Haaverstad R, et al. Expression of neuroendocrine markers in non-small cell lung cancer. APMIS. 2007; 115: 152-63.
240 Sun RF, Liu ZG, Ma G, et al. Prognostic value of neuroendocrine markers for predicting survival in patients with small cell lung cancer. Int J Clin Exp Pathol. 2016; 9: 5970-9.
241 van der Zwan WA, Bodei L, Mueller-Brand J, et al. GEPNETs update: Radionuclide therapy in neuroendocrine tumors. Eur J Endocrinol. 2015; 172: R1-8.
242 Di Florio A, Sancho V, Moreno P, et al. Gastrointestinal hormones stimulate growth of Foregut Neuroendocrine Tumors by transactivating the EGF receptor. Biochim Biophys Acta. 2013; 1833: 573-82.
243 Kashyap R, Hofman MS, Michael M, et al. Favourable outcomes of (177)Lu-octreotate peptide receptor chemoradionuclide therapy in patients with FDG-avid neuroendocrine tumours. Eur J Nucl Med Mol Imaging. 2015; 42: 176-85.
244 Carver BS. Defining and Targeting the Oncogenic Drivers of Neuroendocrine Prostate Cancer. Cancer Cell. 2016; 29: 431-2.
245 Monboisse JC, Oudart JB, Ramont L, et al. Matrikines from basement membrane collagens: a new anti-cancer strategy. Biochim Biophys Acta. 2014; 1840: 2589-98.
246 Chelberg MK, Tsilibary EC, Hauser AR, et al. Type IV collagen-mediated melanoma cell adhesion and migration: involvement of multiple, distinct domains of the collagen molecule. Cancer Res. 1989; 49: 4796-802.
247 Brown E, McKee T, diTomaso E, et al. Dynamic imaging of collagen and its modulation in tumors in vivo using second-harmonic generation. Nat Med. 2003; 9: 796-800.

248 Schminke B, Frese J, Bode C, et al. Laminins and Nidogens in the Pericellular Matrix of Chondrocytes: Their Role in Osteoarthritis and Chondrogenic Differentiation. Am J Pathol. 2016; 186: 410-8.
249 Yamada KM, Olden K. Fibronectins[mdash]adhesive glycoproteins of cell surface and blood. Nature. 1978; 275: 179-84.
250 Kim LB, Shkurupy VA, Putyatina AN. Dynamic Structure of Proteoglycans/Glycosaminoglycans in the Lungs of Mice with Chronic Granulomatous Inflammation. Bull Exp Biol Med. 2016; 160: 435-8.
251 Dahl LB, Dahl IM, Engstrom-Laurent A, et al. Concentration and molecular weight of sodium hyaluronate in synovial fluid from patients with rheumatoid arthritis and other arthropathies. Ann Rheum Dis. 1985; 44: 817-22.
252 Goldberg VM, Buckwalter JA. Hyaluronans in the treatment of osteoarthritis of the knee: evidence for disease-modifying activity. Osteoarthritis Cartilage. 2005; 13: 216-24.
253 Zhu J, Liang L, Jiao Y, et al. Enhanced invasion of metastatic cancer cells via extracellular matrix interface. PLoS One. 2015; 10: e0118058.
254 Yu J, Liu Y, Guo C, et al. Upregulated long non-coding RNA LINC00152 expression is associated with progression and poor prognosis of tongue squamous cell carcinoma. J Cancer. 2017; 8(4):523-30.
255 Zhou YJ, Liao QJ, Li XY, et al. HYOU1, Regulated by LPLUNC1, Is Up-Regulated in Nasopharyngeal Carcinoma and Associated with Poor Prognosis. J Cancer. 2016; 7: 367-76.
256 Zeng Z, Bo H, Gong Z, et al. AFAP1-AS1, a long noncoding RNA upregulated in lung cancer and promotes invasion and metastasis. Tumour Biol. 2016; 37: 729-37.
257 Xiao K, Yu Z, Li X, et al. Genome-wide Analysis of Epstein-Barr Virus (EBV) Integration and Strain in C666-1 and Raji Cells. J Cancer. 2016; 7: 214-24.
258 Song Y, Li X, Zeng Z, et al. Epstein-Barr virus encoded miR-BART11 promotes inflammation-induced carcinogenesis by targeting FOXP1. Oncotarget. 2016; 7: 36783-99.
259 Lian Y, Li XY, Tang YY, et al. Long Non-coding RNAs Function as Competing Endogenous RNAs to Regulate Cancer Progression. Prog Biochem Biophys. 2016; 43: 219-25.
260 He B, Li W, Wu Y, et al. Epstein-Barr virus-encoded miR-BART6-3p inhibits cancer cell metastasis and invasion by targeting long non-coding RNA LOC553103. Cell Death Dis. 2016; 7: e2353.
261 Gong Z, Yang Q, Zeng Z, et al. An integrative transcriptomic analysis reveals p53 regulated miRNA, mRNA, and lncRNA networks in nasopharyngeal carcinoma. Tumour Biol. 2016; 37: 3683-95.
262 Yan Q, Zeng Z, Gong Z, et al. EBV-miR-BART10-3p facilitates epithelial-mesenchymal transition and promotes metastasis of nasopharyngeal carcinoma by targeting BTRC. Oncotarget. 2015; 6: 41766-82.
263 Howlett AR, Bissell MJ. The influence of tissue microenvironment (stroma and extracellular matrix) on the development and function of mammary epithelium. Epithelial Cell Biol. 1993; 2: 79-89.
264 Liao Q, Zeng Z, Guo X, et al. LPLUNC1 suppresses IL-6-induced nasopharyngeal carcinoma cell proliferation via inhibiting the Stat3 activation. Oncogene. 2014; 33: 2098-109.
265 Gong Z, Zhang S, Zeng Z, et al. LOC401317, a p53-regulated long non-coding RNA, inhibits cell proliferation and induces apoptosis in the nasopharyngeal carcinoma cell line HNE2. PLoS One. 2014; 9: e110674.
266 Yang Y, Liao Q, Wei F, et al. LPLUNC1 inhibits nasopharyngeal carcinoma cell growth via down-regulation of the MAP kinase and cyclin D1/E2F pathways. PLoS One. 2013; 8: e62869.
267 Gattazzo F, Urciuolo A, Bonaldo P. Extracellular matrix: a dynamic microenvironment for stem cell niche. Biochim Biophys Acta. 2014; 1840: 2506-19.
268 Popovic ZV, Sandhoff R, Sijmonsma TP, et al. Sulfated Glycosphingolipid as Mediator of Phagocytosis: SM4s Enhances Apoptotic Cell Clearance and Modulates Macrophage Activity. J Immunol. 2007; 179: 6770-82.
269 O'Connell J, O'Sullivan GC, Collins JK, et al. The Fas counterattack: Fas-mediated T cell killing by colon cancer cells expressing Fas ligand. J Exp Med. 1996; 184: 1075-82.
270 Gong ZJ, Huang HB, Xu K, et al. Advances in microRNAs and TP53 Gene Regulatory Network. Prog Biochem Biophys. 2012; 39: 1133-44.
271 Huang HB, Liang F, Xiong W, et al. Bioinformatics Accelerates Drug Repositioning. Prog Biochem Biophys. 2012; 39: 35-44.
272 Huang HB, Deng M, Zheng Y, et al. Innate immune protein lactotransferrin prevents initiation and arrests progression of nasopharyngeal carcinoma. Prog Biochem Biophys. 2013; 40: 319-24.
273 Liang F, Xu K, Gong ZJ, et al. ChIP-seq: a New Technique for Genome-wide Profiling of Protein-DNA Interaction. Prog Biochem Biophys. 2013; 40: 216-27.
274 Tang K, Wei F, Bo H, et al. Cloning and Functional Characterization of a Novel Long Non-coding RNA Gene Associated With Hepatocellular Carcinoma. Prog Biochem Biophys. 2014; 41: 153-62.
275 Yu ZY, Song YL, Gong ZJ, et al. The Mechanism and Tumorigenesis of Oncogenic DNA Virus Integration. Prog Biochem Biophys. 2014; 41: 324-31.
276 Li YW, Wang YM, Zhang XY, et al. Progress of Long Noncoding RNA HOTAIR in Human Cancer. Prog Biochem Biophys. 2015; 42: 228-35.
277 Tu CF, Qi P, Li XY, et al. Tumor Heterogeneity: The Challenge of Precision Medicine. Prog Biochem Biophys. 2015; 42: 881-90.

Patient insights on cancer care: opportunities for improving efficiency

Findings from the international All.Can patient survey

All.Can Changing cancer care together

The All.Can initiative is made possible with financial support from Bristol-Myers Squibb (main sponsor), Amgen, MSD and Johnson & Johnson (sponsors) and Varian (contributor), with additional non-financial (in kind) support from Intacare and Goings-On.

About All.Can

All.Can is an international, multi-stakeholder policy initiative aiming to identify ways we can optimise the use of resources in cancer care to improve patient outcomes.

All.Can comprises leading representatives from patient organisations, policymakers, healthcare professionals, research and industry, and consists of All.Can international, plus All.Can national initiatives currently established in 13 countries.

Members of All.Can international are:

Matti Aapro, Clinique Genolier, Switzerland; **Tit Albreht**, Institute of Public Health, Slovenia; **Neil Bacon**, International Consortium for Health Outcomes Measurement; **Kathleen Barnard**, Save Your Skin Foundation Canada; **Antonella Cardone**, European Cancer Patient Coalition (ECPC); **Szymon Chrostowski**, Polish Cancer Patient Coalition; **David Duplay**, healtheo360; **Alex Filicevas**, ECPC; **Pascal Garel**, HOPE (European Hospital and Healthcare Federation); **Stefan Gijssels**, Digestive Cancers Europe; **Rainer Hess**, GVG-Committee on Health Goals; **Matthew Hickey**, Intacare International Ltd; **Petra Hoogendoorn**, Goings-On; **Vivek Muthu**, Marivek Healthcare Consulting; **Kathy Oliver**, The International Brain Tumour Alliance; **Richard Price**, European CanCer Organisation; **Natalie Richardson**, Save Your Skin Foundation Canada; **Christobel Saunders**, University of Western Australia; **Thomas Szucs**, University of Basel; **Jan van Meerbeeck**, Antwerp University Hospital; **Lieve Wierinck**, Former Member of the European Parliament; **Andy Whitman**, Varian; **Titta Rosvall-Puplett**, Bristol-Myers Squibb; **Karin Steinmann**, Amgen; **Matthijs Van Meerveld**, MSD; **Aleksandra Krygiel-Nael**, Johnson & Johnson; **Shannon Boldon**, The Health Policy Partnership (secretariat); **Suzanne Wait**, The Health Policy Partnership (secretariat)*

* Baxter, Helpsy and Roche joined All.Can international in July 2019 (in addition to the members listed here)

Acknowledgements

This report was developed by All.Can international, with close input from Quality Health, along with All.Can national initiatives in Australia, Belgium, Canada, Italy, Poland, Spain, Sweden and the United Kingdom, and healtheo360 in the United States.

We would like to thank the dedicated team at Quality Health for their work in coordinating all aspects of this survey, and for their continued commitment to enriching the quality of the study and findings.

In addition, we would like to thank the following individuals and organisations for their efforts in helping us review local-language versions of the survey, disseminate the survey at the national level and provide comments on iterative drafts of this report:

All.Can international research and evidence working group
All.Can Australia
All.Can Belgium
All.Can Canada
All.Can Italy
All.Can Poland
All.Can Spain
All.Can Sweden
All.Can UK
European Cancer Patient Coalition (ECPC)
European CanCer Organisation (ECCO)
healtheo360
Save Your Skin Foundation
The International Brain Tumour Alliance (IBTA)
Lesley Millar and the University of Western Australia Medical School
Participants at the 2017 IBTA World Summit
Participants in the All.Can sessions at the European Health Forum Gastein
Benjamin Gandouet, Oncopole Toulouse
Laura Del Campo, Federazione italiana delle Associazioni di Volontariato in Oncologia (FAVO)
Patients Association
Jo's Cervical Cancer Trust
NET Patient Foundation
Leukaemia Care
Cancer 52
Pink Ribbon Foundation
Maggie's
Womb Cancer Support UK
Womb Cancer Info

Table of contents

Glossary of terms

This glossary provides definitions of terms used throughout this report. These may be accepted definitions (referenced as appropriate), or All.Can's internal definitions of terms used in relation to the patient survey. Where text is coloured light blue in the report, it indicates that these terms are explained in the glossary.*

Active treatment

Any range of treatments intended to control or cure cancer (e.g. surgery, radiotherapy, chemotherapy, hormonal therapy or immunotherapy), as opposed to treatments patients may receive in addition to relieve symptoms or side effects of treatment (e.g. pain medication).[1]

Allied health professionals

Health professionals other than those working in medicine, nursing or pharmacy who are involved with the delivery of health or related services. This includes, among others, dietitians, nutritionists, occupational therapists and physiotherapists.[2]

Caregivers

'Any relatives, friends, or partners who have a significant relationship with and provide assistance (i.e. physical, emotional, medical) to a patient with a life-threatening, incurable illness.'[3]

Inefficiency

The allocation of resources to anything that does not focus on what matters to patients.

Integrated care

Care that is 'person-centred, coordinated, and tailored to the needs and preferences of the individual, their caregivers and family. It means moving away from episodic care to a more holistic approach to health, care and support needs, that puts the needs and experience of people at the centre of how services are organised and delivered.'[4]

* Terms relating specifically to All.Can or the reporting of survey findings, such as 'inefficiency' and 'respondents', are not highlighted in light blue throughout the report.

Multidisciplinary cancer care

Care used and implemented by multidisciplinary care teams, which are 'an alliance of all medical and healthcare professionals related to a specific tumour disease whose approach to cancer care is guided by their willingness to agree on evidence-based clinical decisions and to coordinate the delivery of care at all stages of the process, encouraging patients in turn to take an active role in their care.'[5]

Out-of-pocket costs

The entirety of costs related to cancer that patients have to pay for themselves. This includes, for example, costs of diagnostic tests, treatment or care which are not covered by the patient's health insurance, travel costs associated with care, and the cost of childcare or household help.

Palliative care

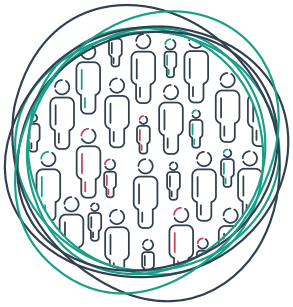
An approach to care 'that improves the quality of life of patients and their families facing the problems associated with life-threatening illness, through the prevention and relief of suffering from pain and other problems, as well as psychosocial and spiritual support.'[6]

Respondents

People who completed the All.Can patient survey. This may include both current and former cancer patients, as well as caregivers who completed the survey on behalf of patients who were themselves unable to do so or had passed away. The term is used in this report in relation to both weighted and unweighted data; for an explanation of data weighting please see **About this survey** (page 11).

Shared decision-making

A process in which 'clinicians and patients work together to choose all aspects of care, based on clinical evidence, patient goals and informed preferences.'[7][8]



Executive summary

Tackling inefficiency in cancer care: the patient perspective

The prevalence, complexity and costs of cancer are rising – yet, across healthcare, up to 20% of expenditure is thought to be spent on interventions that are deemed inefficient.[9] There is thus an urgent need to ensure that cancer care is delivered as efficiently as possible for the sustainability of our healthcare systems.

All.Can defines efficiency as focusing resources on what matters to patients – and our aim is to find practical solutions to improve the efficiency of cancer care. However, in order to tackle inefficiency from the patient's perspective, we believe that we need to gain a better understanding of where patients consider their care is not focused on what matters to them.

The All.Can patient survey was designed with this purpose in mind. It asked patients where they had encountered inefficiency across the entire continuum of cancer care, including diagnosis, treatment and care, ongoing support and the broader impact of cancer on their lives. The survey questionnaire made explicit our definition of inefficiency.

Nearly 4,000 respondents from more than 10 countries participated in the survey from January – November 2018 – making this, to our knowledge, the largest international survey specifically aimed at obtaining patient perspectives on inefficiency in cancer care.

About this survey

The All.Can patient survey was conducted by Quality Health, with close input from the All.Can international research and evidence working group. It was disseminated via patient organisations and social media.

To find out more about the survey and to view additional materials, see www.all-can.org/what-we-do/research/patient-survey/about-the-survey/

Key opportunities identified to improve efficiency in cancer care

Overall, the majority of survey respondents reported that their needs were sufficiently addressed during their care. However, the survey highlighted four crucial areas where respondents reported that they experienced inefficiency and where there are opportunities for improvement.

1. Swift, accurate and appropriately delivered diagnosis

Diagnosis was not always communicated to respondents in the most appropriate way. Respondents sometimes reported a **lack of empathy** from physicians, along with poor timing. For example, some were told they had cancer without a family member present, or had to wait several days to speak to a specialist after receiving their diagnosis.

When asked to select the one area of cancer care where they experienced the most inefficiency, **26%** of respondents chose diagnosis – more than any other area of cancer care.

Across all cancer types, **58%** of respondents had their cancer detected outside of a screening programme. As might be expected, this varied considerably by cancer type.

Among respondents whose cancer was detected outside of a screening programme, **delayed diagnosis** (>6 months) was associated with a more negative patient view of all aspects of care and support.

Time to diagnosis varied significantly by cancer type: nearly **80%** of prostate cancer respondents whose cancer was detected outside of screening said their cancer was diagnosed in less than a month, while for head and neck cancer respondents diagnosed outside of screening this was only **25%.**

Nearly a third (**32%**) of respondents whose cancer was detected outside of a screening programme reported that their **cancer was initially diagnosed as something different** – and, again, this varied considerably by cancer type.

2. Information, support and shared decision-making

In qualitative responses, **respondents said that they felt overwhelmed because too much information was given at once**, and they would have preferred to receive relevant information at appropriate points along the entire care pathway.

Nearly a third of respondents (**31%**) felt they were **not given adequate information** about their cancer care and treatment in a way that they could understand. Additionally, only half of respondents (**53%**) felt they were sufficiently **involved in making decisions** about their care.

Nearly two in five respondents (**39%**) felt they had **inadequate support to deal with ongoing symptoms and side effects**, both during and beyond active treatment. In particular, nearly a third (**31%**) felt that they lacked adequate information and care for dealing with the pain they experienced.

Respondents reported **a lack of information and support on what to expect** after their phase of **active treatment** was over. Specifically, more than a third of respondents (**35%**) felt inadequately informed about how to recognise whether their cancer might be returning or getting worse.

Gaps in information and support along the entire care pathway were more prevalent among those with more advanced cancers – reflecting similar findings in the literature that the support needs of these patients are often less well met than are those of patients with earlier-stage cancer.

3. Integrated multidisciplinary care

A common finding was that respondents felt there was sometimes a **lack of coordination** in their care – for example, they had no written care plan, nor a primary point of contact to whom they could direct questions.

Respondents stated that **cancer specialist nurses played a critical role** in filling this gap, acting as the respondents' companion and 'navigator' through the cancer care journey and helping them adapt all aspects of their lives to cancer – both during and after treatment.

Respondents wanted more **information about what they could do to support their treatment and recovery** in terms of diet, exercise and complementary therapies. In most countries, they had to pay for these services themselves. While three quarters (**76%**) felt that support from **allied health professionals** (dieticians, physiotherapists etc.) was always or sometimes available, nearly a quarter (**24%**) did not.

The majority of respondents (**69%**) said they **needed psychological support during or after their cancer care**, yet one in three (**34%**) of those who needed such support reported that it was unavailable. Even when psychological support was available, it was not always considered helpful. This may reflect the limited availability of specialist psycho-oncology services that are designed with the needs of cancer patients in mind.

In addition, respondents expressed **concerns about the impact of cancer on their families** and wanted psychological support for them as well.

More than two in five respondents (**41%**) were not given information at the hospital about available **peer-support groups**.

4. The financial impact of cancer

Respondents frequently spoke of the financial impact that cancer had on their lives – not just in terms of components of their care they may have had to pay for themselves, but also the cost of travel to medical appointments, childcare or household help, lost income from employment, and difficulties in getting insurance, mortgages or loans as a result of their cancer diagnosis.

More than half of respondents (**51%**) **paid for some part of their overall cancer care themselves**, either **out-of-pocket** or through private insurance. Respondents highlighted that this often created significant financial pressure for their families.

Apart from any possible care costs incurred, **36%** of respondents also referred to significant **travel costs** (i.e. to and from the hospital or clinic), and **26%** to **loss of employment income** – with those who were self-employed and caring for young children being especially vulnerable to financial insecurity due to their cancer diagnosis.

A recurring theme among respondents was that **the financial implications of cancer could last a lifetime**, in terms of people's ability to find or keep employment, reduced productivity for themselves and their **caregivers**, and an inability to get insurance or loans – even years after their treatment was finished.

Conclusions

Findings from this survey identify some important areas where cancer patients have highlighted from personal experience that improvements are needed. They align with other findings from patient surveys in the literature, as well as the views of patient organisations in the All.Can network. These themes are intrinsically important as they represent respondents' perceptions of their care.

As we strive to improve the efficiency of cancer care, we must ensure that the patient perspective is always central to our definitions and aims. Each of the areas identified represents an opportunity to improve cancer care for patients. We need to give these issues due prominence in future cancer plans, policies and investment decisions to build truly patient-driven care. We need to develop integrated health and social policies to address the wide-reaching impact cancer can have on all aspects of people's lives.

Making these changes could lead to real differences – to patients' outcomes, their experience of care and the financial impact cancer has on them, their families and ultimately on the health system and society as a whole.

About this survey

Purpose

The aim of the survey was to obtain patients' perspectives on where they felt they encountered inefficiency in their care, looking at the entire care continuum as well as the broader impact of cancer on their lives. The survey questionnaire made explicit that we defined inefficiency as resources that are not focused on what matters to patients.

Survey conduct and oversight

The design and conduct of the survey was led by Quality Health (quality-health.co.uk), a specialist health and social care survey organisation working with public, private and voluntary sector organisations to understand and improve patients' experience of their care and treatment. The All.Can international research and evidence working group provided close input and validation for all phases of the survey and analysis.

The survey was conducted internationally, with adapted versions in 10 countries (Australia, Belgium, Canada, France, Italy, Poland, Spain, Sweden, the United Kingdom and the United States).

Survey development and patient interviews

The questions included in the survey were developed based on key themes that emerged from an international literature review and five exploratory pre-survey patient interviews. Iterative versions of the survey were revised based on input from the All.Can international research and evidence working group and other professional stakeholders where appropriate.

Country-specific versions of the survey were produced in relevant languages for each country and comprised both common questions and a maximum of five questions specific to the country. All surveys were developed with input from national All.Can initiatives (where they existed) and validated by patient representatives in each country. Additionally, an international 'generic' version of the survey was available in English, French, German and Spanish.

All versions of the survey were reviewed by the All.Can international research and evidence working group to ensure consistency between the different country versions. The survey asked respondents whether they would be willing to take part in a post-survey interview to provide more insights. These interviews were conducted in all participating countries except France, Spain and the United States (as there were no volunteers in those countries). Some of these interviews have been featured as patient stories throughout this report.

Recruitment of respondents

The survey was made available online, with only a few paper copies distributed where they were requested. Respondents were predominantly recruited via patient organisations and social media (Facebook, Twitter and LinkedIn). A notable exception was in Australia, where the survey was also distributed by clinic staff in Western Australia; and the United States, where it was distributed via the healtheo360 online platform. These methods of recruitment were chosen as they offered the most feasible and flexible approach to reaching a wide group of patients within the scope and budget of the project.

The survey was open to current and former cancer patients, irrespective of age and cancer type. Caregivers and former caregivers were also invited to respond on behalf of those patients who were unable to respond personally or who had passed away. Because caregivers were asked to complete the survey on behalf of patients, we use the term 'respondents' in this report when describing the survey results to refer to both patients and caregivers who completed the survey.

The survey ran from January to November 2018.

Respondent characteristics

A total of 3,981 people completed the survey. The number of respondents by country is presented in **Table I**. Overall characteristics of respondents are presented in **Table II**.

Table I. Overview of survey languages and responses (unweighted data)

Country	Survey languages	Number of respondents*	% of total responses
Australia	English	861	22%
Belgium	French, Dutch, German	396	10%
Canada	English, French	342	9%
France	French	55	1%
Italy	Italian	97	2%
Poland	Polish	1135	29%
Spain	Spanish	50	1%
Sweden	Swedish	60	2%
United Kingdom	English, Welsh	360	9%
United States	English	513	13%
International 'generic' version	English, Spanish, German, French	112	3%
Total		**3,981**	**101%****

* The number of respondents for each country shown here represents the international grouping of respondents. Each country survey asked whether the respondent was a resident in that country; if the respondent said no, their response was added to the international sample but not the country-specific sample. Therefore, the number of respondents shown in each country profile (**Appendix 1**) may not match the number of respondents for each country shown here. For more information, please see the full survey methodology at www.all-can.org/what-we-do/research/patient-survey/about-the-survey/

** This comes to a total of 101% due to rounding

Table II. Characteristics of respondents (unweighted data)

		Number	Percentage
Respondents (Age: mean 55 years; median 57 years)*	Patients (current or former)	3,450	89%
	Caregivers filling in the survey on behalf of a patient	432	11%
	Unspecified	99	n/a
Gender distribution	Male	787	20%
	Female	3,092	80%
	Unspecified	102	n/a
Length of time since patients were first treated for this cancer, at the time they completed the survey	Less than 1 year	964	25%
	1–5 years	1,706	44%
	More than 5 years	1,185	30%
	Don't know/can't remember	35	1%
	Unspecified	91	n/a
Patients per cancer type	Brain/central nervous system	97	2%
	Breast	1,656	42%
	Colorectal/bowel	216	6%
	Gastric cancers (oesophageal, stomach, pancreatic, liver or gall bladder)	123	3%
	Gynaecological	415	11%
	Haematological	362	9%
	Head and neck	161	4%
	Lung	167	4%
	Prostate	144	4%
	Sarcoma	72	2%
	Skin	203	5%
	Urological	186	5%
	Other**	115	3%
	Unspecified	64	n/a

* The age distribution of survey respondents is similar to the age distribution of the general cancer patient population

** For a full breakdown of cancer types included in these categories, please see the full methodology at www.all-can.org/what-we-do/research/patient-survey/about-the-survey/

Reporting of quantitative findings

Quantitative findings from the survey are based on responses to the closed-ended questions in the survey. Percentages are calculated after excluding respondents who did not answer each particular question. All percentages are rounded to the nearest whole number, therefore the sum of percentages for all answers to a given question may not total 100%.

As the patient survey welcomed responses from all cancer patients (no quotas were set) in order to be as inclusive as possible, the relative volume of people responding to the survey varied between countries and cancer types. To correct for this, quantitative findings have been weighted by two factors:

- Representative cancer prevalence rates for each cancer type listed within each participating country
- General population statistics for each country as a proportion of the international total.

Where figures and tables in the findings section of this report state a base size, this is always the unweighted base size; however, all other data in these figures and tables have been weighted.

To see the statistics used for these calculations

Please download a copy of the full methodology document at www.all-can.org/what-we-do/research/patient-survey/about-the-survey/

Reporting of qualitative findings

Qualitative findings presented in the report are based on responses to open-ended questions. A thematic analysis was conducted of all qualitative responses to the survey, as well as the in-depth patient interviews. Final themes were agreed by consensus of the All.Can international research and evidence working group and Quality Health. The most relevant and illustrative quotes supporting these themes were then selected to substantiate each section in the report.

Qualitative responses were not quantitatively analysed due to the significant cost that translations and coding would have entailed on such a large sample. In addition, as not all respondents answered the open-ended questions, it would not be possible to give an accurate estimation of what proportion of all respondents might agree with each comment. Therefore, we have expressed these findings throughout the report as 'respondents' in the plural – without quantifying how many this concerned in each instance.

Report structure

The report is organised into four themes that emerged from our findings. These themes closely mirror the closed-ended questions in the survey, which focused on specific areas known from previous research to be important to patient care. However, open-ended free-text questions allowed respondents to mention other areas of importance to them. As these responses were captured in the thematic analysis described above, they also contributed to our selection of the four themes highlighted in this report.

A selection of patient stories based on the post-survey patient interviews are also included throughout the report, providing more context and insights into the relevant sections. Names and some other identifying details have been changed to protect the anonymity of those respondents who shared their stories with us.

Country-level findings

Country-level findings are reported in **Appendix 1**. These findings are unweighted and therefore not directly comparable between countries. Individual country reports were only developed where the survey had more than 50 responses, namely for Australia, Belgium, Canada, Italy, Poland, the United Kingdom and the United States. Country-level reports were not developed for France, Spain and Sweden.

Limitations

Respondents participated in the survey voluntarily, therefore they are self-selected and represent the perspectives of patients who wanted to have their voices heard and were able to complete the survey. They do not necessarily reflect the perspectives of all cancer patients.

As the survey was primarily distributed online, it was limited to those who had access to the internet, were active on social media, or connected with a national or international patient organisation that shared the survey.

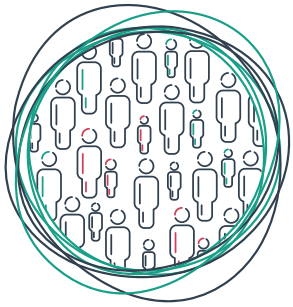
The survey was focused on patient experiences and processes of care and therefore did not include any questions regarding specific treatments or interventions.

Finally, it is important to mention that this report is focused on reporting the findings of the survey, and as such, we have not conducted an in-depth analysis of what improvements in health outcomes and overall efficiency of care could be achieved if the issues highlighted in this report were adequately addressed. All.Can is committed to exploring these questions further and it is our hope that this report may also encourage others within the research and policy community to do the same.

For more information

The survey questionnaires and full methodology may be found on the All.Can website (www.all-can.org/what-we-do/research/patient-survey) along with other survey materials not included in this report – including further patient stories drawn from interviews conducted as part of the survey.

All.Can patient survey: findings



Introduction

The past decade has seen transformational advances in cancer care. As the prevalence of cancer increases, governments and health systems around the world are struggling to fund these advances – and notions of value, efficiency and affordability have become increasingly important in the cancer policy debate. At least one fifth of total healthcare spending is thought to be wasted on inefficient care.[9] Moreover, removing wasteful or ineffective interventions could lead to a gain of approximately two years of life expectancy in industrialised countries.[10] Within this landscape, leading researchers and policy experts are trying to identify where inefficiencies lie, in order to pave the way for sustainable cancer care.[11-13]

Unfortunately, the patient perspective is too often forgotten in current definitions of value and efficiency.[14] Existing definitions are most often driven by health economists and healthcare professionals, with outcomes measures often based on processes that are easily measurable within healthcare systems, rather than on outcomes known to matter to patients.[14 15] Yet patients are, arguably, the only people who have full sight of the impact of their condition and care experience on their physical, emotional and mental wellbeing.[14] Their perspectives must, therefore, be built into any definitions of value and efficiency.

All.Can defines inefficiency as the allocation of resources to anything that does not focus on what matters to patients.[16] Our aim is to find sustainable solutions to improving cancer care. To guide these efforts, we need to gain a better understanding of where patients perceive their care is not focused on what matters to them – and find practical ways to remedy any gaps.

The All.Can patient survey was designed with this purpose in mind: we asked patients where they had encountered inefficiencies in their care, and where efforts were most needed to improve efficiency. We made our definition of inefficiency explicit throughout the survey.

This report presents the main themes that have emerged from our findings. While most respondents reported that their needs were sufficiently addressed, the findings also show that there is clear room for improvement. Each of the themes represents an opportunity for improving cancer care from the perspective of patients.

Nearly 4,000 respondents from more than 10 countries participated in the survey – making this, to our knowledge, the largest international survey specifically aimed at obtaining patient perspectives on inefficiency in cancer care. It is our hope that the insights gathered in this report may help guide patient-driven policies to improve the efficiency and sustainability of cancer care.

Key opportunities

to improve efficiency from the patient perspective:

1. Swift, accurate and appropriately delivered diagnosis
2. Information, support and shared decision-making
3. Integrated multidisciplinary care
4. The financial impact of cancer

1

Swift, accurate
and appropriately delivered diagnosis[i]

Key findings

- The way diagnosis is communicated was found to be important. Respondents reported a lack of empathy from physicians and poor timing – such as being told they had cancer without a family member present, or having to wait several days to speak to a specialist.
- When asked to select the one area of cancer care where they experienced the most inefficiency, **26%** of respondents chose diagnosis – more than any other area of cancer care.
- Across all cancer types, **58%** of respondents had their cancer diagnosed outside of a screening programme.
- Among respondents whose cancer was detected outside of screening:
  - Delayed diagnosis (>6 months) was associated with a more negative respondent view of all aspects of care and support
  - The speed of diagnosis varied significantly by cancer type
  - Nearly a third (**32%**) reported that their cancer was initially diagnosed as something different.

‘No procedure was useless; everything happened very quickly and efficiently. Ultra-fast and professional support.’ Respondent from Belgium

‘Everyone was vague about my diagnosis. No one wanted to commit. I had to press the surgeon for a direct response. It took too long for a final diagnosis.’ Respondent from the United States

i Please note that in this survey, patients were asked only about their experience of diagnosis from the moment they contacted the doctor or were seen as part of a screening programme.

Overview of findings

The way diagnosis was communicated was very important to respondents.

A theme that emerged from qualitative responses was that respondents[ii] sometimes felt that their instincts were not listened to by doctors – even when they themselves thought their symptoms may be related to cancer. This was mentioned particularly by younger respondents.

> ‘After seeing multiple doctors, not one of them thought my symptoms could be related to cancer as I was 15 at the time and “too young” for a cancer diagnosis.’ Respondent from Australia

> ‘I had delays in diagnosis, and, above all, I felt I was made a fool of about the symptoms I had – they were trivialised as an intestinal virus and anxiety.’ Respondent from Italy

In qualitative responses, respondents often reported a lack of attention and empathy in how doctors communicated the news of their diagnosis. Respondents said they would have liked more time to discuss things and digest information.

> ‘Make the diagnosis in a softer way and take a little more time for it.’ Respondent from Belgium

> ‘My GP just told me he would be surprised if I didn’t have leukaemia as he looked at a blood test done for another issue… What was I supposed to do with that information?’ Respondent from Canada

Respondents also expressed concern that information was sometimes withheld from them – including the fact that they had cancer. There were comments indicating that the different steps in their diagnosis were not explained enough, or in an understandable way.

ii As explained on page 14 (About this survey), where this report refers to ‘respondents’ without a specific percentage, we are referring to qualitative findings. These findings cannot be quantified as not all respondents answered the open-ended questions, so it would not be possible to give an accurate estimation of what proportion of all respondents might agree with each comment.

> ‘I wish I had been told the whole truth from the start instead of diminishing it. I was the one to use the word “cancer” for the first time.’ Respondent from Belgium

> ‘Nothing would have changed the diagnosis, but the way I was treated and lack of communication made a difficult time horribly upsetting for me, my friends and family.’ Respondent from the United Kingdom

The timing of delivering the diagnosis is also key. Respondents commented that doctors should make sure people are not alone when receiving their diagnosis and are given a point of contact for any questions that will inevitably arise after they recover from the initial shock.

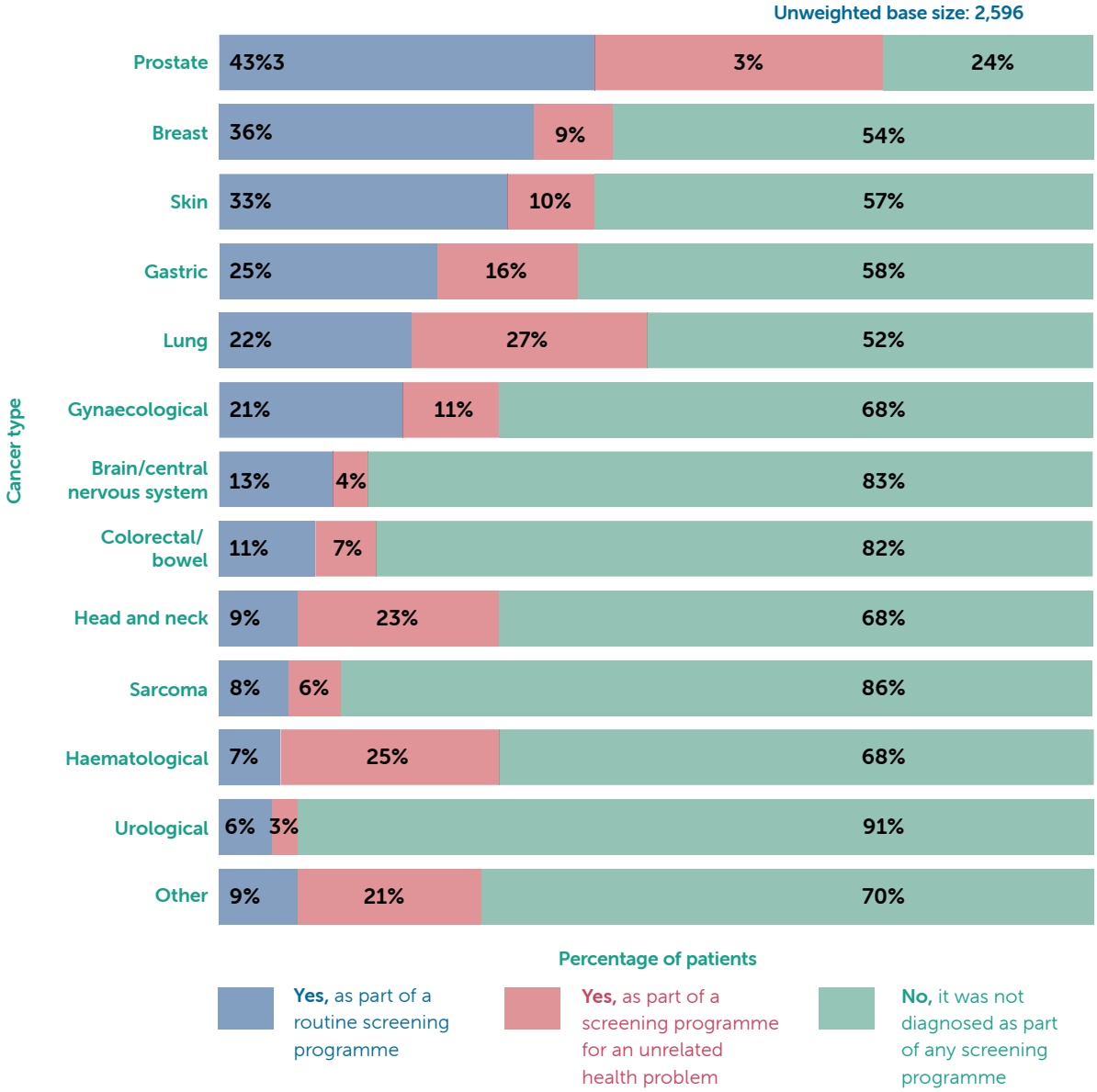
> ‘A nurse called on a Friday and gave me the biopsy results and said a doctor wouldn’t be available to speak to me until Monday. Worst weekend ever.’ Respondent from the United States

> ‘I was told over the phone that it was melanoma and I was being booked with a surgeon, but wasn’t given any other information, so it was extremely stressful.’ Respondent from Canada

The proportion of respondents whose cancer was detected by screening varied by cancer type.

Overall, **26%** of respondents recalled that their cancer was detected through a routine cancer screening programme and **17%** stated their cancer was detected through screening for a health problem unrelated to cancer. The remainder (**58%**) had their cancer detected outside of any screening programme (**Figure 1**).

Figure 1. Was your cancer diagnosed as part of a routine screening programme, or as part of a screening programme for an unrelated health problem?



For all respondents, regardless of whether their cancer was detected through screening, diagnosis was one of the main areas where they reported the greatest inefficiency.

When asked to select the one area of cancer care where they experienced the most inefficiency, **26%** of respondents chose diagnosis – more than any other area of cancer care. As might be expected, this was highest among respondents whose cancer was diagnosed outside of screening (**31%**), compared to **18%** among respondents whose cancer was detected through a routine cancer screening programme and **13%** among respondents whose cancer was detected through screening for an unrelated health problem.

During the whole of your cancer care and treatment, where do you feel there was most inefficiency?

(Respondents were asked to select one option only)

- **26%** my initial diagnosis
- **21%** dealing with the ongoing side effects
- **14%** getting the right treatment for my cancer
- **12%** dealing with the psychological impacts
- **10%** dealing with the financial implications
- **5%** the opportunity to take part in clinical trials
- **2%** access to patient support groups
- **10%** other*

* The most frequently reported 'other' sources of inefficiency included coordination between different elements of the healthcare system (e.g. general practice, social services and hospital), inefficiency around organising appointments, general delays, follow-up care, and getting the right information and communication.

For cancers detected outside of a screening programme, the speed of diagnosis had a major impact on respondents' experience across the entire care pathway.

'I lost valuable time having to wait three months to secure an appointment with the specialist after I noticed symptoms.' Respondent from Belgium

Why it matters

For many cancers, early diagnosis can improve survival[17] – for example, a breast cancer study showed that patients who experienced short delays in diagnosis (under 3 months) had 7% better overall survival compared with those who had longer delays (3–6 months).[18 19]

Early diagnosis is associated with reduced treatment costs – the cost of treating colon, rectal, breast, ovarian and lung cancer at stage IV has been reported as 2–3 times the cost of treating these cancers at stage I.[20]

People whose cancer diagnosis took longer gave more negative scores on virtually every question in the survey, particularly in terms of information and support (**Table 1**).

Table 1. Respondents' negative perceptions of information and support, by time taken to receive diagnosis

Survey questions	Unweighted base size*	% of respondents who answered 'No' to each question**					
		Overall	Respondents whose diagnosis took...				
			< 1 month	1 to 3 months	3 to 6 months	6 months to 1 year	> 1 year
Were you involved as much as you wanted to be in deciding which treatment options were best for you?	3,124	15%	14%	16%	14%	22%	30%
Have you always been given enough information about your cancer care and treatment, in a way that you could understand?	3,650	31%	24%	38%	36%	44%	44%
Have you always been given enough information, in a way that you could understand, about signs and symptoms to look out for that your cancer might be returning/getting worse?	2,627	35%	30%	40%	31%	48%	51%
Were you given information about patient groups, charities and other organisations that might be able to support you through your diagnosis and care?	3,717	41%	40%	43%	41%	54%	52%

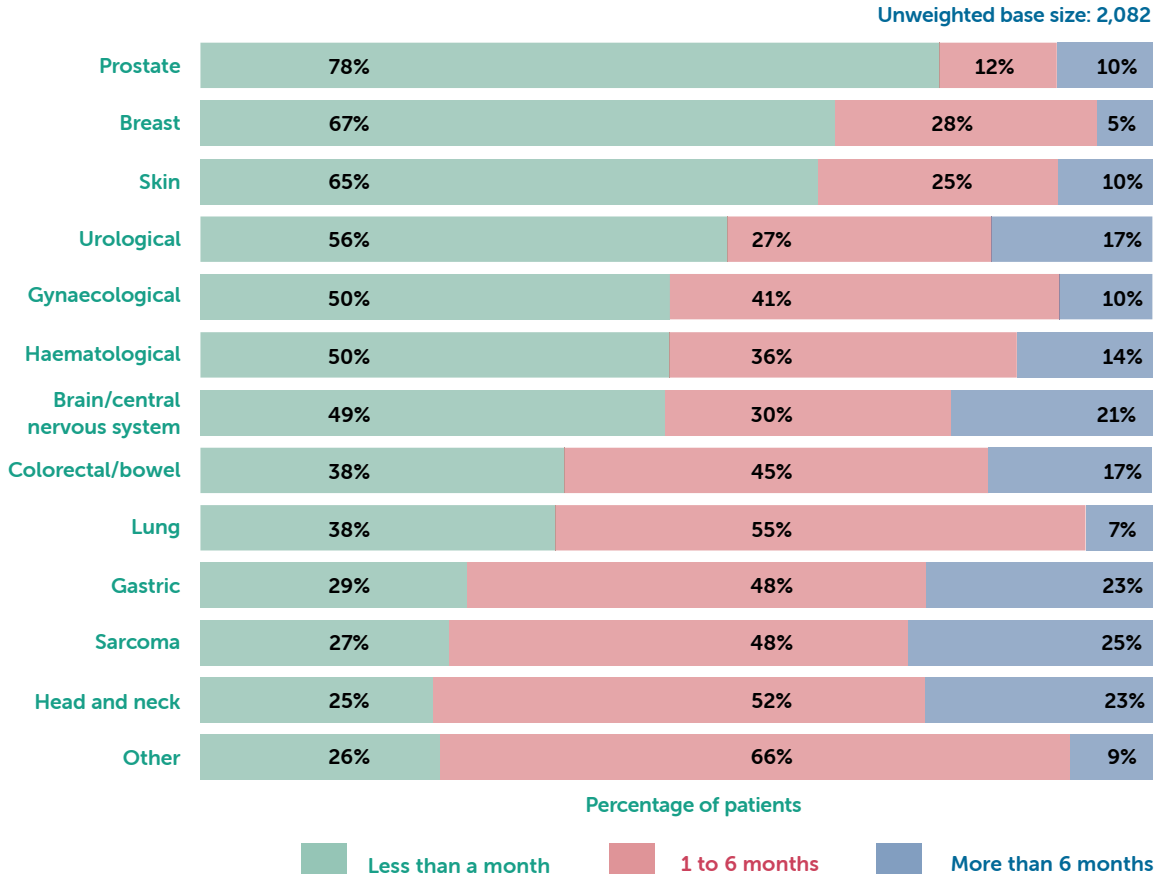
* Total number of people who responded to each question

** **Interpreting this table:** the 'Overall' column gives the proportion of all respondents who answered 'no' to each question in the first column, and the other columns are broken down by the time taken to diagnose the cancer. For example, 15% of respondents overall reported that they were not involved as much as they wanted to be in deciding which treatment options were best for them; for those diagnosed in less than one month, this figure was 14%, but for those whose diagnosis took more than one year, it was 30%.

For cancers detected outside of a screening programme, the speed of diagnosis varied considerably by cancer type.

Nearly **80%** of prostate cancer respondents said their cancer was diagnosed in less than a month, while for head and neck cancer respondents this was only **25%** (**Figure 2**).

Figure 2. Time to diagnosis, by cancer type (among respondents whose cancer was detected outside of a screening programme)



What we know

Late diagnosis and misdiagnosis are common in many cancers and can lead to delays in treatment or limited treatment options, poorer outcomes, lower likelihood of survival and higher costs of care.[16 18]

Diagnosis may be delayed for various reasons, including patient-related factors (e.g. lack of awareness of symptoms) and system-related factors, including availability of specialists, speed of referral, fast access to imaging, pathology capacity and other factors. The complexity of the process of clinical evaluation, diagnosis and staging may also vary by cancer type.[18]

Early diagnosis is not equally feasible for all cancer types. Cancers that have clear signs and symptoms and effective treatments (e.g. breast cancer) tend to benefit most from early diagnosis.[18]

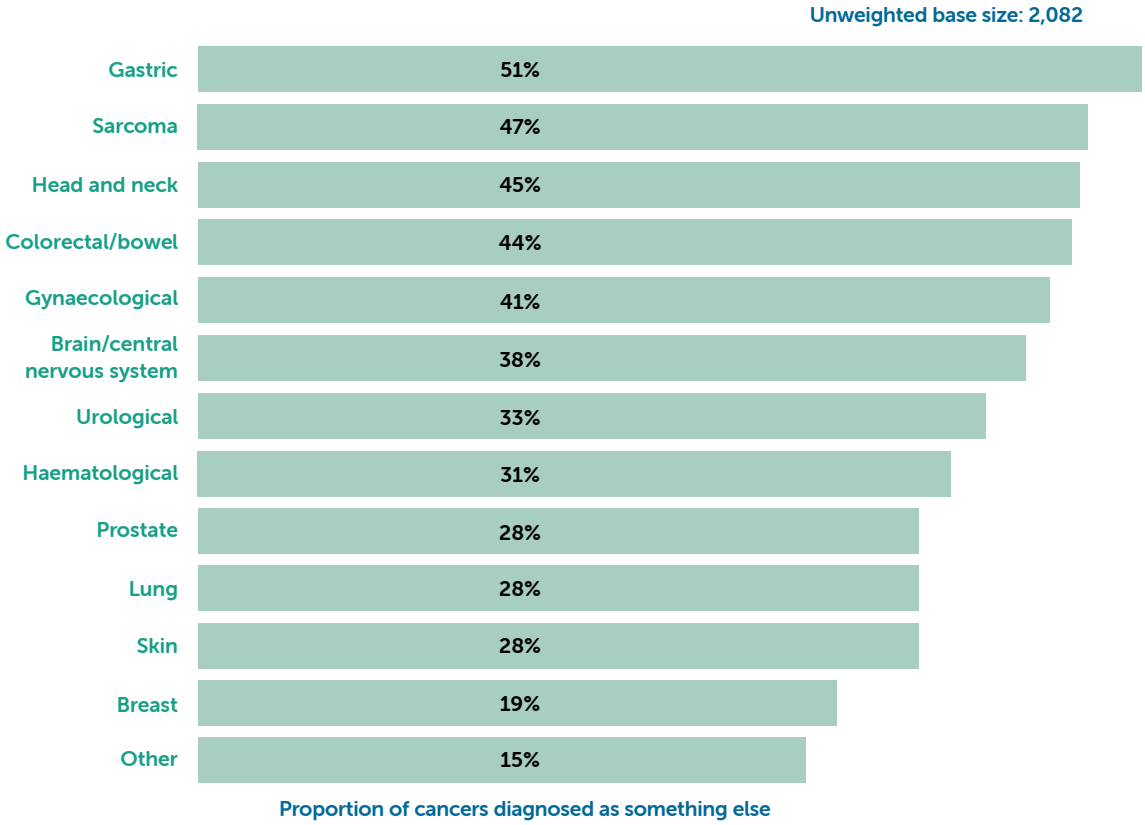
For some cancers (e.g. colorectal), early diagnosis – before symptoms start to show – is crucial to allow time for effective treatment options. This emphasises the importance of screening for early detection.

One reason for delays in diagnosis for respondents whose cancer was detected outside of a screening programme was that they were diagnosed with something else before eventually receiving a correct diagnosis of cancer.

- **32%** of respondents whose cancer was not detected through a screening programme reported that their cancer was diagnosed as something else (initially or multiple times).

This varied between cancer types, with over half (**51%**) of gastrointestinal cancer respondents having been diagnosed with something else, once or many times, whereas for breast cancer respondents this was significantly less at **19%** (**Figure 3**).

Figure 3. Proportion of cancers diagnosed as something else, once or multiple times, by cancer type (among respondents whose cancer was detected outside of a screening programme)



Patient stories

Deborah* (United Kingdom)

I had random abdominal pain, which the GP thought was a kidney infection. When antibiotics didn't help, I was referred to gynaecology at my local hospital. They weren't expecting me when I arrived, and were very rude. I was in so much pain, and they thought I was making it up. The whole experience was horrendous.

It took about a month to work out what it was, when I eventually had the right scan. The doctor said, 'There's a mass near your kidney. There's nothing we can do about it now – it's Friday night. Don't worry about it. Go home and we'll be in touch next week.' He wrote my discharge summary, which obviously the patient isn't supposed to read. It said I had a 10cm tumour – potential lymphoma. I read that in the car on the way home with my children. That's how I found out that things were not good.

The scan was in July, and I had to wait until the end of November before I had surgery. I have a very aggressive form of cancer and to have to wait so long for surgery was completely unacceptable. It took them three months to get all the scans in order because it was the summer holidays.

I had one appointment with my surgeon and the letter was never sent to me. I got a phone call about five days after my diagnosis, asking why I wasn't at my appointment. The letter arrived three days after the appointment was supposed to happen. At that point, you feel like it is the end of the world.

Ten weeks after my surgery, I had another scan. The disease had spread to my bones. They found other tumours, including one in my liver.

I had further major surgery. Then I read about a new drug and proactively referred myself to a medical trial. Surgery is the main option for sarcoma, but you get to the stage where they cannot keep operating. I had to demand to see my oncologist. I never met her before that point – maybe if I had met her after my first surgery, my cancer might not have metastasised. Anyway, they were trialling the new drug at a hospital in another city and I asked if she could refer me. It took a while to get onto the trial. I would have started chemo a lot earlier if I had stayed at my first hospital, but it was definitely a good decision. When you have a rare cancer, you're going to have to travel. It's just what you do to get the best treatment. I was more than happy to go where I was referred.

I was given amazing emotional support through the hospital's charity. I'm having counselling, which I found out about through the research team. But this is the thing: there are all sorts of things available, but it is finding out about them. The counselling only came about from a nurse making a throwaway comment. Similarly, I found out there's an acupuncture team, but only because the radiologists mentioned it.

I've had some hideous experiences – like being told my cancer had spread by someone I'd never met before. Several times, I have been made to feel that I'm making things up. I've had to see doctors who know nothing about my disease and write incorrect follow-up notes. It takes weeks to unpick that sort of stuff – it's a waste of time and energy.

The computer systems are ridiculous. If I have to go into Accident & Emergency, I take a copy of all my scans and notes, as they won't be able to access them. They won't know what drug I'm on because they'll never have heard of it. I've seen about 50 people over the last year. It can't be that difficult to find someone to provide some continuity of care.

Communication between departments shouldn't be siloed. I once had genetic blood tests and they wouldn't fax the form from one hospital to another, so I had to physically drive to one hospital, pick up a piece of paper, drive to the other hospital, wait for two hours for a blood test – and then they lost the test, so I had to do it again. It's archaic – why can't they just email my doctor?

It would have been better if I'd been listened to. When people say you're making it up, and you know something is wrong, you almost need your GP, or someone who knows you, to speak for you. I know that some people do make things up, and they have to deal with that, but I had a 10cm tumour. They should take patients seriously. I was made to feel like I was nothing from the moment I walked into the hospital. I was shouted at! I can still remember the nurse who did it – she was clearly having a really bad day, but that was no excuse. When you go into this profession, you have to be professional. Be nice!

They should treat patients like people – that's their biggest job. That's what I would say to anyone coming into this: make them see you as a person.

* Names and some other identifying elements have been changed to protect patients' anonymity.

2

Information, support
and shared decision-making

Key findings

- Respondents reported that too much information being given at once was sometimes overwhelming, and they would have preferred to receive relevant information at appropriate points along the entire care pathway.
- Almost half of respondents (**47%**) did not feel sufficiently involved in deciding which treatment option was best for them.
- Nearly two in five respondents (**39%**) felt they had inadequate support to deal with ongoing symptoms and side effects.
- Close to a third of respondents (**31%**) felt that they lacked adequate support for dealing with pain.
- More than a third of respondents (**35%**) felt inadequately informed about how to recognise whether their cancer might be returning or getting worse.
- **41%** of respondents were not given information at the hospital about available peer-support groups.
- Gaps in information and support were more prevalent among people with more advanced cancers.

'The disconnect between the language my haematologist uses and common language has been frustrating.' Respondent from Canada

'I don't want more information, but better information.' Respondent from the United States

Overview of findings

Respondents often felt overwhelmed by the information they received.

A recurring theme in qualitative responses was that respondents felt overwhelmed by all the information they received at the point of diagnosis, and would rather have had information provided at each stage of their care.

> 'It would have been good to have access to resources at appropriate points during treatment i.e. before surgery, before radiation. I found I was given all the information at once, which was too much.' Respondent from Australia

Respondents also spoke of a disconnect between the language used by their doctors and what they could understand. They often did not know where to begin or what to ask, as the experience of cancer was new to them.

> 'How can you ask a question when you have never had chemotherapy before? It's like being given a lemon meringue pie and not knowing what it tastes like until you try it!' Respondent from Australia

There were also comments that the information provided was not always tailored to the patient's individual experience or stage of treatment.

> 'Some of the information was not relevant to my situation. To go through all the information was beyond what I was up for, so most of it remained unread.' Respondent from Canada

What we know

Information needs vary from one patient to another and are influenced by many factors.[21 22] They also change along the care pathway.[23]

Many studies show that patients often do not fully comprehend what their diagnosis, prognosis and treatment mean. This can be due to them not fully understanding the terminology used, not receiving all relevant information or not being able to recall what they have been told during medical appointments.[22 24-26]

Why it matters

Part of quality healthcare delivery is understanding what patients want to know and providing that information at the right time in an understandable way.[27 28]

Information can help patients feel in control of their disease, reduce anxiety, create realistic expectations, and promote self-care and engagement in their care.[22 23] Fulfilling patients' needs for information is also associated with improved treatment adherence[21 29 30] and better clinical outcomes.[22 30]

Respondents were often not sufficiently involved in decisions about their care or provided with enough information about their treatment options.

Almost half of respondents (**47%**) did not feel sufficiently involved in deciding which treatment option was best for them, and nearly a third (**31%**) felt they were not always given enough information about their treatment and care.

Have you always been given enough information about your cancer care and treatment, in a way that you could understand?*

- Yes: **69%**
- No, I was given information, but could not understand it all: **14%**
- No, I was not given enough information: **16%**

Were you involved as much as you wanted to be in deciding which treatment options were best for you?

- Yes: **53%**
- Yes, to some extent: **32%**
- No, I would have liked to be more involved: **15%**

*** All percentages are rounded to the nearest whole number, so may not total 100%**

'Options could have been explained a little better. I had a new procedure done and thought I was cured.'
Respondent from the United States

Why it matters

Studies have shown that shared decision-making is associated with improved patient outcomes.[34] Treatment decisions can change after patients become well informed – with many choosing fewer treatments – and there is a substantial gap between the outcomes patients prefer and the outcomes that doctors think patients prefer.[35]

What we know

Patient empowerment – including involving patients in shared decision-making and providing information to help them better understand their condition – is widely recognised as being an enabler of high-quality and sustainable healthcare.[31-33]

Shared decision-making should involve enquiring into patients' goals for their treatment, providing evidence-based information about treatment options, and having systems for recording and implementing patients' treatment preferences.[7 8]

Respondents needed more and better guidance on how to deal with ongoing side effects – especially after treatment was over.

Dealing with ongoing side effects was perceived as a major source of inefficiency, with **21%** of respondents saying it was the greatest source of inefficiency in their care. This was the second highest-reported area of inefficiency overall, after diagnosis (**26%**).

> 'I think we do not take the aftermath into account enough. Treatments... help heal cancer but destroy other things. Life becomes different after and many "little sores" occur, with which one must live.' Respondent from Belgium

In some comments, respondents said they had little warning of what the most common side effects were or how to deal with them – especially once they were no longer having **active treatment**.

> 'The side effects are more than just a nuisance and need real recognition.' Respondent from the United Kingdom

> 'Perhaps a clinician could go over the fine details on the usage of the drugs. I'm having to learn the do's and don'ts via the internet rather than someone attached to my particular case.' Respondent from Canada

What we know

Evidence shows that, at the end of their treatment, cancer patients may be left to deal with consequences of treatment that could have been managed or avoided altogether.[36 37]

Long-term consequences and effects of having cancer and its treatment can include physical effects, chronic fatigue, sexual difficulties, mental health issues and pain. Problems can persist for up to 10 years after treatment, or even longer, and may lead to social isolation and financial difficulties due to disruption to work.[38]

Respondents were not always given adequate information about pain management and palliative care.

- **31%** of respondents said they were never, or only sometimes, given enough information and care to deal with the pain they experienced.

Although **69%** of respondents said they received enough information and care to manage their pain, this was not the case for all respondents. Further, in the qualitative comments, respondents suggested that their worries or the pain they experienced were sometimes dismissed.

> ‘Although I kept saying that my pain was 7–8 out of 10, each time I returned to the room for chemotherapy, I was never advised or directed to solve this problem. I was the one to take charge… but it took (and still takes) a lot of energy.’ Respondent from France

Why it matters

Without adequate assessment of patient needs – both during and after active treatment – suboptimal service use (overuse or underuse) may occur. This can have a negative impact on patient outcomes and costs incurred for healthcare systems.[39]

More individualised approaches to follow-up versus a one-size-fits-all approach may have benefits as well – for example, by supporting patients in self-managing their condition.[37]

In England, it is estimated that follow-up costs £1,554 per patient over a five-year period (equivalent to 4–5% of the total national cancer budget) but one study showed it may be possible to save up to £1,000 per patient through a stratified approach to follow-up, pathway efficiency and better management of comorbidities.[37]

Respondents who had access to comprehensive palliative care services reported great satisfaction with this aspect of their care. However, a number of respondents said that palliative care was not discussed with them as an option when they themselves thought it could be helpful.

> 'I was referred to the palliative service, which provided much more supportive care than I had ever imagined I could receive. I was given support, counselling, specialist advice, which was invaluable.' Respondent from Australia

What we know

Many cancer patients experience unnecessary pain – studies suggest that one in three cancer patients do not receive pain medication appropriate to their pain level.[40]

Palliative care is often assumed to be solely focused on end-of-life care – but, in fact, it is much broader. The World Health Organization defines it as an approach that improves quality of life for people (and their families) with life-threatening illnesses – including pain relief and psychosocial support.[6]

Guidelines recommend that the need for palliative care should be built into treatment plans early in the course of illness, in conjunction with therapies that are intended to prolong life, such as chemotherapy or surgery.[6 41]

Why it matters

Early integration of palliative care can lead to improved symptom control and reduced distress through treatment and care delivery that matches patients' preferences – and overall improvements in patient outcomes, quality of life and survival.[42-44]

It can also significantly improve patients' understanding of their prognosis over time, which may impact treatment decisions about end-of-life care and lead to less aggressive treatment.[44 45]

What we know

Studies have shown that patients' information needs are often highest, and least well met, during the phase following active treatment.[30] An effective handover from secondary care to primary care, with regular and timely follow-up, is therefore necessary for all patients.[39]

Respondents often lacked information about how to tell whether their cancer might be coming back.

Another important gap frequently expressed in qualitative comments was the lack of information on how to deal with possible signs and symptoms that cancer might be recurring. This led to significant fears for respondents, not knowing whether a symptom they experienced was harmless or a cause for greater concern.

> 'I don't think professionals really understand how much we fear recurrence.'
> Respondent from the United Kingdom

> 'I would like to understand better how I will be able to monitor the risk of recurrence when I am no longer receiving regular follow-up after my hormone therapy.'
> Respondent from Belgium

Why it matters

Without appropriate follow-up after discharge, patients can feel lost or abandoned, and ill-prepared to manage their condition, after weeks of intensive treatment and frequent interactions with their care team.[46]

Respondents were not always given information about available patient groups or peer support.

- **41%** of respondents said they were not given any information about patient advocacy groups, charities or other organisations that could support them.

In addition to information and support they may have received from their care team, respondents expressed the value of being able to speak to people who had been through a cancer diagnosis themselves.

> 'I would suggest that anyone with any type of cancer look for others that have that type of cancer, and help each other. Only those who are going through cancer, or have gone through it, really understand.'
> Respondent from the United States

Why it matters

Even with the support of family and friends, many people who have cancer find it helpful and comforting to talk with others who have already gone through the experience first-hand, to discuss all aspects of how to deal with cancer and its impact on life. Patient organisations often help provide this peer support to patients. They can fill important gaps in patients' needs, providing emotional support and financial advice, as well as valuable information about treatment options and available services.[47]

Not all healthcare professionals may feel comfortable or able to distribute patient support-group information. This presents a missed opportunity as doctors are usually the main source of information connecting patients to support groups.[48]

Overall, gaps in information and support were found to be greatest for respondents whose cancers were diagnosed at an advanced stage.

Across almost all questions relating to information, respondents whose cancer was diagnosed at a more advanced stage reported gaps more often than those with earlier-stage cancer (**Table 2**).

Table 2. Respondents' negative perceptions of information and support, by stage of disease at time of diagnosis

Survey responses	Unweighted base size*	% of respondents who answered 'No' to each question**		
		Overall	Respondents whose cancer had not spread to other organs at diagnosis	Respondents whose cancer had spread to other organs at diagnosis
Were you involved as much as you wanted to be in deciding which treatment options were best for you?	3,124	15%	12%	22%
Have you always been given enough information about your cancer care and treatment, in a way that you could understand?	3,650	31%	26%	39%
Have you always been given enough information, in a way that you could understand, about signs and symptoms to look out for that your cancer might be returning/ getting worse?	2,627	35%	31%	39%
Do you feel you have always been given enough support to deal with any ongoing symptoms and side effects, even beyond the phase of 'active' treatment (if applicable)?	3,623	39%	34%	54%

* Total number of people who responded to each question

** **Interpreting this table:** the 'Overall' column gives the proportion of all respondents who answered 'no' to each question in the first column, and the other columns are broken down by how advanced the cancer was at point of diagnosis. For example, 15% of respondents overall reported that they were not involved as much as they wanted to be in deciding which treatment options were best for them; for those whose cancer had not spread at diagnosis, this figure was 12%, but for those whose cancer had already spread by the time of diagnosis, it was 22%.

What we know

There is evidence that support services available for cancer patients may not always be appropriate for patients with more advanced disease. For example, a comprehensive survey of breast cancer patients in Australia found that those with metastatic breast cancer found available support less adapted to their needs compared to patients with earlier-stage disease.[49]

Patient stories

Julie* (Belgium)

Two weeks after noticing a lump in my breast, I saw my GP and was referred for a mammogram. They told me it was probably benign but suggested I get it removed just in case. I had a biopsy, and the people at the research centre said everything looked OK and that I shouldn't be too worried about it – so I felt really reassured. When I got the results a few weeks later from my gynaecologist, he told me they'd found some bad cells in my breast. I didn't know what that meant exactly, but I knew there was something wrong. When I got emotional, he asked, 'Didn't you expect this?' The way he gave the diagnosis was very hard for me; I found it unprofessional and it felt like he didn't take me seriously.

The breast care nurse was at the appointment and, after the conversation with the gynaecologist, she had all the time in the world to answer questions. She was very understanding, gave us a lot of information and made me feel it was OK to be emotional. It still wasn't clear to me whether I had cancer or not, so the nurse had to tell me. Throughout my cancer treatment, the breast care nurses have always been a great support.

I wasn't really involved in discussing my treatment plan. They never gave me the option to choose an alternative location or seek a second opinion. Now, I know that I actually had a choice about certain things, but at the time I just trusted the doctors. It gave me a good feeling that my treatment plan was designed by a team of doctors. I've had various treatments: mastectomy, tissue expansion, radiation, anti-hormonal therapy, chemotherapy and a breast reconstruction. If I had the choice now, I wouldn't have had a breast reconstruction. I wasn't well informed about the rehabilitation. It's been very hard. I have a very tight tummy now, which makes walking difficult and causes heavy back pain. They also removed my lymph nodes, which gave me a very big arm. All these things cause me a lot of stress.

My illness had a huge effect on my marriage, which ended in a divorce. The emotional impact was huge. But I'm very happy with the psychological support I received from the breast care nurses and my friends. I practised mindfulness for cancer patients and my kids got support from the hospital as well.

I had so many questions, but they all needed to be answered by different doctors. It would have been nice to have an appointment with all the doctors at once, so I could ask all my questions at the same time. It would be less time-consuming and would have cost me less energy – energy I didn't have.

I would like to tell other patients that you have a choice about certain treatments. If you make your own decisions, you will probably feel a lot more in control – and that will make you feel stronger.

* Names and some other identifying elements have been changed to protect patients' anonymity.

3

Integrated
multidisciplinary care

Key findings

- Respondents commented that specialist cancer nurses had played a critical role – acting as their 'navigators' and helping them adapt all aspects of their lives to cancer – both during and after treatment.
- Nearly a quarter of respondents (**24%**) felt that support from allied health professionals (dieticians, physiotherapists etc.) was not always available.
- Respondents wanted more information about what they could do to support their treatment and recovery in terms of diet, exercise and complementary therapies.
- **69%** of respondents said they needed psychological support during or after their cancer care. However, of these, **34%** said it was not available.
- Many respondents expressed concern for the impact their cancer had on their families, and wanted psychological support for them as well.

> 'There needs to be a plan made for each cancer patient, so a person doesn't have to explain to each healthcare person what is going on and why the cancer patient needs help.' Respondent from Canada

> 'Psychological support should not just be offered in the form of a brochure stating, "If you need help, you can get it here." Many people will say they are "coping" when, in reality, they need support readily at hand.' Respondent from Australia

Overview of findings

Cancer nurses played a critical role in coordinating care for respondents.

Respondents often reported a lack of communication between their primary care physician and specialists – particularly in countries with a primary-care-led model (e.g. Canada, Australia and the United Kingdom). Respondents reported the impact of this lack of communication from diagnosis onwards – and again after they had left the hospital setting and were in the follow-up stage of care in the community setting.

> 'There needs to be more communication between healthcare providers... don't tell the patient to ask the surgeon, who then refers you back to your doctor, and this keeps going on and on.' Respondent from Canada

> 'I needed one central point of contact for everything but also one place to go for everything too. I have been under the care of three different hospitals with appointments for different procedures, tests etc. at different locations – it takes a lot of energy. I have had to become, in effect, a manager rather than a patient.' Respondent from the United Kingdom

Respondents commented that specialist cancer nurses had played a vital role in remedying these communication and coordination gaps – acting as the patient's companion and 'navigator' through all phases of care.

> 'The reference nurse in oncology has been very helpful and has always directed me to the appropriate specialist doctor without ever underestimating my problems. It is a real asset to have such a reliable person.' Respondent from Belgium

What we know

In 2014, the European CanCer Organisation (ECCO) endorsed cancer nurse specialists as an essential part of the multidisciplinary care team.[50]

Ideally, a cancer nurse specialist acts as a central point of contact for patients, helping them navigate through diagnosis, treatment, follow-up and, if necessary, end-of-life care.[50 51]

Cancer nurses can help ensure clear communication with patients and their families and other healthcare professionals; they can address patients' emotional, psychological, financial and social needs and offer information, advice, support and reassurance.[51 52]

Why it matters

Having a cancer nurse specialist has been shown to improve outcomes for patients and reduce associated costs of care, due to:

- reduction of symptoms[53 54]
- improved patient knowledge and self-management[53 54]
- improved management of chronic problems[54]
- faster care pathways, allowing more patients to be seen[52]
- reduced rates of emergency admissions and shorter hospital stays[54-56]
- fewer follow-up appointments.[54]

Data also suggest that this specialist role may bring overall savings to healthcare systems, with one report in the United Kingdom suggesting that introducing specialist nurses into the cancer care pathway could save about 10% of cancer expenditure.[52]

Access to allied health professionals and complementary care was often perceived as inadequate by respondents.

Almost a quarter of respondents (**24%**) said they did not have access to support from **allied health professionals**. In qualitative findings, respondents commented that they would have liked to be told what role these different professionals or services could play in aiding their recovery.

> ‘It was difficult to find a physiotherapist. The importance of this [role in my recovery] was not explained.’ Respondent from France

> ‘They should actually involve additional specialists (i.e. dietitian, physiotherapist, psychologist) in the treatment of the patient from the moment of diagnosis. The content provided by all physicians should be consistent. I would avoid frightening a patient by focusing on how difficult and demanding their treatment is, and focus more on the goals to be achieved after treatment.’ Respondent from Poland

In addition, respondents reported that they were not always provided with enough information about how they could optimise their care through diet, exercise, mindfulness and complementary therapies. In many countries, respondents had to pay **out-of-pocket** for these services.

> 'I had to source my own information regarding complementary therapies... These were quite expensive, and yet they should be offered as part of cancer treatment, in my opinion.' Respondent from Australia

> 'I would have liked to know more about the food that should be eaten, the quality of life I could expect...' Respondent from Spain

What we know

The added value of complementary therapies is widely recognised among international cancer societies. For example, the American Cancer Society recommends a selection of evidence-based complementary therapies as part of integrated care: music therapy, meditation, yoga and relaxation, massage, acupressure and acupuncture.[57]

Complementary approaches can be important for patients' care, wellbeing and recovery.[58] Doctors do not necessarily need to provide these components of care, but they should be able to signpost patients to relevant services.[49]

Psychological support was often unavailable to respondents.

A recurring comment from respondents was that their psychological or emotional needs were not sufficiently addressed by their cancer care team. More than a third of respondents (**34%**) who wanted psychological support said it was unavailable to them.

- **69%** of respondents said they needed psychological support during or after their cancer care. But **34%** of these respondents said it was not available.

> 'I think the psychological involvement part is forgotten. It is true that the main thing is to survive, but it is also necessary to feel accompanied and understood.' Respondent from Spain

Even when psychological support was available, it was not always felt to be helpful.

In qualitative comments, respondents mentioned being referred to professionals who did not have a sufficient understanding of cancer to provide any meaningful help to them or their families. In some instances, support was inappropriate or even hurtful to respondents.

‘I needed someone to talk to; I was given written information.’
Respondent from France

‘On the emotional side, I had to deal with everything myself. A psychologist, in the hospital after an operation, reduced me to tears by asking questions like, “Do you know that you will be bald and may lose your job?” It sounds unbelievable, but it happened.’
Respondent from Poland

What we know

According to the International Psycho-Oncology Society, 40–60% of cancer patients and family members experience psychological distress that could benefit from intervention, but only a minority receive psychological support and care.[59] This is despite psychological distress screening being recommended for all cancer patients from diagnosis onwards.[60]

These figures are partly due to the fact that significant gaps exist in the availability of psycho-oncology services across the world. A 2015 survey across 27 countries in Europe found that only eight (30%) had nationally recommended psycho-oncology care guidelines, ten (37%) had budgets for psycho-oncology care, and six (22%) had official certifications for psycho-oncology care education.[59 61]

Why it matters

Mental distress is common among cancer patients, and can result in difficulty in processing information, decisional regret about treatment choices, fear of cancer recurrence, chronic pain and difficulty with social reintegration.[60]

Cancer patients are three times more likely to suffer from depression compared with the general population. Cancer patients with depression have 39% higher mortality,[62] higher healthcare utilisation, and higher healthcare expenditure than patients who do not have depression.[63] For example, a study in the United States found that adult cancer survivors had an estimated 31.7% greater total expenditure compared to those without depression.[63]

A higher incidence of anxiety and depression is reported in adolescents and young adult cancer survivors compared with older populations. This can inhibit their ability to get an education and gain employment.[64] Furthermore, psychological care is especially crucial in the post-treatment phase, due to fears over not being able to conceive children, body image dissatisfaction and anxiety.[64]

The impact of cancer on families and loved ones was a common concern for respondents.

In addition to needing psychological support themselves, respondents said that psychological support should be offered to their spouses and children. This comment was made by both patients and caregivers completing the survey.

> ‘I wish more attention was given to the partners of cancer patients. It is often they who suffer most from the treatment.’ Respondent from Belgium

> ‘We, as a family, also felt broken. We had no previous cases of cancer in the family. I don’t know if you know what it feels like, watching someone close to you for 12 months, knowing they are dying.’ Respondent from Poland

Why it matters

Many studies show the negative impact that parental cancer has on the lives of children and the whole family unit, including siblings, necessitating support for the entire family.[65 66]

Caregivers to people with cancer, in particular, have a unique burden placed on them – and their new role in providing practical, emotional and other support can negatively affect their own psychological, physical and financial health. Reasons for experiencing increased burden are multifaceted, and can be due to coping with the emotional impact of a loved one who is suffering, taking on this new 'full-time job' of providing care to a patient with cancer and/or taking on additional household responsibilities with no extra support. On average, these caregivers provide 8.3 hours of care each day for 13.7 months.[3]

As a result, caregivers can have high unmet needs.[3] They frequently report psychological issues, including fear, hopelessness and mood disturbances. Studies also show that rates of anxiety and depression among family caregivers are comparable to, or higher than, those of the patient for whom they provide care. Physical health complications are also common – including sleep difficulties, fatigue, cardiovascular disease, decreased immune function and increased mortality.[3]

In the United States alone, it is estimated that the annual economic value of caregiving is $375 billion across all diseases.[3]

Patient stories

Hanna* (Sweden)

I saw a doctor after discovering blisters on the roof of my mouth. I had to fight to get an examination and was eventually sent to an ear, nose and throat clinic. It was easy for the doctor to spot the tumour and I was sent home; later, my doctor called me and told me it was malignant. I would rather have spoken face to face, so that I could have had someone to answer my questions.

I was not given a clinical nurse or doctor after my diagnosis, and I had no one to talk to. At the hospital they did not show much sympathy for me and I felt I was annoying when I was seeking information.

I was never involved in any discussion about different treatments. Everything went so quickly, and I had no other information – I had to search online for a lot of information about my diagnosis myself.

After my surgery, my speech was very affected, which was a complete shock. This might have been communicated to me, but if it was, it was not in a way that I was able to understand due to the circumstances. I had severe issues with swallowing and could only consume liquids. I had to contact a nutritionist myself as I became malnourished because I couldn't eat properly. I was given a speech therapist, who has been an enormous support.

I wasn't given any psychological support at all after the surgery – even my surgeon never found the time to see me afterwards – and I really needed someone because what had happened to my speech was so traumatic. Throughout my career, communication has been hugely important, and I am a very social person.

The dates of my follow-up appointments are constantly changing, which is very hard for me as I am very anxious and nervous before them – if it was once or twice it would have been fine, but it happens all the time.

As I haven't been given any psychological support, it has affected my mental health. I feel like I haven't been listened to; I have felt very lonely and not cared for. It has now been more than four and a half years, and I still haven't been offered any support at all. I am so grateful to my family and friends for their support – I don't know what I would have done without them. But I need someone to talk to outside of my friends and family. This is something that has never been discussed during my follow-ups.

My treatment was very efficient – everything happened so fast, from my first visit to the surgery, so that worked out very well. But if I could change something, it would be that I would have had someone to talk to. That's something I still need.

* Names and some other identifying elements have been changed to protect patients' anonymity.

Patient stories

Ray* (United Kingdom)

From the first time I saw my GP to being diagnosed with cancer took six months. It's far too long – shockingly long.

I think GPs' knowledge needs to be improved, and if they don't know they should send people for a biopsy. A biopsy is zero inconvenience to me, but maximum efficiency for results. Looking back now, I should have demanded a referral. But I also think the doctor should say that they don't know what it is and send you to get a biopsy done.

Unless you have been told the words – 'You've got cancer' – you have no idea what it means to hear them. I can't even explain it now. It's everything you understand about life: you as a person, your whole existence, all your experiences – when you hear those words, 'you've got cancer', it just goes out the window. Everything. You can't hear anything else.

I had surgery, but the cancer came back a few years later. With my secondary cancer, I wasn't happy after treatment. Maybe I was a bit depressed – I wasn't in a good place. And then I found out about a holistic centre, where I had some psychology appointments. They were absolutely fantastic, and that turned my life around. It wasn't just psychology, there was reiki, aromatherapy and all sorts – all provided by volunteers. The thing that annoys me, though, is that I had to ask for it. No one told me this was available until I told them I needed someone to help me with my head. I wish I'd had it the first time around as well.

The best emotional support I got in the hospital, the people that spent time with me to make me feel safe and secure, were the healthcare assistants. They're worth their weight in gold. When I was crying in bed, they used to sit there and literally hold my hand. It's those small things that make so much difference.

The main thing I would change about my cancer treatment would be the psychological side of things. Your emotional needs are not acknowledged or addressed at all. And it shouldn't just be aftercare, it should be right the way through treatment.

The National Health Service is fantastic at the clinical side of it – they do that brilliantly. But we really need to concentrate on the psychological impact. It's not just medication, surgery and treatment. The psychological trauma of cancer is massive, but no one ever tells you that. It can only be supported through good psychological care, and that needs to be provided as part of your cancer treatment. I got it as part of my cancer treatment – but only because I asked for it.

You do sort of get used to having cancer, and I have lived with it successfully, I think. For the first five years I was running away from it, but I'm not running away anymore. It's part of me. I've learnt that if you call it a 'battle', it's almost like you're going to lose. It's not about winning or losing a battle; it's about living. I'm not scared of dying – I'm just scared of not living well. And living well doesn't mean it has to be hedonistic; it can just be sitting with your wife and kids watching TV, laughing, having quality of life, and feeling safe and secure.

My health is not bad now. You can't compare it, my pre-cancer life and post-cancer life. I've had lymph nodes and muscle removed, and blood clots in my lungs. Health-wise, physically, I'm probably 70% of what I used to be. I used to run a lot of marathons; I was lean. Now I'm a bit rotund, which upsets me a bit.

Mentally, though, I'm the strongest I've ever been. That's the positive side. But I've had to do that through psychology, just for myself as well. I can't stress that enough: people need to put the effort into their own heads. No one can make you feel happy apart from yourself, so you can be your own worst enemy. You've got to be your own best friend.

* Names and some other identifying elements have been changed to protect patients' anonymity.

4

The financial impact
of cancer

Key findings

- Just over half of respondents (**51%**) paid for some part of their care, either out-of-pocket or through private insurance.
- Travel costs (**36%**) and loss of employment income (**26%**) were the most frequently reported non-treatment-related costs for respondents.
- Cancer had a negative, and often long-term, impact on productivity for respondents and their caregivers. In some cases, a diagnosis of cancer created lifelong financial insecurity.

‘The running costs of cancer are generally ignored. There are a huge number of hospital visits involved in chemo and oncologist appointments, with travel costs (petrol and parking), as well as the dislocation and stress to the driver/carer’s life.’
Respondent from the United Kingdom

‘I was not really given any info about how long to recover from chemo or returning to work. I had to devise my own return-to-work plan.’
Respondent from Canada

Overview of findings

Many respondents were shocked by the dramatic financial impact cancer had on their lives.

Respondents were asked about the financial impact of cancer on their lives, and in the qualitative responses many reported that they had incurred significant costs due to cancer.

Even in countries with publicly funded healthcare systems, respondents frequently reported having to pay for some of their cancer care themselves. Reasons differed but included wanting to avoid delays or because a particular aspect of care was not covered by their insurance system.

> ‘I paid for several exams out of my own pocket to speed things up.’ Respondent from Italy

> ‘I did the biopsy privately because the National Health Fund doctor said she did not see a reason to do it.’ Respondent from Poland

For some respondents, cancer continued to have a financial impact for many years after they had completed treatment, such as having to pay for complementary care not covered by their health insurance, or not being able to fully return to work.

The financial impact of cancer was sometimes devastating, as respondents had to make huge sacrifices to pay for their care and the associated travel.

> ‘I had to sell an investment property to pay for my cancer treatment, as I had to travel, and stay away, for seven weeks for radiation treatment.’ Respondent from Australia

What we know

There is growing evidence from different countries that many patients face a ‘cascade of financial burden’ due to cancer:

- **An Australian study found that cancer patients may spend up to 15% of their lifetime income on their disease.[67]**
- **A study of working-age cancer survivors in the US found that one third had gone into debt, and 55% incurred costs of $10,000 or more due to their cancer.[68]**
- **A French national study (VICAN 2) found that, two years after diagnosis, 25% of people living with cancer were below the poverty threshold, compared with 14% of the general population.[69]**
- **Patients living in rural or remote areas may be most affected by the financial impact of cancer due to the need to travel a long way to specialist centres.[49]**
- **Even when cancer patients are eligible for support, they may not be aware of financial assistance to which they are entitled, and navigating existing benefit systems can be difficult.[70]**

Travel costs and loss of employment were the most frequently reported non-treatment-related costs for respondents.

Were there any other financial implications of your cancer care and treatment?*

- Travel costs: **36%**
- Loss of employment: **26%**
- Loss of insurance: **10%**
- Childcare costs: **8%**

* This was a 'tick all that apply' question, therefore results do not equal 100%

'Cost of parking, especially when attending the hospital for appointments up to three times a week. Paying up to $100 a week.' Respondent from Australia

What we know

With the growing number of cancer survivors, there is increasing recognition of the need for social policies to help former cancer patients return to work after their care and protect them from financial insecurity.[71]

In Italy, for example, there is a law allowing patients to switch from full-time to part-time work while undergoing treatment, and go back to full-time work as soon as they are able. It has been recommended that all countries implement similar measures.[72] The Netherlands, meanwhile, is one of the first countries with a government Plan of Action for 'Cancer & Work'.[73]

Unfortunately, not all countries have legal frameworks for the reintegration of cancer survivors into the workplace, although more countries are developing legislation to support and protect this right to return to work.[72]

For some respondents, cancer had a negative, and often long-term, impact on productivity for them and their caregivers.

Over a quarter of respondents (**26%**) reported that they had suffered financially due to loss of employment related to their cancer.

Respondents sometimes reported not only a loss of their own income, but reduced income for their **caregivers**, who had to assume greater responsibility by caring for a spouse with cancer alongside maintaining daily household tasks and often caring for children on their own. Self-employed respondents and caregivers felt the impact of this most strongly.

‘My husband has cancer, and he’s self-employed (no work means no money). I was denied paid leave (I’m a caregiver). I can’t take unpaid leave (no work means no money).’
Respondent from Italy

‘I have no family nearby and my husband’s employers were not understanding. He had to take unpaid leave to take me for treatment and tests, and visit me during surgery and recovery. This not only caused financial strain but added to stress as he could have lost his job due to absences.’
Respondent from the United Kingdom

Why it matters

Although many cancer patients are able to return to work after their care, this is not the case for all. Lost productivity due to cancer is estimated to cost €52 billion per year in the EU.[74]

People surviving cancer are 1.4 times more likely to be unemployed and three times more likely to receive disability benefits than the general population.[75] Based on the French VICAN 2 study, 22% of those aged 18–57 reported losing their job when their cancer was diagnosed, rising to 92% 15 months after diagnosis.[69]

A study in the United Kingdom found that almost one in three people living with cancer (30%) had a loss of income as a result of their diagnosis and lost £860 a month on average. A third of people (33%) stopped working permanently or temporarily.[70]

These data underline the need for social policies that protect patients from financial insecurity during and after their cancer care.

A prior diagnosis of cancer created lifelong financial insecurity for some respondents.

In addition to the cost burden of cancer itself, respondents sometimes commented that having had cancer had a long-term impact on their ability to get a mortgage or affordable insurance – and many worried about their future financial security as a result.

> 'It's impossible to get a bank loan, however necessary at this moment... One is punished for being ill, and it all comes down to luck. In my case, I had never neglected my health.' Respondent from Belgium

> 'Cannot get a new mortgage insurance, new travel insurance or new life insurance. We have become outcasts in the eyes of insurance companies.' Respondent from Canada

What we know

In France, research found that disclosure of a childhood cancer was associated with difficulties in obtaining insurance and loans later in life.[76]

As a result of these findings, France put into place in 2016 the first anti-discrimination laws to relieve cancer survivors of having to disclose their history of cancer to insurers, as long as it had been at least five years since the end of their active treatment. These laws – called 'The right to be forgotten' (Le Droit à l'Oubli) – are now also being implemented in Belgium.

Patient stories

Sofia* (Italy)

During one year, I consulted three specialists at my own expense. After three negative medical opinions, I had peace of mind. But when I eventually saw another specialist, he immediately arranged an operation. A week later, he phoned to confirm that it was cancer, and that I needed a second operation. I considered that phone call a gesture of kindness; he wanted to inform me immediately, as I had asked. But the diagnosis could have been made a year earlier.

I chose to do all the diagnosis and the surgical operations at my own expense, because I found it difficult to get an appointment in a public hospital – especially at 8.30am to fit around work.

Cancer treatments have had a heavy impact on my work. My job required me to travel a lot and did not allow for long absences. After the first two operations to remove the primary cancer, I had another seven preventive operations. After each operation, I needed to be off work for two weeks.

The psychological impact has also been very hard. I had to face fears and make tough decisions. Each operation left marks on my body, which has been difficult to accept. During these years of analysis and examinations, I have never been offered any psychological support, nor had I been advised about patient support groups. I paid for psychological aid at my own expense.

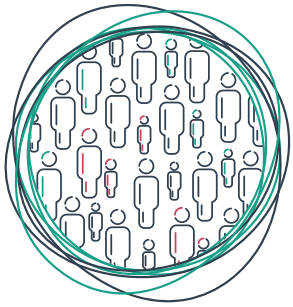
Through personal online research, I discovered support networks. I have become an active member of an association that offers support. Meeting other patients, who understand and know exactly what you are experiencing, is of enormous importance.

The financial impact of my cancer has been significant. At the time, I had private health insurance – an optional benefit of my job. It covered about 40% of the total expenses, and I paid the rest out of my own pocket.

But after quitting my job, I had to subscribe to new health insurance. It includes oncologic expenses in the case of primary tumours, but not any risks related to previously diagnosed cancer – unless you pay impossible premiums.

The experience of having cancer in my 30s changed my perspective on life. I decided to make a career change; now I work with my partner, so I can spend more time with him and I have a more peaceful approach to work. I began to ask myself: how do I want to live my life from now on? In my case, it led to a profound change of my priorities and a reorganisation of my life – to give more value to myself rather than other people's expectations.

* Names and some other identifying elements have been changed to protect patients' anonymity.



Conclusions

This report presents findings from nearly 4,000 respondents on where they felt inefficiency occurred in their care.

These findings are intrinsically important, as they represent patients' perceptions about the efficiency of their care. Patients live the reality of healthcare delivery. Their insights are both unique and valuable. If we want to be true to our aim of delivering care focused on what matters to patients, we must consider these patient insights alongside economic and clinical data, and ensure that we account for them in our definitions of efficiency and inefficiency. From a policy perspective, we should not only be looking at health system reform; we should also look at how policies and societies need to adapt to adequately provide for people living with and beyond cancer.

The All.Can patient survey findings reflect similar findings from surveys and reviews in the literature[38 49 77 78] and reveal a number of opportunities where improvement is needed from the patient perspective:

1. **Ensure swift, accurate and appropriately delivered diagnosis.** When asked to select the one area of cancer care where they experienced the most inefficiency, 26% of respondents chose diagnosis – more than any other area of cancer care. Even in countries where another area of cancer care was reported as most inefficient, diagnosis was always among the top three areas of inefficiency reported by respondents. For respondents whose cancer was diagnosed outside of a screening programme, speed of diagnosis had an impact on their entire experience of care.

2. **Improve information-sharing, support and shared decision-making.** Respondents expressed the need for better information and support to help them feel more engaged in their care. Information on what to expect in terms of side effects and risk of recurrence, and what to do after the phase of active treatment was over, was particularly needed.

3. **Make integrated multidisciplinary care a reality for all patients.** Respondents asked for more focus on the emotional and psychological impact of cancer and better integration of allied health and complementary services into their care. Cancer nurse specialists were often cited as playing an essential navigator role for respondents and their families.

4. **Address the financial implications of cancer.** Respondents commented on the significant and lasting economic burden often caused by cancer, and the need for greater support early in their care to facilitate their return to work and adapt their lives following cancer care.

Throughout the survey, respondents' comments underscored the wide-reaching impact cancer can have on all aspects of their lives. This is also reflected in economic data: social costs represent 60% of the total cost of cancer.[74] Integrated health and social policies that recognise the broad impact of cancer on individuals will be essential[72] if we are to curb the costs of cancer on our society.

Finally, we should not forget that simple solutions can often go a long way in improving efficiency – leading not only to economic gains but, most importantly, to better outcomes for patients.

Find out more

All.Can is eager to continue working with others based on these survey findings. More patient stories will be posted on our website, and we would be happy to share further information about the survey. **To find out more, please contact us at secretariat@all-can.org.**

References

1. mediLexicon. Active treatment. Available from: https://www.medilexicon.com/dictionary/93463 [Accessed 31/05/19]
2. Association of Schools of Allied Health Professionals. What is allied health? Available from: http://www.asahp.org/what-is [Accessed 31/05/19]
3. Applebaum AJ, Breitbart W. 2013. Care for the cancer caregiver: a systematic review. Palliative & supportive care 11(3): 231-52
4. NHS England. Integrated care and support. Available from: https://www.england.nhs.uk/ourwork/part-rel/transformation-fund/ [Accessed 08/06/19]
5. European CanCer Organisation (ECCO). 2014. Policy Statement on Multidisciplinary Cancer Care. Available from: https://www.ecco-org.eu/Policy/Positions-and-publications/Policy-Statement-on-Multidisciplinary-Cancer-Care [Accessed 24/05/19]
6. World Health Organization. WHO Definition of Palliative Care. Available from: https://www.who.int/cancer/palliative/definition/en/ [Accessed 24/05/19]
7. The Health Foundation. 2013. The MAGIC programme: Evaluation. London: The Health Foundation
8. Elwyn G, Durand MA, Song J, et al. 2017. A three-talk model for shared decision making: multistage consultation process. BMJ 359: j4891
9. World Health Organization. 2010. Health systems financing: the path to universal coverage. World health report 2010. Geneva: WHO
10. Medeiros J, Schwierz C. 2015. Efficiency estimates of health care systems. Brussels: European Commission Directorate-General for Economic and Financial Affairs
11. Choosing Wisely. Homepage. Available from: https://www.choosingwisely.org/ [Accessed 29/03/19]
12. Choosing Wisely. 2013. New ASCO Choosing Wisely List details five cancer tests and treatments routinely performed despite lack of evidence. Available from: http://www.choosingwisely.org/new-asco-choosing-wisely-list-details-five-cancer-tests-and-treatments-routinely-performed-despite-lack-of-evidence/ [Accessed 29/03/19]
13. Organisation for Economic Co-operation and Development. 2017. Tackling Wasteful Spending on Health. Paris: OECD
14. Addario BJ, Fadich A, Fox J, et al. 2018. Patient value: Perspectives from the advocacy community. Health Expectations 21(1): 57-63
15. Porter ME. 2010. What Is value in health care? New England Journal of Medicine 363(26): 2477-81

16. All.Can. 2017. Towards sustainable cancer care: Reducing inefficiencies, improving outcomes. London: All.Can
17. Cancer Research UK. 2018. Why is early diagnosis important? [Updated 26/06/18]. Available from: https://www.cancerresearchuk.org/about-cancer/cancer-symptoms/why-is-early-diagnosis-important [Accessed 10/02/19]
18. World Health Organization. 2018. Guide to Cancer Early Diagnosis. Geneva: WHO
19. Richards MA, Westcombe AM, Love SB, et al. 1999. Influence of delay on survival in patients with breast cancer: a systematic review. The Lancet 353(9159): 1119-26
20. Birtwistle Mike, Alexandra E. 2014. Saving lives, averting costs - An analysis of the financial implications of achieving earlier diagnosis of colorectal, lung and ovarian cancer. London: Cancer Research UK
21. Husson O, Mols F, van de Poll-Franse LV. 2011. The relation between information provision and health-related quality of life, anxiety and depression among cancer survivors: a systematic review. Ann Oncol 22(4): 761-72
22. Jefford M, Tattersall MHN. 2002. Informing and involving cancer patients in their own care. The Lancet Oncology 3(10): 629-37
23. Treacy JT, Mayer DK. 2000. Perspectives on cancer patient education. Seminars in Oncology Nursing 16(1): 47-56
24. Pieterse AH, Jager NA, Smets EM, et al. 2013. Lay understanding of common medical terminology in oncology. Psychooncology 22(5): 1186-91
25. Chapman K, Abraham C, Jenkins V, et al. 2003. Lay understanding of terms used in cancer consultations. Psycho-Oncology 12(6): 557-66
26. Epstein AS, Prigerson HG, O'Reilly EM, et al. 2016. Discussions of Life Expectancy and Changes in Illness Understanding in Patients With Advanced Cancer. J Clin Oncol 34(20): 2398-403
27. Campbell-Enns HJ, Woodgate RL, Chochinov HM. 2017. Barriers to information provision regarding breast cancer and its treatment. Supportive care in cancer : official journal of the Multinational Association of Supportive Care in Cancer 25(10): 3209-16
28. Rutten LJF, Arora NK, Bakos AD, et al. 2005. Information needs and sources of information among cancer patients: a systematic review of research (1980–2003). Patient Education and Counseling 57(3): 250-61
29. Tokdemir G, Kav S. 2017. The Effect of Structured Education to Patients Receiving Oral Agents for Cancer Treatment on Medication Adherence and Self-efficacy. Asia Pac J Oncol Nurs 4(4): 290-98
30. Halbach SM, Ernstmann N, Kowalski C, et al. 2016. Unmet information needs and limited health literacy in newly diagnosed breast cancer patients over the course of cancer treatment. Patient Education and Counseling 99(9): 1511-18
31. All Party Parliamentary Groups (APPG). 2014. Patient empowerment: for better quality, more sustainable health services globally.
32. European Health Parliament. 2015. Patient empowerment and centredness. Available from: http://www.healthparliament.eu/wp-content/uploads/2017/09/EHP-papers_Patients-empowerment.pdf

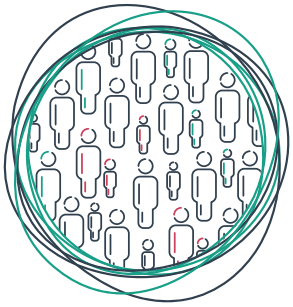
33. European Patients' Forum (EPF). 2017. EPF Campaign on Patient Empowerment: Roadmap for Action. Available from: http://www.eu-patient.eu/globalassets/campaign-patient-empowerment/roadmap/roadmap_patient-empowerment_-epf_2017.pdf [Accessed 10/07/19]
34. Kehl KL, Landrum MB, Arora NK, et al. 2015. Association of Actual and Preferred Decision Roles With Patient-Reported Quality of Care: Shared Decision Making in Cancer Care. JAMA Oncology 1(1): 50-58
35. Mulley A, Trimble C, Elwyn G. 2012. Patients' preferences matter. London: The King's Fund
36. European Society for Medical Oncology. 2010. Improving Rare Cancer Care in Europe: Recommendations on Stakeholder Actions and Public Policies. 10
37. National Cancer Survivorship Initiative (NCSI). 2013. Living with and beyond cancer: taking action to improve outcomes. London: NHS England
38. Macmillan Cancer Support. 2013. Cured but at what cost? London: Macmillan Cancer Support
39. Macmillan Cancer Support. 2014. Assessment and care planning for cancer survivors: A concise evidence review. London University of the West of Scotland
40. Cancer World. 2017. Pain! The Denial needs to end. Available from: http://cancerworld.net/cover-story/pain-the-denial-needs-to-end/ [Accessed 10/07/19]
41. Ferrell BR, Temel JS, Temin S, et al. 2017. Integration of Palliative Care Into Standard Oncology Care: American Society of Clinical Oncology Clinical Practice Guideline Update. Journal of Clinical Oncology 35(1): 96-112
42. ESMO. 2012A. Gastrointestinal stromal tumors: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 23 (7): vii49-55
43. Hoerger M, Greer JA, Jackson VA, et al. 2018. Defining the Elements of Early Palliative Care That Are Associated With Patient-Reported Outcomes and the Delivery of End-of-Life Care. Journal of Clinical Oncology 36(11): 1096-102
44. Temel JS, Greer JA, Muzikansky A, et al. 2010. Early palliative care for patients with metastatic non-small-cell lung cancer. New England Journal of Medicine 363(8): 733-42
45. Temel JS, Greer JA, Admane S, et al. 2011. Longitudinal perceptions of prognosis and goals of therapy in patients with metastatic non-small-cell lung cancer: results of a randomized study of early palliative care. J Clin Oncol 29(17): 2319-26
46. Mages NL, Castro JR, Fobair P, et al. 1981. Patterns of psychosocial response to cancer: Can effective adaptation be predicted? International Journal of Radiation Oncology*Biology*Physics 7(3): 385-92
47. European Society for Medical Oncology (ESMO). 2017. Patient Guide on Survivorship. Available from: https://www.esmo.org/Patients/Patient-Guides/Patient-Guide-on-Survivorship [Accessed 01/04/19]
48. Oliver K. 2019. Personal communication: 13/03/19
49. Breast Cancer Network Australia. 2018. State of the Nation Report. Camberwell, Victoria: BCNA

50. Borras JM, Albreht T, Audisio R, et al. 2014. Policy statement on multidisciplinary cancer care. European Journal of Cancer 50(3): 475-80
51. Charalambous A, Wells M, Campbell P, et al. 2018. A scoping review of trials of interventions led or delivered by cancer nurses. Int J Nurs Stud 86: 36-43
52. All.Can. 2017. Spotlight on cancer nurses: the heart of high-quality cancer care. Available from: http://www.all-can.org/wp-content/uploads/2017/05/AllCan_spotlight_cancer_nurses.pdf [Accessed 10/07/19]
53. Sussman J, Howell D, Bainbridge D, et al. 2011. The impact of specialized oncology nursing on patient supportive care outcomes. J Psychosoc Oncol 29(3): 286-307
54. Campbell P, Torrens C, Kelly D, et al. 2017. Recognizing European cancer nursing: Protocol for a systematic review and meta-analysis of the evidence of effectiveness and value of cancer nursing. Journal of Advanced Nursing 73(12): 3144-53
55. ESMO. 2012. Soft tissue and visceral sarcomas: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 23 (7): vii92-9
56. National Institute for Health and Care Excellence. 2006. Improving Outcomes for People with Sarcoma: the Manual. Cardiff: National Collaborating Centre for Cancer
57. Greenlee H, DuPont-Reyes MJ, Balneaves LG, et al. 2017. Clinical practice guidelines on the evidence-based use of integrative therapies during and after breast cancer treatment. CA Cancer J Clin 67(3): 194-232
58. Walker SP. 2019. The ROC Curve Redefined — Optimizing Sensitivity (and Specificity) to the Lived Reality of Cancer. New England Journal of Medicine 380(17): 1594-95
59. Travado L, Breitbart W, Grassi L, et al. 2017. 2015 President's Plenary International Psycho-oncology Society: psychosocial care as a human rights issue—challenges and opportunities. Psycho-Oncology 26(4): 563-69
60. Alias A, Henry M. 2018. Psychosocial Effects of Head and Neck Cancer. Oral and Maxillofacial Surgery Clinics of North America 30(4): 499-512
61. Grassi L, Fujisawa D, Odyio P, et al. 2016. Disparities in psychosocial cancer care: a report from the International Federation of Psycho-oncology Societies. Psycho-Oncology 25(10): 1127-36
62. Smith HR. 2015. Depression in cancer patients: Pathogenesis, implications and treatment (Review). Oncology Letters 9(4): 1509-14
63. Pan X, Sambamoorthi U. 2015. Health care expenditures associated with depression in adults with cancer. J Community Support Oncol 13(7): 240-47
64. Ismail Y, Hendry J. 2018. Support needs of adolescents' post-cancer treatment: A systematic review. Radiography 24(2): 175-83
65. Visser A, Huizinga G, Van der Graaf W, et al. 2005. The impact of parental cancer on children and the family: A review of the literature. Cancer Treat Rev 30(8): 683-94
66. Shah BK, Armaly J, Swieter E. 2017. Impact of Parental Cancer on Children. Anticancer research 37(8): 4025-28

67. The Pharmacy Guild of Australia. 2017. Half of Australian cancer survivors shouldering financial stress - and it's on the rise. [Updated 21/07/19]. Available from: https://www.guild.org.au/news-events/blog/2017/half-of-australian-cancer-survivors-shouldering-financial-stress-and-its-on-the-rise [Accessed 18/07/19]
68. Banegas MP, Guy GP Jr., de Moor JS, et al. 2016. For Working-Age Cancer Survivors, Medical Debt And Bankruptcy Create Financial Hardships. Health affairs (Project Hope) 35(1): 54-61
69. Albreht T, Kiasuwa R, Van den Bulcke M. 2017. CanCon: European Guide on Quality Improvement in Comprehensive Cancer Control. Ljubljana: National Institute of Public Health
70. Macmillan Cancer Support. 2015. Cancers hidden price tag: revealing the costs behind the illness. London: Macmillan Cancer Support
71. Braspenning I, Tamminga S, Frings-Dresen M, et al. 2018. Rehabilitation and return to work after cancer — instruments and practices. European Risk Observatory report. Luxembourg: Publications Office of the European Union
72. European Society for Medical Oncology (ESMO) and European Cancer Patient Coalition (ECPC). 2017. Survivorship - ESMO Patient Guide Series. Brussels: ECPC
73. oPuce. Volwaardig werk na kanker. Available from: http://opuce.nl [Accessed 25/03/19]
74. Luengo-Fernandez R, Leal J, Gray A, et al. 2013. Economic burden of cancer across the European Union: a population-based cost analysis. Lancet Oncology 14(12): 1165-74
75. European Agency for Safety and Health at Work. 2018. How can cancer survivors best be supported to return to work? [Updated 25/05/18]. Available from: https://osha.europa.eu/en/about-eu-osha/press-room/how-can-cancer-survivors-best-be-supported-return-work [Accessed 01/04/19]
76. Dumas A, Allodji R, Fresneau B, et al. 2017. The right to be forgotten: a change in access to insurance and loans after childhood cancer? Journal of cancer survivorship: research and practice 11(4): 431-37
77. Youth Cancer Europe. 2018. White paper on the needs of young people living with cancer. Available from: https://www.youthcancereurope.org/wp-content/uploads/2018/10/YouthCancerEurope_Brussels_2018_WhitePaper-sm.pdf [Accessed 10/07/19]
78. Chadder J, Zomer S, Lockwood G, et al. 2018. Understanding the Experiences of Cancer Patients as They Transition From Treatment to Primary and Community Care: A Pan-Canadian Study of Over 13,000 Cancer Survivors. Journal of Global Oncology 4(Supplement 2)

Appendix 1: country findings


United States
Canada
Belgium
Poland
United Kingdom
Italy
Australia



Introduction

This Appendix contains overviews of findings of the All.Can patient survey in seven participating countries.

All.Can international and Quality Health worked with individual All.Can national initiatives and associated member organisations to develop tailored surveys for each participating country. Most questions remained the same across countries, but additional questions were added to suit local country contexts and at the request of each country. In some cases, the wording of questions was adapted to accommodate local differences. Surveys were produced in English, and then translated into local languages. All translations were verified by a representative of the country's national All.Can initiative. The surveys were hosted online, and links were distributed.

To download a copy of the survey questions used in each country, please visit the All.Can international website (www.all-can.org/what-we-do/research/patient-survey/about-the-survey/).

For more information on All.Can national initiatives involved in the patient survey, please visit http://www.all-can.org/national-initiatives/.

Interpretation of findings

The survey was conducted in 10 countries: Australia, Belgium, Canada, France, Italy, Poland, Spain, Sweden, the United Kingdom and the United States. We have excluded findings from France, Spain and Sweden from this section due to small sample sizes (<50 responses each).

Country findings presented in this section are unweighted and therefore represent the actual responses from current and former cancer patients and/or caregivers who completed the survey in each country. As they represent different populations in each country, **the country findings in this Appendix must be considered independently and cannot be directly compared with those of other countries**. Further country comparisons will form part of the next phase of our research.

For more information

For more detailed findings from each country, please contact the All.Can international secretariat: secretariat@all-can.org. Further country-level analyses will be disseminated by each All.Can national initiative throughout 2019.

Australia

1 in 2 Australians will be diagnosed with cancer by the age of 85 and, in 2019, it is estimated 145,000 new cases will be diagnosed and 50,000 deaths will occur.[i]

About this survey in Australia

The survey in Australia was conducted in partnership with All.Can Australia and the University of Western Australia. Data collection took place from 12 July – 30 November 2018.

A total of 850 people took part in the Australian survey.

Respondent profile

- **Cancer type:** breast **68%**; lymphoma **7%**; prostate **5%**; other (various cancer types, all <5%) **20%**
- **Gender:** female **89%**; male **11%**
- **Age:** 0–24 **1%**; 25–64 **67%**; 65+ **32%**

Please note: as the majority of respondents in Australia were breast cancer patients, the key findings (page 67) consider the results for breast cancer patients against the results for all other cancer types. This applies only to the Australian findings.

Where did inefficiencies occur most?

- My initial cancer diagnosis: **23%**
- Dealing with ongoing side effects: **19%**
- Dealing with the psychological impacts: **15%**

‘My cancer nurse was, and still is, the most amazing support we could have asked for. She has a wealth of knowledge and helped us out and at any time of the day or night!’

‘Some initial psychological assistance might be useful, even if the patient doesn’t request it – sometimes one doesn’t realise one needs it!’

i Cancer Council Australia. 2019. Cancer in Australia. Available online at https://www.cancer.org.au/about-cancer/what-is-cancer/facts-and-figures.html

Australia: key findings

Swift, accurate and appropriately delivered diagnosis

- **28%** of respondents whose cancer was detected outside of a screening programme said that their cancer was diagnosed as something different – either initially or multiple times. This occurred less frequently for respondents with breast cancer (**18%**) than for all other tumour types (**43%**)
- The largest difference between cancer types was expressed in diagnosis: **91%** of breast cancer respondents whose cancer was detected outside of a screening programme were diagnosed within three months, compared to an average of **69%** in all other tumour types
- **12%** of respondents whose cancer was detected outside of a screening programme waited more than six months to be diagnosed with cancer. In breast cancer this was **5%**, while the average across all other tumour types was **22%**

Information, support and shared decision-making

- **35%** did not feel involved enough in deciding which treatment options were best for them
- **28%** were not given enough information (in a way they could understand) about their cancer care and treatment
- **50%** did not receive enough support to deal with ongoing symptoms and side effects – including beyond the active phase of their treatment
- **41%** did not receive enough information (in a way they could understand) about the signs and symptoms indicating that their cancer might be returning or getting worse
- **31%** were not given information about patient groups, charities and other organisations that might be able to support them

Integrated multidisciplinary care

- **30%** did not have access to a specialist cancer nurse, either immediately after their diagnosis or during treatment
- **19%** said that supported from allied health professionals was not available when they needed it
- **63%** were not offered complementary therapies (e.g. massage, meditation, acupuncture, aromatherapy and/or other non-traditional therapies) as part of their cancer treatment
- **64%** reported that they needed some sort of psychological support during/after their cancer care but, of those, **35%** said it was not available

The financial impact of cancer

- **79%** reported out-of-pocket costs, **32%** reported travel costs (**11%** had to travel for 1–2 hours to attend appointments or receive treatment, **7%** for more than 2 hours, and **9%** required an overnight stay because it was too far from home), **27%** reported a loss of employment, **8%** a loss of insurance and **4%** childcare costs

Belgium

An estimated 3% of the Belgian population were living with and beyond cancer between 2004 and 2013.[i] There are over 70,000 new cases of cancer each year.[ii]

About this survey in Belgium

The survey in Belgium was conducted in partnership with All.Can Belgium. Data collection took place from 28 August – 30 November 2018.

A total of 391 people took part in the Belgian survey.

Respondent profile

- **Cancer type**: breast **47%**; haematological **9%**; bowel/colorectal **7%**; lung **7%**; other (various cancer types, all <5%) **29%**
- **Gender**: female **75%**; male **25%**
- **Age**: 0–24 **1%**; 25–64 **61%**; 65+ **34%**; not specified **3%**
- **Language**: Dutch **63%**; French **37%**; German **0.26%**

Where did inefficiencies occur most?

- Dealing with ongoing side effects: **28%**
- My initial cancer diagnosis: **17%**
- Getting the right treatment for my cancer: **15%**

‘My son’s diagnostic process was very fast and efficient. Even after our first emergency visit, the follow-up (to make sure we did not stop the medical exams) was impeccable.’

‘I had to ask for psychological help myself. This wasn’t the priority of the treating physician, but I am very glad that I insisted. The doctor was not against it, but didn’t think it was a priority!’

[i] Belgian Cancer Registry. Cancer burden 2004-2013. Available online at https://kankerregister.org/media/docs/publications/BCR_publicatieCancerBurden2016_web160616.pdf

[ii] Belgian Cancer Registry. Annual tables. Available online at https://kankerregister.org/default.aspx?PageId=643

Belgium: key findings

Swift, accurate and appropriately delivered diagnosis

- **26%** of respondents whose cancer was detected outside of a screening programme said their cancer was diagnosed as something different – either initially or multiple times
- **13%** of respondents whose cancer was detected outside of a screening programme waited more than six months to be diagnosed with cancer

Information, support and shared decision-making

- **45%** did not feel involved enough in deciding which treatment options were best for them
- **24%** were not given enough information (in a way they could understand) about their cancer care and treatment
- **42%** did not receive enough support to deal with ongoing symptoms and side effects – including beyond the active phase of their treatment
- **40%** did not receive enough information (in a way they could understand) about the signs and symptoms indicating that their cancer might be returning or getting worse
- **50%** were not given information about patient groups, charities and other organisations that might be able to support them

Integrated multidisciplinary care

- **53%** always felt supported by those involved in their care (e.g. surgeons, oncologists, radiologists, nurses and other specialists)
- **11%** said that support from allied health professionals was not available when they needed it
- **71%** reported that they needed some form of psychological support during/after their cancer care but, of those, **23%** said it was not available
- **80%** of those who received psychological support found it helpful
- **6%** had to miss or cancel their own appointments three or more times at short notice, whereas **2%** reported this was done by their hospital or clinic

The financial impact of cancer

- **21%** reported travel costs, **10%** a loss of employment, **2%** childcare costs, **2%** loss of insurance and **15%** other

Canada

Cancer is the leading cause of death in Canada – responsible for 30% of all deaths. One in every two Canadians is expected to develop cancer during their lifetime, and one in four Canadians will die from cancer. In 2015, 2.1 million people in Canada (aged 12 and over) reported living with and beyond cancer.[i]

About this survey in Canada

The survey in Canada was conducted in partnership with All.Can Canada, led by Save Your Skin Foundation. Data collection took place from 13 June – 30 November 2018.

A total of 314 people took part in the Canadian survey.

Respondent profile

- **Cancer type**: breast **37%**; haematological **13%**; skin **11%**; other (various cancer types, all <5%) **39%**
- **Gender**: female **81%**; male **19%**
- **Age**: 0–24 **1%**; 25–64 **61%**; 65+ **37%**
- **Language**: English **87%**; French **13%**

Where did inefficiencies occur most?

- My initial cancer diagnosis: **25%**
- Dealing with ongoing side effects: **17%**
- Dealing with the psychological impacts: **15%**

‘Having a Nurse Practitioner assigned to my case to help me through the initial trauma of a cancer diagnosis would have been very helpful. Doctors don’t always have time to provide patients with all the support they need.’

i Government of Canada. 2018. Fact sheet: Cancer in Canada. Available online at https://www.canada.ca/en/public-health/services/publications/diseases-conditions/fact-sheet-cancer-canada.html

Canada: key findings

Swift, accurate and appropriately delivered diagnosis

- **27%** of respondents whose cancer was detected outside of a screening programme said that their cancer was diagnosed as something different – either initially or multiple times
- **14%** of respondents whose cancer was detected outside of a screening programme waited more than six months to be diagnosed with cancer

Information, support and shared decision-making

- **44%** did not feel involved enough in deciding which treatment options were best for them
- **35%** were not given enough information (in a way they could understand) about their cancer care and treatment
- **48%** did not receive enough information (in a way they could understand) about the signs and symptoms indicating that their cancer might be returning or getting worse
- **43%** were not given information about patient groups, charities and other organisations that might be able to support them
- Of those who knew about patient groups, **62%** used them a lot, or some of the time
- **88%** would like their health data shared with larger data bodies for the purpose of research and the ability to track long-term patient outcomes

Integrated multidisciplinary care

- **18%** said that support from allied health professionals was not available when they needed it
- **61%** reported that they needed some form of psychological support during/after their cancer care but, of those, **35%** said it was not available
- **27%** were left with unused medicine at the end of their treatment because they were given too much

The financial impact of cancer

- **48%** paid for some part of their cancer care: **25%** for private insurance, **10%** for care not covered by the national health system, and **6%** to avoid delays
- **43%** paid for medicines, **38%** paid for alternative treatment and support (homeopathy, naturopathy, psychosocial support, physiotherapy) and **19%** paid for some part of their diagnosis not covered by a private or public insurance plan
- **46%** reported travel costs, **28%** reported loss of employment, **6%** reported loss of insurance, **5%** reported childcare costs and **18%** reported other non-care-related costs

Italy

Cancer represents the second leading cause of death in Italy. In 2018, there were 3.3 million people living with and beyond cancer, and an incidence of 373,000 new cases that year.[i]

About this survey in Italy

The survey in Italy was conducted in partnership with All.Can Italy. Data collection took place from 27 September – 30 November 2018.

A total of 96 people took part in the Italian survey.

Respondent profile

- **Cancer type**: breast **25%**; urological **25%**; skin **22%**; other (various cancer types, all <5%) **29%**
- **Gender**: female **66%**; male **34%**
- **Age**: 0–24 **2%**; 25–64 **79%**; 65+ **17%**; not specified **1%**

Where did inefficiencies occur most?

- Dealing with the psychological impacts: **25%**
- My initial cancer diagnosis: **24%**
- Dealing with ongoing side effects: **11%**
- Access to patient support groups: **11%**

‘Cancer is a disease that must be addressed with a 360-degree view, and psychological support is necessary for the patient or for their partner.’

i AIOM. 2018. I numeri del cancro in Italia. Available online at https://www.fondazioneaiom.it/wp-content/uploads/2018/10/2018_NumeriCancro-pazienti.pdf

Italy: key findings

Swift, accurate and appropriately delivered diagnosis

- **22%** of respondents whose cancer was detected outside of a screening programme said that their cancer was diagnosed as something different – either initially or multiple times
- **9%** of respondents whose cancer was detected outside of a screening programme waited more than six months to be diagnosed with cancer

Information, support and shared decision-making

- **50%** did not feel involved enough in deciding which treatment options were best for them
- **32%** were not given enough information (in a way they could understand) about their cancer care and treatment
- **44%** did not receive enough support to deal with ongoing symptoms and side effects – including beyond the active phase of their treatment
- **59%** were not given information about patient groups, charities and other organisations that might be able to support them
- **94%** of those who used patient groups, charities and other organisations said they benefitted from them

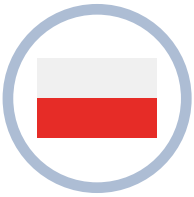
Integrated multidisciplinary care

- **76%** were treated by a multidisciplinary team (e.g. surgeons, oncologists, radiologists, nurses and other specialists)
- **70%** saw a physician within 24 hours if any complications occurred during treatment
- **68%** reported that they needed some form of psychological support during/after their cancer care but, of those, **46%** said that it was not available
- Of those who received psychological support, **71%** received it in a hospital setting and **19%** from a voluntary organisation

The financial impact of cancer

- **62%** incurred expenses because of their cancer: **57%** paid to speed-up waiting times, **9%** paid for private insurance, and **33%** for other reasons
- **48%** reported travel costs, **15%** a loss of insurance, and **13%** a loss of employment

Poland



In Poland each year, over 163,000 people are diagnosed with cancer, and more than 100,000 people die from cancer.[i]

About this survey in Poland

The survey in Poland was conducted in partnership with All.Can Poland. Data collection took place from 7 June – 30 November 2018.

A total of 1,135 people took part in the Polish survey.

Respondent profile

- **Cancer type**: breast **39%**; gynaecological **14%**; haematological **8%**; other (various cancer types, all <5%) **39%**
- **Gender**: female **83%**; male **17%**
- **Age**: 0–24 **3%**; 25–64 **75%**; 65+ **22%**

Where did inefficiencies occur most?

- Dealing with ongoing side effects: **27%**
- Dealing with the psychological impacts: **18%**
- My initial cancer diagnosis: **14%**

'There is always something to improve – definitely faster start of treatment from the first diagnosis, because undoubtedly the whole process takes too long. During this time, the patient and family do not know what to do with themselves, and the cancer progresses'.

i Najwyższa Izba Kontroli (NIK) 2018. How to prevent and cure cancer more effectively? Available online at https://www.nik.gov.pl/plik/id,16371,vp,18897.pdf

Poland: key findings

Swift, accurate and appropriately delivered diagnosis

- **27%** of respondents whose cancer was detected outside of a screening programme said that their cancer was diagnosed as something different – either initially or multiple times
- **12%** of respondents whose cancer was detected outside of a screening programme waited more than six months to be diagnosed with cancer

Information, support and shared decision-making

- **52%** did not feel involved enough in deciding which treatment options were best for them
- **53%** were not given enough information (in a way they could understand) about their cancer care and treatment
- **63%** were not informed in a way they could understand about possible treatments, including those that were not reimbursed or available in other centres
- **69%** did not receive enough information on how to deal with pain
- **69%** did not receive enough support to deal with ongoing symptoms and side effects – including beyond the active phase of their treatment
- **75%** were not given information about patient groups, charities and other organisations that might be able to support them

Integrated multidisciplinary care

- **50%** said that support from allied health professionals was not available when they needed it
- **89%** reported that they needed some form of psychological support during/after their cancer care but, of those, **41%** said it was not available

The financial impact of cancer

- **47%** paid for some cancer care and treatment themselves: **33%** to avoid delays, **15%** for treatment not covered by the national health system, and **4%** for private insurance
- **63%** reported that they had to purchase additional medicines, **43%** reported an absence from work, and **43%** reported additional costs to fulfil family and social roles (i.e. running a home, taking care of children)

United Kingdom

1 in 2 people in the United Kingdom will be diagnosed with cancer in their lifetime.[i] In 2015, it was estimated that more than 2.5 million people in the United Kingdom were living with cancer, and there were 359,960 new cases of cancer that year.[ii iii]

About this survey in the United Kingdom

The survey in the United Kingdom was conducted in partnership with All.Can UK. Data collection took place from 31 January – 23 August 2018.

A total of 322 people took part in the United Kingdom survey.

Respondent profile

- **Cancer type:** breast **28%**; gynaecological **20%**; bowel/colorectal **9%**; other (various cancer types, all <5%) **43%**
- **Gender:** female **79%**; male **21%**
- **Age:** 0–24 **1%**; 25–64 **62%**; 65+ **37%**

Where did inefficiencies occur most?

- My initial cancer diagnosis: **36%**
- Dealing with ongoing side effects: **19%**
- Dealing with the psychological impacts: **15%**

‘In addition to support to manage the anxiety of having a life-threatening condition, I think that more needs to be done to support people [with cancer] managing at work, i.e. managing time off, talking to your employer, understanding your rights.’

‘I would like more information about mental health support. I asked repeatedly (oncologists, nurse and GP) and nobody was able to give me any information. I knew I had a problem, but it was extremely difficult for me to find professional support.’

i Cancer Research UK. **Lifetime risk of cancer.** Available online at https://www.cancerresearchuk.org/health-professional/cancer-statistics/risk/lifetime-risk#heading-Zero

ii Cancer Research UK. **Cancer incidence statistics.** Available online at https://www.cancerresearchuk.org/health-professional/cancer-statistics/incidence#heading-Zero

iii Macmillan Cancer Support (2015). **2.5 million people now living with cancer in UK.** Available online at https://www.macmillan.org.uk/aboutus/news/latest_news/25millionpeoplenowlivingwithcancerinukmacmillanrevealstoday.aspx

United Kingdom: key findings

Swift, accurate and appropriately delivered diagnosis

- **40%** of respondents whose cancer was detected outside of a screening programme said that their cancer was diagnosed as something different – either initially or multiple times
- **21%** of respondents whose cancer was detected outside of a screening programme waited more than six months to be diagnosed with cancer

Information, support and shared decision-making

- **52%** did not feel involved enough in deciding which treatment options were best for them
- **38%** were not given enough information (in a way they could understand) about their cancer care and treatment
- **58%** did not receive enough support to deal with ongoing symptoms and side effects – including beyond the active phase of their treatment
- **48%** did not receive enough information (in a way they could understand) about the signs and symptoms indicating that their cancer might be returning or getting worse
- **40%** were not given information about patient groups, charities and other organisations that might be able to support them

Integrated multidisciplinary care

- **67%** reported that they needed some form of psychological support during/after their cancer care but, of those, **50%** said it was not available
- **34%** said they were given too much medication and therefore had excess amounts left over at the end of their treatment

The financial impact of cancer

- **14%** paid for some or all of their cancer care themselves (either because the care and treatment they wanted was not available via the National Health Service, or because they wanted to avoid delays in treatment)
- **47%** reported travel costs, **27%** reported loss of employment, **12%** reported loss of insurance and **5%** reported childcare costs

United States

In 2018, an estimated 1,735,350 new cases of cancer were diagnosed in the United States, with 609,640 people dying from the disease.[i]

About this survey in the United States

Respondents were recruited through the healtheo360 online platform. Data collection took place from 1 June – 17 August 2018.

A total of 497 people took part in the United States survey.

Respondent profile

- **Cancer type**: breast **25%**; skin **11%**; gynaecological **11%**, head and neck **11%**; other (various cancer types, all <5%) **41%**
- **Gender**: female **63%**; male **37%**
- **Age**: 0–24 **1%**; 25–64 **87%;** 65+ **12%**

Where did inefficiencies occur most?

- My initial cancer diagnosis: **31%**
- Dealing with ongoing side effects: **23%**
- Dealing with the financial implications: **15%**

> 'More follow-up care would be important. I ended up in a fairly serious bout of depression a year or so after my treatment ended. It came out of nowhere – luckily, I sought help from a therapist and was able to get through it with medication. The therapist said this is quite common in cancer patients – it would have been helpful to know this and to know what to expect'.

> 'Because I became too sick to work, I lost my job. Because I lost my job, I lost my health insurance'.

i National Cancer Institute. 2018. Cancer Statistics. Available online at https://www.cancer.gov/about-cancer/understanding/statistics

United States: key findings

Swift, accurate and appropriately delivered diagnosis

- **31%** of respondents whose cancer was detected outside of a screening programme said that their cancer was diagnosed as something different – either initially or multiple times
- **12%** of respondents whose cancer was detected outside of a screening programme waited for more than six months to be diagnosed with cancer

Information, support and shared decision-making

- **41%** did not feel involved enough in deciding which treatment options were best for them
- **25%** were not given enough information (in a way they could understand) about their cancer care and treatment
- **31%** did not receive enough support to deal with ongoing symptoms and side effects – including beyond the active phase of their treatment
- **30%** did not receive enough information (in a way they could understand) about the signs and symptoms indicating that their cancer might be returning or getting worse
- **23%** were not given information about patient groups, charities and other organisations that might be able to support them

Integrated multidisciplinary care

- **59%** reported that they needed some form of psychological support during/after their cancer care but, of those, **31%** said that it was not available

The financial impact of cancer

- **68%** paid for some part of their cancer care: **55%** for private health insurance, **6%** for care and treatment not covered by insurance, and **4%** to avoid delays
- **44%** reported travel costs, **31%** reported loss of employment, **10%** reported loss of insurance and **9%** reported childcare costs

Published in July 2019

All.Can Changing cancer care together

The All.Can initiative is made possible with financial support from Bristol-Myers Squibb (main sponsor), Amgen, MSD and Johnson & Johnson (sponsors) and Varian (contributor), with additional non-financial (in kind) support from Intacare and Goings-On.

Nearly half of cancer patients don't feel involved in treatment decisions, survey finds

CARLOTTA JARACH

29 July 2019 News 0

Almost half (47%) of patients with cancer do not think that they have been sufficiently involved in the treatment decision process. This is what the international All.Can cancer initiative reveals in its report *Patient insights on cancer care: opportunities for improving efficiency*, along with other patients' outlooks concerning support and adequate information and care beyond the disease.

The All.Can patient survey was conducted internationally, with adapted versions in 10 countries, and aims to obtain patients' perspectives on where they felt they faced inadequacy in their care, looking at the entire care continuum as well as the broader impact of cancer on their lives.

"Patients are often forgotten when it comes to cancer care planning," said Alex Filicevas, Head of EU Affairs at the European Cancer Patient Coalition and member of All.Can international's steering committee. "With the prevalence, complexity and costs of cancer rising across the globe, it is imperative to listen to what patients say would improve their experience of care. Ignoring the findings of this report would be a missed opportunity to do the right thing by patients and make changes that could make a real difference."

Almost 4,000 respondents took part who were affected by different cancers, and based on their answers four areas where to intervene were identified. The first area is the diagnosis itself: for many cancers, an early diagnosis can improve survival, but according to the respondents almost a third (32%) of whose cancer was diagnosed outside of a screening programme said their cancer was diagnosed as something else, either once or even multiple times, and a quarter (26%) stated their initial diagnosis was the most inefficient part of their cancer care journey. Another possible theme is the cited information-sharing process, where 39% of respondents were mot given enough support to deal with any ongoing symptoms and side effects. In that, the issue was more prevalent among people with more advanced cancers. Integrated care is not a reality for everyone yet: 69% patients in fact said they needed psychological support either during or after their cancer care, but a third (34%) of them said it was 'not available'. The fourth and last area identified by the survey, was regarding the financial issues: 26% of the respondents reported a loss of employment income and 36% of them cited travel costs as a financial implication of their cancer care and treatment.

“It is so important that, as physicians, we listen to what patients are telling us in this survey,” said All.Can international member Christobel Saunders, breast cancer surgeon and Professor of Surgical Oncology at the University of Western Australia. “Each of the areas identified represents an opportunity to improve cancer care for patients and provide truly patient-driven care.”

https://cancerworld.net/news/nearly-half-of-cancer-patients-dont-feel-involved-in-treatment-decisions-survey-finds/

Research Article

A study of coagulation profile in neoplastic conditions

Sarmishtha M Patel, Swati Gupta, Mandakini M Patel, Juhi D Mahadik, Kinjal A Patel, Avani S Patel

Department of Pathology, Government Medical College, Surat, Gujarat, India.
Correspondence to: Swati Gupta, E-mail:swatinteracts@yahoo.com

Received July 18, 2015. Accepted August 4, 2015

Abstract

Background: Malignant tumors are often associated with thromboembolic episodes and disturbed coagulative processes. Plasma D-dimer and other coagulation parameters form a simple panel of tests for the assessment of the intravascular coagulation and fibrinolysis (ICF) syndrome. The abnormal hemostatic results obtained in a proper clinical setting pave for the suspicion of the ICF syndrome. Moreover, precautionary measures can be taken to avoid its complication by the use of mild anticoagulants.

Objective: To evaluate the changes in hemostatic–clotting parameters in patients with malignancies and elucidate the association of ICF with malignant tumors.

Materials and Methods: This study comprised 60 cases, including apparently normal controls and patients of benign and malignant lesions. After the processing of blood samples, tests such as platelet count, prothrombin time, activated partial thromboplastin time, fibrinogen, fibrin degradation products, and D-dimer were done.

Result: A total of 60 cases were evaluated in our study. A strong association was seen between the malignancies and the elevated D-dimer elucidating the presence of ICF in these patients along with other altered coagulation parameters in comparison with apparently normal controls in the study.

Conclusion: Increased D-dimer and altered coagulation parameters significantly correlate with malignant behavior of tumors and their spread. They might be useful indicators of aggressive tumor biology and behavior.

KEY WORDS: D-dimer, malignancy, coagulation

Introduction

According to Armand Trousseau, "if the diagnosis of a suspected carcinoma of an internal organ could not be verified, spontaneous appearance of thrombophlebitis afforded necessary proof for diagnosis" and described "phlegmasia alba dolens" as a presenting symptom of occult cancer.[1]

Malignancies show an increased susceptibility to thromboembolic events when compared with benign tumors and the general population. Ovarian, pancreatic, prostatic, and lung cancers and mucin-producing carcinomas of gastrointestinal tract are among the malignancies often associated with thromboembolic episodes.[2]

Thrombosis occurs spontaneously, after surgery, radiation therapy, and anticancer treatment and might be the first manifestation of underlying cancer.[3]

In this study, changes in hemostatic parameters and their relation to cancer are analyzed.

Access this article online	
Website: http://www.ijmsph.com	Quick Response Code:
DOI: 10.5455/ijmsph.2016.1807201565	

Materials and Methods

This study was conducted during a period of 6 months from August 2013 to January 2014. A total of 60 cases were included in this study, including apparently normal controls and patients with benign and malignant tumors; all the patients were admitted to New Civil Hospital, Surat, Gujarat, India.

After taking consent, complete history and clinical findings with details regarding the tumor nature and laboratory data were collected.

For the evaluation of hematological parameters, 2 mL blood sample was collected in EDTA Vacuette and run on Sysmex Kx 21 for the evaluation of Hb, total count, and platelets.

For coagulation parameters, 2 mL blood sample was collected in the Citrate Vacuette. Plasma thus isolated was evaluated for prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen (FIB), fibrin degradation products (FDP), and D-dimer.

- Platelet-poor plasma was prepared from citrated samples immediately after venipuncture by centrifugation for 10 min at 1,500 rpm at room temperature.
- Plasma was transferred to plastic tubes, frozen, and stored at –40°C until evaluated.
- Evaluation was done on fully auto coagulometer (Stago STA compact CT coagulation analyzer).
- FDP was performed using latex agglutination test kit (TULIP XL FDP).
- D-dimer levels were measured by a quantitative latex assay (STA-LIA test D-DI).
- Pooled plasma from healthy individuals were prepared and divided into aliquots, each containing 1 mL, and stored at –40ºC to be used simultaneously with patients' plasma. Positive and negative control plasma samples (for D-dimer test) were supplied with plasma D-dimer kit.
- all the laboratory tests were performed in the laboratory of the hospital.

The reference ranges were established by our laboratory, which is NABL accredited. The reference ranges were:

- Platelet count: 150,000–400,000/mm^3
- PT = 11–15 s
- APTT = 29–35 s,
- fibrinogen = 250–450 mg/dL,
- Plasma D-dimer concentration < 0.5µg/mL.
- FDP, being a qualitative test, gives result as either positive or negative.

The concept of "intravascular coagulation and fibrinolysis (ICF) syndrome" was introduced by Owen and Bowie, and the purpose was to examine the incidence and type of hemostatic derangement in patients with malignancies.[4]

Patients were considered to have ICF if their D-dimer was more than 0.5 µg/dL.

The concept of ICF of Owen and Bowie was adopted and their classification of overcompensated, compensated, and decompensated ICF was tested with an attempt to identify key tests that might help to ascertain patients with coagulation problems.

The D-dimer and platelets were used as indicators and to separate the patients into four groups:

1. patients with no ICF (normal D-dimer);
2. those with overcompensated ICF (elevated D-dimer and elevated platelets count);
3. those with compensated ICF (elevated D-dimer but normal platelets count); and
4. those with decompensated ICF (elevated D-dimer and decreased platelets count)[2].

This has been correlated with acute, subacute, and chronic disseminated intravascular coagulation (DIC) and decompensated, overcompensated, and compensated DIC, respectively.

- A set of hemostatic tests were applied to these four groups, and many tests were done. (PT, APTT, fibrinogen, and D-dimer).
- In this study, D-dimer test has been considered for the diagnosis of ICF, because it is presently regarded to be more specific for fibrin degradation products, while the formation of FDP, X, Y, D, and E fragments, may be either fibrinogen or fibrin derived following the plasmin digestion.[5,6]

Statistical Analysis

Data were entered into a computerized database for statistical analysis. The mean, standard deviation, standard error of mean, standard error of difference, *t*-value, and 95% confidence intervals of various variables were calculated. The statistical significance of difference in the rate of an outcome between the two groups was assessed by χ^2-test.

Result

In our study, 37.14% of malignancies showed thrombocytosis, indicating slight tendency toward thrombocytosis (n = 13/16) [Table 1].

Benign lesions showed PT from 11 to15 s (85.71%). Malignancies predominantly showed APTT > 35 s (85.71%) [Table 2].

Benign lesions showed APTT from 29 to 35 s (92.86%). Malignancies predominantly showed APTT > 35 s (85.71%). Thus malignancies exhibited elevated PT and APTT when compared with control group and benign lesions.

In our study, control group and patients with benign lesions showed exclusively to have fibrinogen from 250 to 450 mg/dL (100%). Patients with malignancies predominantly showed fibrinogen within 250 and 450 mg/dL (45.71%). However, hypofibrinogenemia (17.14%) and hyperfibrinogenemia (37.14%) were seen only in patients with malignancies. Malignancies tended to show abnormal fibrinogen (54.29%) when compared with benign lesions [Table 3].

In our study, control group and benign lesions showed negative FDP test (100%). Malignancies predominantly revealed positive FDP test (80%). Malignancies tended to show a positive FDP test when compared with control group and benign lesions [Table 4].

In our study, control group and patients with benign lesions showed D-dimer between 0.2 and 0.5 μg/mL (100% and 92.86%, respectively). Patients with malignancies predominantly showed D-dimer > 0.5μg/mL (88.57%). Such patients with elevated D-dimer are said to have ICF syndrome.

Malignancies tended to show elevated D-dimer when compared with benign lesions.

A singular case of elevated D-dimer in benign lesions was of recently operated intestinal polyp explaining the elevated D-dimer in this case [Table 5].

Table 1: Distribution according to the platelet count

Platelet count, ×10³/mm³	Number of cases			
	Normal, *N* (%)	Benign, *N* (%)	Malignant, *N* (%)	Total (%)
<150	2 (18.18)	3 (21.43)	2 (5.71)	7 (11.67)
150–400	8 (72.73)	9 (64.29)	20 (57.14)	37 (61.67)
>400	1 (9.09)	2 (14.29)	13 (37.14)	16 (26.67)
Total	11 (100)	14 (100)	35 (100)	60 (100)

Table 2: Distribution according to the PT

PT (s)	Number of cases			
	Normal, *N* (%)	Benign, *N* (%)	Malignant, *N* (%)	Total (%)
11–15	10 (90.91)	12 (85.71)	8 (22.86)	30 (50)
>15	1 (9.09)	2 (14.29)	27 (77.14)	30 (50)
Total	11 (100)	14 (100)	35 (100)	60 (100)

Table 3: Distribution according to FIB

FIB, mg/dL	Number of cases			
	Normal, *N* (%)	Benign, *N* (%)	Malignant, *N* (%)	Total (%)
<250	0 (0)	0 (0)	6 (17.14)	6 (10)
250–450	11 (100)	14 (100)	16 (45.71)	41 (68.33)
>450	0 (0)	0 (0)	13 (37.14)	13 (21.67)
Total	11 (100)	14 (100)	35 (100)	60 (100)

Table 4: Distribution according to FDP

FDP	Number of cases			
	Normal, *N* (%)	Benign, *N* (%)	Malignant, *N* (%)	Total (%)
Positive	0 (0)	0 (0)	28 (80)	28 (46.67)
Negative	11 (100)	14 (100)	7 (20)	32 (53.33)
Total	11 (100)	14 (100)	35 (100)	60 (100)

Table 5: Distribution according to D-dimer

D-dimer, μg/mL	Number of cases			
	Normal, *N* (%)	Benign, *N* (%)	Malignant, *N* (%)	Total (%)
0.2–0.5	11 (100)	13 (92.86)	4 (11.43)	28 (46.66)
>0.5 (ICF)	0 (0)	1 (7.14)	31 (88.57)	32 (53.33)
Total	11 (100)	14 (100)	35 (100)	60 (100)

Table 6: Distribution of the ICF syndrome cases into decompensated, compensated, and overcompensated

Platelets (×10³/mm³)	Number of cases		
	Benign, *N* (%)	Malignant, *N* (%)	Total (%)
150–400 (compensated)	1 (5)	199 (95)	20 (62.50)
<150 (decompensated)	0 (0)	2 (100)	2 (6.25)
>400 (overcompensated)	0 (0)	10 (100)	10 (31.25)
Total	1	31	32 (100)

Table 7: Lymph node involvement status

Lymph nodes	No. of cases	Mean D-dimer (µg/mL)
Involved	12	2.325
Not involved	7	1.85
Total	19	100

Table 8: Statistical comparison between apparently normal controls and patients with malignancies

Variables	Malignancy			Normal			*t*	df	95%CI		SED	*P*
	Mean	SD	SEM	Mean	SD	SEM			LL	UL		
PLT	334.14	104.56	17.67	273.73	126.52	38.15	1.5899	44	–16.17	137	38.001	0.119
PT	23.151	17.48	2.955	13.691	0.887	0.267	1.7805	44	–1.248	20.169	5.313	0.0819
APTT	46.426	18.796	3.177	32.945	2.25	0.678	2.3553	44	1.946	25.015	5.723	0.023
FIB	409.51	163.44	27.63	341.18	66.43	20.03	1.3437	44	–34.16	170.8	50.855	0.1859
D-dimer	2.7246	2.9599	0.5003	0.3682	0.061	0.0184	2.6199	44	0.5437	4.169	0.899	0.012

Table 9: FDP status

FDP	Normal controls	Malignant
Positive	0	28
Negative	11	7
Total	11	35

ICF syndrome is classified into compensated, decompensated, and overcompensated types based on low, adequate, and high platelet levels of the patient, respectively. In our study, the majority of patients with malignant lesions with elevated D-dimer showed compensated type of ICF (61.29%), followed by overcompensated type (32.26%) and decompensated type (6.45%) [Table 6].

In our study, the status of lymph node involvement in 19 patients with malignancies was available. Of the 19 cases, 12 showed tumor involvement while seven of them did not. The mean D-dimers were 2.325µg/mL and 1.85µg/mL, respectively [Table 7].

Statistical tests were done.The mean, standard deviation (SD), and standard error of mean (SEM) for various variables were calculated in the normal and malignancies groups. Statistical comparison was done between the normal group and malignancies group. The *t* value, degree of freedom (*df*), 95% confidence interval (CI), standard error of difference (SED), and *P* value were calculated for each of the variables.

P value of < 0.05 was considered to be statistically significant. [Table 8].

The χ^2-test was done for FDP, thus comparing the malignancies vs. normal controls group and malignant vs. benign lesions group [Table 9].

The *P* values for the above-mentioned comparison is <0.0001, which is extremely statistically significant.

Discussion

Cancer is a prothrombotic state. Experimental and clinical studies have shown an association between cancer and haemostasis, which is altered and provides a growth benefit to tumors, although clinical symptoms occur less often.[7] The tumors, through the production of procoagulant factors, and the host, through its inflammatory response, participate in the process.

Abnormal coagulation activation encourages endothelial adhesion, metastatic spread, tumor cell growth, and survival.[2]

In this study we studied the hemostatic and coagulation profile in 60 cases (35 showed malignancies, 14 showed benign lesions, and 11 were apparently normal controls). Complete blood count, PT, APTT, Fibrinogen, D-Dimer, and FDP were done.

Amin et al.[7] showed that the percentage of patients with abnormal coagulation was 88%. Mohammed et al.[2] showed that the coagulation abnormalities were in 80% patients. In our study, 94.28% malignancies showed coagulation abnormalities comparable with the above studies.

Advanced cancer shows increased platelet activation, indicated by increased platelet turnover and decreased platelet survival time.[8]

Mohammed et al.[2] showed that mean platelet count in cancer was 317.8 ± 23.46 ×10^3/cm and in control group was 260.7 ± 7.96 ×10^3/cm. Amin et al.[7] showed that the mean platelet count = 286 ± 144 × 10^3/cm in malignancies in comparison with the control group (212 ± 46 × 10^3/cm). However, Omer and Abdalla[9] showed the mean platelet count in cancer was 249.6 ± 142.3 × 10^3/cm, while for the control group, it was 279.7 ± 77.9 × 10^3/cm. Suega and Bakta[10] showed the mean platelet = 365 × 10^3/cm in malignancies.

In our study, the mean platelet count in malignancies was 334.14 ± 104.56 × 10^3/cm, which was higher when compared with apparently normal controls (273.73 ± 126.52 × 10^3/cm).

Mohammed et al.[2] showed that mean PT in cancer was 15 ± 0.32 s and in control group was 12.9 ± 0.27 s. Amin et al.[7] showed that the mean PT was15 ± 3s in malignancies group when compared with the control group with a mean PT of 13 ± 1 s. Omer and Abdalla[9] showed that the mean PT in cancer was 13.7 ± 1.3 s, while in control group, it was 12.2 ± 0.8 s.

In our study, the mean PT in malignancies was 23.15 ± 17.48 s, higher in comparison with the patients with benign lesions and apparently normal controls (14.28 ± 1.91 s and 13.69 ± 0.89 s, respectively).

Mohammed et al.[2] showed that mean APTT in cancer was 37.9 ± 0.31 s and in control group was 35.1 ± 0.56 s. Omer and Abdalla[9] showed that the mean APTT in cancer was 35.7 ± 6.6 s and in the control group was 29.6 ± 2.2 s.

In our study, the mean APTT in malignancies was 46.43 ± 18.8 s, higher in comparison with benign lesions and apparently normal controls (33.66 ± 4.26 s and 32.95 ± 2.25 s, respectively).

In our study, there is a significant difference in the mean APTT values of the control group and patients with malignancies, the difference being statistically significant ($p = 0.023$).

Mohammed et al.[2] showed that the mean fibrinogen in cancer was 310 ± 15 mg/L and in control group was 300 ± 8 mg/dL. Amin et al.[7] showed that the mean fibrinogen was 300 ± 100 mg/dL in malignancies in comparison with the control group (230 ± 60 mg/dL).

In our study, the mean fibrinogen in malignancies was 409.51 ± 163.44 mg/dL, higher in comparison with benign lesions and apparently normal controls (346 ± 57.94 mg/dL and 341.18 ± 66.43 mg/dL, respectively).

As the half-life of fibrinogen is 4 days, a 50% or greater decrease in fibrinogen over 1 day is convincing evidence of DIC or fibrinolysis, despite the final value being within normal range.[2]

D-dimer, the main breakdown fragment of fibrin, is a biochemical marker of thrombogenesis and fibrin turnover. High D-dimer is an indirect marker of hypercoagulation activation and thrombolysis. Procoagulant factors in cancer cause constitutive activation of the coagulation cascade leading to thrombin and fibrin generation. Fibrin formation and remodeling process provides extra cellular matrix essential for the initial step of cancer cell to migrate, invade, and metastasize. It is summarized that more advanced the cancer, more D-dimer is produced as an marker for coagulation activation.[10]

Amin et al.[7] showed the mean D-dimer was 3.708 ± 3.236 µg/mL in malignancies in comparison with the control group (0.325 ± 0.365 µg/mL). Omer and Abdalla[9] showed the mean D-dimer in cancer was 2.19632 ± 2.11095 µg/mL, while for the control group mean D-dimer was 0.21365 ± 0.10357 µg/mL. Mohammed et al.[2] showed D-dimer in cancer was 2–4 µg/mL and in control group was <0.5 µg/mL. Suega and Bakta[10] showed the mean D-dimer was 1.260 µg/mL in malignancies.

In our study, a statistically significant difference in the mean D-dimer of patients with malignancies and the control group is seen ($p = 0.012$).

However, high D-dimer can be seen in DIC, vaso-occlusive crisis in sickle cell disease, thromboembolic events, myocardial infarction, surgery, inflammatory processes, smoking, senility, pregnancy, trauma, and infection.

Plasma D-dimer correlates with tumor burden, no. of metastatic sites, progression kinetics, cytokines related to angiogenesis,[11] invasion depth, lymph node metastasis, peritoneal dissemination, distant metastasis, tumor size, and TNM stage.[12] The D-dimer and platelets were used as markers to separate the patients into four groups of ICF.[5,13]

Patients with no ICF, those with overcompensated, compensated, and decompensated ICF.

Mohammed et al.[2] showed that the patients with ICF was 45% (n = 18/40). Those with overcompensated ICF was 38.88% (n = 7/18), with compensated ICF was 38.88% (n = 7/18), and with decompensated ICF was 22.22% (n = 4/18).

In our study, patients with malignancies showing ICF was 88.57% (n = 31/35). Those with overcompensated ICF was 32.26% (n = 10/31), with compensated ICF was 61.29% (n= 19/31), and with decompensated ICF was 6.45% (n = 2/31).

This variation is probably owing to the difference in the type of patients that were studied in the two studies. The percentage of patients with compensated ICF is comparable in both the studies.

Suega and Bakta[10] found patients with ICF was 75.94% (n = 60/79), patients with overcompensated ICF was 40% (n = 24/60), which is comparable with our study. Omer and Abdalla[9] found ICF in 88% patients (n = 53/60). In this study most of the patients (87%) had normal platelets counts, 10% had thrombocytopenia and 3% had an elevated count suggesting a compensated ICF in majority of the patients which is also comparable to our study.

Advance cancer stage with high tumour load and elevated proliferation rate is associated with high coagulation activation, its duration and severity.

Blackwell et al.[14] showed 75.75% (n = 25/33) of patients with involved lymph nodes had elevated D dimer, which is comparable with our study. In our study patients of malignancies with lymph node involvement showed elevated D- dimer (83.33%, n = 10/12).

In our study, there were 19 patients with malignancies in which the status of lymph node involvement was available. Of the 19 cases, 12 showed tumor involvement in lymph nodes, while 7 were negative. The mean D-dimers was 2.325 ± 3.33 µg/mL and 1.85 ± 3.33 µg/mL, respectively, which showed a significant difference.

Given its sensitivity for predicting positive lymph node involvement, a role of D-dimer, along with other predictive factors to decide whether or not axillary lymph node dissection is needed may be used.[14]

The χ^2-test was applied on the FDP values, thus comparing malignant lesions vs. normal controls group.

The *P* value for the above comparison is <0.0001, which is extremely statistically significant.

Conclusion

Malignant cells can interact with the haemostatic system in several ways, but the two major interactions are the capacity to produce and release procoagulant, fibrinolytic activities, and inflammatory cytokines; and direct interaction with other blood cells (i.e., endothelial cells, platelets, and monocytes).

Abnormal coagulation activation encourages endothelial adhesion and metastatic spread, tumor cell growth, and their survival. Despite the well-established link between cancer and venous thrombosis, anticoagulation is not the standard treatment for these patients. Assessment of the coagulation profile in cancer might help understanding their relationship with coagulation abnormalities and in the prediction as well as management of complications arising from them. Disruption of blood coagulation impairs metastasis. Use of mild anticoagulants in the setting of cancer with DIC might be considered hoping that antithrombotic treatment may have a positive result on tumor growth and propagation.

Our study implies a relation between activation of hemostasis mirrored by elevated D-dimer and malignancy. D-dimer might be used as a universal surrogate indicator of the relation between cancer and the activation of hemostasis and fibrinolysis, with elevated D-dimer levels symbolizing the pathogenesis of a more aggressive malignant process associated with poor clinical results.

References

1. Jurasz P, Alonso-Escolano D, Radomski MW. Platelet–cancer interactions: mechanisms and pharmacology of tumour cell-induced platelet aggregation. Br J Pharmacol 2004;143(7):819–26.
2. Mohammed MR, Mansoor SS, Taher MG. Hemostatic derangements in patients with solid malignant tumors. J Pioneering Med Sci 2013;3(1):1–9.
3. Hussain A. Haemostatic issues in cancer development and progression:? the role of coagulation and haemostatic factors in cancer development and metastasis. Pécsi Egyetemi Archívum 2007;
4. Hagedorn AB, Bowie EJ, Elveback LR, Owen CA Jr. Coagulation abnormalities in patients with inoperable lung cancer. Mayo Clin Proc 1974;49(9):647–53.
5. Seligsohn URI. Disseminated intravascular coagulation In: *Williams Manual of Hematology*, 5th edn., Beutler E, Lichtman MA, Coller BS (Eds.). USA: McGraw–Hill Professional, 1995. pp. 1497–516.
6. Elms MJ, Bunce IH, Bundesen PG, Rylatt DB, Webber AJ, Masci PP, et al. Measurement of cross linked fibrin degradation products—an immunoassay using a monoclonal antibodies. Thromb Haemost 1983;50(2):591–4.
7. Amin AM, Getta HA, Abdulsalam AH. Diagnosis of chronic disseminated intravascular coagulation in 72 cancer patients according to the International Society on thrombosis and hemostasis score system. Iraqi J Comm Med 2012;(2).
8. Caine GJ, Stonelake PS, Lip GY, Kehoe ST. The hypercoagulable state of malignancy: pathogenesis and current dsebate. Neoplasia 2002;4(6):465–73.
9. Omer AOA, Abdalla MHA. Evaluation of haemostatic abnormalities among Sudanese patients with haematologic and solid malignancy. Am J Med Med Sci 2014;4(5):150–3.
10. Suega K, Bakta IM. Correlation between clinical stage of solid tumor and D-dimer as a marker of coagulation activation. Acta Med Indones 2011;43(3):162–7.
11. Dirix LY, Salgado R, Weytjens R, Colpaert C, Benoy I, Huget P, et al. Plasma fibrin D-dimer levels correlate with tumour volume, progression rate and survival in patients with metastatic breast cancer; Br J Cancer 2002;86(3):389–95.
12. Liu L, Zhang X, Yan B, Gu Q, Zhang X, Jiao J, Sun D, Wang N, et al. Elevated plasma D-dimer levels correlate with long term survival of gastric cancer patients. PLoS One 2014;9(3):e90547.
13. Sun NC, MCAfee WM, Hum GJ, Weiner JM. Haemostatic abnormalities in malignancy, a prospective study of one hundred eight patients. part 1. Coagulation studies. Am J Clin Pathol 1979;71(1):10–6.
14. Blackwell K, Haroon Z, Broadwater G, Berry D, Harris L, Iglehart D, et al. Plasma D-dimer levels in operable breast cancer patients correlate with clinical stage and axillary lymph node status. J Clin Oncol 2000;18(3):600–08.

How to cite this article: Patel SM, Gupta S, Patel MM, Mahadik JD, Patel KA, Patel AS. A study of coagulation profile in neoplastic conditions. Int J Med Sci Public Health 2016;5:402-407

Source of Support: Nil, **Conflict of Interest:** None declared.

Molecular Cancer Therapeutics

Small Molecule Therapeutics

Urolithin A, a Novel Natural Compound to Target PI3K/AKT/mTOR Pathway in Pancreatic Cancer

Tulasigeri M. Totiger, Supriya Srinivasan, Venkatakrishna R. Jala, Purushottam Lamichhane, Austin R. Dosch, Alexander A. Gaidarski III, Chandrashekhar Joshi, Shobith Rangappa, Jason Castellanos, Praveen Kumar Vemula, Xi Chen, Deukwoo Kwon, Nilesh Kashikar, Michael VanSaun, Nipun B. Merchant, and Nagaraj S. Nagathihalli

DOI: 10.1158/1535-7163.MCT-18-0464 Published February 2019

Abstract

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive malignancy and is highly resistant to standard treatment regimens. Targeted therapies against *KRAS*, a mutation present in an overwhelming majority of PDAC cases, have been largely ineffective. However, inhibition of downstream components in the KRAS signaling cascade provides promising therapeutic targets in the management of PDAC and warrants further exploration. Here, we investigated Urolithin A (Uro A), a novel natural compound derived from pomegranates, which targets numerous kinases downstream of KRAS, in particular the PI3K/AKT/mTOR signaling pathways. We showed that treatment of p70S6K *in vitro,* successfully inhibited the growth of L-Kras$^{G12D/+}$;Tgfbr2$^{flox/flox}$ (PKT) mice compared of these Uro A–treated tumor samples confirmed AKT and p70S6K, reduced proliferation, and models. In addition, Uro A treatment reprogrammed infiltrating immunosuppressive cell populations such ages, and regulatory T cells. Overall, this work s a therapeutic agent in PDAC through suppression

Footnotes

- **Note:** Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Received May 1, 2018.

Revision received September 6, 2018.

Accepted October 29, 2018.

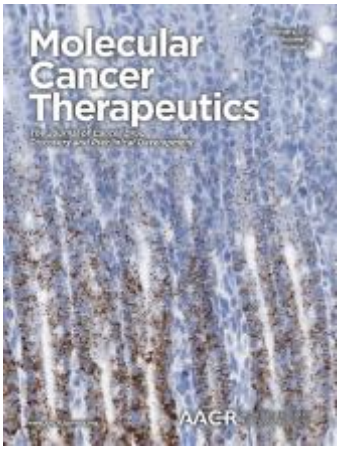
Published first November 7, 2018.

View Full Text

Previous

Next

Back to top



February 2019
Volume 18, Issue 2
Table of Contents
Table of Contents (PDF)
About the Cover
Index by Author
Editorial Board (PDF)

Molecular Cancer Therapeutics
eISSN: 1538-8514
ISSN: 1535-7163

MEDICAL SCIENCE MONITOR

LAB/IN VITRO RESEARCH

e-ISSN 1643-3750
© Med Sci Monit, 2018; 24: 846-854
DOI: 10.12659/MSM.907814

Received: 2017.10.30
Accepted: 2018.01.16
Published: 2018.02.11

Ursolic Acid Attenuates High Glucose-Mediated Mesangial Cell Injury by Inhibiting the Phosphatidylinositol 3-Kinase/Akt/Mammalian Target of Rapamycin (PI3K/Akt/mTOR) Signaling Pathway

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEF Er-Min Wang
AG Qiu-Ling Fan
BC Yuan Yue
CF Li Xu

Department of Nephrology, First Hospital of China Medical University, Shenyang, Liaoning, P.R. China

Corresponding Author: Qiu-Ling Fan, e-mail: cmufql@163.com
Source of support: National Natural Science Foundation of China (81770724,81270808); Natural Science Foundation of Liaoning Province (201602821); and the Science and Technology Planning Program of Shenyang City (F16-205-1-40)

Background: To investigate the protective effect of ursolic acid (UA) on high glucose (HG)-induced human glomerular mesangial cell injury and to determine whether UA inhibits cell proliferation and reactive oxygen species (ROS) production by suppressing PI3K/Akt/mTOR pathway activation.

Material/Methods: Human mesangial cells were cultured with normal glucose (NG group), high glucose (HG group), mannitol (mannitol hypertonic control group), or high glucose with different concentrations (0.5, 1.0, and 2.0 mmol/L) of UA (HG+UA groups). Cell proliferation and intracellular ROS levels were assessed by methyl thiazolyl tetrazolium (MTT) and dichloro-dihydro-fluorescein diacetate (DCFH-DA) flow cytometry assays, respectively. Western blotting was used to detect mesangial cell expression of PI3K/Akt/mTOR pathway components, including Akt, p-Akt, mTOR, and p-mTOR, and proteins related to cell injury, including TGF-β1 and fibronectin (FN). mRNA expression of TGF-β1 and FN were evaluated using real-time quantitative polymerase chain reaction (PCR).

Results: Abnormal proliferation was observed in human glomerular mesangial cells at 48 h after treatment with HG, and UA suppressed the HG-induced proliferation of mesangial cells in a dose-dependent manner. UA inhibited ROS generation and oxidative stress in mesangial cells and mitigated mesangial cell injury. Treatment with UA reduced Akt and mTOR phosphorylation levels in mesangial cells exposed to HG ($p<0.05$ *vs.* HG) and downregulated protein and mRNA expression of TGF-β1 and FN in these cells ($p<0.05$ *vs.* HG).

Conclusions: UA attenuated mesangial cell proliferation and ROS generation by inhibiting HG-mediated PI3K/Akt/mTOR pathway activation, thereby ameliorating mesangial cell damage.

MeSH Keywords: Glucose • Mesangial Cells • Phosphatidylinositol 3-Kinases

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/907814

2529 1 6 20

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

Background

The morbidity of diabetes has increased rapidly in the past decade. Diabetic nephropathy (DN) is a serious complication of diabetes and is the most common cause of end-stage renal disease [1]. A typical pathological feature of DN [2], glomerulosclerosis is primarily manifested as deposition of extracellular matrix (ECM) proteins, such as collagen and fibronectin (FN), in the mesangial area, and the resulting reduced filtration surface area of glomerular capillaries leads to further progression of DN [3]. PI3K/Akt/mTOR signaling is a classical pathway that regulates many cellular functions, such as glucose metabolism, glycogen synthesis, protein synthesis, cell proliferation, cell hypertrophy, and cell death [4], and previous studies have shown that the PI3K/Akt/mTOR pathway is involved in the pathogenesis of DN. High glucose (HG) levels activate phosphatidylinositol-3-kinase (PI3K) in mesangial cells, which stimulates phosphoinositide-dependent kinase (PDK) and protein kinase B (PKB) and subsequently upregulates expression of RhoA, Rac, and type I collagen, inducing an inflammatory response in these cells [5], which leads to damage.

The plant-derived pentacyclic triterpene ursolic acid (UA), which is found widely in berries, fruits, and herbs, exhibits anti-tumor, anti-liver fibrosis, hypoglycemic, hypolipidemic, and anti-atherosclerotic effects [6–11]. Previous studies have found that UA inhibits overexpression of inducible nitric oxide synthase (iNOS) by suppressing STAT-3, ERK1/2, and JNK pathway activation and alleviates glomerular hypertrophy and the increased ECM deposition typically observed in a streptozotocin-induced DN rat model, thus delaying DN progression [12]. However, the detailed mechanism of UA-mediated renal protection remains unclear and requires in-depth analysis.

This study aimed to investigate the effect of UA on the proliferation of human mesangial cells cultured *in vitro* under HG conditions, to determine whether UA has a protective effect on mesangial cell injury under diabetic conditions and to determine whether the mechanism occurs through regulation of the PI3K/Akt/mTOR pathway.

Material and Methods

Materials

Cells and reagents

The following cells, reagents, and antibodies were used in this study: human glomerular mesangial cells (ScienCell Research Laboratories); 4201 standard mesangial cell medium (MCM; ScienCell Research Laboratories); UA (Sigma, US); methyl thiazolyl tetrazolium (MTT) powder (Sigma, US); dichloro-dihydro-fluorescein diacetate (DCFH-DA) Reactive Oxygen Species (ROS) Detection Kit (Wuhan Beyotime); TRIzol RNA Extraction Kit (Invitrogen, US); GoTaq Quantitative Polymerase Chain Reaction (qPCR) Master Mix (Promega, US); rabbit anti-Akt, anti-p-Akt (p-Ser473), anti-mTOR and anti-p-mTOR (Ser2448) polyclonal antibodies (Cell Signaling Technology, US); rabbit anti-TGFβ1, anti-FN, anti-Bax, anti-Samd2/3, anti-Samd7 and anti-GAPDH polyclonal antibodies (Proteintech, US); and horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (Abcam, US).

Methods

Mesangial cell culture: Human mesangial cells were thawed and cultured in MCM 4201 at 37°C in an incubator with 5% CO_2 and saturated humidity for cell adherence to the culture dish. The culture medium was changed every other day. The cells were trypsinized with 0.25% trypsin for passaging. Cells in exponential growth phase from the 5th to 9th passages were used for subsequent experiments after they had attached to the culture dish and reached 70–80% confluence.

Groups: Cells were divided into the following groups at 24 h after synchronization in serum-free culture medium: (1) normal glucose (NG) group (5.5 mmol/L glucose); (2) HG group (30.0 mmol/L glucose); (3) UA group (30.0 mmol/L glucose+0.5, 1.0, or 2.0 mmol/L UA); and (4) mannitol hypertonic control group (5.5 mmol/L glucose+24.5 mmol/L mannitol, MA).

Detection of cell proliferation using MTT assays: Cells in exponential growth phase were collected. After adjusting the cell density of the suspension, 150 ml of 4201 culture medium was added to each experimental well in a 96-well plate. The cells were seeded in the plate and cultured at 37°C with 5% CO_2 until they had fully covered the bottom of each well; 150 ml of stimulant was added to each well, and each sample was repeated in 3 wells. The cells were then cultured at 37°C with 5% CO_2 and observed under an inverted microscope after 24, 48, and 72 h of culturing. MTT solution (20 ml; 5 mg/mL, or 0.5% MTT) was then added to each well, and the cells were cultured for another 4 h. The medium in each well was carefully removed, followed by the addition of 150 ml dimethyl sulfoxide and incubation on a shaker at low speed for 10 min to fully dissolve the crystalized precipitate. The absorbance of each well was measured at 490 nm using a microplate reader.

DCFH-DA flow cytometry to detect ROS production in cells: Cells in exponential growth phase were collected. After adjusting the cell density of the suspension, 5 mL of 4201 culture medium was added to each flask, and the density of the cells to be tested was adjusted to 5×10^5/flask. The cells were cultured at 37°C with 5% CO_2 until they had adhered to the flask wall, and the culture medium was replaced with medium

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

Table 1. PCR primer sequences.

Primer	Sequence	Product size
β-actin	5'-CCATGTACGTTGCTATCCAGG-3' 5'-TCTCCTTAATGTCACGCACGA-3'	252 bp
FN	5'-CCGCCATTAATGAGAGTGAT-3' 5'-AGTTAGTTGCGGCAGGAGAAG-3'	133 bp
TGF-β	5'-GCCCTGGACACCAACTATTGC-3' 5'-AGGCTCCAAATGTAGGGGCAGG-3'	161 bp

containing different stimulants. The cells were cultured at 37°C with 5% CO_2 for 24, 48, and 72 h, collected with 600 ml trypsin and counted; 20 000 cells/mL were centrifuged at 1000 rpm and 4°C for 5 min, and the supernatant was discarded. The cells were gently resuspended in 1 mL of 4201 culture medium containing 10 mmol/L DCFH-DA, followed by culturing in an incubator at 37°C for 30 min. The cells and probe were mixed thoroughly for 30 min by inverting the flask once every 3–5 min. The cell suspension was then centrifuged at 1000 rpm for 5 min at 4°C, and the supernatant was discarded. The cells were resuspended and washed 3 times with 1 mL serum-free medium to completely remove the DCFH-DA that did not enter the cells. The cells were then centrifuged again at 1000 rpm and 4°C for 5 min, and the supernatant was discarded, followed by the addition of 500 ml phosphate-buffered saline (PBS) to resuspend the cells. After 30 min, the cells were subjected to flow cytometry (using the parameters set for FITC) at an excitation wavelength of 488 nm and an emission wavelength at 525 nm to detect the fluorescence intensity before and after stimulation.

Western blotting was used to detect the activity of the PI3K/Akt/mTOR and TGF-β1/Samd pathways and expression of TGF-β1, FN, Smad2/3, Samd7 proteins: Cells were collected from each group, and 60 μL protein lysis buffer and 0.6 μL protease inhibitor were added, followed by ultrasonication for 20 min and centrifugation at 1200×g for 5 min. The supernatant was collected as the total protein sample, and the bicinchoninic acid (BCA) method was used to measure the protein concentration, which was then adjusted to 5 μg/μL. The protein samples were separated by 7.5%, 10%, and 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% skim milk powder or 3% bovine serum albumin (BSA) at room temperature for 2 h, followed by incubation with the primary antibody at 4°C overnight. The membrane was then washed with Tris-buffered saline-Tween 20 (TBST) and incubated with the secondary antibody at room temperature for 2 h. The membrane was washed again with TBST, followed by the addition of enhanced electrochemiluminescence (ECL) reagent and observation.

Real-time qPCR was performed to detect TGF-β1 and FN mRNA expression: Total RNA was extracted from mesangial cells using TRIzol reagent (Invitrogen, US) according to the manufacturer's manual, and 4 μg total RNA was taken from each sample to synthesize cDNA using random primers and Moloney murine leukemia virus (MMLV) reverse transcriptase. Real-time qPCR was performed using the Rotor-Gene 3000 PCR system. The primers were synthesized by TaKaRa (Dalian, China) (Table 1). In brief, 2 μL cDNA was added to a 20-μL reaction system (containing 2.5 μL 2.5 nmol/μL deoxynucleotides (dNTPs), 10 μL 10× SYBR Green I PCR buffer, 1.5 μL 25 mmol/L $MgCl_2$, 1 μL each of upstream and downstream primers, and 1 U Taq polymerase). The reaction proceeded at 94°C for 5 min for predenaturation, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. β-Actin was used as the internal standard, and the experimental data were processed using the $2^{-\Delta\Delta}$CT method to calculate the mRNA/β-actin mRNA ratio for each sample. The experiment was repeated 3 times for each experimental group.

Statistical analysis: All data were analyzed using SPSS17.0 software. The experimental data are expressed as the mean ± standard deviation ($\chi \pm s$). Univariate analysis of variance (ANOVA) was used for intergroup comparisons, and the least significant difference (LSD) method was employed for pairwise comparison. Differences were considered statistically significant at $p<0.05$.

Results

Comparison of the mesangial cell proliferation rate in different groups

The proliferation rates of mesangial cells in each group were measured using an MTT assay after 24, 48, 72 h of culturing. Based on the results, the difference in the proliferation rate was most obvious in the 48-h group, and no statistically significant difference in proliferation rate was found for the hypertonic control group compared with the NG group ($p>0.05$). The proliferation rate of cells in the HG group was significantly higher than that in the NG group ($p<0.05$). Compared with

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

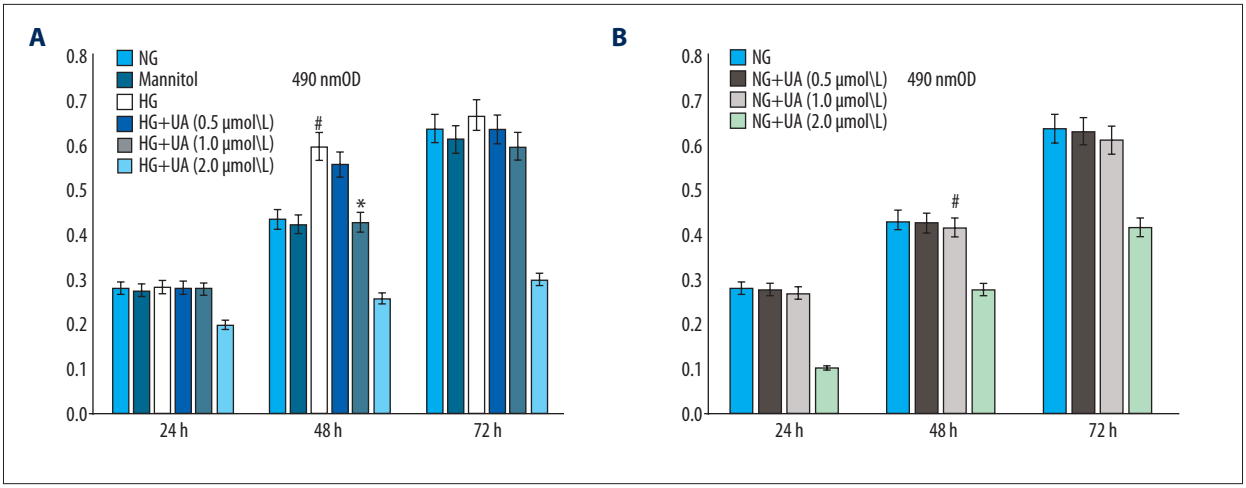


Figure 1. (**A**) UA inhibited the mesangial cell proliferation induced by high glucose (determined by an MTT assay). # p<0.01 compared with the NG group; * p<0.05 compared with the HG group. (**B**) UA (1.0 umol/L) did not affect the mesangial cell growth induced by normal glucose. NG – normal glucose culture group; Mannitol – hypertonic control group; HG – high glucose culture group; UA – ursolic acid.

the HG group, mesangial cells exhibited a dose-dependent reduction in proliferation at 48 h after treatment with different concentrations of UA (0.5, 1.0, and 2.0 mmol/L; Figure 1A). In contrast, the normal growth rate of mesangial cells was not affected after treatment with different concentrations of UA (0.5 and 1.0 umol/L) in the NG group (Figure 1B).

Changes in ROS production in mesangial cells

A DCFH-DA probe was used to detect ROS levels in mesangial cells via flow cytometry, and the results revealed higher ROS production in cells of the HG group than in NG group cells (p<0.05). HG+UA group (1.0 mmol/L) cells exhibited significantly decreased ROS production compared with the HG group (p<0.05; Figure 2).

Changes in p-Akt/Akt and p-mTOR/mTOR protein expression

After 48 h of culturing, markedly higher levels of p-Akt (ser473) and p-mTOR (ser2448) (p<0.05) proteins were observed in HG group mesangial cells than in cells of the NG group. UA (1.0 mmol/L) significantly inhibited HG-induced Akt and mTOR phosphorylation in mesangial cells (Figure 3).

Changes in mRNA and protein expression of TGFβ1 in mesangial cells detected by real-time PCR and Western blotting

The results of real-time PCR and Western blotting showed that TGFβ1 mRNA and protein expression in mesangial cells increased significantly (p<0.05) in the HG group after 48 h of culturing. UA (1.0 mmol/L) significantly inhibited HG-induced TGFβ1 mRNA and protein expression in mesangial cells (p<0.05; Figure 4).

Changes in FN mRNA and protein expression detected by RT-PCR and Western blotting

Real-time PCR and immunoblotting revealed significantly increased FN mRNA and protein expression in HG group mesangial cells (p<0.05) after 48 h of culturing. UA (1.0 mmol/L) significantly inhibited HG-induced FN mRNA and protein expression in mesangial cells (p<0.05; Figure 5).

Changes in Smad2/3 and Samd7 protein expression detected by Western blotting

Immunoblotting revealed significantly increased Smad2/3 protein expression and significantly decreased Smad7 in HG group mesangial cells (p<0.05) after 48 h of culturing. UA (1.0 mmol/L) significantly inhibited HG-induced Smad2/3 protein expression in mesangial cells (p<0.05). In contrast, UA (1.0 mmol/L) significantly promoted HG-induced Smad7 protein expression in mesangial cells (p<0.05; Figure 6).

Discussion

PI3K/Akt/mTOR signaling is a classical pathway widely found in eukaryotic cells that regulates a variety of cellular functions, such as glucose metabolism, glycogen synthesis, protein synthesis, cell proliferation, cell hypertrophy, and cell death [4]. In this study, we observed elevated p-Akt and p-mTOR expression in human mesangial cells exposed to HG stimulation for 48 h, along with activation of the PI3K/Akt/mTOR pathway, abnormal

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

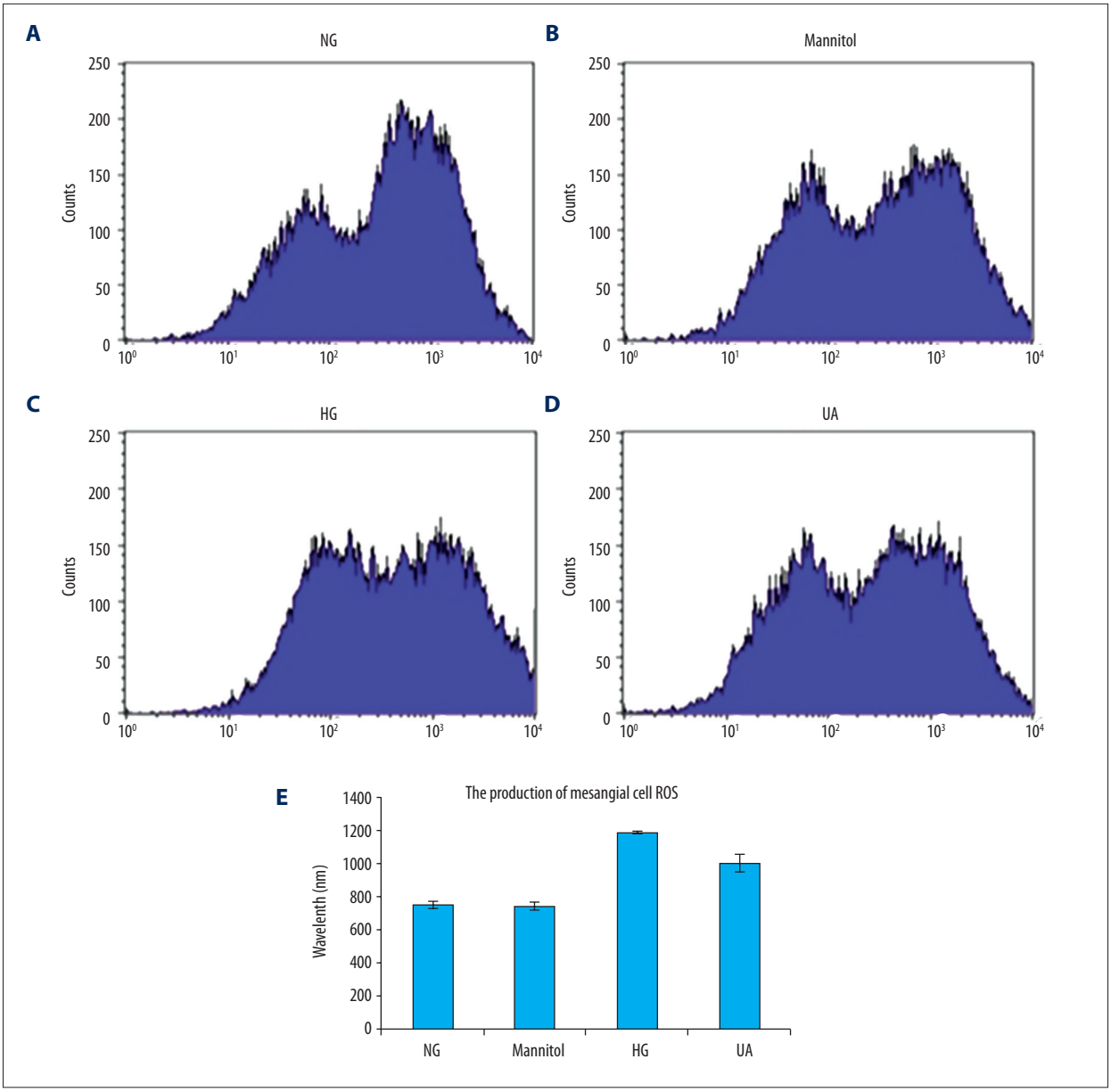


Figure 2. Ursolic acid (UA) significantly inhibited the ROS production in mesangial cells induced by high glucose levels. (**A**) NG, normal glucose group (5 mmol/L glucose); (**B**) Mannitol, hypertonic control group (5 mmol/L glucose+24.5 mmol/L mannitol); (**C**) HG, high glucose group (30 mmol/L glucose); (**D**) UA, UA treatment group (30 mmol/L glucose+1.0 mmol/L UA); (**E**) semi-quantitative analysis. # $p<0.01$ compared with the NG group; * $p<0.05$ compared with the HG group.

proliferation, and overexpression of the cytokine TGFβ1 and ECM protein FN, suggesting that PI3K/Akt/mTOR signaling is involved in HG-mediated mesangial cell damage. Consistent with our results, the *in vitro* study by Chen et al. [13] also demonstrated that HG activated the PI3K/Akt signaling pathway, downregulated downstream FoxO3α expression, promoted oxidative stress, and enhanced ECM deposition, suggesting that this pathway may be involved in the pathogenesis of DN [14].

Oxidative stress refers to excessive production of ROS and reactive nitrogen species (RNS) in response to various harmful stimuli that exceeds the oxygen-scavenging capacity of the body and causes an imbalance between the oxidation and antioxidant systems, leading to tissue damage. In recent years, many studies have suggested that stimulation by a variety of factors, such as HG, the inflammatory response, mechanical traction, and lipid deposition, induces oxidative stress in glomerular mesangial cells and promotes the development and progression of glomerulosclerosis [15,16]. Moreover,

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

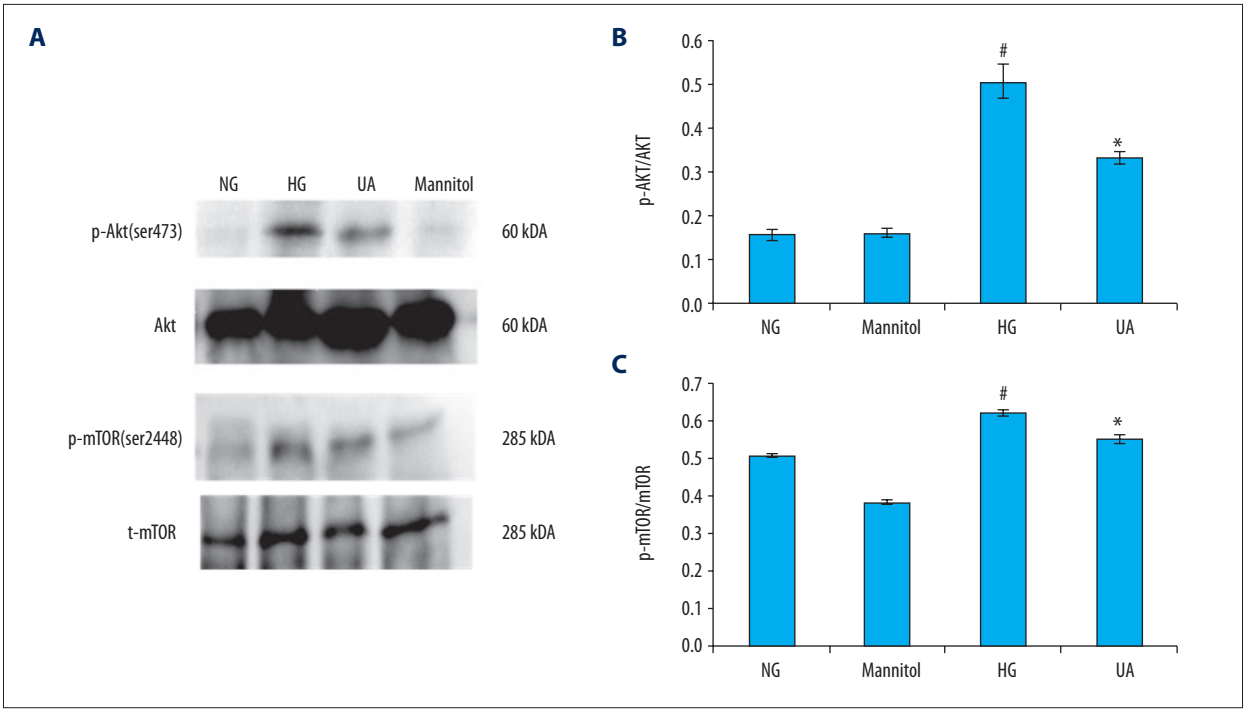


Figure 3. (**A–C**) Western blotting was used to detect PI3K/Akt/mTOR pathway activity in mesangial cells of each group. NG: normal glucose group (5 mmol/L glucose); Mannitol: hypertonic control group (5 mmol/L glucose+24.5 mmol/L mannitol); HG: high glucose group (30 mmol/L glucose); HG+UA: ursolic acid treatment group (30 mmol/L glucose+1.0 mmol/L ursolic acid). # p<0.05 compared with the NG group; * p<0.05 compared with the HG group.

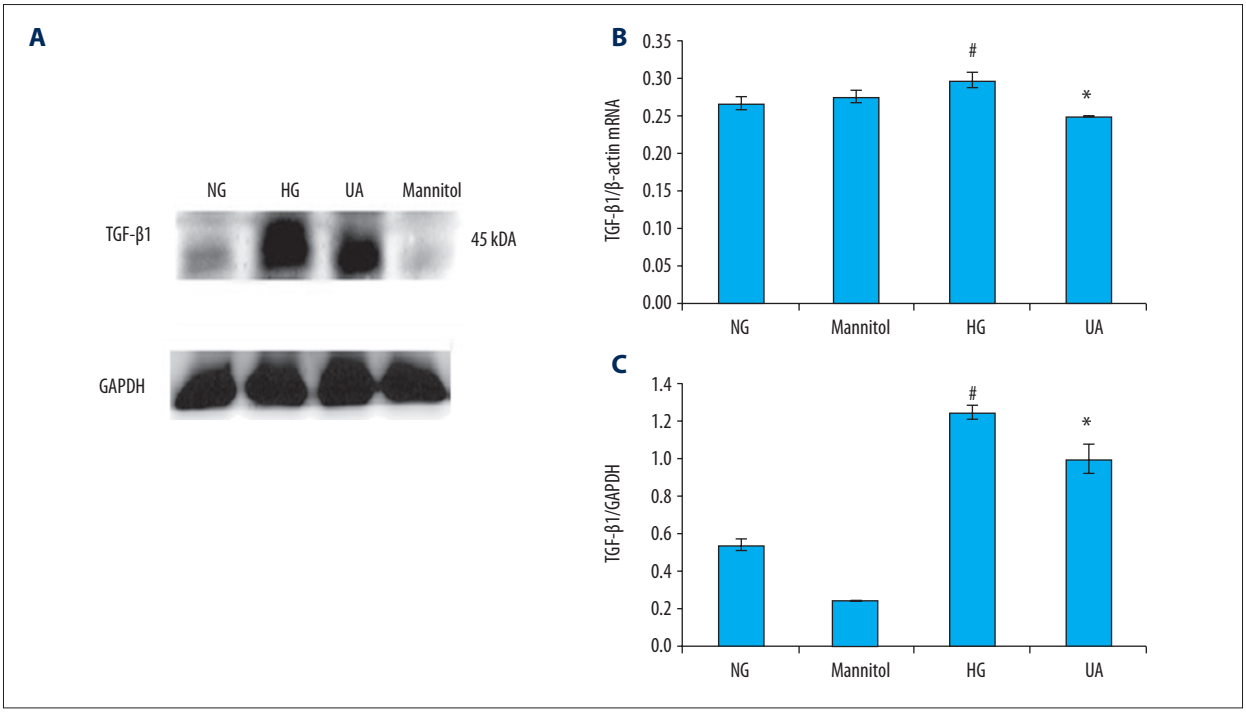


Figure 4. (**A–C**) mRNA and protein expression of TGFβ1 in mesangial cells detected by RT-PCR and Western blotting. NG: normal glucose group (5 mmol/L glucose); Mannitol: hypertonic control group (5 mmol/L glucose+24.5 mmol/L mannitol); HG: high glucose group (30 mmol/L glucose); HG+UA: ursolic acid treatment group (30 mmol/L glucose+1.0 mmol/L ursolic acid). # p<0.05 compared with the NG group; * p<0.05 compared with the HG group.

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

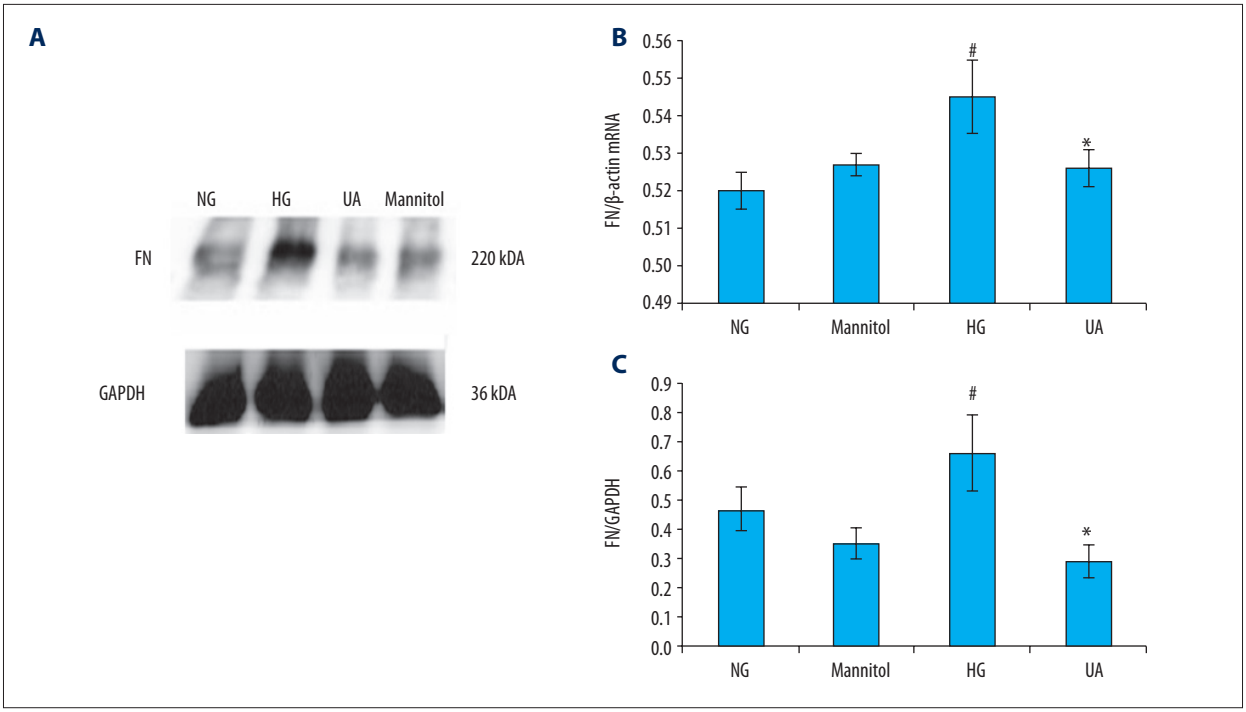


Figure 5. (**A–C**) FN mRNA and protein expression in mesangial cells detected by real-time PCR and Western blotting. NG: normal glucose group (5 mmol/L glucose); Mannitol: hypertonic control group (5 mmol/L glucose+24.5 mmol/L mannitol); HG: high glucose group (30 mmol/L glucose); HG+UA: ursolic acid treatment group (30 mmol/L glucose+1.0 mmol/L ursolic acid). # p<0.05 compared with the NG group; * p<0.05 compared with the HG group.

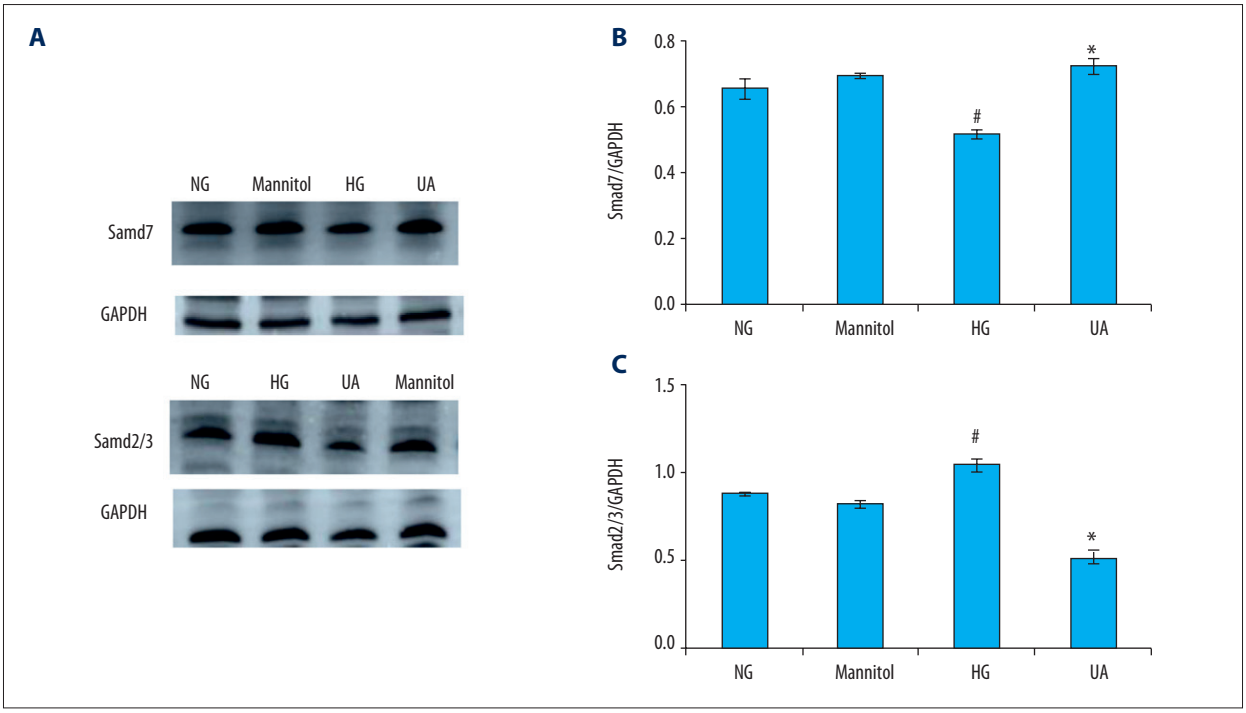


Figure 6. (**A–C**) Smad2/3 and Samd7 protein expression detected by Western blotting. NG: normal glucose group (5 mmol/L glucose); Mannitol: hypertonic control group (5 mmol/L glucose+24.5 mmol/L mannitol); HG: high glucose group (30 mmol/L glucose); HG+UA: ursolic acid treatment group (30 mmol/L glucose+1.0 mmol/L ursolic acid). # p<0.05 compared with the NG group; * p<0.05 compared with the HG group.

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

overproduction of ROS, which are important intracellular signaling messengers that activate multiple signal transduction pathways, can indirectly lead to tissue and cell damage. In this study, we found that HG increased ROS production and oxidative stress, induced excessive TGFβ1 secretion, and enhanced FN expression in mesangial cells. Similarly, Jeong et al. revealed that HG activates the PI3K/Akt-ERK1/2 and p38 MAPK pathways, promotes ROS production, and enhances the activity of NADPH oxidase, resulting in ECM accumulation [17]. In this study, we found that HG increased Samd2/3 production and decreased Samd7 production, inducing excessive TGFβ1 secretion. Barnes et al. also found that ROS induce excessive TGFβ1 secretion from mesangial cells by activating the angiotensin II-TGFβ1-Smad pathway, promoting ECM deposition, and reducing ECM degradation [18,19].

UA is a plant-derived pentacyclic triterpene that is widely found in berries, fruits, and herbs. UA has anti-tumor, anti-liver fibrosis, hypoglycemic, hypolipidemic, and anti-atherosclerotic effects [6–11], and previous studies have found that this triterpene inhibits proliferation of cultured Jurkat cells (malignant tumor cells) and induces their apoptosis by suppressing PI3K/Akt pathway activation [20]. Thus, UA is expected to become a new treatment for hematological malignancies. Furthermore, UA inhibits proliferation and induces apoptosis of human hepatic stellate cells by reducing TGF-β mRNA expression, thereby mitigating the vicious cycle of hepatic fibrosis. UA and its homolog oleanolic acid decrease levels of fasting blood glucose, glycosylated hemoglobin, and urinary albumin in diabetic mice in a dose-dependent manner, possibly by increasing blood insulin levels and suppressing renal aldose reductase (AR) activation. In addition, UA regulates transcription of phosphoenolpyruvate carboxykinase (PEPCK) and phosphorylation of its downstream effector insulin receptor substrate-2 (IRS-2) by enhancing expression of the PPARα protein in liver tissues; this affects cytokine and free fatty acid (FFA) levels in serum, as well as TNF-α and adiponectin levels, thereby improving insulin resistance and lowering blood lipid levels in KKAy mice. UA was also found to inhibit mononuclear cell aggregation in blood and atherosclerosis in a dose-dependent manner in low-density lipoprotein (LDL) receptor-deficient mice induced by streptozotocin, likely by inhibiting the diabetes-induced pro-inflammatory response. However, the protective effect of UA in DN and the associated mechanism have not yet been fully clarified; therefore, further studies are needed.

Conclusions

In this study, we found that UA inhibited the mesangial cell proliferation induced by HG in a dose-dependent manner, suppressed expression of the cytokine TGF-β1, and reduced FN expression and ECM deposition. Aside from the TGF-β1/Samd signaling pathway, the protective effect of UA is also likely achieved through inhibition of PI3K/Akt/mTOR pathway activation. Furthermore, UA contains a hydroxyl group in its C_3 side chain that has antioxidant activity, thereby allowing UA to inhibit ROS production. The findings of this study further our understanding of the pharmacological effects of UA and provide new strategies and methods for treatment of DN using Chinese herbal medicines.

References:

1. El-Haddad B, Reule S, Drawz PE: Dual renin-angiotensin-aldosterone system inhibition for the treatment of diabetic kidney disease: Adverse effects and unfulfilled promise. Curr Diab Rep, 2015; 15(10): 70
2. Slyne J, Slattery C, McMorrow T, Ryan MP: New developments concerning the proximal tubule in diabetic nephropathy: *In vitro* models and mechanisms. Nephrol Dial Transplant, 2015; 30(4): 60–67
3. Uttarwar L, Gao B, Ingram AJ, Krepinsky JC: SREBP-1 activation by glucose mediates TGF-beta upregulation in mesangial cells. Am J Physiol Renal Physiol, 2012; 302(3): F329–41
4. Luna-Acosta JL, Alba-Betancourt C, Martínez-Moreno CG et al: Direct anti-apoptotic effects of growth hormone are mediated by PI3K/Akt pathway in the chicken bursa of Fabricius. Gen Comp Endocrinol, 2015; 224: 148–59
5. Wang C, Liu X, Ke Z et al: Mesangial medium from IgA nephropathy patients induces podocyte epithelial-to-mesenchymal transition through activation of the phosphatidyl inositol-3-kinase/Akt signaling pathway. Cell Physiol Biochem, 2012; 29(5–6): 743–52
6. Jamal M, Imam SS, Aqil M et al: Transdermal potential and anti-arthritic efficacy of ursolic acid from niosomal gel systems. Int Immunopharmacol, 2015; 29(2): 361–69
7. Sundaresan A, Radhiga T, Pugalendi KV: Effect of ursolic acid and Rosiglitazone combination on hepatic lipid accumulation in high fat diet-fed C57BL/6J mice. Eur J Pharmacol, 2014; 741: 297–303
8. Lee J, Lee HI, Seo KI et al: Effects of ursolic acid on glucose metabolism, the polyol pathway and dyslipidemia in non-obese type 2 diabetic mice. Indian J Exp Biol, 2014; 52(7): 683–91
9. Castro AJ, Frederico MJ, Cazarolli LH et al: The mechanism of action of ursolic acid as insulin secretagogue and insulinomimetic is mediated by cross-talk between calcium and kinases to regulate glucose balance. Biochim Biophys Acta, 2015; 1850(1): 51–61
10. Jyoti MA, Zerin T, Kim TH et al: *In vitro* effect of ursolic acid on the inhibition of *Mycobacterium tuberculosis* and its cell wall mycolic acid. Pulm Phaimacol Ther, 2015; 33: 17–24
11. Nelson AT, Camelio AM, Claussen KR et al: Synthesis of oxygenated oleanolic and ursolic acid derivatives with anti-inflammatory properties. Bioorg Med Chem Lett, 2015; 25(19): 4342–46
12. Zhang T, Su J, Guo Bet al: Ursolic acid alleviates early brain injury after experimental subarachnoid hemorrhage by suppressing TLR4-mediated inflammatory pathway. Int, Immunopharmacol, 2014; 23(2): 585–91
13. Chen G, Wang T, Uttarwar L et al: SREBP-1 is a novel mediator of TGFβ1 signaling in mesangial cells. J Mol Cell Biol, 2014; 6(6): 516–30
14. Zhang L, Pang S, Deng B et al: High glucose induces renal mesangial cell proliferation and fibronectin expression through JNK/NF-kappaB/NADPH oxidase/ROS pathway, which is inhibited by resveratrol. Int J Biochem Cell Biol, 2012; 44(4): 629–38
15. Jeong SI, Kim SJ, Kwon TH et al: Schizandrin prevents damage of murine mesangial cells via blocking NADPH oxidase-induced ROS signaling in high glucose. Food Chem Toxicol, 2012; 50(3–4): 1045–53
16. Teles F, da Silva TM, da Cruz Júnior FP et al: Brazilian red propolis attenuates hypertension and renal damage in 5/6 renal ablation model. PloS One, 2015; 10(1): e0116535

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

17. Wang Y, Wang Y, Liu D et al: Cordyceps sinensis polysaccharide inhibits PDGF-BB-induced inflammation and ROS production in human mesangial cells. Carbohydr Polym, 2015; 125: 135–45
18. Alique M, Sánchez-López E, Rayego-Mateos S et al: Angiotensin II, via angiotensin receptor type 1/nuclear factor-κB activation, causes a synergistic effect on interleukin-1-β-induced inflammatory responses in cultured mesangial cells. J Renin Angiotensin Aldosterone Syst, 2015; 16(1): 23–32
19. Ding K, Wang Y, Jiang W et al: Qian Yang Yu Yin Granule-containing serum inhibits angiotensin II-induced proliferation, reactive oxygen species production, and inflammation in human mesangial cells via an NADPH oxidase 4-dependent pathway. BMC Complement Altern Med, 2015; 15: 81
20. Ou X, Liu M, Luo H et al: Ursolic acid inhibits leucine-stimulated mTORC1 signaling by suppressing mTOR localization to lysosome. PLoS One, 2014; 9(4): e95395

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

Biomed Pharmacother. 2018 Jul;103:699-707. doi: 10.1016/j.biopha.2018.04.072. Epub 2018 Apr 24.

Inhibition of PI3K/Akt/mTOR pathway by apigenin induces apoptosis and autophagy in hepatocellular carcinoma cells.

Yang J[1], Pi C[1], Wang G[2].

Author information

Abstract

Apigenin is a dietary flavonoid with known antioxidant and antitumor effects against several types of cancers by promoting cell death and inducing cell cycle arrest. Apigenin also regulates a variety of intracellular signal transduction pathways during apoptosis or autophagy. However, the precise mechanism underlying the anticancer effects of apigenin in liver cancer remains poorly understood. In this study, we demonstrated that apigenin has anticancer activity against hepatocellular carcinoma cells. Apigenin inhibited the cell growth and induced cell death in a dose- and time-dependent manner in HepG2 cells. We found that apigenin treatment increased the expression of LC3-II and the number of GFP-LC3 puncta. Moreover, inhibition of autophagy with 3-MA and Atg5 gene silencing strengthened apigenin-induced proliferation inhibition and apoptosis. Our data has indicated that apigenin-induced autophagy has a protective effect against cell death. Additionally, apigenin induced apoptosis and autophagy through inhibition of PI3K/Akt/**mTOR** pathway. Most importantly, in vivo data showed that administration of apigenin decreased tumor growth and autophagy inhibition by 3-MA significantly enhanced the anticancer effect of apigenin. Collectively, our results reveal that apigenin inhibits cell proliferation and induces autophagy via suppressing the PI3K/Akt/**mTOR** pathway. Our results also

suggest combination of autophagy inhibitors and apigenin would be a potential chemotherapeutic strategy against hepatocellular carcinoma.

KEYWORDS: Apigenin; Apoptosis; Autophagy; PI3K/Akt/**mTOR**

PMID: 29680738 DOI: 10.1016/j.biopha.2018.04.072

[Indexed for MEDLINE]

MeSH terms, Substances

LinkOut - more resources

REVIEW

The mTOR Signalling Pathway in Cancer and the Potential mTOR Inhibitory Activities of Natural Phytochemicals

Heng Kean Tan[1], Ahmed Ismail Hassan Moad[2], Mei Lan Tan[1,2]*

Abstract

The mammalian target of rapamycin (mTOR) kinase plays an important role in regulating cell growth and cell cycle progression in response to cellular signals. It is a key regulator of cell proliferation and many upstream activators and downstream effectors of mTOR are known to be deregulated in various types of cancers. Since the mTOR signalling pathway is commonly activated in human cancers, many researchers are actively developing inhibitors that target key components in the pathway and some of these drugs are already on the market. Numerous preclinical investigations have also suggested that some herbs and natural phytochemicals, such as curcumin, resveratrol, timosaponin III, gallic acid, diosgenin, pomegranate, epigallocatechin gallate (EGCC), genistein and 3,3'-diindolylmethane inhibit the mTOR pathway either directly or indirectly. Some of these natural compounds are also in the clinical trial stage. In this review, the potential anti-cancer and chemopreventive activities and the current status of clinical trials of these phytochemicals are discussed.

Keywords: mTOR signalling pathway - PI3K/Akt/mTOR - natural compounds - mTOR inhibitors

Asian Pac J Cancer Prev, **15 (16)**, 6463-6475

Introduction

The mammalian target of rapamycin (mTOR) kinase is a conserved serine/threonine protein kinase that plays an important role in regulating many fundamental molecules mediating cell growth and cell cycle progression in response to cellular signals in eukaryotes (Liu et al., 2009b; Houghton, 2010). The mTOR signalling pathway has a central role in cellular processes such as cell survival, cell growth and proliferation, cell death, and tumor angiogenesis. This pathway is frequently hyper-activated in several human malignancies and therefore is considered to be an interesting and attractive therapeutic target for anti-cancer therapy.

The mTOR is also known as FKBP12-rapamycin associated protein (FRAP), or rapamycin and FKBP12 target (RAFT), or rapamycin target (RAPT), or sirolimus effector protein (SEP). The mTOR gene is located on human chromosome 1 in location 1p36.2 (Huang and Houghton, 2003). It is identified in mammalian cells as a 289 kDa serine/threonine kinase consisting of 2549 amino acids and the structural domains of mTOR, are evolutionarily conserved, comprising of six functional domains (Sabatini et al., 1994; Sabers et al., 1995; Abraham, 1998). The domains comprise of (1) HEAT (Huntingtin elongation factor 3, a subunit of protein phosphatase 2A and TOR1) domain, which mediates protein-protein interactions; (2) FAT (FRAP-ATM-TRAPP) domain; (3) FRB (FKBP12-rapamycin binding) domain, which mediates the inhibitory action of rapamycin on Raptor-bound mTOR; (4) PIKK (PI3-kinase-related kinase) domain, serine phosphorylation sites (S2035 and S2481); (5) RD (Repressor domain); and (6) the carboxy-terminal FATC domain (Kirken and Wang, 2003; Asnaghi et al., 2004).

The mTOR kinase plays a crucial role in regulating cell growth, cell proliferation, cell survival, protein synthesis and autophagy. It regulates and controls the transcription of ribosomal proteins and the synthesis of rRNA and tRNA (Hardwick et al., 1999; Powers and Walter, 1999). In general, the activity of mTOR is regulated by insulin and other growth factors via the phosphatidylinositol 3-kinase (PI3K)–Akt pathway (Kadowaki and Kanazawa, 2003).

In eukaryotic cells, mTOR exists as two different complexes: mTORC1; a rapamycin-sensitive complex defined by its interaction with Raptor (regulatory-associated protein of mTOR) and mTORC2; a rapamycin-insensitive complex defined by its interaction with Rictor (rapamycin-insensitive companion of mTOR) (Bharti and Aggarwal, 2002; Loewith et al., 2002; Sarbassov et al., 2004). Raptor is the first protein shown to bind directly to mTOR that is required to mediate mTOR regulation of p70 ribosomal S6 kinase (p70S6K) and the binding protein of eukaryotic translation initiation factor 4E (4E-BP1) activities (Bharti and Aggarwal, 2002; Kim et al., 2002a). On the other hand, PRAS40 and Deptor are identified as

*[1]Malaysian Institute of Pharmaceuticals and Nutraceuticals, Ministry of Science, Technology and Innovation (MOSTI), Halaman Bukit Gambir, [2]Advanced Medical and Dental Institute, Universiti Sains Malaysia, Pulau Pinang, Malaysia *For correspondence: tanml@usm.my, drtanmelan@yahoo.com*

distinct negative regulators of mTORC1 (Sancak et al., 2007; Peterson et al., 2009).

In the rapamycin-sensitive mTOR signalling pathway, rapamycin binds to FK506-binding protein of 12 kDa (FKBP12), and subsequently, the complex binds to the FRB domain of mTORC1. This weakens the interaction between mTOR and Raptor and subsequently inhibits the mTORC1 functions (Kirken and Wang, 2003; Guertin et al., 2004; Hay and Sonenberg, 2004). However, the mechanisms on how rapamycin and several rapamycin derivatives bind to FKBP12 to inhibit mTORC1 signalling remain poorly defined (Dowling et al., 2010). Starvation or lack of nutrients such as amino acids and/or glucose appears to mimic rapamycin treatment which causes rapid inactivation of p70S6K and hypophosphorylation of the 4E-BP1 (Proud, 2002).

The activity of mTOR is regulated by various growth factors such as insulin, insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF), transforming growth factor (TGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and platelet-derived growth factor (PDGF) (Gomez-Pinillos and Ferrari, 2012). Growth factor-induced activation of mTOR is mediated by Class I PI3K which has the unique ability to generate oncogenic phosphatidylinositol-3,4,5-triphosphate (PIP3). Class II and Class III PI3Ks lack this ability and therefore have not been linked to cancer (Vogt et al., 2010). Class I PI3Ks are further divided into Class IA PI3Ks and Class IB PI3K. Class IA PI3Ks are heterodimers consisting of a p85 regulatory subunit that associates with p110α, β or δ catalytic subunit and are involved primarily in the pathogenesis of human cancer (Rodon et al., 2013).

Following growth factor binding to its cognate receptor tyrosine kinase (RTK), Class IA PI3Ks are recruited to the cell membrane by direct interaction of the p85 subunit with the activated receptors or by interaction with adaptor proteins associated with the receptors. Binding removes the inhibitory effect of p85 on p110, resulting in activation of p110 catalytic subunit. The activated p110 subunit catalyses the phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP2) to PIP3 at the membrane. PIP3 is an important second messenger in the cell and is the predominant mediator of PI3K activity. PIP3 acts as docking sites for signalling proteins that have pleckstrin homology (PH) domain, including Akt and 3-phosphoinositide-dependent kinase 1 (PDK1) (Vogt et al., 2010; Baselga, 2011). Figure 1 illustrates the mTOR signalling pathway in general.

The serine/threonine protein kinase Akt, also known as protein kinase B (PKB), a downstream effector of PI3K, is a critical mediator of mTOR activity (Hay and Sonenberg, 2004). Akt activation is initiated by translocation to the plasma membrane, which is mediated by docking of Akt to PIP3 on the membrane. Akt is then phosphorylated on Thr308 by PDK1 and on Ser473 by putative PDK2. A number of potential PDK2s have been identified, including integrin-linked kinase (ILK), protein kinase C β2, DNA-dependent protein kinase (DNA-PK), ataxia telangiectasia mutated (ATM), Akt itself and mTORC2. Both phosphorylation events are required for full activation of Akt. Once Akt has been phosphorylated and activated, it phosphorylates many other proteins, thereby regulating a wide range of cellular processes involved in protein synthesis, cell survival, proliferation and metabolism. Akt activates mTOR either by direct phosphorylation of mTOR at Ser2448 (Nave et al., 1999) or by indirect phosphorylation and inhibition of tuberous sclerosis complex 2 (TSC2) (Inoki et al., 2002). Akt phosphorylation of TSC2 represses GTPase-activating protein (GAP) activity, thereby allowing GTP-bound

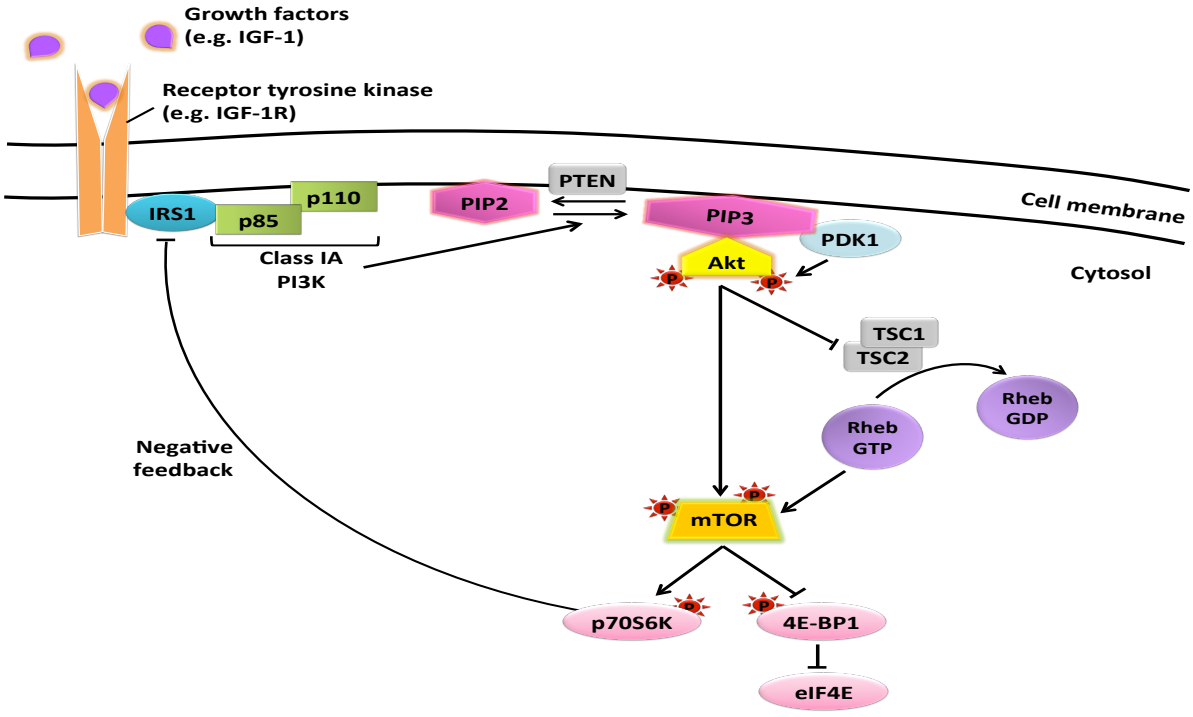


Figure 1. The mTOR Signalling Pathway and Regulatory Feedback Loop

active Ras homolog enriched in brain (Rheb) to activate mTOR (Plas and Thompson, 2005). Phosphorylation of mTOR at Ser2481 (an autophosphorylation site) correlates to the activation of mTOR catalytic activity (Caron et al., 2010; Soliman et al., 2010).

When conditions are favourable for cell growth, activated mTORC1 phosphorylates several substrates to promote anabolic processes (such as ribosome biogenesis, translation and the synthesis of lipids and nucleotides) and suppress catabolic processes (such as autophagy) (Fruman and Rommel, 2014). The mTORC1 regulates protein synthesis through the phosphorylation and inactivation of the repressor of mRNA translation, 4E-BP1 and through the phosphorylation and activation of p70S6K. Phosphorylation of 4E-BP1 releases eukaryotic translation initiation factor 4E (eIF4E), allowing it to interact with eIF4G to initiate cap-dependent translation. Activated p70S6K regulates cell growth via increased translation of 5'TOP (terminal oligopyrimidine tract) mRNAs, which encode components of the translation machinery, such as ribosomal proteins and elongation factors. Through the phosphorylation of several other effectors, mTORC1 promotes lipid biogenesis and metabolism, and suppresses autophagy (Hay and Sonenberg, 2004; Gomez-Pinillos and Ferrari, 2012; Laplante and Sabatini, 2013). In contrast, mTORC2 does not have direct role in regulating protein translation. However, mTORC2 is found to phosphorylate serum and glucocorticoid-regulated kinase 1 (SGK1), protein kinase C (PKC), and also Akt at Ser473, which in turn regulates cell cycle progression, cell survival, metabolism and cytoskeletal organization (Gomez-Pinillos and Ferrari, 2012; Laplante and Sabatini, 2012).

The tumour suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is the most important negative regulator of the PI3K signalling pathway. PTEN is a phosphatidylinositol-3 phosphatase that antagonizes PI3K activity by dephosphorylating PIP3 that is generated by PI3K (Abdulkareem and Blair, 2013). Loss of PTEN results in an unrestrained signalling of the PI3K pathway, leading to the formation of cancer. It is also associated with many types of cancers, including breast cancer (Vivanco and Sawyers, 2002; Sansal and Sellers, 2004). Another important protein involved in the regulation of mTORC1 activity is the tuberous sclerosis complex (TSC), which is a heterodimer of two proteins, TSC1 (also known as hamartin) and TSC2 (also known as tuberin) (Hay and Sonenberg, 2004). TSC1 and TSC2 functions as a GAP that negatively regulates a small GTPase called Rheb, transforming Rheb into its inactive GDP-bound state which subsequently unable to activate mTOR (Hay and Sonenberg, 2004). Finally, regulatory feedback loop exists as an intrinsic mechanism of self-control to refrain further activation of mTOR pathway. Following mTOR phosphorylation, activated p70S6K phosphorylates and destabilizes insulin receptor substrate 1 (IRS1), thereby inhibiting PI3K activation and blocking upstream overstimulation of the PI3K/Akt/mTOR cascade (Gomez-Pinillos and Ferrari, 2012; Shimobayashi and Hall, 2014) (Figure 1).

One of most studied and important pathways involved in the regulation of autophagy is the PI3K/Akt/mTOR signalling pathway. Inhibition of mTOR by nutrient-depletion, starvation or rapamycin leads to the induction of autophagy. Increased levels of the mTOR kinase are found to inhibit the autophagy process, resulting in excessive cell growth and tumor development. Studies have shown that mTORC1 controls autophagy through the regulation of a protein complex composed of ULK1 (unc-51-like kinases), mAtg13 and FIP200 (Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009). ULK kinase complex is directly controlled by mTOR, of which maintains the hyperphosphorylation state of mAtg13 and suppresses the induction of autophagy (Galluzzi et al., 2008). Inhibition of mTOR by rapamycin leads to dephosphorylation of ULK1, ULK2, and mAtg13 and activates ULK to phosphorylate FIP200, which suggests that ULK-Atg13-FIP200 complexes are direct targets of mTOR and important regulators of autophagy in response to mTOR signalling (Jung et al., 2009).

In contrast to mTORC1, relatively little is known regarding the regulatory pathway of mTORC2. The mTOR-Rictor complex, unlike mTOR-Raptor, does not bind to FRB domain and is insensitive to rapamycin treatment (Loewith et al., 2002; Sarbassov et al., 2004). The mTORC2 complex promotes cell signalling through phosphorylation and activation of the pro-survival and pro-proliferative kinase Akt, which positively regulates cell survival, proliferation and metabolism (Sarbassov et al., 2006; Manning and Cantley, 2007). The molecular mechanism by which mTORC2 regulates cytoskeletal organization has not been clearly defined, although many different studies have reported that knocking down mTORC2 components affects actin polymerization and disrupts cell morphology (Jacinto et al., 2004; Sarbassov et al., 2004). In another study, depletion of mTOR and Rictor, but not Raptor, impairs actin polymerization in neutrophils stimulated with chemoattractants and that small Rho GTPases Rac and Cdc42 serve as downstream effectors of Rictor to regulate actin assembly and organization in neutrophils (He et al., 2013).

The mTOR Signalling Pathway and Cancer

The mTOR pathway is a key regulator of cell proliferation and several upstream activators and downstream effectors of mTOR are known to be deregulated in some cancers such as renal cell carcinoma, non-small cell lung cancer, breast cancer, sarcomas, colorectal and gastrointestinal tumors (Law, 2005; Tokunaga et al., 2008; Li et al., 2013; Takahashi et al., 2014; Wang and Zhang, 2014). The mTOR signalling is constitutively activated in many tumor types, suggesting that mTOR is an attractive target for cancer drug development and therapy (Yu et al., 2001; Chan, 2004; Shor et al., 2009; Han et al., 2013; Pandurangan, 2013). The mTOR signalling network consists of a number of tumor suppressor genes and proto-oncogenes, thereby explains that aberrant activities of these genes will promote the formation of cancerous cells.

The signalling network defined by PI3K, Akt and mTOR controls most hallmarks of cancer, including cell cycle, survival, metabolism, motility and genomic

instability. Cancer genetic studies suggest that the PI3K pathway is the most frequently altered pathway in human tumours, where the PIK3CA gene (which encodes the PI3K p110α catalytic isoform) is the second most frequently mutated oncogene, and PTEN is among the most frequently mutated tumour suppressor genes (Fruman and Rommel, 2014). Therefore, PI3K pathway is probably one of the most important pathways in cancer metabolism and growth, and has been identified as an important target in breast cancer research (Baselga, 2011).

The p110α and p110β isoforms of Class I PI3Ks are expressed in almost all tissues and cell types, both of which play important roles in regulating cell growth and metabolism (Vogt et al., 2010). The p110α isoform is the most important subunit in PI3K as it is important for the growth and maintenance of numerous tumours that feature PI3K activation. Ablation of p110α resulted in substantially reduced Akt phosphorylation in response to stimulation by various growth factors (Zhao et al., 2006; Pal and Mandal, 2012). Of the four Class I PI3K catalytic isoforms, only PIK3CA (encoding p110α) is frequently mutated in human cancer. Mutations in Class I PI3K regulatory subunit genes are also found in cancer cells and cause increased PI3K activity (Fruman and Rommel, 2014). PIK3CA and PIK3R1 (which encodes p85 regulatory subunit) are mutated at frequencies ranging from 5%-25% in several common cancers, including cancers of the breast, endometrium and large intestine (Vogt et al., 2010). Overall, 20%-25% of breast tumors exhibit PIK3CA mutation (Baselga, 2011). PIK3CA mutation has been shown to increase PIP3 level, activate Akt signalling and promote oncogenic transformation (Baselga, 2011).

Akt is frequently and constitutively active in many types of human cancer. Constitutive Akt activation can occur as a result of amplification of Akt genes or due to mutations in components of the signalling pathway that activate Akt. Constitutive Akt signalling is believed to promote proliferation and increase cell survival, thereby contributing to cancer progression (Nicholson and Anderson, 2002). Amplification of Akt1, Akt2 and Akt3 has been reported in breast, ovarian, pancreatic and gastric cancers (Rodon et al., 2013). Activating mutation in Akt1, which results in growth factor-independent membrane translocation of Akt and increased Akt phosphorylation, was identified in breast, melanoma, colorectal and ovarian cancers. Phosphorylation of Akt at Ser473 has been associated with poor prognosis in human cancers, including breast cancer (LoPiccolo et al., 2008). Transgenic mice generated by expressing myristoylated-Akt1 (myr-Akt1) under the control of the MMTV-LTR promoter revealed that expression of myr-Akt1 in mammary glands alone did not increase the frequency of tumor formation. However, there was an increased susceptibility of forming mammary tumors induced by DMBA in the transgenic mice, especially in post-lactation mice, indicating that Akt1 accelerates carcinogen-induced tumorigenesis (Wu et al., 2014). Interestingly, although mutations in PDK1 are rarely found in human cancer, amplification or overexpression of PDK1 was found in ~20% of breast cancers (Liu et al., 2009a).

Aberrant activation of mTOR has been implicated in certain cancers. Activation of mTOR provides tumour cells with a growth advantage by promoting protein synthesis and contributes to the genesis of cancer through its effect on cell cycle progression (Fingar et al., 2004). The effects of mTOR on cell cycle progression is mediated, at least in part, by the increased translation of positive regulators of cell cycle progression, such as cyclin D1 and Myc, and by decreased translation of negative regulators thereof, such as p27kip1 (Gera et al., 2004; Hay and Sonenberg, 2004). On the other hand, tumor suppressor PTEN is frequently mutated in advanced stages of human cancers, particularly glioblastoma, endometrial and prostate cancers. Germline mutations in the PTEN gene give rise to Cowden's disease, which is associated with an increased risk of developing breast cancer and other cancers (Nicholson and Anderson, 2002). Somatic loss of PTEN by gene mutation or deletion frequently occurs in human cancers. PTEN is deleted or mutated in approximately 45% of uterine endometrial cancers, 30% of glioblastomas and spinal tumors, and less commonly in cancers of the prostate, bladder, adrenal glands, thyroid, breast, skin (melanomas) and colon (Abdulkareem and Blair, 2013).

Clinical Development of PI3K/Akt/mTOR (PAM) Inhibitors

Since mTOR signalling pathway is one of the most commonly activated signalling networks in human cancers and that kinases are amenable to pharmacological intervention, many pharmaceutical companies and academic laboratories are actively developing inhibitors that target key components in the pathway (Moschetta et al., 2014). Many of the agents developed and evaluated in early stage clinical trials have been shown to be safe, well tolerated and effective in multiple tumor types. Current PAM inhibitors in early development include reversible ATP-competitive inhibitors of the four p110 isoforms of Class I PI3K (also known as pan-PI3K inhibitors), the irreversible pan-PI3K inhibitors, p110 isoform-specific inhibitors, dual pan-PI3K-mTOR inhibitors, Akt inhibitors and mTOR inhibitors (Rodon et al., 2013; Porta et al., 2014).

Wortmannin and LY294002 are two well known, first generation pan-PI3K inhibitors. Wortmannin and LY294002 are effective inhibitors of PI3K and have shown anti-proliferative and apoptotic effects *in vitro* and *in vivo*. However, the use of these two compounds is limited to the preclinical level due to their instability in aqueous solutions, toxic side effects, poor pharmaceutical properties and lack of selectivity for individual PI3K p110 isoforms (Pal and Mandal, 2012). Isoform-specific inhibitors are of particular interest because agents that target single isoform may produce fewer side effects and less toxicity to the immune system due to the fact that p110α and p110β play important roles in multiple cellular processes while p110γ and δ isoforms are important in the immune system. Some inhibitors of Akt are being tested clinically, although the development of Akt-specific and isozyme-selective inhibitors was predicted to be difficult due to high degree of homology in the ATP binding pocket

between Akt, protein kinase A (PKA) and PKC (Rodon et al., 2013).

Rapamycin, also known as sirolimus, is a prototypical mTOR inhibitor. It is an antibiotic macrolide derived from bacterium *Streptomyces hygroscopius*, and first isolated in 1975 (Sehgal et al., 1975; Vezina et al., 1975). Rapamycin was first developed as immunosuppressant by Wyeth pharmaceutical company in 1997 and more recently presented as anti-cancer agents in the form of various analogues (Liu et al., 2009b). Rapamycin binds to its intracellular receptor FKBP12, and subsequently attaches to the mTORC1 and suppresses mTOR-mediated phosphorylation of p70S6K and 4E-BP1. Rapamycin has been precluded from clinical development due to its poor aqueous solubility and chemical instability (Hidalgo and Rowinsky, 2000; Mita et al., 2003). Rapamycin analogues (also known as rapalogues) inhibit mTOR through the same mechanism as rapamycin, but have better pharmacological properties for clinical use in cancer. In general, the therapeutic effects of rapamycin analogues are similar to rapamycin (Tsang et al., 2007). Rapamycin analogues with improved stability and pharmacological properties have been significantly tolerated by patients in Phase I trials, and the agents have shown promising antitumor effect in many types of cancers including breast cancer (Noh et al., 2004).

Temsirolimus (CCI-779) and everolimus (RAD001) are two rapamycin analogues that have been developed as anti-cancer drugs (Hasskarl, 2014). Temsirolimus is the first mTOR inhibitor approved by FDA, USA for the treatment of advanced renal cell carcinoma in 2007. This is followed by the approval of everolimus for the treatment of adults with advanced and recurrent renal cell carcinoma (2009); adults with progressive neuroendocrine tumors of pancreatic origin (2011); adults with tuberous sclerosis complex (TSC) who have renal angiomyolipomas not requiring immediate surgery (2012); children with TSC who have a rare brain tumor called subependymal giant cell astrocytoma (2012); and for use in combination with exemestane to treat certain postmenopausal women with advanced hormone receptor positive, HER2-negative breast cancer (2012) (Hasskarl, 2014).

Nevertheless, rapalogues are not broadly effective as single agents, although they have been approved for the treatment of a few tumour types for which modest therapeutic effects can be achieved (Fruman and Rommel, 2014). Preclinical studies demonstrated that Akt activation was triggered after blockade of mTORC1 by rapamycin and rapalogues (Sun et al., 2005; O'Reilly et al., 2006; Wan et al., 2007). Clinically, upon mTOR blockade with everolimus, Akt phosphorylation was upregulated in 50% of the treated tumors (Tabernero et al., 2008). The increased Akt activity can ultimately enhance tumour growth. This limited anti-tumour activity of mTOR inhibitors is suspected to be related to the fact that these agents only inhibit the mTORC1 complex. The blockade of mTOR and the resulting inhibition of p70S6K relieves regulatory feedback loop, which results in IGF-1R-mediated feedback activation of Akt (Baselga, 2011; Rodon et al., 2013). Therefore, agents targeting both mTORC1 and mTORC2, and dual pan-class I PI3K-mTOR inhibitors are being developed (Rodon et al., 2013). In addition, preclinical models have shown that combining mTOR inhibitors and IGF-1R antibodies/inhibitors result in blockage of mTOR inhibitor-induced Akt activation (Wan et al., 2007), and this combination is currently being explored in clinical trials (Chen and Sharon, 2013). In the pre-clinical and clinical studies, the inhibitors targeting the different members of mTOR pathway have been used alone or in combination with other targeted agents for the treatment of breast cancer (Ghayad and Cohen, 2010).

Although the mTOR-targeting therapy was based on the premise that an essential PI3K effector Akt activates the rapamycin-sensitive mTORC1 pathway, new data suggests that rapamycin-insensitive mTORC2 phosphorylates Akt on a key activation site, providing some knowledge that the relationship between mTOR and PI3K signalling is complex (Guertin and Sabatini, 2009). Inhibitors that target both mTORC1 and mTORC2 would be expected to block activation of the PI3K pathway more effectively than rapamycin and its analogues (Liu et al., 2009b). Current evidences from the analyses of some solid tumors also suggests that dual PI3K/mTOR inhibitors, which bind to and inactivate both PI3K and mTOR, may achieve better outcomes among resistant cancers (Tang and Ling, 2014). Currently, OSI-027 (OSI Pharmaceuticals, USA), AZD8055 (Astra Zeneca, UK), and INK128 (Intellikine, USA) are the first three ATP-competitive mTOR inhibitors to enter clinical trials in patients with advanced solid tumors and lymphoma (Liu et al., 2009a; Garcia-Echeverria, 2010; Houghton, 2010). OSI-027 is the first orally bioavailable small-molecule mTORC1/mTORC2 inhibitor, a semi-synthetic compound with the ability of eliciting both tumor cell apoptosis and autophagy and halting tumor cell proliferation (Yap et al., 2008; Vakana et al., 2010).

Natural Phytochemicals as mTOR Inhibitors

Numerous important anticancer drugs in the market are either obtained from natural sources, by structural modification of natural compounds, or by synthesis of new compounds using natural compound as lead (Cragg et al., 1997; da Rocha et al., 2001). Therefore, sourcing out new drugs and the continuous interest in using natural compounds for cancer therapy is a global effort. Numerous preclinical investigations have shown that some herbs and natural phytochemicals, such as curcumin, resveratrol, timosaponin III, gallic acid, diosgenin, pomegranate, epigallocatechin gallate (EGCC), genistein, and 3,3'-diindolylmethane inhibit mTOR pathway either directly or indirectly (Table 1). Some of them are undergoing clinical trials as chemotherapeutic agents, chemopreventive compounds and/or combination therapy to improve the efficacy of the standard chemotherapy. These natural phytochemicals with mTOR inhibitory activities have great potential in cancer prevention. This is in view that higher consumption of fruits and vegetables was associated with lower risk of cancer (Gullett et al., 2010).

Curcumin, a polyphenol natural compound extracted from the plant *Curcuma longa* L., is commonly used

as spice in India and Southeast Asia. It is used as food additive and traditional Indian medicine for the treatment of various diseases such as biliary disorders, anorexia, cough, diabetic wounds, hepatic disorders, rheumatism and sinusitis (Shishodia et al., 2007). Curcumin has shown exceptional chemopreventive and anti-tumor activities in some pre-clinical studies. In HCT116 colorectal cancer cells, curcumin downregulates protein and mRNA expression of mTOR, Raptor and Rictor, suggesting that curcumin exerts its anti-proliferative effects by inhibiting the mTOR signalling pathway and thus may represent a novel class of mTOR inhibitor (Johnson et al., 2009). In human Rh1 and Rh30 rhabdomyosarcoma cells, DU145 prostate cancer cells, MCF-7 breast cancer cells and Hela cervical cancer cells, curcumin rapidly inhibits the phosphorylation of mTOR and its downstream effector molecules such as p70S6K and 4E-BP1, indicating that curcumin may execute its anticancer activity primarily by blocking mTOR-mediated signalling pathways in these tumor cells (Beevers et al., 2006). Furthermore, curcumin induces apoptosis, inhibits cell growth and inhibits the basal or type I insulin-like growth factor-induced motility of the Rh1 and Rh30 cells (Beevers et al., 2006). Curcumin is found to dissociate Raptor, at low concentration, and Rictor, at high concentration, from mTOR complex. However, it is unclear if curcumin disrupts the mTOR complex by direct binding to mTOR or to a component of the mTOR complexes (Beevers et al., 2009). In human PC3 prostate cancer cells, curcumin suppresses murine double minute 2 (MDM2) oncogene expression through the erythroblastosis virus transcription factor 2 (EST2) by modulating PI3K/mTOR/ETS2 signalling pathway (Li et al., 2007a). In both human U87-MG and U373-MG malignant glioma cells, curcumin inhibits the Akt/mTOR/p70S6K pathway and activates the extracellular signal-regulated kinase (ERK) pathway, resulting in the induction of autophagy. On the other hand, activation of Akt pathway by recombinant full-length human active Akt1 protein (rAkt1) inhibited curcumin-induced autophagy and decreased curcumin-inhibited phosphorylation of Akt and p70S6K, suggesting that curcumin-induced inactivation of Akt/mTOR/p70S6K pathway plays a role in induction of autophagy (Aoki et al., 2007). As combined treatment, curcumin and dual PI3K/Akt and mTOR inhibitor induce apoptosis through p53-dependent Bcl-2 mRNA down-regulation at the transcriptional level and Mcl-1 protein down-regulation at the post-transcriptional level in human renal carcinoma Caki cells (Seo et al., 2014).

The promising effect of curcumin at the preclinical phases has led to the initiation of several clinical trials. In Phase I clinical studies, it has been shown that curcumin is not toxic to human; and in Phase II clinical trial, curcumin is well tolerated and produces some biological activity in patients with advanced pancreatic cancer (Cheng et al., 2001; Sharma et al., 2001; Lao et al., 2006; Dhillon et al., 2008). Curcumin taken orally for 3 months produces histologic improvement of precancerous lesions in 1 out of 2 patients with recently resected bladder cancer, 2 out of 7 patients of oral leucoplakia, 1 out of 6 patients of intestinal metaplasia of the stomach, 1 out of 4 patients with uterine cervical intraepithelial neoplasm (CIN) and 2 out of 6 patients with Bowen's disease (Cheng et al., 2001). Radiologically stable colorectal cancer was demonstrated in 5 out of 15 patients after 2-4 months of treatment with curcuma extract at doses between 440 and 2200 mg/day, containing 36-180 mg of curcumin (Sharma et al., 2001). In a Phase II, nonrandomized, open-label clinical trial in 44 eligible smokers with eight or more aberrant crypt foci (ACF) on screening colonoscopy, a significant 40% reduction in ACF number occurred with the 4-g dose of curcumin for 30 days. The ACF reduction in the 4-g group was associated with a significant, five-fold increase in post-treatment plasma curcumin/conjugate levels (Carroll et al., 2011). A Phase I/II study of gemcitabine-based chemotherapy plus curcumin for patients with gemcitabine-resistant pancreatic cancer reported that 8 g oral curcumin daily with gemcitabine-based chemotherapy was safe and feasible in patients with pancreatic cancer (Kanai et al., 2011). However, all these are short term studies and the unremarkable response rates were not surprising and it certainly warrants longer trials.

Interestingly, a randomized, double-blind, placebo-controlled clinical trial of 30 breast cancer patients revealed that oral curcumin, 6.0 g daily during radiotherapy, reduced the severity of radiation dermatitis in breast cancer patients (Ryan et al., 2013). Curcumin in improved formulations have also proven to be safe and acceptable among patients in pilot studies (Irving et al., 2013; Kanai et al., 2013). Other ongoing clinical trials include Phase II combination therapy with standard radiation therapy and chemotherapy (capecitabine) in rectal cancer, Phase II trial to prevent colon cancer in smokers with aberrant crypt foci, Phase II trial in patients with pancreatic cancer, Phase II trial in patients with colorectal cancer, Phase I trial in patients with advanced cancer as well as Phase I trial to prevent colorectal cancer in patients undergoing colorectal endoscopy or colorectal surgery (Table 1).

Resveratrol is a polyphenolic compound present in grapes and red wine with potential anti-inflammatory and anticancer properties (Pervaiz, 2003; Marques et al., 2009). It is used in traditional Chinese and Japanese medicine to treat dermatitis, gonorrhea, athlete's foot and hyperlipemia (Aggarwal et al., 2004). In human LNCaP prostate carcinoma cells, resveratrol inhibits PI3K/Akt signalling pathway and induces apoptosis (Aziz et al., 2006). Resveratol is also shown to down-regulate the PI3K/Akt/mTOR signalling pathway, and combination with rapamycin further enhances the resveratrol-induced cell death in human U251 glioma cells (Jiang et al., 2009). In smooth muscle cells (SMC), resveratrol blocks the oxidized LDL (oxLDL)-induced activation of the mTOR pathway via PI3K/PDK1/Akt, thereby inhibiting oxLDL-induced SMC proliferation (Brito et al., 2009). In MDA-MB-231 and MCF-7 human breast cancer cells, resveratrol decreases mTOR and p70S6K phosphorylation, and in combination with rapamycin, suppresses the phosphorylation of Akt. An additive effect of resveratrol and rapamycin combination suggests some therapeutic value in breast cancer (He et al., 2010). In both estrogen receptor (ER)-positive and ER-negative breast cancer cells, resveratrol activates AMP-activated kinase (AMPK) and subsequently downregulates mTOR, 4E-BP1

Table 1. The List Of Natural Compounds And Clinical Trial Phases

No	Natural compounds	Target	Natural Source	Clinical trial phase	Reference	Status
1	Curcumin	Akt and mTOR	*Curcuma longa* L.	In Phase I-II for pancreatic cancer[1], colorectal cancer[2], colon cancer[3], rectal cancer[4], advanced cancer[5]. In clinical trial for familial adenomatous polyposis[6]	1. http://clinicaltrials.gov/show/NCT00094445 2. http://clinicaltrials.gov/show/NCT00118989 3. http://clinicaltrials.gov/show/NCT00365209 4. http://clinicaltrials.gov/show/NCT00745134 5. http://clinicaltrials.gov/show/NCT01201694 6. http://clinicaltrials.gov/show/NCT00641147	1-5: ongoing, but not recruiting participants 6: currently recruiting participants
2	Resveratrol	PI3K, Akt and mTOR	Grapes and red wine	In clinical trial for neuroendocrine tumor[7]	7. http://clinicaltrials.gov/show/NCT01476592	7: ongoing, but not recruiting participants
3	Pomegranate	PI3K, Akt and mTOR	*Punica granatum* L.	In Phase II for prostate cancer[8,9,10]	8. http://clinicaltrials.gov/show/NCT00060086 9. http://clinicaltrials.gov/show/NCT00731848 10. http://clinicaltrials.gov/show/NCT00732043	8-10: ongoing, but not recruiting participants
4	Genistein	Akt and mTOR	*Glycine max* (L.) Merr. and several plants	In Phase I-II for prostate cancer[11,12], breast cancer[13, 16], pancreatic cancer[14] bladder cancer[15], endometrial cancer[16]	11. http://clinicaltrials.gov/show/NCT00499408 12. http://clinicaltrials.gov/show/NCT01126879 13. http://clinicaltrials.gov/show/NCT00244933 14. http://clinicaltrials.gov/show/NCT00376948 15. http://clinicaltrials.gov/show/NCT00118040 16. http://clinicaltrials.gov/show/NCT00099008	11, 13-16: completed 12: currently recruiting participants
5	3, 3-diindolylmethane	PI3K, Akt and mTOR	Cruciferous vegetables	In Phase II-III for breast cancer[17] and prostate cancer[18]	17. http://clinicaltrials.gov/show/NCT01391689 18. http://clinicaltrials.gov/show/NCT00888654	17-18: currently recruiting participants
6	Green tea extract or polyphenon E	Akt and mTOR	Green tea	In Phase I-II for breast cancer[19,20], leukemia[21], monoclonal gammopathy of undetermined significance and/or smoldering multiple myeloma[22], prostatic hyperplasia[23], premalignant lesions of the head and neck[24]	19. http://clinicaltrials.gov/show/NCT00516243 20. http://clinicaltrials.gov/show/NCT00676793 21. http://clinicaltrials.gov/show/NCT00262743 22. http://clinicaltrials.gov/show/NCT00942422 23. http://clinicaltrials.gov/show/NCT00596011 24. http://clinicaltrials.gov/show/NCT01116336	19,20,22,23: ongoing, but not recruiting participants 21: completed 24: currently recruiting participants
7	Epigallocatechin gallate (EGCG)	Akt and mTOR	Green tea	In preclinical study for human hepatoma cells and keloid fibroblast	Huang et al., 2009; Zhang et al., 2006	
8	Timosaponin AIII	Akt and mTOR	Anemarrhena asphodeloides Bunge	In pre-clinical study for BT-549 and MDAM231 breast cancer cells	King et al., 2009	
9	Gallic acid	Akt and mTOR	*Phaleria macrocarpa* (Scheff.) Boerl.	In pre-clinical study for TE-2 esophageal cancer cells	Faried et al., 2007	
10	Diosgenin	Akt and mTOR	*Dioscorea* spp.	In pre-clinical study for AU565 breast adenocarcinoma cells	Chiang et al., 2007	

and mRNA translation (Lin et al., 2010).

Resveratrol has undergone numerous clinical investigations for its putative cancer chemopreventive properties. A pilot study of SRT501, a micronized resveratrol preparation, given as 5.0 g daily for 14 days, to patients with colorectal cancer and hepatic metastases scheduled to undergo hepatectomy, revealed a marked increase of cleaved caspase-3, a marker of apoptosis, in malignant hepatic tissue compared with tissue from the placebo-treated patients (Howells et al., 2011). In healthy volunteers, the ingestion of resveratrol caused a significant decrease in circulating IGF-1 and IGFBP-3 in all volunteers, suggesting chemopreventive activities (Brown et al., 2010). In another study with healthy volunteers, daily intake of 1 g of resveratrol for 4 weeks revealed an induction of GST-pi level and UGT1A1 activity in individuals with low baseline enzyme level/activity, indicating that resveratrol can modulate enzyme systems involved in carcinogen activation and detoxification, suggesting a possible mechanism by which resveratrol inhibits carcinogenesis (Chow et al., 2010).

Unfortunately, a Phase II study of SRT501 (resveratrol) with bortezomib in patients with relapsed and/or refractory multiple myeloma has to be terminated recently (Popat et al., 2013). Out of 24 patients, 9 patients receiving SRT501 and bortezomib were withdrawn from the study, mainly due to serious adverse reactions. The predominant study finding was an unexpected renal toxicity and low efficacy of SRT501 with nausea and vomiting which could have

resulted in disease progression and dehydration. This study has demonstrated an unacceptable safety profile and minimal efficacy in patients with relapsed/refractory multiple myeloma (Popat et al., 2013). At least two more clinical trials on colorectal cancer were completed but no published data was noted on the outcome. Currently an intervention study to examine the effects of resveratrol on neuroendocrine tumor is ongoing (Table 1).

Pomegranate, an ancient and mystical fruit of the tree *Punica granatum* L., has been used for centuries for the treatment of inflammatory diseases and disorders of the digestive tract (Faria and Calhau, 2010). In A/J mice, pomegranate fruit extract decreases carcinogen-induced lung tumorigenesis. Analysis of the murine lung tissue sample showed that pomegranate fruit extract down-regulates mTOR signalling by inhibiting the phosphorylation of PI3K, Akt and mTOR, and downstream molecules such as p70S6K and 4E-BP1 (Khan et al., 2007a). Other anti-carcinogenic effects of pomegranate fruit in numerous animal and cell culture models are well demonstrated in various studies (Kim et al., 2002b; Malik et al., 2005; Khan et al., 2007b).

In a Phase II clinical trial for men with rising PSA (prostate serum antigen) after surgery or radiotherapy for localized prostate cancer, patients were treated with 8 ounces of pomegranate juice daily (Pantuck et al., 2006). This study shows statistically significant prolongation on PSA doubling time over a period of 13 months. However, it was uncertain if improvements in biomarker like PSA doubling time are likely to serve as surrogate for clinical benefit. In a randomized Phase II study of pomegranate extract for men with rising PSA following initial therapy for localized prostate cancer, pomegranate extract treatment was associated with more than 6 months increase in PSA doubling time without adverse effects. Unfortunately, the significance of slowing of PSA doubling time remains unclear (Paller et al., 2013). Currently, clinical trials using either pomegranate juice or extract on prostate cancer patients are still ongoing (Table 1).

Genistein, the predominant isoflavone found in soybean (*Glycine max* (L.) Merr.), was found to have potent anti-tumor effects on prostate, brain, breast and colon cancers (Ravindranath et al., 2004; Hwang et al., 2009; Nakamura et al., 2009; Das et al., 2010; Sakamoto et al., 2010). In Hela and CaSki cervical cancer cells, genistein inhibits cell growth by modulating various mitogen-activated protein kinases (MAPK) and inhibiting Akt phosphorylation (Kim et al., 2009). In MCF-7 breast cancer cells, genistein decreases protein expression of total Akt and phosphorylated Akt, suggesting that genistein could offer protection against breast cancer through down-regulation of the PI3K/Akt signalling pathway (Anastasius et al., 2009). Combination of genistein and indol-3-carbinol induces apoptosis and autophagy in HT-29 colon cancer cells by inhibiting Akt and mTOR phosphorylation (Nakamura et al., 2009). In addition, it inhibits Akt kinase activity and abrogates the EGF-induced activation of Akt in PC3 prostate cancer cells (Li and Sarkar, 2002). Genistein is also found to augment the efficacy of cisplatin in pancreatic cancer by down-regulating Akt expression (Banerjee et al., 2007).

The promising anti-cancer effects of genistein has led to Phase II clinical trials involving combination therapy of genistein with gemcitabine hydrochloride in stage IV breast cancer, genistein with gemcitabine and erlotinib in locally advanced or metastatic pancreatic cancer as well as genistein with vitamin D in men with early stage prostate cancer (Table 1). Other clinical trials of genistein include Phase II study in patients who are undergoing surgery for bladder cancer, Phase II study in patients with prostate cancer as well as Phase I study of genistein in preventing breast or endometrial cancer in healthy postmenopausal women (Table 1). A Phase II randomized, placebo-controlled trial was carried out to investigate whether daily, oral genistein (300 or 600 mg/d) as purified soy extract for 14 to 21 days before surgery alters molecular pathways in bladder epithelial tissue in 59 subjects diagnosed with urothelial bladder cancer (Messing et al., 2012). Overall, genistein treatment was well tolerated and the observed toxicities were primarily mild to moderate. A significant reduction in bladder cancer tissue p-EGFR staining was observed in low dose treatment group as compared with placebo. However, there were no significant differences in tumor tissue staining between treatment groups for COX-2, Ki-67, activated caspase-3, Akt, p-Akt and MAPK (Messing et al., 2012).

3,3'-diindolylmethane is a potential anticancer component found in cruciferous vegetables with anti-proliferative and antiandrogenic properties in human prostate cancer cells (Le et al., 2003; Garikapaty et al., 2006). In DU145 human prostate cancer cells, the anti-proliferative effect of 3,3'-diindolylmethane was mediated by downregulation of PI3K, total Akt and phosphorylated Akt (Garikapaty et al., 2006). BR-DIM, a formulated 3,3'-diindolylmethane with higher bioavailability, inhibits phosphorylation of Akt in C4-2B prostate cancer cells (Li et al., 2007b) and inhibits phosphorylation of Akt, mTOR, 4E-BP1 and p70S6K in platelet-derived growth factor-D-overexpressing PC3 prostate cancer cells (Kong et al., 2008). A Phase I dose-escalation study of oral BR-DIM in castrate-resistant, non-metastatic prostate cancer patients revealed that BR-DIM was well tolerated and modest efficacy was demonstrated (Heath et al., 2010). In a pilot study to demonstrate the protective effect of BR-DIM supplements in postmenopausal women with a history of early-stage breast cancer, daily DIM (108 mg DIM/day) supplements for 30 days increased the 2-hydroxylation of estrogen urinary metabolites (Dalessandri et al., 2004). Currently, Phase II/III studies in patients with breast cancer and Phase II study in patients with stage I or stage II prostate cancer undergoing radical prostatectomy are ongoing (Table 1).

EGCG, a polyphenolic compound, is the major catechin found in green tea (Nagle et al., 2006). High consumption of green tea is associated with decreased risk of carcinogenesis and EGCG is a potent antioxidant that may have anticancer properties (Nagle et al., 2006; Katiyar et al., 2007; Pyrko et al., 2007). EGCG induces AMPK in both p53 positive and negative human hepatoma cells, resulting in the suppression of mTOR and 4E-BP1, and a general decrease in mRNA translation (Huang et al., 2009).

In keloid fibroblast, EGCG inhibits the phosphorylation of Akt, p70S6K and 4E-BP1 (Zhang et al., 2006). Further studies are needed to establish the relationship between EGCG and PI3K/Akt/mTOR pathway and to determine whether mTOR mediates the effects of EGCG in treating brain, prostate, cervical and bladder cancers (Hsieh and Wu, 2009; Philips et al., 2009; Qiao et al., 2009; Das et al., 2010). However, many current clinical studies focus on using green tea extract or polyphenon E in a wide range of cancers such as breast cancer, leukemia, multiple myeloma and head and neck lesions (Table 1).

Timosaponin AIII is a steroidal saponin isolated from *Anemarrhena asphodeloides* Bunge (Liliaceae), a traditional Chinese medicine with anti-diabetic, anti-platelet aggregation and diuretic activities (Zhang et al., 1999). Timosaponin AIII has been reported to exhibit cytotoxicity towards HeLa cervical cancer cells and HCT-15 human colorectal cancer cells (Sy et al., 2008; Kang et al., 2011). Timosaponin AIII selectively induces cell death in BT474 and MDAM231 breast carcinoma cells, but not in normal MCF10A immortalized mammary epithelial cells. It exerts its anti-proliferative activity by inhibiting phosphorylation of Akt and mTOR, as well as p70S6K and 4E-BP1 (King et al., 2009). This compound is still in pre-clinical stages and has not progressed into clinical trials.

Gallic acid is a natural antioxidant polyhydroxyphenolic compound found in various plants and fruits (Chu et al., 2002; Sun et al., 2002). Gallic acid is also isolated from *Phaleria macrocarpa* (Scheff.) Boerl, an Indonesian medicinal plant which is used in traditional medicine to control cancer, impotency, hemorrhoids, diabetes mellitus, allergies, liver and heart disease. In preclinical studies, gallic acid induces apoptosis and inhibits cell growth of various cancer cell lines, including human TE-2 esophageal cancer, MKN-28 gastric cancer, HT-29 and Colo201 colon cancer, MCF-7 breast cancer, CaSki cervix cancer and mouse colon-26 colon cancer cells (Faried et al., 2007). It up-regulates the pro-apoptotic Bax protein, induces the caspase-cascade and down-regulates anti-apoptotic protein such as Bcl-2 (Faried et al., 2007). In human TE-2 esophageal cancer cells, gallic acid reduces the phosphorylation of Akt, mTOR and p70S6K, suggesting that the inhibitory effect of gallic acid was mediated by down-regulation of Akt/mTOR pathway (Faried et al., 2007).

Diosgenin is a naturally occurring plant steroid with potential antineoplastic activities as it induces apoptosis in various human cancer cell lines (Moalic et al., 2001; Liu et al., 2005). In human AU565 HER2-overexpressing breast adenocarcinoma cells, diosgenin down-regulates protein levels of fatty acid synthase (FAS), phosphorylated Akt and phosphorylated mTOR, suggesting that diosgenin may suppress FAS expression in AU565 cells through PI3K/Akt/mTOR signal transduction pathway (Chiang et al., 2007). High levels of FAS are associated with poor prognosis in human cancers, and it is highly elevated in HER2-overexpressing breast cancer cells (Kuhajda, 2000; Kumar-Sinha et al., 2003). In another study to determine effect of diosgenin on breast cancer cells, diosgenin is found to inhibit p-Akt expression and Akt kinase activity without affecting PI3 kinase levels. It causes G1 cell cycle arrest by down-regulating cyclin D1, cdk-2 and cdk-4 expression in breast tumor cells, resulting in inhibition of cell proliferation and induction of apoptosis. Interestingly, no significant toxicity was seen in the normal breast epithelial cells (MCF-10A). *In vivo* tumor studies indicate that diosgenin significantly inhibits tumor growth in both MCF-7 and MDA-231 xenografts in nude mice, indicating that it is a potential chemotherapeutic agent (Srinivasan et al., 2009). Diosgenin, timosaponin AIII and gallic acid are still in pre-clinical stages and have not progressed to clinical trials.

Conclusion

Hyperactivation of the PI3K/Akt/mTOR signalling pathway is a prominent hallmark of cancer and is frequently implicated in resistance to anticancer therapies such as biologics, tyrosine kinase inhibitors, radiation, and cytotoxics (Ballou and Lin, 2008). In therapeutic sensitivity restoration, inhibitors of the PI3K/Akt/mTOR pathway are often evaluated in combination with the other anticancer therapies in preclinical models and in clinical studies. Current preclinical and clinical evidences suggest that inhibitors of the PI3K/Akt/mTOR pathway in combination with other anticancer therapies are able to circumvent resistance by cancer cells. One of the important considerations of mTOR inhibitors would be the general tolerability and safety profile of the drugs. Although most of the reported toxicities are mild to moderate in severity and can be managed clinically by dose modification and supportive measures, efforts should continue to optimize leads with greater safety and better pharmacological profile. It is quite interesting that mTOR signalling pathway is not only implicated in various cancers but appears to be involved in multiple disease conditions. For example, rapamycin was also investigated for its longevity activity and lifespan extension possibilities. The relationship between age-associated diseases with mTOR and its signalling systems are intriguing. The mTOR signalling pathway clearly offers tremendous opportunities for discovery of new drugs that target both aging and its associated diseases (Sharp and Richardson, 2011).

Rapamycin and its analogues are versatile drugs with proven efficacy in cancer and new drugs produced promising results in various cancer-related clinical trials. Potential chemopreventive activities of some natural phytochemicals such as curcumin, green tea extract and pomegranate are convincing as more and more trials were carried out to provide evidence-based data to advocate chemoprevention of cancer. The challenge for the future will be to further dissect the molecular signalling pathway to fully understand the mechanisms underpinning sensitivity or resistance to mTOR inhibition. The uncover of these pathways and identification of novel drug targets will provide insight into rational combinations of mTOR inhibitors with classic cytotoxic agents, radiation, and other molecular targeted therapies in the treatment and prevention of cancer as well as to discover novel uses of this class of drugs.

References

Abdulkareem IH, Blair M (2013). Phosphatase and tensin homologue deleted on chromosome 10. *Niger Med J,* **54**, 79-86.

Abraham RT (1998). Mammalian target of rapamycin: immunosuppressive drugs uncover a novel pathway of cytokine receptor signalling. *Curr Opin Immunol,* **10**, 330-6.

Aggarwal BB, Bhardwaj A, Aggarwal RS, et al (2004). Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies. *Anticancer Res,* **24**, 2783-840.

Anastasius N, Boston S, Lacey M, Storing N, Whitehead SA (2009). Evidence that low-dose, long-term genistein treatment inhibits oestradiol-stimulated growth in MCF-7 cells by down-regulation of the PI3-kinase/Akt signalling pathway. *J Steroid Biochem Mol Biol,* **116**, 50-5.

Aoki H, Takada Y, Kondo S, et al (2007). Evidence that curcumin suppresses the growth of malignant gliomas *In vitro* and *In vivo* through induction of autophagy: role of Akt and extracellular signal-regulated kinase signalling pathways. *Mol Pharmacol*, **72**, 29-39.

Asnaghi L, Bruno P, Priulla M, et al (2004). mTOR: a protein kinase switching between life and death. *Pharmacol Res* **50**, 545-9.

Aziz MH, Nihal M, Fu VX, et al (2006). Resveratrol-caused apoptosis of human prostate carcinoma LNCaP cells is mediated via modulation of phosphatidylinositol 3'-kinase/Akt pathway and Bcl-2 family proteins. *Mol Cancer Ther* **5**, 1335-41.

Ballou LM, Lin RZ (2008). Rapamycin and mTOR kinase inhibitors. *J Chem Biol*, **1**, 27-36.

Banerjee S, Zhang Y, Wang Z, et al (2007). *In vitro* and *In vivo* molecular evidence of genistein action in augmenting the efficacy of cisplatin in pancreatic cancer. *Int J Cancer*, **120**, 906-17.

Baselga J (2011). Targeting the phosphoinositide-3 (PI3) kinase pathway in breast cancer. *Oncologist*, **16**, 12-9.

Beevers CS, Chen L, Liu L, et al (2009). Curcumin disrupts the mammalian target of rapamycin-raptor complex. *Cancer Res,* **69**, 1000-8.

Beevers CS, Li F, Liu L, et al (2006). Curcumin inhibits the mammalian target of rapamycin-mediated signalling pathways in cancer cells. *Int J Cancer*, **119**, 757-64.

Bharti AC, Aggarwal BB (2002). Nuclear factor-kappa B and cancer: its role in prevention and therapy. *Biochem Pharmacol*, **64**, 883-8.

Brito PM, Devillard R, Negre-Salvayre A, et al (2009). Resveratrol inhibits the mTOR mitogenic signalling evoked by oxidized LDL in smooth muscle cells. *Atherosclerosis* **205**, 126-34.

Brown VA, Patel KR, Viskaduraki M, et al (2010). Repeat dose study of the cancer chemopreventive agent resveratrol in healthy volunteers: safety, pharmacokinetics, and effect on the insulin-like growth factor axis. *Cancer Res,* **70**, 9003-11.

Caron E, Ghosh S, Matsuoka Y, et al (2010). A comprehensive map of the mTOR signalling network. *Mol Syst Biol,* **6**, 453.

Carroll RE, Benya RV, Turgeon DK, et al (2011). Phase IIa clinical trial of curcumin for the prevention of colorectal neoplasia. *Cancer Prev Res*, **4**, 354-64.

Chan S (2004). Targeting the mammalian target of rapamycin (mTOR): a new approach to treating cancer. *Br J Cancer* **91**, 1420-4.

Chen HX, Sharon E (2013). IGF-1R as an anti-cancer target--trials and tribulations. *Chin J Cancer,* **32**, 242-52.

Cheng AL, Hsu CH, Lin JK, et al (2001). Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. *Anticancer Res*, **21**, 2895-900.

Chiang CT, Way TD, Tsai SJ, et al (2007). Diosgenin, a naturally occurring steroid, suppresses fatty acid synthase expression in HER2-overexpressing breast cancer cells through modulating Akt, mTOR and JNK phosphorylation. *FEBS Lett*, **581**, 5735-42.

Chow HH, Garland LL, Hsu CH, et al (2010). Resveratrol modulates drug- and carcinogen-metabolizing enzymes in a healthy volunteer study. *Cancer Prev Res,* **3**, 1168-75.

Chu Y-F, Sun J, Wu X, et al (2002). Antioxidant and antiproliferative activities of common vegetables. *J Agr Food Chem*, **50**, 6910-6.

Cragg GM, Newman DJ, Snader KM (1997). Natural products in drug discovery and development. *J Nat Prod*, **60**, 52-60.

da Rocha AB, Lopes RM, Schwartsmann G (2001). Natural products in anticancer therapy. *Curr Opin Pharmacol*, **1**, 364-9.

Dalessandri KM, Firestone GL, Fitch MD, et al (2004). Pilot study: effect of 3,3'-Diindolylmethane supplements on urinary hormone metabolites in postmenopausal women with a history of early-stage breast cancer. *Nutr Cancer*, **50**, 161-7.

Das A, Banik NL, Ray SK (2010). Flavonoids activated caspases for apoptosis in human glioblastoma T98G and U87MG cells but not in human normal astrocytes. *Cancer*, **116**, 164-76.

Dhillon N, Aggarwal BB, Newman RA, et al (2008). Phase II trial of curcumin in patients with advanced pancreatic cancer. *Clin Cancer Res*, **14**, 4491-9.

Dowling RJ, Topisirovic I, Fonseca BD, et al (2010). Dissecting the role of mTOR: lessons from mTOR inhibitors. *Biochim Biophys Acta,* **1804**, 433-9.

Faria A, Calhau C (2010). Chapter 36 - Pomegranate in human health: an overview (W. Ronald Ross and R. P. Victor, Ed.^ Eds). Academic Press, San Diego, **pp**. 551-63.

Faried A, Kurnia D, Faried LS, et al (2007). Anticancer effects of gallic acid isolated from Indonesian herbal medicine, Phaleria macrocarpa (Scheff.) Boerl, on human cancer cell lines. *Int J Oncol*, **30**, 605-13.

Fingar DC, Richardson CJ, Tee AR, et al (2004). mTOR controls cell cycle progression through its cell growth effectors S6K1 and 4E-BP1/eukaryotic translation initiation factor 4E. *Mol Cell Biol,* **24**, 200-16.

Fruman DA, Rommel C (2014). PI3K and cancer: lessons, challenges and opportunities. *Nat Rev Drug Discov*, **13**, 140-56.

Galluzzi L, Vicencio JM, Kepp O, et al (2008). To die or not to die: that is the autophagic question. *Curr Mol Med*, **8**, 78-91.

Ganley IG, Lam du H, Wang J, et al (2009). ULK1.ATG13. FIP200 complex mediates mTOR signalling and is essential for autophagy. *J Biol Chem*, **284**, 12297-305.

Garcia-Echeverria C (2010). Allosteric and ATP-competitive kinase inhibitors of mTOR for cancer treatment. *Bioorg Med Chem Lett*, **20**, 4308-12.

Garikapaty VP, Ashok BT, Tadi K, et al (2006). 3,3'-Diindolylmethane downregulates pro-survival pathway in hormone independent prostate cancer. *Biochem Biophys Res Commun*, **340**, 718-25.

Gera JF, Mellinghoff IK, Shi Y, et al (2004). AKT activity determines sensitivity to mammalian target of rapamycin (mTOR) inhibitors by regulating cyclin D1 and c-myc expression. *J Biol Chem*, **279**, 2737-46.

Ghayad SE, Cohen PA (2010). Inhibitors of the PI3K/Akt/mTOR pathway: new hope for breast cancer patients. *Recent Pat Anticancer Drug Discov*, **5**, 29-57.

Gomez-Pinillos A, Ferrari AC (2012). mTOR signalling pathway and mTOR inhibitors in cancer therapy. *Hematol Oncol Clin North Am*, **26**, 483-505.

Guertin DA, Kim D-H, Sabatini DM (2004). Growth control through the mTOR network. In 'Cell Growth: Control of Cell

Size' (Hall MN et al., eds), Cold Spring Harbor Laboratory Press, pp. 193-234.
Guertin DA, Sabatini DM (2009). The pharmacology of mTOR inhibition. *Sci Signal*, **2**, 24.
Gullett NP, Ruhul Amin AR, Bayraktar S, et al (2010). Cancer prevention with natural compounds. *Semin Oncol*, **37**, 258-81.
Han D, Li SJ, Zhu YT, et al (2013). LKB1/AMPK/mTOR signalling pathway in non-small-cell lung cancer. *Asian Pac J Cancer Prev*, **14**, 4033-9.
Hardwick JS, Kuruvilla FG, Tong JK, et al (1999). Rapamycin-modulated transcription defines the subset of nutrient-sensitive signalling pathways directly controlled by the Tor proteins. *Proc Natl Acad Sci USA*, **96**, 14866-70.
Hasskarl J (2014). Everolimus. *Recent Results Cancer Res*, **201**, 373-92.
Hay N, Sonenberg N (2004). Upstream and downstream of mTOR. *Genes Dev*, **18**, 1926-45.
He X, Wang Y, Zhu J, et al (2010). Resveratrol enhances the anti-tumor activity of the mTOR inhibitor rapamycin in multiple breast cancer cell lines mainly by suppressing rapamycin-induced AKT signalling. *Cancer Lett*, **28**, 168-76
He Y, Li D, Cook SL, et al (2013). Mammalian target of rapamycin and Rictor control neutrophil chemotaxis by regulating Rac/Cdc42 activity and the actin cytoskeleton. *Mol Biol Cell*, **24**, 3369-80.
Heath EI, Heilbrun LK, Li J, et al (2010). A Phase I dose-escalation study of oral BR-DIM (BioResponse 3,3'-Diindolylmethane) in castrate-resistant, non-metastatic prostate cancer. *Am J Transl Res*, **2**, 402-11.
Hidalgo M, Rowinsky EK (2000). The rapamycin-sensitive signal transduction pathway as a target for cancer therapy. *Oncogene*, **19**, 6680-6.
Hosokawa N, Sasaki T, Iemura S, et al (2009). Atg101, a novel mammalian autophagy protein interacting with Atg13. *Autophagy*, **5**, 973-9.
Houghton PJ (2010). mTOR and Cancer Therapy: General Principles (V. A. Polunovsky and P. J. Houghton, Ed.^ Eds.), pp. 113-31. Humana Press, Springer New York Dordrecht Heidelberg London.
Howells LM, Berry DP, Elliott PJ, et al (2011). Phase I randomized, double-blind pilot study of micronized resveratrol (SRT501) in patients with hepatic metastases--safety, pharmacokinetics, and pharmacodynamics. *Cancer Prev Res*, **4**, 1419-25.
Hsieh TC, Wu JM (2009). Targeting CWR22Rv1 prostate cancer cell proliferation and gene expression by combinations of the phytochemicals EGCG, genistein and quercetin. *Anticancer Res*, **29**, 4025-32.
Huang CH, Tsai SJ, Wang YJ, et al (2009). EGCG inhibits protein synthesis, lipogenesis, and cell cycle progression through activation of AMPK in p53 positive and negative human hepatoma cells. *Mol Nutr Food Res*, **53**, 1156-65.
Huang S, Houghton PJ (2003). Targeting mTOR signalling for cancer therapy. *Curr Opin Pharmacol*, **3**, 371-7.
Hwang YW, Kim SY, Jee SH, et al (2009). Soy food consumption and risk of prostate cancer: a meta-analysis of observational studies. *Nutr Cance*r, **61**, 598-606.
Inoki K, Li Y, Zhu T, et al (2002). TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat Cell Biol*, **4**, 648-57.
Irving GR, Howells LM, Sale S, et al (2013). Prolonged biologically active colonic tissue levels of curcumin achieved after oral administration--a clinical pilot study including assessment of patient acceptability. *Cancer Prev Res (Phila)*, **6**, 119-28.
Jacinto E, Loewith R, Schmidt A, et al (2004). Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nat Cell Biol*, **6**, 1122-8.
Jiang H, Shang X, Wu H, et al (2009). Resveratrol downregulates PI3K/Akt/mTOR signalling pathways in human U251 glioma cells. *J Exp Ther Oncol*, **8**, 25-33.
Johnson SM, Gulhati P, Arrieta I, et al (2009). Curcumin inhibits proliferation of colorectal carcinoma by modulating Akt/mTOR signalling. *Anticancer Res*, **29**, 3185-90.
Jung CH, Jun CB, Ro SH, et al (2009). ULK-Atg13-FIP200 complexes mediate mTOR signalling to the autophagy machinery. *Mol Biol Cell*, **20**, 1992-2003.
Kadowaki M, Kanazawa T (2003). Amino acids as regulators of proteolysis. *J Nutr*, **133**, 2052-6.
Kanai M, Otsuka Y, Otsuka K, et al (2013). A Phase I study investigating the safety and pharmacokinetics of highly bioavailable curcumin (Theracurmin) in cancer patients. *Cancer Chemother Pharmacol*, **71**, 1521-30.
Kanai M, Yoshimura K, Asada M, et al (2011). A phase I/II study of gemcitabine-based chemotherapy plus curcumin for patients with gemcitabine-resistant pancreatic cancer. *Cancer Chemother Pharmacol,* **68**, 157-64.
Kang Y-J, Chung H-J, Nam J-W, et al (2011). Cytotoxic and antineoplastic activity of timosaponin a-iii for human colon cancer cells. *J Nat Prod*, **74**, 701-6.
Katiyar S, Elmets CA, Katiyar SK (2007). Green tea and skin cancer: photoimmunology, angiogenesis and DNA repair. *J Nutr Biochem*, **18**, 287-96.
Khan N, Afaq F, Kweon MH, et al (2007a). Oral consumption of pomegranate fruit extract inhibits growth and progression of primary lung tumors in mice. *Cancer Res*, **67**, 3475-82.
Khan N, Hadi N, Afaq F, et al (2007b). Pomegranate fruit extract inhibits prosurvival pathways in human A549 lung carcinoma cells and tumor growth in athymic nude mice. *Carcinogenesis*, **28**, 163-73.
Kim DH, Sarbassov DD, Ali SM, et al (2002a). mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell*, **110**, 163-75.
Kim ND, Mehta R, Yu W, et al (2002b). Chemopreventive and adjuvant therapeutic potential of pomegranate (Punica granatum) for human breast cancer. *Breast Cancer Res Treat*, **71**, 203-17.
Kim SH, Kim YB, Jeon YT, et al (2009). Genistein inhibits cell growth by modulating various mitogen-activated protein kinases and AKT in cervical cancer cells. *Ann NY Acad Sci*, **1171**, 495-500.
King FW, Fong S, Griffin C, et al (2009). Timosaponin AIII is preferentially cytotoxic to tumor cells through inhibition of mTOR and induction of ER stress. *PloS one*, **4**, 7283.
Kirken RA, Wang YL (2003). Molecular actions of sirolimus: sirolimus and mTor. *Transplant Proc*, **35**, 227-30.
Kong D, Banerjee S, Huang W, et al (2008). Mammalian target of rapamycin repression by 3,3'-Diindolylmethane inhibits invasion and angiogenesis in platelet-derived growth factor-D-overexpressing PC3 cells. *Cancer Res*, **68**, 1927-34.
Lao CD, Ruffin MTt, Normolle D, et al (2006). Dose escalation of a curcuminoid formulation. *BMC Complement Altern Med*, **6**, 10.
Laplante M, Sabatini DM (2012). mTOR signalling in growth control and disease. *Cell*, **149**, 274-93.
Laplante M, Sabatini DM (2013). Regulation of mTORC1 and its impact on gene expression at a glance. *J Cell Sci*, **126**, 1713-9.
Law BK (2005). Rapamycin: an anti-cancer immunosuppressant? *Crit Rev Oncol Hematol*, **56**, 47-60.
Le HT, Schaldach CM, Firestone GL, et al (2003). Plant-derived 3,3'-Diindolylmethane is a strong androgen antagonist in human prostate cancer cells. *J Biol Chem*, **278**, 21136-45.

Li JC, Zhu HY, Chen TX, et al (2013). Roles of mTOR and p-mTOR in gastrointestinal stromal tumors. *Asian Pac J Cancer Prev*, **14**, 5925-8.

Li M, Zhang Z, Hill DL, et al (2007). Curcumin, a dietary component, has anticancer, chemosensitization, and radiosensitization effects by down-regulating the MDM2 oncogene through the PI3K/mTOR/ETS2 pathway. *Cancer Res*, **67**, 1988-96.

Li Y, Sarkar FH (2002). Inhibition of nuclear factor kappaB activation in PC3 cells by genistein is mediated via Akt signalling pathway. *Clin Cancer Res*, **8**, 2369-77.

Li Y, Wang Z, Kong D, et al (2007). Regulation of FOXO3a/beta-catenin/GSK-3beta signalling by 3,3'-Diindolylmethane contributes to inhibition of cell proliferation and induction of apoptosis in prostate cancer cells. *J Biol Chem*, **282**, 21542-50.

Lin JN, Lin VC, Rau KM, et al (2010). Resveratrol modulates tumor cell proliferation and protein translation via SIRT1-dependent AMPK activation. *J Agric Food Chem*, **58**, 1584-92.

Liu MJ, Wang Z, Ju Y, et al (2005). Diosgenin induces cell cycle arrest and apoptosis in human leukemia K562 cells with the disruption of Ca_2+ homeostasis. *Cancer Chemother Pharmacol,* **55**, 79-90.

Liu P, Cheng H, Roberts TM, et al (2009a). Targeting the phosphoinositide 3-kinase pathway in cancer. *Nat Rev Drug Discov*, **8**, 627-44.

Liu Q, Thoreen C, Wang J, Sabatini D, Gray NS (2009b). mTOR mediated anti-cancer drug discovery. *Drug Discov Today Ther Strateg*, **6**, 47-55.

Loewith R, Jacinto E, Wullschleger S, et al (2002). Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Mol Cell*, **10**, 457-68.

LoPiccolo J, Blumenthal GM, Bernstein WB, et al (2008). Targeting the PI3K/Akt/mTOR pathway: effective combinations and clinical considerations. *Drug Resist Updat*, **11**, 32-50.

Malik A, Afaq F, Sarfaraz S, et al (2005). Pomegranate fruit juice for chemoprevention and chemotherapy of prostate cancer. *Proc Natl Acad Sci USA*, **102**, 14813-8.

Manning BD, Cantley LC (2007). AKT/PKB signalling: navigating downstream. *Cell*, **129**, 1261-74.

Marques FZ, Markus MA, Morris BJ (2009). Resveratrol: cellular actions of a potent natural chemical that confers a diversity of health benefits. *Int J Biochem Cell Biol*, **41**, 2125-8.

Messing E, Gee JR, Saltzstein DR, et al (2012). A phase 2 cancer chemoprevention biomarker trial of isoflavone G-2535 (genistein) in presurgical bladder cancer patients. *Cancer Prev Res*, **5**, 621-30.

Mita MM, Mita A, Rowinsky EK (2003). The molecular target of rapamycin (mTOR) as a therapeutic target against cancer. *Cancer Biol Ther*, **2**, 169-77.

Moalic S, Liagre B, Corbiere C, et al (2001). A plant steroid, diosgenin, induces apoptosis, cell cycle arrest and COX activity in osteosarcoma cells. *FEBS Lett*, **506**, 225-30.

Moschetta M, Reale A, Marasco C, et al (2014). Therapeutic targeting of the mTOR-signalling pathway in cancer: benefits and limitations. *Br J Pharmacol*, [Epub ahead of print]

Nagle DG, Ferreira D, Zhou YD (2006). Epigallocatechin-3-gallate (EGCG): chemical and biomedical perspectives. *Phytochemistry*, **67**, 1849-55.

Nakamura Y, Yogosawa S, Izutani Y, et al (2009). A combination of indol-3-carbinol and genistein synergistically induces apoptosis in human colon cancer HT-29 cells by inhibiting Akt phosphorylation and progression of autophagy. *Mol Cancer*, **8**, 100.

Nave BT, Ouwens M, Withers DJ, et al (1999). Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. *Biochem J*, **344**, 427-31.

Nicholson KM, Anderson NG (2002). The protein kinase B/Akt signalling pathway in human malignancy. *Cell Signal* **14**, 381-95.

Noh WC, Mondesire WH, Peng J, et al (2004). Determinants of rapamycin sensitivity in breast cancer cells. *Clin Cancer Res*, **10**, 1013-23.

O'Reilly KE, Rojo F, She QB, et al (2006). mTOR inhibition induces upstream receptor tyrosine kinase signalling and activates Akt. *Cancer Res*, **66**, 1500-8.

Pal I, Mandal M (2012). PI3K and Akt as molecular targets for cancer therapy: current clinical outcomes. *Acta Pharmacol Sin*, **33**, 1441-58.

Paller CJ, Ye X, Wozniak PJ, et al (2013). A randomized Phase II study of pomegranate extract for men with rising PSA following initial therapy for localized prostate cancer. *Prostate Cancer Prostatic Dis*, **16**, 50-5.

Pandurangan AK (2013). Potential targets for prevention of colorectal cancer: a focus on PI3K/Akt/mTOR and Wnt pathways. *Asian Pac J Cancer Prev,* **14**, 2201-5.

Pantuck AJ, Leppert JT, Zomorodian N, et al (2006). Phase II study of pomegranate juice for men with rising prostate-specific antigen following surgery or radiation for prostate cancer. *Clin Cancer Res*, **12**, 4018-26.

Pervaiz S (2003). Resveratrol: from grapevines to mammalian biology. *FASEB J*, **17**, 1975-85.

Peterson TR, Laplante M, Thoreen CC, et al (2009). DEPTOR is an mTOR inhibitor frequently overexpressed in multiple myeloma cells and required for their survival. *Cell*, **137**, 873-86.

Philips BJ, Coyle CH, Morrisroe SN, et al (2009). Induction of apoptosis in human bladder cancer cells by green tea catechins. *Biomed Res*, **30**, 207-15.

Plas DR, Thompson CB (2005). Akt-dependent transformation: there is more to growth than just surviving. *Oncogene*, **24**, 7435-42.

Popat R, Plesner T, Davies F, et al (2013). A phase 2 study of SRT501 (resveratrol) with bortezomib for patients with relapsed and or refractory multiple myeloma. *Br J Haematol* **160**, 714-7.

Porta C, Paglino C, Mosca A (2014). Targeting PI3K/Akt/mTOR signalling in cancer. *Fron Oncol*, **4**, 64.

Powers T, Walter P (1999). Regulation of ribosome biogenesis by the rapamycin-sensitive TOR-signalling pathway in Saccharomyces cerevisiae. *Mol Biol Cell*, **10**, 987-1000.

Proud CG (2002). Regulation of mammalian translation factors by nutrients. *Eur J Biochem*, **269**, 5338-49.

Pyrko P, Schonthal AH, Hofman FM, et al (2007). The unfolded protein response regulator GRP78/BiP as a novel target for increasing chemosensitivity in malignant gliomas. *Cancer Res*, **67**, 9809-16.

Qiao Y, Cao J, Xie L, et al (2009). Cell growth inhibition and gene expression regulation by (-)-epigallocatechin-3-gallate in human cervical cancer cells. *Arch Pharm Res*, **32**, 1309-15.

Ravindranath MH, Muthugounder S, Presser N, et al (2004). Anticancer therapeutic potential of soy isoflavone, genistein. *Adv Exp Med Biol*, **546**, 121-65.

Rodon J, Dienstmann R, Serra V, et al (2013). Development of PI3K inhibitors: lessons learned from early clinical trials. *Nat Rev Clin Oncol*, **10**, 143-53.

Ryan JL, Heckler CE, Ling M, et al (2013). Curcumin for radiation dermatitis: a randomized, double-blind, placebo-

controlled clinical trial of thirty breast cancer patients. *Radiat Res*, **180**, 34-43.

Sabatini DM, Erdjument-Bromage H, Lui M, et al (1994). RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. *Cell*, **78**, 35-43.

Sabers CJ, Martin MM, Brunn GJ, et al (1995). Isolation of a protein target of the FKBP12-rapamycin complex in mammalian cells. *J Biol Chem*, **270**, 815-22.

Sakamoto T, Horiguchi H, Oguma E, et al (2010). Effects of diverse dietary phytoestrogens on cell growth, cell cycle and apoptosis in estrogen-receptor-positive breast cancer cells. *J Nutr Biochem*, **21**, 856-64.

Sancak Y, Thoreen CC, Peterson TR, et al (2007). PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. Mol. *Cell*, **25**, 903-15.

Sansal I, Sellers WR (2004). The biology and clinical relevance of the PTEN tumor suppressor pathway. *J Clin Oncol*, **22**, 2954-63.

Sarbassov DD, Ali SM, Kim DH, et al (2004). Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr Biol*, **14**, 1296-302.

Sarbassov DD, Ali SM, Sengupta S, et al (2006). Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol Cell*, **22**, 159-68.

Sehgal SN, Baker H, Vezina C (1975). Rapamycin (AY-22,989), a new antifungal antibiotic. II. Fermentation, isolation and characterization. *J Antibiot (Tokyo)*, **28**, 727-32.

Seo BR, Min KJ, Cho IJ, et al (2014). Curcumin significantly enhances dual PI3K/Akt and mTOR inhibitor NVP-BEZ235-induced apoptosis in human renal carcinoma Caki cells through down-regulation of p53-dependent Bcl-2 expression and inhibition of Mcl-1 protein stability. *PloS one*, **9**, 95588.

Sharma RA, McLelland HR, Hill KA, et al (2001). Pharmacodynamic and pharmacokinetic study of oral curcuma extract in patients with colorectal cancer. *Clin Cancer Res*, **7**, 1894-900.

Sharp Z, Richardson A (2011). Aging and cancer: can mTOR inhibitors kill two birds with one drug? *Target Oncol*, **6**, 41-51.

Shimobayashi M, Hall MN (2014). Making new contacts: the mTOR network in metabolism and signalling crosstalk. *Nat Rev Mol Cell Biol*, **15**, 155-62.

Shishodia S, Chaturvedi MM, Aggarwal BB (2007). Role of curcumin in cancer therapy. *Curr Probl Cance*, **31**, 243-305.

Shor B, Gibbons JJ, Abraham RT, et al (2009). Targeting mTOR globally in cancer: thinking beyond rapamycin. *Cell Cycle* **8**, 3831-7.

Soliman GA, Acosta-Jaquez HA, Dunlop EA, et al (2010). mTOR Ser-2481 autophosphorylation monitors mTORC-specific catalytic activity and clarifies rapamycin mechanism of action. *J Biol Chem*, **285**, 7866-79.

Srinivasan S, Koduru S, Kumar R, et al (2009). Diosgenin targets Akt-mediated prosurvival signalling in human breast cancer cells. *Int J Cancer*, **125**, 961-7.

Sun J, Chu Y-F, Wu X, et al (2002). Antioxidant and antiproliferative activities of common fruits. *J Agric Food Chem*, **50**, 7449-54.

Sun SY, Rosenberg LM, Wang X, et al (2005). Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition. *Cancer Res*, **65**, 7052-8.

Sy LK, Yan SC, Lok CN, et al (2008). Timosaponin A-III induces autophagy preceding mitochondria-mediated apoptosis in HeLa cancer cells. *Cancer Res*, **68**, 10229-37.

Tabernero J, Rojo F, Calvo E, et al (2008). Dose- and schedule-dependent inhibition of the mammalian target of rapamycin pathway with everolimus: a Phase I tumor pharmacodynamic study in patients with advanced solid tumors. *J Clin Oncol* **26**, 1603-10.

Takahashi Y, Kohashi K, Yamada Y, et al (2014). Activation of the Akt/mammalian target of rapamycin pathway in myxofibrosarcomas. *Human pathol*, **45**, 984-93.

Tang KD, Ling MT (2014). Targeting Drug-Resistant Prostate Cancer with Dual PI3K/MTOR Inhibition. *Curr Med Chem*, [Epub ahead of print].

Tokunaga E, Oki E, Egashira A, et al (2008). Deregulation of the Akt pathway in human cancer. *Curr Cancer Drug Targets*, **8**, 27-36.

Tsang CK, Qi H, Liu LF, et al (2007). Targeting mammalian target of rapamycin (mTOR) for health and diseases. *Drug Discov Today*, **12**, 112-24.

Vakana E, Sassano A, Platanias LC (2010). Induction of autophagy by dual mTORC1-mTORC2 inhibition in BCR-ABL-expressing leukemic cells. *Autophagy*, **6**, 966-7.

Vezina C, Kudelski A, Sehgal SN (1975). Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. *J Antibiot*, **28**, 721-6.

Vivanco I, Sawyers CL (2002). The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer*, **2**, 489-501.

Vogt PK, Hart JR, Gymnopoulos M, et al (2010). Phosphatidylinositol 3-kinase: the oncoprotein. *Curr Top Microbiol Immunol*, **347**, 79-104.

Wan X, Harkavy B, Shen N, et al (2007). Rapamycin induces feedback activation of Akt signalling through an IGF-1R-dependent mechanism. *Oncogene*, **26**, 1932-40.

Wang XW, Zhang YJ (2014). Targeting mTOR network in colorectal cancer therapy. *World J Gastroenterol*, **20**, 4178-88.

Wu Y, Kim J, Elshimali Y, et al (2014). Activation of Akt1 accelerates carcinogen-induced tumorigenesis in mammary gland of virgin and post-lactating transgenic mice. *BMC Cancer*, **14**, 266.

Yap TA, Garrett MD, Walton MI, et al (2008). Targeting the PI3K-AKT-mTOR pathway: progress, pitfalls, and promises. *Curr Opin Pharmacol*, **8**, 393-412.

Yu K, Toral-Barza L, Discafani C, et al (2001). mTOR, a novel target in breast cancer: the effect of CCI-779, an mTOR inhibitor, in preclinical models of breast cancer. *Endocr Relat Cancer*, **8**, 249-58.

Zhang J, Meng Z, Zhang M, et al (1999). Effect of six steroidal saponins isolated from anemarrhenae rhizoma on platelet aggregation and hemolysis in human blood. *Clin Chim Acta*, **289**, 79-88.

Zhang Q, Kelly AP, Wang L, et al (2006). Green tea extract and (-)-epigallocatechin-3-gallate inhibit mast cell-stimulated type I collagen expression in keloid fibroblasts via blocking PI-3K/AkT signalling pathways. *J Invest Dermatol*, **126**, 2607-13.

Zhao JJ, Cheng H, Jia S, et al (2006). The p110α isoform of PI3K is essential for proper growth factor signalling and oncogenic transformation. *Proc Natl Acad Sci USA*, **103**, 16296-300.

Hua et al. Journal of Hematology & Oncology (2019) 12:71
https://doi.org/10.1186/s13045-019-0754-1

Journal of Hematology & Oncology

REVIEW Open Access

Targeting mTOR for cancer therapy

Hui Hua[1], Qingbin Kong[2], Hongying Zhang[2], Jiao Wang[3], Ting Luo[4] and Yangfu Jiang[2*]

Abstract

Mechanistic target of rapamycin (mTOR) is a protein kinase regulating cell growth, survival, metabolism, and immunity. mTOR is usually assembled into several complexes such as mTOR complex 1/2 (mTORC1/2). In cooperation with raptor, rictor, LST8, and mSin1, key components in mTORC1 or mTORC2, mTOR catalyzes the phosphorylation of multiple targets such as ribosomal protein S6 kinase β-1 (S6K1), eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1), Akt, protein kinase C (PKC), and type-I insulin-like growth factor receptor (IGF-IR), thereby regulating protein synthesis, nutrients metabolism, growth factor signaling, cell growth, and migration. Activation of mTOR promotes tumor growth and metastasis. Many mTOR inhibitors have been developed to treat cancer. While some of the mTOR inhibitors have been approved to treat human cancer, more mTOR inhibitors are being evaluated in clinical trials. Here, we update recent advances in exploring mTOR signaling and the development of mTOR inhibitors for cancer therapy. In addition, we discuss the mechanisms underlying the resistance to mTOR inhibitors in cancer cells.

Keywords: Cancer, Drug resistance, mTOR, Oncogene, Targeted therapy

Introduction

The mechanistic target of rapamycin (mTOR) is a dual-specificity protein kinase phosphorylating serine/threonine as well as tyrosine residues [1]. Since the catalytic domain of mTOR resembles that of lipid kinases such as phosphoinositide 3-kinase (PI3K), mTOR is considered as an atypical protein kinase belonging to the PI3K-related kinase family [2]. As a core component of several distinct complexes including mTOR complex 1 (mTORC1), mTOR complex 2 (mTORC2), and a putative mTOR complex 3 (mTORC3), mTOR has critical roles in diverse biological processes, such as cell proliferation, survival, autophagy, metabolism, and immunity [2, 3]. While mTOR and mammalian lethal with SEC13 protein 8 (mLST8) are common members of both mTORC1 and mTORC2, regulatory-associated protein of mTOR (raptor), the 40 kDa proline-rich Akt substrate (PRAS40), and DEP domain-containing protein 6 (DEPTOR) are specific members of mTORC1 [1, 2]. Instead, rapamycin-insensitive companion of mTOR (rictor) and mammalian stress-activated protein kinase-interacting protein 1 (mSIN1 or MAPKAP1) are unique components in mTORC2 but not mTORC1 [1]. Another rapamycin-insensitive complex, mTORC3, consists of ETV7, mTOR, and other undefined components [3]. mTORC1 senses nutrients, growth factors, and cellular energy to orchestrate nucleotide, lipid, and protein synthesis; inhibit autophagy; and stimulate cell growth [2]. mTORC2 is not only regulated by growth factors, but also activates type I insulin-like growth factor receptor (IGF-IR) and insulin receptor (InsR) through the tyrosine kinase activity of mTOR [1]. Besides, mTORC2 regulates the actin polarization and endocytosis [4, 5].

The mTOR signaling pathway has critical roles in mammalian metabolism and physiology. The de-regulated activity of mTOR is involved in many pathophysiological conditions, such as aging, Alzheimer's disease, diabetes, obesity, and cancer [2]. As a natural inhibitor of mTORC1, rapamycin is able to increase lifespan in mice [6, 7]. mTOR activity is frequently de-regulated in a variety of human cancers, such as breast, prostate, lung, liver, and renal carcinomas. Upregulation of mTOR signaling can promote tumor growth and progression through diverse mechanisms including the promotion of growth factor receptor signaling, angiogenesis, glyolytic metabolism, lipid metabolism, cancer cell migration, and suppression of autophagy [1, 2]. Hence, mTOR is a promising target for cancer therapy. In this review, we discuss the roles of mTOR in human cancer and the rationales and challenges for developing mTOR inhibitors to treat cancer.

* Correspondence: jyangfu@scu.edu.cn
[2]Laboratory of Oncogene, Cancer Center, West China Hospital, Sichuan University, Chengdu, China
Full list of author information is available at the end of the article

The assembly of mTOR complexes

The studies of mTORC1 structure demonstrate that mTORC1 adopts a dimeric architecture with an overall size of (280~300) × (200~210) × (100~130) $Å^3$ [8, 9]. mTOR and LST8 form the core of mTOR complex that contains raptor and other regulatory proteins [8]. The human mTOR contains 2549 amino acids that form several domains including the NH_2-terminal HEAT (N-HEAT), middle HEAT (M-HEAT), FAT, and kinase domain with a FRB insertion (Fig. 1). Raptor also contains a HEAT domain, as well as WD40 and caspase-like domain [8, 9]. Besides, LST8 has WD40 domain. The HEAT motifs have conserved Asp and Arg residues at positions 19 and 25, respectively. A signature motif of WD40 repeats is ~ 40 amino acids often ending with a tryptophan-aspartic acid (W-D) dipeptide [10]. The HEAT repeats 12–13 in one mTOR interact with the HEAT repeats 20–23 in the M-HEAT domain of another mTOR, thereby forming a dimer [8]. Raptor may stabilize the dimer by binding the HEAT repeats 11–13 in one mTOR and repeats 20–22 in another mTOR [8, 11]. In addition, raptor is required for recruiting substrates to mTORC1 [12, 13]. Both mTOR and raptor are subjected to phosphorylation at multiple residues (Fig. 1a), which positively or negatively regulates mTORC1 activity.

The assembly of mTORC2 and *Saccharomyces cerevisiae* TORC2 follows a similar principle to mTORC1. The human mTORC2 structure reveals a hollow rhombohedral fold with overall dimensions of ~ 220 × 200 × 130 ($Å^3$) [14]. A dimer of mTOR is located in the core of this complex, while each mTOR or TOR heterodimerizes with rictor and mSIN1 [14, 15]. Rictor has an NH_2-terminal armadillo (ARM) repeat cluster (~ 900 residues), and the rest of the rictor is largely unstructured (Fig. 1b) [16]. Interestingly, ARM and HEAT domains have similar conserved residues that form the hydrophobic domain core and may have a common phylogenetic origin [17]. In addition, mSin1 has a CRIM, a Ras-binding domain

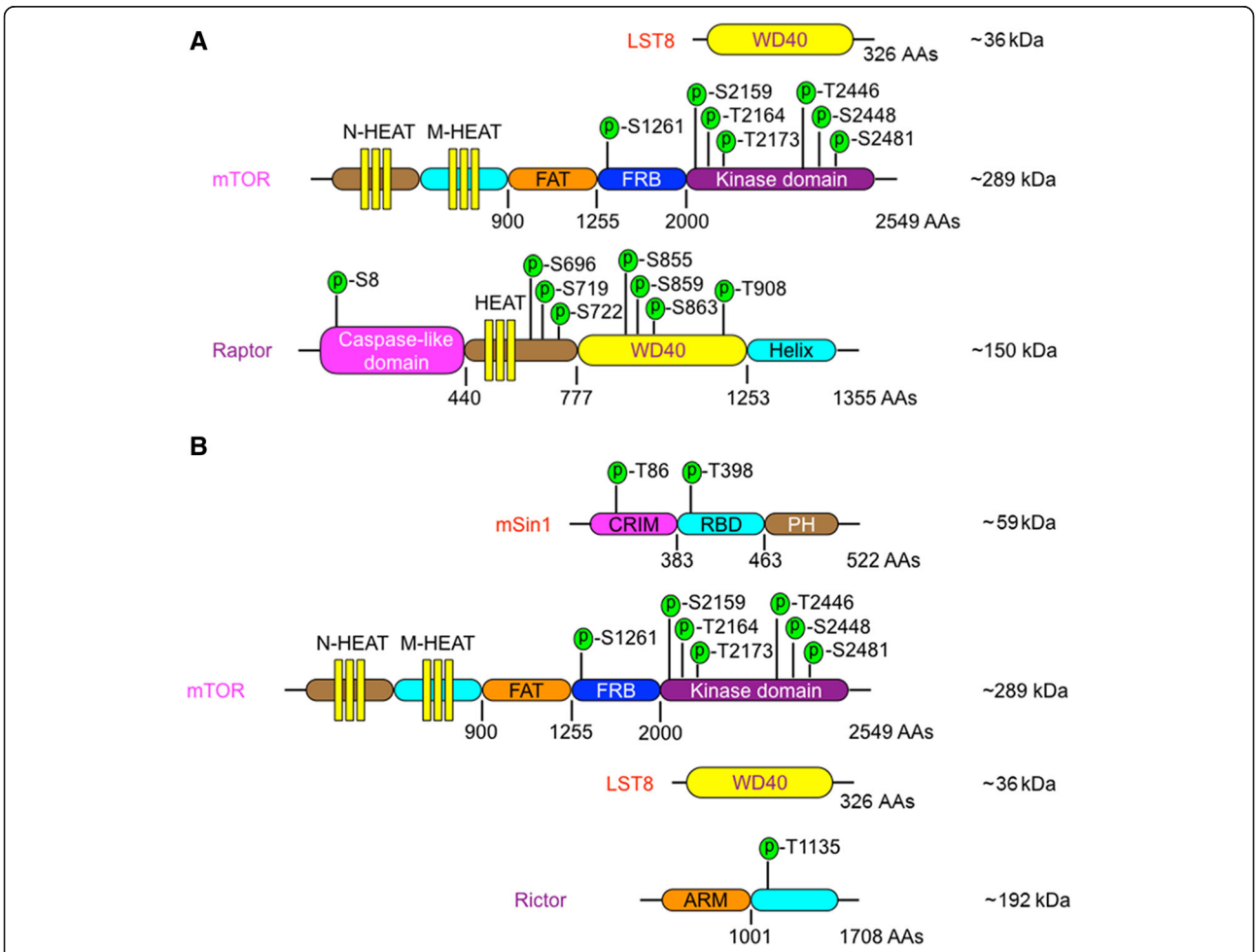


Fig. 1 The domains in key components of mTORC1 and mTORC2. **a** The molecular weight, domains, and phosphorylation sites in key components of mTORC1, including mTOR, LST8, and raptor. **b** The molecular weight, domains, and phosphorylation sites in key components of mTORC2, including mTOR, mSin1, and rictor

(RBD), and a pleckstrin homology (PH) domain [18]. During the assembly of mTORC2, the FRB domain of mTOR binds to mSin1 and the carboxy terminal region of rictor, while the NH_2-terminal portion (residues 506–516) of rictor interacts with the COOH-terminal region (residues 1186-1218) of M-HEAT of mTOR [14]. In addition, mSin1 directly binds to rictor. Both rictor and mSin1 are responsible for recruiting substrates to mTORC2. Of note, both rictor and mSin1 have mTOR-independent partners. For example, rictor interacts with integrin-linked kinase and promotes its phosphorylation of Akt [19], while mSin1 interacts with Ras and inhibits ERK1/2 phosphorylation [20]. Thus, the outcome from the manipulation of rictor or mSin1 alone may not exactly reflect the function of mTORC2.

Regulation of mTORC1 activity

The activity of mTORC1 is regulated by growth factors, cellular energy, stresses and nucleotides, etc. The lysosomes are primary sites for mTORC1 activation. The activation of mTORC1 by growth factors is dependent on Ras homolog enriched in the brain (RHEB), a lysosomal GTPase that directly interacts with mTOR and activates it [21]. Upon binding to growth factors such as epidermal growth factor (EGF) and insulin-like growth factor (IGF), the growth factor receptors (EGFR, IGFR, etc.) are activated, which in turn activate PI3K-PDK1-Akt signaling pathway. Active Akt phosphorylates tuberous sclerosis complex 2 (TSC2) and inhibits the TSC complex, a GTPase-activating protein (GAP) complex consisting of TSC1/2 and TRE2-BUB2-CDC16 domain family member 7 (TBC1D7) [22, 23]. The TSC complex can inactivate RHEB thereby inhibiting mTOR [24]. Therefore, the activation of Akt leads to the depression of RHEB and then activates mTORC1. Moreover, the ubiquitination of RHEB regulates its ability to activate mTORC1 [21]. The E3 ubiquitin ligase RNF152 catalyzes RHEB ubiquitination, leading to an increase in the interaction between RHEB and TSC [21]. In contrast, Akt can phosphorylate the deubiquitinase USP4 that promotes RHEB deubiquitination thereby releasing RHEB from TSC [21].

Downstream of the growth factor receptors, the mitogen-activated protein kinase (MAPK) also upregulates mTORC1 activity. Mechanistically, MEK1/2 promotes raptor phosphorylation through ERK1/2 and p90 ribosomal S6 kinase (RSK1/2). ERK1/2 directly phosphorylates raptor at S8, S696, and S863, while RSK1/2 phosphorylates raptor at S719/722 [25, 26]. Meanwhile, the intestinal cell kinase (ICK), a MAPK-related kinase, phosphorylates raptor at T908 [27]. Phosphorylation of raptor by ERK/RSK/ICK promotes the activation of mTORC1.

mTORC1 not only senses growth factors, but also responds to cellular energy. Low cellular energy results in an increase in AMP/ATP ratio, which activates the energy sensor AMP-dependent kinase (AMPK). AMPK stimulates the GAP activity of TSC and then promotes the inhibition of RHEB by TSC, leading to the downregulation of mTORC1 [28]. In addition, the TCA cycle metabolite ketoglutarate inhibits mTORC1 through repressing ATP synthase, increasing AMP/ATP ratio and activating AMPK [29]. Cellular energy deficiency usually leads to endoplasmic reticulum stress, which in turn induces the unfolded protein response (UPR). Ire1, ATF6, and PERK are three major mediators of the UPR. Upon ER stress, ATF6 can induce RHEB expression, which in turn promotes mTORC1 activation and cell survival [30]. However, overactivated mTORC1 is also harmful to cell survival under ER stress. Mutations in TSC1/2 or activation of RHEB renders cells hypersensitive to ER stress-induced apoptosis, which may be due to the downregulation of ATF4/6 by mTOR [31]. Therefore, mTORC1 may have versatile effects on cell survival under ER stress.

While the regulation of mTORC1 by growth factors is dependent on RHEB and the TSC complex, amino acids can stimulate mTORC1 independent of TSC. The regulation of mTORC1 by amino acids is very complicated, involving multiple amino acid sensors and protein machinery [32]. The lysosomal Ragulator (RAG) guanosine triphosphatases (GTPases) play key roles in the activation of mTORC1 by amino acids. RAGA or RAGB heterodimerizes with RAGC or RAGD [33]. Further, RAG proteins form a large complex with LAMTOR1/2/3/4/5, which recruit RAG and mTORC1 to the lysosomal surface [34]. The activity of RAG is regulated by two complexes, GATOR1 and GATOR2. GATOR1, which is composed of DEPDC5, NPRL2, and NPRL3, inhibits the GTPase-activated protein (GAP) activity of RAGA/B thereby repressing the activation of mTORC1 by amino acids [35]. Instead, GATOR2, a protein complex consisting of MIOS, WDR24, WDR59 SEH1L, and SECB, negatively regulates GATOR1 by inducing DEPDC5 degradation [35]. Furthermore, KICSTOR, a large complex consisting of KPTN, ITFG2, C12ORF66, and seizure threshold 2 (SZT2), recruits GATOR1 to the lysosomal surface and mediates the interaction between GATOR1 and RAG [36, 37].

Sestrin (SESN) is another category of negative inhibitors of amino acid-induced mTORC1 activation. Mechanistically, SESNs interact with GATOR2, leading to the release of GATOR1 from GATOR2. The released GATOR1 in turn inhibits RAG and mTORC1 [38–40]. Of note, SESN2 is known as a leucine sensor in mTORC1 signaling. Leucine directly binds to SESN2, leading to the dissociation of SESN2 from GATOR2. The released GATOR2 binds to GATOR1 and then prevents the inhibition of RAG by GATOR1. These sequential processes result in RAG-mediated mTORC1 activation [41].

To prevent the overactivation of mTORC1 by amino acids, there are negative feedback pathways to RAG-mediated mTORC1 activation. Two E3 ubiquitin ligases, RNF152 and SKP2, reportedly induce RAGA ubiquitination and potentiate the binding of RAGA to GATOR1 [42, 43]. While leucine sufficiency is sensed by SESN2, the stimulation of mTORC1 by arginine is mediated by SLC38A9 [44]. Moreover, the ubiquitin ligase TRAF6 can catalyze K63 ubiquitination of both Akt and mTOR thereby promoting the activation of Akt and mTORC1 by amino acids [45, 46].

In addition, mTOR may be activated by lipid and cholesterol. Fatty acid metabolism leads to the de novo synthesis of phosphatidic acid (PA), which stabilizes both mTORC1 and mTORC2 [47]. Moreover, cholesterol can stimulate mTORC1 activation and growth signaling. Mechanistically, SLC38A9 acts as a lysosomal cholesterol sensor to stimulate the activation of mTORC1 by RAG complex [48]. Recently, it was reported that mTORC1 is also responsive to the levels of purine nucleotides [49]. While adenylate stimulates mTORC1 by inhibiting TSC, guanylate downregulates RHEB and then inhibits mTORC1 [49]. The mechanisms underlying the regulation of TSC and RHEB by adenylate and guanylate remain to be known.

Regulation of mTORC2 activity

Although mTORC1 and mTORC2 are distinct complexes, there is a crosstalk between these two complexes. On one hand, mTORC2 can activate IGF-IR-Akt axis thereby upregulating mTORC1 [1]. On the other hand, mTORC1 feeds back to inhibit mTORC2 via S6K1, one of the substrates of mTORC1. Once activated by mTORC1, S6K1 phosphorylates rictor and mSin1 on T1135 and T86/398, respectively, leading to the impairment of mTORC2 integrity [50–52].

While mTORC2 directly activates IGF-IR and InsR, receptor tyrosine kinases such as EGFR, PDGFR, and IGF-IR can activate mTORC2 via PI3K. Mechanistically, PI3K-induced PtdIns (3,4,5) P3 (PIP3) binds to the PH domain of mSin1 and then disables the inhibition of mTOR kinase domain by mSin1, thereby activating mTORC2 [18]. In addition, PI3K promotes the association of mTORC2 with ribosome, where mTORC2 is activated [53]. Therefore, mTORC2 also responds to growth factors. Notably, another study suggests that mTORC2 activity is localized in the plasma membrane, mitochondria, and endosomal vesicles, and the activity of mTORC2 via the mSin1-PH domain at the plasma membrane is PI3K- and growth factor-independent [54]. In addition, IKKα interacts with mTORC2 and enhances its kinase activity towards Akt [55]. These data suggest that the activation of mTORC2 involves multiple location and different mechanisms.

How does mTORC2 respond to cellular energy and nutrients? The energy sensor AMPK inhibits mTORC1 and then releases the suppression of mTORC2 by mTORC1, leading to the activation of mTORC2 [56]. Thus, upregulation of mTORC2 may help cells adapt to low levels of cellular energy. Moreover, mTORC2 is activated by glutamine starvation. Activated mTORC2 upregulates the expression and phosphorylation of glutamine:fructose-6-phosphate amidotransferase 1 (GFAT1), the rate-limiting enzyme of the hexosamine biosynthesis pathway (HBP) [57, 58]. A study of budding yeast demonstrates that the LKB1-ELM1-GIN4/HSL1 axis is required for coordinating TORC2 signaling to the changes in carbon source [59]. It remains to know if similar pathway works in human cancer cells.

Similar to mTORC1, mTORC2 is also stabilized by phosphatidic acid (PA), a central metabolite in the synthesis of membrane phospholipids [60]. The generation of PA is catalyzed by the phospholipase D, diacylglycerol kinases, and lysophosphatidic acid acyltransferases. Moreover, the activity of mTORC1 and mTORC2 is regulated by mLST8 ubiquitination. It has been reported that the E3 ubiquitin ligase TRAF2 positively regulates K63-linked polyubiquitination of mLST8, which impairs its interaction with mSin1 and compromises the mTORC2 integrity, but enhances the assembly of mTORC1 [61]. On the contrary, the deubiquitinase OTUDB7 removes polyubiquitin chains from $G_{\beta}L$ to promote $G_{\beta}L$ interaction with mSin1 and the integrity of mTORC2 [61]. Besides, the exchange factor found in platelets, leukemic, and neuronal tissues (XPLN) interacts with mTORC2 and negatively regulates mTORC2 activity [62]. Lastly, mTOR is a target of proteasomal degradation when it is ubiquitinated by FBXW7 [63].

Targets of mTORC1 and mTORC2

As a protein kinase, mTOR catalyzes the phosphorylation of its targets and regulates their activity. mTORC1 and mTORC2 have different substrates. While the repertoire of mTOR substrates keeps increasing, there are more targets remaining to be identified. S6K1 and 4E-BP1 are two well-known mTORC1 targets. mTORC1 phosphorylates S6K1 at T389 and 4E-BP1 at multiple residues [64]. Phosphorylation of S6K1 by mTORC1 leads to increased protein and nucleotide synthesis. While 4E-BP1 is a negative regulator of 5′cap-dependent mRNA translation, phosphorylation of 4E-BP1 by mTORC1 induces its dissociation from eIF4E, thereby relieving its inhibition of protein synthesis [65]. To cope with increased protein synthesis, mTORC1 also promote ribosome biogenesis by inducing ribosomal RNA transcription. Mechanistically, mTORC1 may translocate to the nucleus, where it binds to ribosomal DNA promoter [66–68]. Nuclear mTOR also phosphorylates TFIIIC and Maf1, thereby promoting

tRNA gene transcription [69]. In fact, nuclear mTOR regulates RNA polymerase 1/2/3-driven transcription. In addition, mTORC1 phosphorylates the E3 ubiquitin ligase SKP2 at S64 and then inhibits SKP2 ubiquitination and degradation [70]. Given that SKP2 promotes the degradation of many proteins, mTORC1 may regulate the turnover of SKP2 substrates indirectly. Thus, mTORC1 not only promotes protein synthesis, but also regulates protein degradation.

Following the identification of mTORC2, it was found that protein kinase C (PKC) α/β were the substrates of mTORC2 that regulates the actin cytoskeleton [4, 71]. Moreover, mTORC2 phosphorylates and activates other AGC kinases, such as serum and glucocorticoid-induced kinase (SGK) and Akt. mTORC2 phosphorylates Akt at S473, leading to allosteric activation of Akt in cooperation with the catalytic activation by PDK1, which phosphorylates Akt at T308 [72]. During the synthesis of nascent proteins, mTORC2 can co-translationally phosphorylate some polypeptides while they are attached to the ribosome. IGF2 mRNA-binding protein (IMP) is responsible for the splicing and translation of IGF2 mRNA. mTORC2 co-translationally phosphorylates IMP1 at S181 and then promotes IMP1 binding to the untranslated region of IGF2 mRNA and enables translational initiation by internal ribosomal entry [73]. mTORC2 not only enhances the production of IGF2 protein, but also phosphorylates and activates IGF-IR and insulin receptor [1]. In contrast to mTORC1's activity as a ser/thr kinase, mTORC2 has tyrosine kinase activity towards IGF-IR/InsR [1].

mTOR inhibitors for cancer therapy

The activity of mTOR is frequently upregulated in human cancer. The aberrant activation of mTOR in human cancer may be attributed to mTOR pathway-activating mutations, amplification, or overexpression of the components of mTOR complexes and mutations or loss of negative regulators of mTOR. PIK3CA mutations are frequently detected in human cancer. Activation of PI3K promotes both mTORC1 and mTORC2 activation. In addition, mutations in KRAS and BRAF may lead to mTORC1 activation. Especially, KRAS can directly bind to PIK3CA (p110α) and activates PI3K pathway, leading to mTOR activation [74]. mTOR-activating mutations are observed in kidney cancer. While mTOR activity is usually upregulated by growth factors and amino acids, activating mutations in mTOR may result in RAG- and RHEB-independent mTOR hyperactivation, thus loss of the dependency on growth factors and amino acids [75]. Point mutations in RHEB and GATOR1 were also detected in renal cancer and endometrial cancer [76]. RHEB1 is overexpressed in acute myeloid leukemia (AML) and promotes AML progression [77]. Whereas mTOR amplification is rare in human cancer, rictor amplification is detected in various kinds of cancer, such as breast cancer, gastric cancer, and liver cancer [78, 79]. Moreover, rictor is overexpressed in human cancers of the brain, breast, lung, gastric, colon, liver, and tongue [80, 81].

Given that mTOR has critical roles in tumor progression, mTOR inhibitors hold promise in cancer therapy. Indeed, rapamycin analogs (rapalog) have been approved for treating cancer in the clinic. In addition, many mTOR inhibitors with different mechanisms of action have been developed, some of which are undergoing clinical trials in variety types of human cancer.

Rapalog

Rapamycin was originally identified as an antifungal, immunosuppressive, and antiproliferative agent. Later studies revealed that rapamycin binds to the 12 kDa FK506-binding protein (FKBP12) and then inhibits mTORC1 [82]. Since rapamycin has poor solubility and pharmacokinetics, it is not suitable for treating human cancer. So far, several water-soluble rapamycin analogs have been developed. For example, temsirolimus and everolimus exhibit tumor-suppressive effects in vivo. Both temsirolimus and everolimus have been used to treat advanced renal cell carcinoma (RCC) in the clinic. Moreover, everolimus is prescribed for treating pancreatic neuroendocrine tumors and advanced breast cancer [83]. Besides, there are many clinical trials to evaluate the efficacy of rapalogs in treating other types of human cancer, such as advanced gastric cancer, hepatocellular carcinoma, non-small cell lung cancer, endometrial cancer, and mantle cell lymphoma (clinicaltrials.gov).

Of particular note, the effect of rapalog monotherapy on solid tumors is modest in the clinic. The incomplete inhibition of mTOR by rapalogs may result in limited clinical success. On the other hand, inhibition of mTORC1 may lead to feedback activation of IGF-IR and Akt, which compromises the anti-cancer effect of rapalogs [1]. Taking into account the complexity of mTOR signaling networks, it is not hard to understand that the response to rapalogs varies in patients with cancer, such as metastatic RCC. It is desirable that there are biomarkers to predict the responses to mTOR inhibition. KRAS, BRAF, and TSC mutations are known as resistant markers for mTOR inhibitors, whereas PIK3CA mutations are sensitive marker [84, 85]. However, the roles of TSC1/2 and mTOR mutations in responding to rapalogs remain controversial. Although it has been reported that mutations in TSC1/2 and mTOR are more frequent in RCC patients who respond well to rapalogs, the majority of rapalog responders have no mutations in mTOR pathway, suggesting that other factors are also involved in rapalog sensitivity [86]. Notably, rapalogs usually arrest cell proliferation but does not induce

apoptosis. Despite the initial response, tumors frequently develop resistance to these agents.

ATP-competitive mTOR inhibitors

To more completely inhibit mTOR, a number of ATP-competitive mTOR inhibitors have been developed to target both mTORC1 and mTORC2. Tumors that are addicted to the mTOR signaling pathway may be sensitive to this kind of inhibitors. Unlike rapalogs, ATP-competitive mTOR inhibitors can not only arrest cell growth, but also induce apoptosis. MLN0128 (also called INK128, sapanisertib, TAK-228) is a pan-mTOR inhibitor that has potent in vitro and in vivo anti-tumor effects, and has underwent clinical trials for solid tumors such as bone and soft tissue sarcoma, breast cancer, and primary effusion lymphoma, a non-Hodgkin B cell lymphoma that usually results from infection of Kaposi sarcoma-associated herpesvirus [87–90]. MLN0128 also reduces tumor growth in CD44-high HCC xenografts and resensitizes HCC to sorafenib [91]. Of note, MLN0128 is an effective agent even in tumors that are resistant to rapamycin or chemotherapy. A recent study demonstrates that MLN0128 can overcome resistance to everolimus and reduce tumor size by 20% in *PIK3CA*-mutant colorectal cancers [92]. In addition, MLN0128 can induce tumor shrinkage in patient-derived xenograft model of pancreatic neuroendocrine tumors, even in everolimus-resistant tumors [93].

PP242 (Tokinib) is another selective ATP-competitive inhibitor of mTOR that has a promising anti-cancer activity over several cancer types, such as leukemia, gastric cancer, and colon cancer [94, 95]. Given that the Akt-mTOR signaling pathway is upregulated in platinum-resistant cancer cells, studies demonstrate that mTORC1/2 inhibitor, such as PP242 and MLN0128, can re-sensitize platinum-resistant ovarian cancer cells to carboplatin in vitro and in vivo [96, 97]. Mechanistically, mTOR inhibition leads to a sharp decrease in the translation of DNA damage and repair response and pro-survival mRNAs, including CHK1 [98]. Consistent with the inhibition of DNA repair, mTOR inhibitors are also effective in enhancing radiosensitivity or restoring radiosensitivity in radioresistant tumors [99, 100]. Moreover, inhibition of mTORC1/C2 signaling improves anti-leukemia efficacy of JAK/STAT blockade in CRLF2-rearranged and/or JAK-driven Philadelphia chromosome-like acute B cell lymphoblastic leukemia [101].

Both AZD2014 (vistusertib) and its analog AZD8055, two ATP-competitive mTORC1/2 inhibitors, are highly effective in treating estrogen receptor (ER)-positive breast cancer. Moreover, AZD2014 and AZD8055 can suppress breast cancer with acquired resistance to endocrine therapy, rapalogs, and paclitaxel [102, 103]. In addition, a combination of AZD2014 with paclitaxel reduces tumor volume in cisplatin-resistant ovarian cancer model [104]. Similar to PP242, AZD2014 enhances the radiosensitivity of glioblastoma stem-like cells [105]. Based on the above-described studies, it appears that the pan-mTORC1/2 inhibitors generally reverse rapalog resistance, endocrine resistance, chemoresistance, and radioresistance.

Dual PI3K/mTOR inhibitors

Although inhibition of mTORC1 and mTORC2 can downregulate Akt S473 phosphorylation, mTOR inhibitors may paradoxically enhance the PI3K/PDK1 axis. Thus, an inhibitor targeting both PI3K and mTOR may have better anti-cancer activity compared to targeting mTOR alone [106, 107]. Due to the similarity between PI3K and mTOR, some chemicals can inhibit both PI3K and mTOR. NVP-BEZ235 (dactolisib) inhibits the activity of multiple class I PI3K isoforms, mTOR and ataxia telangiectasia, and Rad3-related protein (ATR) and has potent anti-cancer activity [108]. Notably, NVP-BEZ235 can penetrate the blood-brain barrier after systemic administration [109]. Therefore, it can be used to treat glioma and reverse temozolomide resistance [110]. In addition, NVP-BEZ235 can suppress paclitaxel-resistant gastric cancer, which exhibits increased PI3K/mTOR activity [111].

LY3023414, a complex fused imidazoquinolinone, is an oral PI3K/mTOR and DNA-PK inhibitor that has anti-tumor effects in animal models. Combination of LY3023414 with standard chemotherapeutic drugs has additive anti-tumor activity [112, 113]. Another dual PI3K/mTOR inhibitor voxtalisib (SAR245409, XL765), a pyridopyrimidinone derivative, significantly inhibits tumor growth in multiple human xenograft models [114]. Combination of voxtalisib and the MEK inhibitor pimasertib synergistically inhibits certain endometrial cancer cells growth [115]. Other dual PI3K/mTOR inhibitors include PQR309, XH00230381967, SN20229799306, GSK2126458 (omipalisib), and PKI-587.

Of note, PQR309 is a 4,6-dimorpholino-1,3,5-triazine-based, brain-penetrant, and orally bioavailable PI3K/mTOR inhibitor [116]. PQR309 effectively inhibits lymphoma in monotherapy and in combination therapy with other drugs, such as the BCL2 inhibitor venetoclax, the HDAC inhibitor panobinostat, the Bruton's tyrosine kinase inhibitor ibrutinib, lenalidomide, the BET proteolysis-targeting chimera ARV-825, the proteasome inhibitor marizomib, and the anti-CD20 monoclonal antibody rituximab [117]. Moreover, PQR309 can suppress cancer cells with primary or secondary resistance to the PI3Kδ. PQR620 and the PI3K/mTORC1/2 inhibitor PQR530 effectively cross the blood-brain barrier [118].

The dual specificity PI3K/mTOR inhibitor gedatolisib (PKI-587, PF05212384) is a bis(morpholino-1,3,5-triazine) derivative [119]. Gedatolisib inhibits tumor growth

in breast, colon, lung, and glioma xenograft models and displays efficacy against T cell acute lymphoblastic leukemia (T-ALL) and Philadelphia chromosome (Ph)-like B cell acute lymphoblastic leukemia (Ph-like ALL) [107, 120]. Combination of gedatolisib with ruxolitinib or dasatinib has superior efficacy than a single agent in CRLF2/JAK-mutant models and ABL/PDGFR-mutant models, respectively [120]. In addition, gedatolisib sensitizes head, neck, and nasophageal carcinoma to radiation therapy [121, 122] and sensitizes EGFR-resistant head and neck carcinoma to cetuximab [123]. Thus, gedatolisib may be a candidate sensitizer to radiotherapy and targeted therapy.

GSK2126458 (omipalisib) is an orally bioavailable inhibitor of PI3Kα and mTOR [124]. Omipalisib potently inhibits FGFR4-V550E tumor-derived cell and human rhabdomyosarcoma cell viability and reduces the growth of rhabdomyosarcoma in vivo [125]. In addition, a combination of the PI3K/mTOR inhibitor VS-5584 and the Wnt inhibitor ICG-001 synergistically inhibits AML with high PRL-3 expression [126]. Finally, the efficacy of mTOR inhibitor may be enhanced by linking the kinase inhibitor to rapamycin (RapaLink) [127]. EZH2 (Y641X)-mutant lymphomas show increased sensitivity to RapaLink-1 [128]. Given that RapaLink integrates the activity of both rapamycin and mTOR kinase inhibitor, it is worthwhile looking forward to the efficacy in clinical trials. Lastly, there are many drugs that may indirectly inhibit mTOR, such as aspirin and metformin [129–131].

Principle mechanisms of mTOR inhibitor resistance in cancer

Drug resistance is a serious problem in treating cancer. Although there may be an initial response, long-lasting treatment with chemotherapeutic or molecular-targeted drugs often faces the challenge of drug resistance. Due to the tumor heterogeneity, some tumors do not respond to a given drug at all. Clonal selection, adaptive evolution, and resistance to cell death are general mechanisms for drug resistance. Due to the complexity and crosstalk in signaling networks, cancer cells may adapt to an inhibitor that targets a given signaling pathway via the compensatory activation of other pathways. Although mTOR inhibitors exhibit potent anti-cancer effects in many preclinical models, resistance does occur. As described below, there are multiple mechanisms underlying the resistance to mTOR inhibitors (Fig. 2).

Drug efflux by ATP binding cassette transporters

ATP-binding cassette (ABC) transporters constitute drug efflux pumps that decrease the intracellular levels of drugs, leading to poor treatment outcome. Overexpression of ABC transporters is a general mechanism for multi-drug resistance in cancer. The same may be true for mTOR inhibitor resistance. In fact, the mTOR inhibitors rapamycin and NVP-BEZ235 are substrates of ABCB1 (P-glycoprotein) and ABCG2 (also called breast cancer resistance protein, BCRP), respectively [132]. In addition, AZD8055 is transported by both ABCB1 and ABCG2 [132].

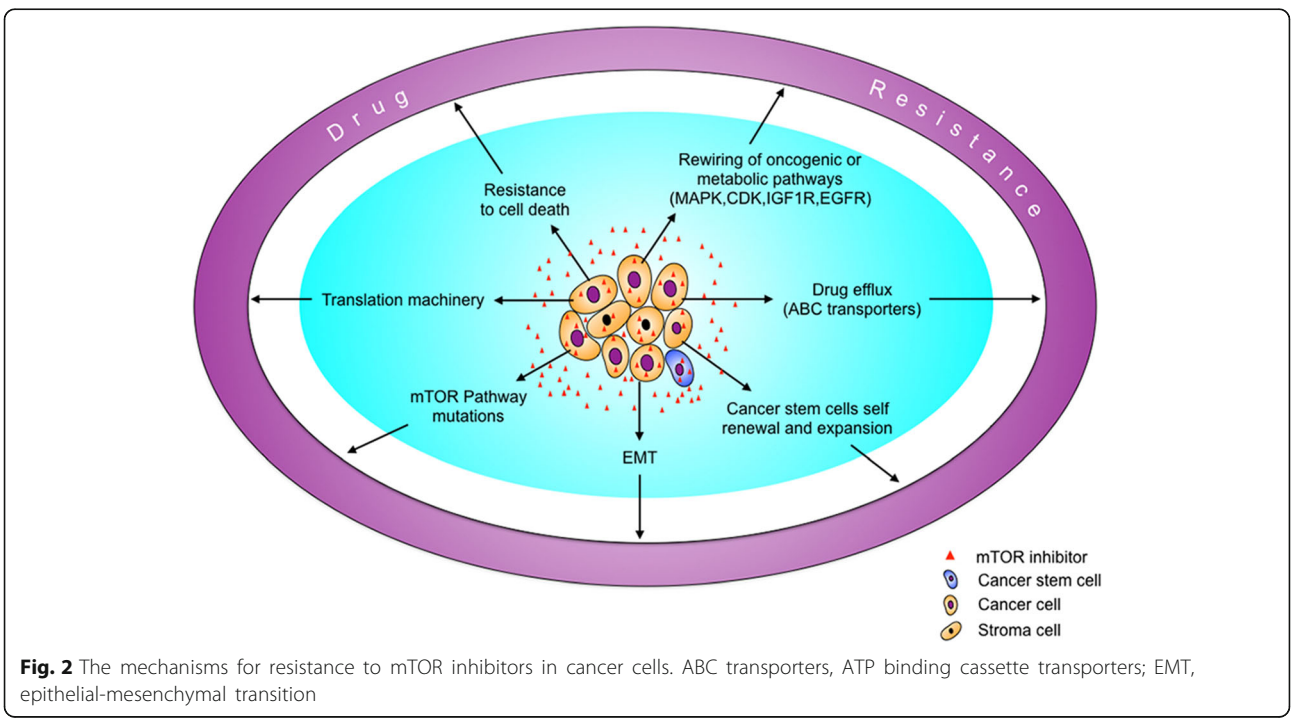


Fig. 2 The mechanisms for resistance to mTOR inhibitors in cancer cells. ABC transporters, ATP binding cassette transporters; EMT, epithelial-mesenchymal transition

Studies show that ABCB1 is overexpressed in luminal breast cancer cell lines that are resistant to everolimus [133]. Also, ABCB1 inhibits brain accumulation of everolimus [134]. Overexpression of ABCG2 in cancer cells confers significant resistance to PF-4989216, which can be reversed by an inhibitor or competitive substrate of ABCG2 [135]. Moreover, GDC-0980 is subject to active efflux by ABCB1 and BCRP, which limits its efficacy [136]. The affinity for ABC transporters may vary among different mTOR inhibitors. Lowering the affinity for ABC transporters or inhibiting ABC transporters may enhance the efficacy of mTOR inhibitors.

Cancer stem cells

Cancer stem cells (CSCs) are a subpopulation in tumor mass that is extremely resistant to standard cancer therapy. Slow-cycling CSC is one of the major obstacles to eradicate tumor [137]. It is generally thought that the mTOR pathway is hyperactivated in CSC. Transforming growth factor-β (TGF-β) can induce epithelial-mesenchymal transition (EMT), which enhances cancer stem cell generation. mTOR is one of the mediators in TGF-β signaling pathways that enhances cancer stemness and drug resistance [138]. The inhibitory effect on CSCs has already been shown for some mTOR inhibitors [139]. Rapamycin, everolimus, and PF-04691502 suppress tamoxifen-induced activation of breast cancer stem cells [140]. Inhibition of mTOR restores tamoxifen resistance in breast cancer cells [141]. Moreover, the ATP-competitive mTOR inhibitor Torin1 and PI3K/mTOR inhibitor VS-5584 preferentially reduce CSC levels in multiple mouse xenograft models of human cancer [142, 143].

However, the interplay between mTOR inhibitors and CSC is complex. Previous studies show that expansion of CSC promotes the resistance to mTOR inhibitor in leiomyosarcoma [144]. PDK1 signaling toward PLK1-MYC activation leads to tumor-initiating cell activation and resistance to mTOR inhibition [145]. Inhibition of EZH2, a catalytic component of polycomb repressive complex which plays a critical role in stem cell maintenance, restores sensitivity to PI3K/mTOR pathway inhibition. It appears that the sensitivity to mTOR inhibitors in CSC may be context- or cell type-dependent. Of note, one study demonstrates that TP53 mutation and BCL2 phosphorylation affect the sensitivity of glioblastoma stem-like cells to mTOR inhibitor [146]. BCL2 (T56/S70) phosphorylation in TP53 wild-type glioblastoma stem-like cells is responsible for the lower sensitivity to the mTORC1/2 inhibitor AZD8055, as - compared to TP53-mutated glioblastoma stem-like cells [146]. In addition, while mTOR inhibitors reportedly suppress CSC, one study demonstrates that treatment of TNBC cell lines with PI3K/mTOR inhibitor or TORC1/2 inhibitor expands CSC population through upregulating FGF1-FGFR-Notch1 axis [147]. Blocking FGFR or Notch1 may prevent resistance to TORC1/2 inhibitors by abrogating the expansion of drug-resistant CSCs in TNBC [49]. Moreover, another dual PI3K/mTOR inhibitor PF-04691502 can induce a stem cell-like gene expression signature in KRAS-mutant colorectal cancer models [148]. Together, these data suggest that the effects of mTOR inhibitors on CSC may be dependent on the genetic background and rewiring of cancer stemness pathways.

Assembly of the translation machinery

Eukaryotic protein synthesis is regulated by several mechanisms including cap-dependent and cap-independent translation. The cap-dependent pathway involves many eukaryotic initiation factors (eIF), such as eIF1, eIF2, eIF3, eIF4A, eIF4B, eIF4E, eIF4H, eIF5, and eIF6. The protein synthesis is initiated by the association of the 40S ribosome subunit with eIF1A and eIF3, followed by binding of the eIF2-GTP-methionine tRNA complex to 40S subunit and then forming a 43S subunit [149]. The eIF4F complex, which consists of eIF4E, eIF4A, and eIF4G, binds to the m^7G cap at the 5′ end of mRNA and then activates mRNA. The activated mRNA is recruited to the 43S complex and then subjected to ATP-dependent scanning of mRNA to locate the initiating AUG code [150]. Finally, the 60S ribosome subunit is associated with the 40S subunit to form the 80S initiation complex, possibly assisted by eIF5. For the initiation of cap-independent protein synthesis, the 40S ribosome subunit binds to an internal region of mRNA, which is referred to as internal ribosome entry sites (IRES), or the untranslated regions of mRNA.

Given that stimulation of cap-dependent translation is one of the major functions of mTORC1, the status of the translation machinery and modes of protein translation may impact on the efficacy of mTOR inhibitors. 4E-BPs are phosphorylated and inactivated by mTORC1. The sensitivity to PP242 is correlated with the extent to which 4E-BP1 phosphorylation is inhibited by this drug [151]. Loss of 4E-BPs in tumor cells results in the resistance to mTOR inhibition. The transcription factor Snail directly represses 4E-BP1 transcription and compromises the anticancer effects of mTOR inhibitors [152]. Of note, Snail is translationally regulated by eIF4E, which is exactly the target of 4E-BP. Phosphorylation of eIF4E (S209, etc.) promotes Snail synthesis [153]. Therefore, 4E-BP and eIF-4E can disable each other. Overexpression of eIF4E or phosphorylation of eIF4E (S209) by MAP kinase-interacting kinase 1 (Mnk1/2) leads to a shift from cap-dependent to cap-independent translation and then renders cancer cells insensitive to mTOR inhibition [154, 155]. Thus, inhibition of Mnk1/2 or its upstream kinase ERK1/2 may restore cap-dependent translation and the sensitivity of mTOR inhibitors [155]. On the other hand, inhibition of mTORC1 may

lead to paradoxical phosphorylation of eIF4E in PI3K- and Mnk-dependent manner and promote cap-independent translation [156]. Hence, a combination of mTOR and Mnk inhibitors is an effective therapeutic strategy for cancer [157].

Notably, 4E-BP1 is not only phosphorylated by mTORC1, but also phosphorylated and inactivated by other kinases such as CDK1, CDK12, and GSK3 . CDK1 can substitute mTORC1 to phosphorylate 4E-BP1 and activate cap-dependent translation, which is resistant to mTOR inhibition [158]. In addition, CDK12 cooperates with mTORC1 to phosphorylate 4E-BP1 and releases it from mTORC1 target mRNAs thereby promoting their translation [159]. Therefore, combinatorial inhibition of mTOR and CDK1/12 may be synthetically lethal to cancer cells. Furthermore, GSK3β can directly phosphorylate4E-BP1 at the same residues (T37/46) that are phosphorylated by mTOR and CDK1 [160]. Given that mTORC2 positively regulates Akt, the negative regulator of GSK3β, mTOR kinase inhibitor may paradoxically activate GSK3. Hence, combinatorial inhibition of mTOR and GSK3β may synergistically suppress tumorigenesis.

mTOR mutations

Gene mutations may affect the sensitivity of a drug that targets the protein encoded by this gene. More than 30 activating mutations of mTOR have been reported in human cancer, such as L1460P, C1483F, E1799K, F1888L, T1977R, V2006I, V2046A, S2215Y, L2230V, E2388Q, I2500F, R2505P, and D2512H [127, 161]. Cancer cells that harbor a subset of those mutations, including C1483F, E1799K, and S2215Y, are hypersensitive to rapamycin, whereas three mutations (A2034V, F2018L, and S2035F) in the FRB domain of mTOR are associated with rapamycin resistance [162, 163]. While tumor cells with mutations in the kinase domain are still responsive to rapalogs [161], mutations in the kinase domain of mTOR, such as M2327I, S2215Y, L2230V, E2388Q, and V2046A, may be responsible for the resistance to the ATP-competitive inhibitor MLN0128 [127]. It remains to know whether activating mutations in the kinase domain of mTOR are responsible for the resistance to allosteric mTOR kinase inhibitors other than MLN0128. In addition, there are recurrent mutations in other mTOR pathway genes, such as *raptor*, *rictor*, and *RHEB* [163]. RHEB-Y35N mutant gains the function to activate mTORC1 [161]. It warrants further studies to clarify which cancer-associated mutations in raptor, rictor, and RHEB may be associated with mTOR inhibitors resistance.

Rewiring of oncogenic or metabolic pathways

The sensitivity to mTOR inhibitors is regulated by other oncogenic pathways, such as PI3K, MAPK, AURKA, and NF-kB signaling [164, 165]. Both the Ras/MAPK and PI3K/Akt/mTOR pathways are tightly involved in tumorigenesis. While tumors with PIK3CA/PTEN mutations or Akt hyperactivation usually are sensitive to mTOR inhibitors, KRAS/BRAF mutations are predictive biomarkers of mTOR inhibitor resistance [148, 166–169]. In addition, mTOR inhibition may lead to the activation of the MEK-Erk pathway. Combination of RAF/MEK inhibitors and mTOR inhibitors may be a strategy to treat KRAS-mutated cancer [170, 171]. Besides, the activation of Erk in response to mTOR inhibition can be abrogated by the CDK4/6 inhibitor palbociclib [172]. Combination of CDK4/6 and mTOR inhibitors synergistically inhibits tumor growth [172, 173]. Alternatively, combined inhibition of wee1, a protein kinase that regulates the G2 checkpoint in the cell cycle, with mTOR inhibition may selectively treat RAS-mutated cancer [174]. Lastly, treatment with everolimus or AZD8055 increases epidermal growth factor receptor (EGFR) activation in tumor cells, leading to drug resistance [175].

Although PIK3CA-mutated cancer is usually sensitive to mTOR inhibition, activation of GSK3β in response to PI3K/mTOR inhibition may lead to the resistance to PI3K/mTOR inhibitors in PIK3CA-mutated cancer [176]. A recent study demonstrates that lung squamous cell carcinoma adapt to chronic mTOR inhibition through the GSK3α/β signaling pathway, which involves the metabolic reprogramming via increased glutaminolysis [177]. One study also reveals that glutaminase (GLS) and glutamate levels are elevated in glioblastoma after treating with mTOR inhibitor [178]. Treatment with GSK3 inhibitors or the glutaminase inhibitor effectively overcomes the resistance to mTOR inhibition [176–178]. Moreover, the activation of the purine salvage pathway due to increased expression of hypoxanthine phosphoribosyl transferase 1 leads to the resistance to the dual PI3K/mTOR inhibitor gedatolisib [179]. In fact, mTOR is tightly involved in purine metabolism. mTORC1 is not only activated by purine nucleobases or nucleosides [49], but also promotes purine synthesis by ATF4-mediated upregulation of the mitochondrial tetrahydrofolate (mTHF) cycle enzyme methylenetetrahydrofolate dehydrogenase 2 (MTHFD2) [180]. Moreover, mTORC1 promotes de novo pyrimidine biosynthesis by S6K1-mediated phosphorylation of carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase (CAD) [181, 182]. Therefore, the increased expression of hypoxanthine phosphoribosyl transferase 1 may rescue the defect in purine synthesis due to mTOR inhibition and help cancer cells adapt to mTOR inhibition.

Another compensatory response to mTORC1 inhibition is the upregulation of transglutaminase 2, a multifunctional enzyme that is involved in cross-linking polypeptide chains with e-(c-glutamyl)-lysine, apoptosis, signal transduction, cell migration, cell adhesion, and extracellular

matrix remodeling [183–185]. Inhibition of transglutaminase 2 potently sensitizes mTORC1-hyperactive cancer cells to rapamycin in vitro and in vivo [183]. Moreover, mitochondria homeostasis is critical for cell growth and survival. Mitochondrial hyperfusion is an adaptive response to mTOR inhibition. Mechanistically, the translation of mitochondrial fission process 1 (MTFP1) is suppressed by mTOR inhibitors, which eventually results in mitochondrial hyperfusion, a process that antagonizes apoptosis [186].

Clinical testing of mTOR inhibitors

Given that preclinical studies demonstrate the anti-cancer efficacy of mTOR inhibitors alone or in combination with chemotherapy, radiotherapy, and targeted therapy, there are many completed or ongoing clinical trials to test the efficacy of mTOR inhibitors for treating various types of human cancer (Table 1). In general, most of mTOR inhibitors are well tolerated, while there are some common adverse effects including fatigue, rash, mucositis, and metabolic complications. mTOR inhibitors are associated with a significantly increased risk of hyperglycemia, hypertriglyceridemia, and hypercholesterolemia [187]. Other adverse events of everolimus are thrombocytopenia, anemia, nausea, and stomatitis [188]. Ridaforolimus is orally bioavailable and better tolerated in children than the adults [189]. Deforolimus was well tolerated and showed encouraging anti-tumor activity across a broad range of malignancies when administered intravenously, and a dose of 12.5 mg/day is being evaluated in phase II trials [190].

Moreover, MLN0028-treated patients may suffer from anorexia, dyspenea and macunopapular rash [191]. In clinical trials of solid tumors, the PI3K/mTOR inhibitor NVP-BEZ235 (twice daily) is poorly tolerated, which leads to treatment discontinuation in some patients and limits its efficacy in treating cancer [192, 193]. Apitolisib (GDC-0980), another dual pan-PI3K/mTOR inhibitor, also has grade 3–4 adverse effects and is less effective than everolimus [194]. GSK2126458 (GSK458) plus trametinib has poor tolerability, due to skin and gastrointestinal toxicities such as diarrhea [195]. Daily oral administration of PF-04691502 (8 mg/day) has adverse events including fatigue, nausea, vomiting, hyperglycemia, and rash [196]. The occurrence of the above-mentioned adverse effects following treatment with mTOR inhibitors may be due to the critical roles of mTOR in metabolism and immunity.

mTOR inhibitors monotherapy

Everolimus has been approved by the FDA for the treatment of advanced renal cell carcinoma, pancreatic neuroendocrine tumors, and advanced breast cancer [83]. Everolimus significantly improves progression-free survival (PFS) among patients with progressive advanced pancreatic neuroendocrine tumors [197]. As registered in clinicaltrials.gov, there are more than 80 clinical trials for mTOR inhibitor monotherapy in cancer patients. A phase 2 trial of everolimus in patients with recurrent adult low-grade gliomas demonstrates a high degree of disease stability [198]. Moreover, everolimus has a promising effect in patients with heavily pretreated, relapsed, or refractory classical Hodgkin's lymphoma, with an overall response rate (ORR) of 45.6%, a median PFS of 8 months, and a long-term response (≥ 12 months) rate of 12% [188]. Of note, everolimus exhibits clinical activity as the first-line monotherapy in a phase 2 clinical trial in 27 patients with advanced biliary tract cancer [199]. Another phase 2 clinical trial in 35 patients with thyroid cancer demonstrates that everolimus has clinical benefit in patients with advanced differentiated thyroid cancer [200]. Also, single-agent ridaforolimus has anti-tumor activity and acceptable tolerability in advanced endometrial cancer patients [201]. These observations need to be validated in a large scale of randomized clinical trials.

Based on a phase 2 trial in 167 patients, oral administration of the mTOR kinase inhibitor voxtalisib (50 mg, twice daily) exhibits a promising efficacy in patients with follicular lymphoma but limited efficacy in patients with mantle cell lymphoma, diffuse large B cell lymphoma, or chronic lymphocytic leukemia/small lymphocytic lymphoma [202]. Of note, serious adverse events occurred in 58.1% of patients [202]. In contrast, the clinical efficacy of MLN0128 in patients with metastatic castration-resistant prostate cancer is limited, possibly due to the dose reductions secondary to toxicity [191]. Although it is expected that mTOR kinase inhibitor may have superior efficacy than rapalogs, a randomized phase 2 trial in patients with metastatic clear cell renal cancer demonstrated that the PFS and OS of AZD2014 were less than that of everolimus [203]. While the PI3K/mTOR inhibitor NVP-BEZ235 is poorly tolerated in cancer patients, a clinical trial in patients with recurrent endometrial cancer demonstrated that weekly intravenous administration of another P3K/mTOR inhibitor gedatolisib achieved moderate anti-cancer activity with tolerable toxicity [204].

mTOR inhibitors in combination therapy

While mTOR inhibitor monotherapy has efficacy in some type of cancer, preclinical studies demonstrate strong rationales for combinatorial treatment with mTOR inhibitors and other drugs. For example, inhibition of both Akt/mTOR and WNT/β-catenin pathways synergistically suppresses AML [205]. As registered in clinicaltrials.gov, there are many clinical trials to test the efficacy of mTOR inhibitors in combination with other molecular targeted or chemotherapeutic agents. For example, everolimus is combined with one or several chemotherapeutic agents,

Table 1 Clinical evaluation of mTOR inhibitors

mTOR inhibitor	Category	Combination	Cancer type	Phase	Response	PFS (months)	OS (months)	Ref. or trial ID*
Everolimus (RAD001)	Rapalog	None	Thyroid cancer	2	No CR/PR; SD (> 24 weeks) 58%	9 (95% CI 4–14)	18 (95% CI 7–29)	200
Everolimus	Rapalog	Letrozole	Relapsed ER(+) high-grade ovarian cancer	2	CR 0; PR 16%; SD 37%	3.9 (95% CI 2.8–11); 3-month rate, 47%; 6-month rate, 32%	13; 6-month OS rate, 84%	209
Everolimus	Rapalog	Exemestrane	ER(+) locally advanced or metastatic breast cancer	3	CBR 33.4% vs 18% (control; placebo plus exemestrane)	6.93 (95% CI 6.44–8.05) vs 2.83 (95% CI 2.74–4.14) (placebo plus exemestrane)	30.98 (95% CI 27.96–34.56) vs control 26.05 (95% CI 22.57–33.08)	NCT00863655
Everolimus	Rapalog	None	Advanced neuroendocine tumor	3	Not available	11.04 (95% CI 8.41–13.86) vs placebo 4.6 (95% CI 3.06–5.49)	44.02 (95% CI 35.61–51.75) vs placebo 37.68 (95% CI 29.14–45.77)	NCT00510068
Everolimus	Rapalog	Rituximab	Diffuse large B cell lymphoma	2	ORR 38% (90% CI 21–56%); CR 3/24; PR 6/24	2.9 (90% CI 1.8–3.8)	8.6 (90% CI 4.9–16.3)	212 NCT00869999
MLN0128	ATP-competitive	Paclitaxel and trastuzumab	Advanced solid tumors	1	CR 0; PR 8/54; SD (> 6 months) 6/54	Not available	Not available	87 NCT01351350
AZD2014 (Vistusertib)	ATP-competitive	None	Metastatic clear cell renal cancer	2	Response rate 4% for AZD1024, 13% for everolimus Progressive disease 69% vs 13% for everolimus treatment	1.8 vs 4.6 for everolimus treatment	4.9 for AZD1024	203
Voxtalisib (SAR24540; XL765)	ATP-competitive	None	Relapsed or refractory non-Hodgkin lymphoma or chronic lymphocytic lymphoma	2	CR 8/164 (4.9%); PR 22/164 (13.4%); SD 55/164 (33.5%); ORR 18.3% (40.3% for follicular lymphoma)	1.9 for follicular lymphoma Overall progression-free rate at 24 weeks, 38·6% (95% CI 30·9–46·3)	Not available	202 NCT01403636
Gedatolisib (PKI-587; PF05212384)	ATP-competitive	None	Recurrent endometrial cancer	2	CR 1/38 (3%); PR 5/38 (13%); SD > 16 weeks, 24% (37% for stathmin-low cancer, 11% for stathmin-high cancer)	3.7 (95% CI 2–5.6) for stathmin-low cancer; 3 (95% CI 1.87–5.7) for stathmin-high cancer	Not available	204 NCT01420081

CR complete response, *CBR* clinical benefit rate, *ORR* overall response rate, *OS* overall survival, *PFS* progression-free survival, *PR* partial response, *SD* stable disease. *, Registration number in ClinicalTrials.gov

such as taxol, cisplatin, carboplatin, oxaliplatin, irinotecan, temozolomide, and gemcitabine.

The phase 3 BOLERO-2 trial in patients with ER-positive/HER2-negative advanced or metastatic breast cancer demonstrates that a combination of everolimus and the aromatase inhibitor exemestane significantly improves PFS, while the OS is not improved [206, 207]. Accordingly, a combination of everolimus and exemestane has been approved as a guideline for treating ER-positive/HER2-negative advanced or metastatic breast cancer [208]. In a phase 2 clinical trial, a combination of everolimus and the aromatase inhibitor letrozole achieved a 12-week PFS rate of 47% in patients with ER-positive relapsed high-grade ovarian cancer [209]. In addition, the combination of everolimus with trastuzumab and paclitaxel has a promising efficacy in patients with highly resistant HER2-positive advanced breast cancer (Table 1). This combination is currently under investigation in the BOLERO-1 phase 3 trial [210]. Moreover, a combination of everolimus with carboplatin is efficacious in treating metastatic triple-negative breast cancer, with a median PFS of 3 months (95% CI 1.6 to 4.6 months) and overall survival (OS) of 16.6 months [211]. In contrast, a combination of everolimus with gemcitabine/cisplatin has no synergistic effect in patients with metastatic triple-negative breast cancer. Hence, this combination still needs validation in more patients.

The CD20-targeted monoclonal antibody rituximab is a treatment for low-grade or follicular CD20-positive non-Hodgkin's lymphoma. Diffuse large B cell lymphoma (DLBCL) is the most common type of non-Hodgkin's lymphoma. A phase 2 study of everolimus (10 mg/day) in combination with rituximab demonstrated an overall response rate of 38%, a complete response rate of 12.5%, and a partial response rate of 25% among 24 patients with heavily pretreated DLBCL [212]. In addition, the combination of everolimus with rituximab or rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) was well tolerated in DLBCL patients [212, 213]. It warrants further study to determine if the combination of everolimus with R-CHOP has a better response in patients with DLBCL. In addition, the combination of mTORC1/2 inhibitor with other targeted cancer drugs has been tested in clinical trials. Among 54 cancer patients who were treated with MLN0128 and trastuzumab/paclitaxel, 14.8% (8/54) of them achieved a partial response, and near 11% (6/54) cases had stable disease for more than 6 months [87]. According to a phase 1 trial (NCT02193633), the combination of paclitaxel and vistusertib is highly active and well tolerated in patients with high-grade serous ovarian cancer and squamous non-small cell lung cancer [214].

Given that IGF-IR signaling may induce mTORC1 inhibitor resistance, the combination of cixutumumab, a humanized monoclonal antibody against IGF-1R, and temsirolimus was tested in a clinical setting. This combination shows clinical activity in patients with sarcoma and adrenocortical carcinoma [215, 216]. In addition, a combination of everolimus (5 mg daily) and the multi-kinase inhibitor sorafenib (400 mg twice daily) exhibits anti-tumor activity in previously untreated patients with metastatic renal cell carcinoma with tolerable toxicity [217]. However, a combination of sorafenib and everolimus fails to achieve the target of 6 month PFS of 50% or greater among patients with unresectable high-grade osteosarcoma progressing after standard treatment [218]. For patients with recurrent glioblastoma, a combination of sorafenib (200 mg twice daily) and temsirolimus (20 mg weekly) is associated with considerable toxicity and poor efficacy [219].

In patients with metastatic castration-resistant prostate cancer, a combination of everolimus and the EGFR inhibitor gefitinib has no significant anti-tumor activity [220]. According to a phase 2 trial, a combination of sunitinib and everolimus as the first-line therapy exhibits poor efficacy in treating advanced renal cell carcinoma [221]. However, another phase 2 trial in patients with metastatic renal carcinoma demonstrates that the first-line sunitinib treatment followed by everolimus achieves a longer OS than the first-line everolimus followed by sunitinib, suggesting that the sequence may affect the outcome [222]. Moreover, a combination of imatinib and everolimus has limited activity in the treatment of patients with advanced chordoma [223]. The combination of pimasertib and voxtalisib showed a poor long-term tolerability and limited anti-tumor activity in patients with advanced solid tumors [224].

Concluding remarks

The discovery of TOR in yeast and mTOR in mammals is a fundamental breakthrough in understanding cell and organism growth, metabolism, and diseases. In-depth studies to clarify the regulators and effectors of mTOR signaling have revealed multiple networks that work together to integrate growth factors, nutrients, sterols, and nucleotides signaling. The identification of the critical roles of mTOR and its regulators in tumorigenesis has driven the development of the ever-growing list of mTOR inhibitors. While some of the mTOR inhibitors have been approved to treat cancer patients, more mTOR inhibitors are under check to fulfill their promise for cancer therapy.

It appears that mTOR inhibitors have mixed efficacy in patients with distinct kinds of cancer and among patients with the same kind of cancer. Recent studies reveal that tumor organoids may help drug testing [225, 226]. Tumor organoids may be used to test the

response of a given tumor to mTOR inhibitors. Alternatively, patient-derived tumor grafts may be transplanted to animals, followed by testing their response to mTOR inhibitors [227]. It would be of interest to determine if these emerging technologies are clinically relevant.

In the era of precise medicine, it needs to determine if there are predictive biomarkers that may guide the stratification of patients in clinical trials or help identify the patients who most likely benefit from treatment with mTOR inhibitors in a clinical setting. Gene testing is a promising approach to achieve this goal. The candidates for gene testing may include mTOR, PIK3CA, GATOR, KRAS, and BRAF. Mutations in PIK3CA and GATOR have been associated with higher sensitivity to mTOR inhibition in preclinical studies. Hence, PIK3CA mutations may be potential sensitive markers. In contrast, KRAS/BRAF mutations may be resistant biomarkers. Both DNA from tumor samples and ctDNA from the blood may be subject to testing of gene mutations. In addition, gene mutations in the tumors may be dynamic during cancer evolution or regression [228]. It remains to determine if dynamic testing of ctDNA during the course of therapy may monitor cancer evolution and better predict drug resistance, thereby adjusting the treatment regimen in time. Recent progress in liquid biopsy may help address this critical issue [229, 230]. In addition to gene testing, the solvable factors in the blood may be potential biomarkers as well. Of particular note, the mechanisms underlying the varied responsiveness to mTOR inhibitors in cancer patients may be complex. Rather than a single or few biomarkers, a set of biomarkers may be more powerful and accurate to meet the challenge.

Moreover, toxicity is a critical problem that precludes the clinical administration of drugs. Although mTOR inhibitors exhibit a promising efficacy in preclinical studies, some inhibitors have serious adverse effects in patients and have to be discontinued. Hence, elucidation of the mechanisms underlying these adverse effects may help manage them in the clinic.

Drug resistance is a serious challenge to successful cancer therapy. As discussed above, the mechanisms for mTOR inhibitor resistance are complex. Further studies to elucidate the diverse mechanisms may help design strategies to overcome the resistance to mTOR inhibition. Mechanism-based combination of mTOR inhibitors with chemotherapeutic agents or molecular-targeted drugs may be practical in the clinic. We expect the results from many ongoing clinical trials to validate the most powerful regimens that include mTOR inhibitors.

Abbreviations

4E-BP1: Eukaryotic translation initiation factor 4E binding protein 1; DEPTOR: DEP domain-containing protein 6; IGF-IR: Type-I insulin-like growth factor receptor; MAPK: Mitogen-activated protein kinase; mLST8: Mammalian lethal with SEC13 protein 8; mSIN1: Mammalian stress-activated protein kinase-interacting protein 1; mTOR: Mechanistic target of rapamycin; PI3K: Phosphoinositide 3-kinase; PKC: Protein kinase C; PRAS40: 40 kDa proline-rich Akt substrate; Raptor: Regulatory-associated protein of mTOR; RCC: Renal cell carcinoma; RHEB: Ras homolog enriched in the brain; Rictor: Rapamycin-insensitive companion of mTOR; S6K1: Ribosomal protein S6 kinase β-1; TSC: Tuberous sclerosis complex

Acknowledgements

We would like to thank Qiulin Tang for her assistance in preparing the manuscript.

Authors' contributions

HH and YJ conceived the review and wrote the manuscript. QK and JW prepared the figures. HZ edited the references. TL was involved in editing the manuscript. All authors read and approved the final manuscript.

Funding

This work was supported by grants 81672814 and 81872388 from the National Natural Science Foundation of China and grant 2018SCUH0009 from the Fundamental Research Fund for the Central Universities.

Availability of data and materials

Not applicable.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]State Key Laboratory of Biotherapy, Laboratory of Stem Cell Biology, National Clinical Research Center for Geriatrics, West China Hospital, Sichuan University, Chengdu 610041, China. [2]Laboratory of Oncogene, Cancer Center, West China Hospital, Sichuan University, Chengdu, China. [3]School of Basic Medicine, Chengdu University of Traditional Chinese Medicine, Chengdu, China. [4]Cancer Center, West China Hospital, Sichuan University, Chengdu, China.

Received: 22 April 2019 Accepted: 14 June 2019
Published online: 05 July 2019

References

1. Yin Y, Hua H, Li M, Liu S, Kong Q, Shao T, et al. mTORC2 promotes type I insulin-like growth factor receptor and insulin receptor activation through the tyrosine kinase activity of mTOR. Cell Res. 2016;26:46–65.
2. Saxton RA, Sabatini DM. mTOR signaling in growth, metabolism, and disease. Cell. 2017;168:960–76.
3. Harwood FC, Klein Geltink RI, O'Hara BP, Cardone M, Janke L, Finkelstein D, et al. ETV7 is an essential component of a rapamycin-insensitive mTOR complex in cancer. Sci Adv. 2018;4:eaar3938.
4. Jacinto E, Loewith R, Schmidt A, Lin S, Rüegg MA, Hall A, et al. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nat Cell Biol. 2004;6:1122–8.
5. Rispal D, Eltschinger S, Stahl M, Vaga S, Bodenmiller B, Abraham Y, et al. Target of rapamycin complex 2 regulates actin polarization and endocytosis via multiple pathways. J Biol Chem. 2015;290:14963–78.
6. Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, et al. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature. 2009;460:392–5.
7. Miller RA, Harrison DE, Astle CM, Fernandez E, Flurkey K, Han M, et al. Rapamycin-mediated lifespan increase in mice is dose and sex dependent and metabolically distinct from dietary restriction. Aging Cell. 2014;13:468–77.
8. Yang H, Wang J, Liu M, Chen X, Huang M, Tan D, et al. 4.4 Å resolution cryo-EM structure of human mTOR complex 1. Protein Cell. 2016;7:878–87.

9. Yip CK, Murata K, Walz T, Sabatini DM, Kang SA. Structure of the human mTOR complex I and its implications for rapamycin inhibition. Mol Cell. 2010;38:768–74.
10. Neer EJ, Schmidt CJ, Nambudripad R, Smith TF. The ancient regulatory-protein family of WD-repeat proteins. Nature. 1994;371:297–300.
11. Aylett CH, Sauer E, Imseng S, Boehringer D, Hall MN, Ban N, et al. Architecture of human mTOR complex 1. Science. 2016;351:48–52.
12. Schalm SS, Fingar DC, Sabatini DM, Blenis J. TOS motif-mediated raptor binding regulates 4E-BP1 multisite phosphorylation and function. Curr Biol. 2003;13:797–806.
13. Nojima H, Tokunaga C, Eguchi S, Oshiro N, Hidayat S, Yoshino K, et al. The mammalian target of rapamycin (mTOR) partner, raptor, binds the mTOR substrates p70 S6 kinase and 4E-BP1 through their TOR signaling (TOS) motif. J Biol Chem. 2003;278:15461–4.
14. Chen X, Liu M, Tian Y, Li J, Qi Y, Zhao D, et al. Cryo-EM structure of human mTOR complex 2. Cell Res. 2018;28:518–28.
15. Karuppasamy M, Kusmider B, Oliveira TM, Gaubitz C, Prouteau M, Loewith R, et al. Cryo-EM structure of Saccharomyces cerevisiae target of rapamycin complex 2. Nat Commun. 2017;8:1729.
16. Zhou P, Zhang N, Nussinov R, Ma B. Defining the domain arrangement of the mammalian target of rapamycin complex component Rictor protein. J Comput Biol. 2015;22:876–86.
17. Andrade MA, Perez-Iratxeta C, Ponting CP. Protein repeats: structures, functions, and evolution. J Struct Biol. 2001;134:117–31.
18. Liu P, Gan W, Chin YR, Ogura K, Guo J, Zhang J, et al. PtdIns(3,4,5)P3-dependent activation of the mTORC2 kinase complex. Cancer Discov. 2015;5:1194–209.
19. McDonald PC, Oloumi A, Mills J, Dobreva I, Maidan M, Gray V, et al. Rictor and integrin-linked kinase interact and regulate Akt phosphorylation and cancer cell survival. Cancer Res. 2008;68:1618–24.
20. Schroder WA, Buck M, Cloonan N, Hancock JF, Suhrbier A, Sculley T, et al. Human Sin1 contains Ras-binding and pleckstrin homology domains and suppresses Ras signalling. Cell Signal. 2007;19:1279–89.
21. Deng L, Chen L, Zhao L, Xu Y, Peng X, Wang X, et al. Ubiquitination of Rheb governs growth factor-induced mTORC1 activation. Cell Res. 2019;29:136–50.
22. Inoki K, Li Y, Zhu T, Wu J, Guan KL. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol. 2002;4:648–57.
23. Potter CJ, Pedraza LG, Xu T. Akt regulates growth by directly phosphorylating Tsc2. Nat Cell Biol. 2002;4:658–65.
24. Tee AR, Manning BD, Roux PP, Cantley LC, Blenis J. Tuberous sclerosis complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. Curr Biol. 2003;13:1259–68.
25. Carriere A, Romeo Y, Acosta-Jaquez HA, Moreau J, Bonneil E, Thibault P, et al. ERK1/2 phosphorylate Raptor to promote Ras-dependent activation of mTOR complex 1 (mTORC1). J Biol Chem. 2011;286:567–77.
26. Carrière A, Cargnello M, Julien LA, Gao H, Bonneil E, Thibault P, et al. Oncogenic MAPK signaling stimulates mTORC1 activity by promoting RSK-mediated raptor phosphorylation. Curr Biol. 2008;18:1269–77.
27. Wu D, Chapman JR, Wang L, Harris TE, Shabanowitz J, Hunt DF, et al. Intestinal cell kinase (ICK) promotes activation of mTOR complex 1 (mTORC1) through phosphorylation of Raptor Thr-908. J Biol Chem. 2012;287:12510–9.
28. Inoki K, Ouyang H, Zhu T, Lindvall C, Wang Y, Zhang X, et al. TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth. Cell. 2006;126:955–68.
29. Chin RM, Fu X, Pai MY, Vergnes L, Hwang H, Deng G, et al. The metabolite α-ketoglutarate extends lifespan by inhibiting ATP synthase and TOR. Nature. 2014;510:397–401.
30. Schewe DM, Aguirre-Ghiso JA. ATF6 alpha-Rheb-mTOR signaling promotes survival of dormant tumor cells in vivo. Proc Natl Acad Sci USA. 2008;105:10519–24.
31. Kang YJ, Lu MK, Guan KL. The TSC1 and TSC2 tumor suppressors are required for proper ER stress response and protect cells from ER stress-induced apoptosis. Cell Death Differ. 2011;18:133–44.
32. Shimobayashi M, Hall MN. Multiple amino acid sensing inputs to mTORC1. Cell Res. 2016;26:7–20.
33. Sancak Y, Bar-Peled L, Zoncu R, Markhard AL, Nada S, Sabatini DM. Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. Cell. 2010;141:290–303.
34. de Araujo MEG, Naschberger A, Fürnrohr BG, Stasyk T, Dunzendorfer-Matt T, Lechner S, et al. Crystal structure of the human lysosomal mTORC1 scaffold complex and its impact on signaling. Science. 2017;358:377–81.
35. Bar-Peled L, Chantranupong L, Cherniack AD, Chen WW, Ottina KA, Grabiner BC, et al. A tumor suppressor complex with GAP activity for the Rag GTPases that signal amino acid sufficiency to mTORC1. Science. 2013;340:1100–6.
36. Wolfson RL, Chantranupong L, Wyant GA, Gu X, Orozco JM, Shen K, et al. KICSTOR recruits GATOR1 to the lysosome and is necessary for nutrients to regulate mTORC1. Nature. 2017;543:438–42.
37. Peng M, Yin N, Li MO. SZT2 dictates GATOR control of mTORC1 signalling. Nature. 2017;543:433–7.
38. Chantranupong L, Wolfson RL, Orozco JM, Saxton RA, Scaria SM, Bar-Peled L, et al. The Sestrins interact with GATOR2 to negatively regulate the amino-acid-sensing pathway upstream of mTORC1. Cell Rep. 2014;9:1–8.
39. Parmigiani A, Nourbakhsh A, Ding B, Wang W, Kim YC, Akopiants K, et al. Sestrins inhibit mTORC1 kinase activation through the GATOR complex. Cell Rep. 2014;9:1281–91.
40. Peng M, Yin N, Li MO. Sestrins function as guanine nucleotide dissociation inhibitors for Rag GTPases to control mTORC1 signaling. Cell. 2014;159:122–33.
41. Wolfson RL, Chantranupong L, Saxton RA, Shen K, Scaria SM, Cantor JR, et al. Sestrin2 is a leucine sensor for the mTORC1 pathway. Science. 2016;351:43–8.
42. Deng L, Jiang C, Chen L, Jin J, Wei J, Zhao L, et al. The ubiquitination of rag A GTPase by RNF152 negatively regulates mTORC1 activation. Mol Cell. 2015;58:804–18.
43. Jin G, Lee SW, Zhang X, Cai Z, Gao Y, Chou PC, et al. Skp2-mediated RagA ubiquitination elicits a negative feedback to prevent amino-acid-dependent mTORC1 hyperactivation by recruiting GATOR1. Mol Cell. 2015;58:989–1000.
44. Wang S, Tsun ZY, Wolfson RL, Shen K, Wyant GA, Plovanich ME, et al. Lysosomal amino acid transporter SLC38A9 signals arginine sufficiency to mTORC1. Science. 2015;347:188–94.
45. Song P, Yang S, Hua H, Zhang H, Kong Q, Wang J, et al. The regulatory protein GADD34 inhibits TRAIL-induced apoptosis via TRAF6/ERK-dependent stabilization of myeloid cell leukemia 1 in liver cancer cells. J Biol Chem. 2019;294:5945–55.
46. Linares JF, Duran A, Yajima T, Pasparakis M, Moscat J, Diaz-Meco MT. K63 polyubiquitination and activation of mTOR by the p62-TRAF6 complex in nutrient-activated cells. Mol Cell. 2013;51:283–96.
47. Menon D, Salloum D, Bernfeld E, Gorodetsky E, Akselrod A, Frias MA, et al. Lipid sensing by mTOR complexes via de novo synthesis of phosphatidic acid. J Biol Chem. 2017;292:6303–11.
48. Castellano BM, Thelen AM, Moldavski O, Feltes M, van der Welle RE, Mydock-McGrane L, et al. Lysosomal cholesterol activates mTORC1 via an SLC38A9-Niemann-Pick C1 signaling complex. Science. 2017;355:1306–11.
49. Hoxhaj G, Hughes-Hallett J, Timson RC, Ilagan E, Yuan M, Asara JM, et al. The mTORC1 signaling network senses changes in cellular purine nucleotide levels. Cell Rep. 2017;21:1331–46.
50. Dibble CC, Asara JM, Manning BD. Characterization of Rictor phosphorylation sites reveals direct regulation of mTOR complex 2 by S6K1. Mol Cell Biol. 2009;29:5657–70.
51. Julien LA, Carriere A, Moreau J, Roux PP. mTORC1-activated S6K1 phosphorylates Rictor on threonine 1135 and regulates mTORC2 signaling. Mol Cell Biol. 2010;30:908–21.
52. Liu P, Gan W, Inuzuka H, Lazorchak AS, Gao D, Arojo O, et al. Sin1 phosphorylation impairs mTORC2 complex integrity and inhibits downstream Akt signalling to suppress tumorigenesis. Nat Cell Biol. 2013;15:1340–50.
53. Zinzalla V, Stracka D, Oppliger W, Hall MN. Activation of mTORC2 by association with the ribosome. Cell. 2011;144:757–68.
54. Ebner M, Sinkovics B, Szczygieł M, Ribeiro DW, Yudushkin I. Localization of mTORC2 activity inside cells. J Cell Biol. 2017;216:343–53.
55. Dan HC, Antonia RJ, Baldwin AS. PI3K/Akt promotes feedforward mTORC2 activation through IKKα. Oncotarget. 2016;7:21064–75.
56. Gao M, Kong Q, Hua H, Yin Y, Wang J, Luo T, et al. AMPK-mediated up-regulation of mTORC2 and MCL-1 compromises the anti-cancer effects of aspirin. Oncotarget. 2016;7:16349–61.
57. Moloughney JG, Kim PK, Vega-Cotto NM, Wu CC, Zhang S, Adlam M, et al. mTORC2 responds to glutamine catabolite levels to modulate the hexosamine biosynthesis enzyme GFAT1. Mol Cell. 2016;63:811–26.

58. Moloughney JG, Vega-Cotto NM, Liu S, Patel C, Kim PK, Wu CC, et al. mTORC2 modulates the amplitude and duration of GFAT1 Ser-243 phosphorylation to maintain flux through the hexosamine pathway during starvation. J Biol Chem. 2018;293:16464–78.
59. Alcaide-Gavilán M, Lucena R, Schubert KA, Artiles KL, Zapata J, Kellogg DR. Modulation of TORC2 signaling by a conserved Lkb1 signaling axis in budding yeast. Genetics. 2018;210:155–70.
60. Foster DA. Phosphatidic acid and lipid-sensing by mTOR. Trends Endocrinol Metab. 2013;24:272–8.
61. Wang B, Jie Z, Joo D, Ordureau A, Liu P, Gan W, et al. TRAF2 and OTUD7B govern a ubiquitin-dependent switch that regulates mTORC2 signalling. Nature. 2017;545:365–9.
62. Khanna N, Fang Y, Yoon MS, Chen J. XPLN is an endogenous inhibitor of mTORC2. Proc Natl Acad Sci U S A. 2013;110:15979–84.
63. Mao JH, Kim IJ, Wu D, Climent J, Kang HC, DelRosario R, et al. FBXW7 targets mTOR for degradation and cooperates with PTEN in tumor suppression. Science. 2008;321:1499–502.
64. Gingras AC, Raught B, Gygi SP, Niedzwiecka A, Miron M, Burley SK, et al. Hierarchical phosphorylation of the translation inhibitor 4E-BP1. Genes Dev. 2001;15:2852–64.
65. Wang L, Rhodes CJ, Lawrence JC Jr. Activation of mammalian target of rapamycin (mTOR) by insulin is associated with stimulation of 4EBP1 binding to dimeric mTOR complex 1. J Biol Chem. 2006;281:24293–303.
66. Gentilella A, Kozma SC, Thomas G. A liaison between mTOR signaling, ribosome biogenesis and cancer. Biochim Biophys Acta. 1849;2015:812–20.
67. Giguère V. Canonical signaling and nuclear activity of mTOR-a teamwork effort to regulate metabolism and cell growth. FEBS J. 2018;285:1572–88.
68. Iadevaia V, Liu R, Proud CG. mTORC1 signaling controls multiple steps in ribosome biogenesis. Semin Cell Dev Biol. 2014;36:113–20.
69. Kantidakis T, Ramsbottom BA, Birch JL, Dowding SN, White RJ. mTOR associates with TFIIIC, is found at tRNA and 5S rRNA genes, and targets their repressor Maf1. Proc Natl Acad Sci U S A. 2010;107:11823–8.
70. Geng Q, Liu J, Gong Z, Chen S, Chen S, Li X, et al. Phosphorylation by mTORC1 stabilizes Skp2 and regulates its oncogenic function in gastric cancer. Mol Cancer. 2017;16:83.
71. Sarbassov DD, Ali SM, Kim DH, Guertin DA, Latek RR, Erdjument-Bromage H, et al. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol. 2004;14:1296–302.
72. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science. 2005;307:1098–101.
73. Dai N, Christiansen J, Nielsen FC, Avruch J. mTOR complex 2 phosphorylates IMP1 cotranslationally to promote IGF2 production and the proliferation of mouse embryonic fibroblasts. Genes Dev. 2013;27:301–12.
74. Rodriguez-Viciana P, Warne PH, Dhand R, Vanhaesebroeck B, Gout I, Fry MJ, et al. Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature. 1994;370:527–32.
75. Xu J, Pham CG, Albanese SK, Dong Y, Oyama T, Lee CH, et al. Mechanistically distinct cancer-associated mTOR activation clusters predict sensitivity to rapamycin. J Clin Invest. 2016;126:3526–40.
76. Ghosh AP, Marshall CB, Coric T, Shim EH, Kirkman R, Ballestas ME, et al. Point mutations of the mTOR-RHEB pathway in renal cell carcinoma. Oncotarget. 2015;6:17895–910.
77. Gao Y, Gao J, Li M, Zheng Y, Wang Y, Zhang H, et al. Rheb1 promotes tumor progression through mTORC1 in MLL-AF9-initiated murine acute myeloid leukemia. J Hematol Oncol. 2016;9:36.
78. El Shamieh S, Saleh F, Moussa S, Kattan J, Farhat F. RICTOR gene amplification is correlated with metastasis and therapeutic resistance in triple-negative breast cancer. Pharmacogenomics. 2018;19:757–60.
79. Kim ST, Kim SY, Klempner SJ, Yoon J, Kim N, Ahn S, et al. Rapamycin-insensitive companion of mTOR (RICTOR) amplification defines a subset of advanced gastric cancer and is sensitive to AZD2014-mediated mTORC1/2 inhibition. Ann Oncol. 2017;28:547–54.
80. Masri J, Bernath A, Martin J, Jo OD, Vartanian R, Funk A, et al. mTORC2 activity is elevated in gliomas and promotes growth and cell motility via overexpression of rictor. Cancer Res. 2007;67:11712–20.
81. Gkountakos A, Pilotto S, Mafficini A, Vicentini C, Simbolo M, Milella M, et al. Unmasking the impact of Rictor in cancer: novel insights of mTORC2 complex. Carcinogenesis. 2018. https://doi.org/10.1093/carcin/bgy086.
82. Brown EJ, Albers MW, Shin TB, Ichikawa K, Keith CT, Lane WS, et al. A mammalian protein targeted by G1-arresting rapamycin-receptor complex. Nature. 1994;369:756–8.
83. Roskoski R. Properties of FDA-approved small molecule protein kinase inhibitors. Pharmacol Res. 2019. https://doi.org/10.1016/j.phrs.2019.03.006.
84. Yeung Y, Lau DK, Chionh F, Tran H, Tse JWT, Weickhardt AJ, et al. K-Ras mutation and amplification status is predictive of resistance and high basal pAKT is predictive of sensitivity to everolimus in biliary tract cancer cell lines. Mol Oncol. 2017;11:1130–42.
85. Bodnar L, Stec R, Cierniak S, Synowiec A, Wcisło G, Jesiotr M, et al. Clinical usefulness of PI3K/Akt/mTOR genotyping in companion with other clinical variables in metastatic renal cell carcinoma patients treated with everolimus in the second and subsequent lines. Ann Oncol. 2015;26:1385–9.
86. Kwiatkowski DJ, Choueiri TK, Fay AP, Rini BI, Thorner AR, de Velasco G, et al. Mutations in TSC1, TSC2, and MTOR are associated with response to rapalogs in patients with metastatic renal cell carcinoma. Clin Cancer Res. 2016;22:2445–52.
87. Burris HA 3rd, Kurkjian CD, Hart L, Pant S, Murphy PB, Jones SF, et al. TAK-228 (formerly MLN0128), an investigational dual TORC1/2 inhibitor plus paclitaxel, with/without trastuzumab, in patients with advanced solid malignancies. Cancer Chemother Pharmacol. 2017;80:261–73.
88. Slotkin EK, Patwardhan PP, Vasudeva SD, de Stanchina E, Tap WD, Schwartz GK. MLN0128, an ATP-competitive mTOR kinase inhibitor with potent in vitro and in vivo antitumor activity, as potential therapy for bone and soft-tissue sarcoma. Mol Cancer Ther. 2015;14:395–406.
89. Gökmen-Polar Y, Liu Y, Toroni RA, Sanders KL, Mehta R, Badve S, et al. Investigational drug MLN0128, a novel TORC1/2 inhibitor, demonstrates potent oral antitumor activity in human breast cancer xenograft models. Breast Cancer Res Treat. 2012;136:673–82.
90. Caro-Vegas C, Bailey A, Bigi R, Damania B, Dittmer DP. Targeting mTOR with MLN0128 overcomes rapamycin and chemoresistant primary effusion lymphoma. MBio. 2019;10:e02871–18.
91. Badawi M, Kim J, Dauki A, Sutaria D, Motiwala T, Reyes R, et al. CD44 positive and sorafenib insensitive hepatocellular carcinomas respond to the ATP-competitive mTOR inhibitor INK128. Oncotarget. 2018;9:26032–45.
92. Fricke SL, Payne SN, Favreau PF, Kratz JD, Pasch CA, Foley TM, et al. MTORC1/2 inhibition as a therapeutic strategy for PIK3CA mutant cancers. Mol Cancer Ther. 2019;18:346–55.
93. Chamberlain CE, German MS, Yang K, Wang J, VanBrocklin H, Regan M, et al. A patient-derived xenograft model of pancreatic neuroendocrine tumors identifies sapanisertib as a possible new treatment for everolimus-resistant tumors. Mol Cancer Ther. 2018;17:2702–9.
94. Rashid MM, Lee H, Jung BH. Metabolite identification and pharmacokinetic profiling of PP242, an ATP-competitive inhibitor of mTOR using ultra high-performance liquid chromatography and mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2018;1072:244–51.
95. Feldman ME, Apsel B, Uotila A, Loewith R, Knight ZA, Ruggero D, et al. Active-site inhibitors of mTOR target rapamycin-resistant outputs of mTORC1 and mTORC2. PLoS Biol. 2009;7:e38.
96. Musa F, Alard A, David-West G, Curtin JP, Blank SV, Schneider RJ. Dual mTORC1/2 inhibition as a novel strategy for the resensitization and treatment of platinum-resistant ovarian cancer. Mol Cancer Ther. 2016;15:1557–67.
97. David-West G, Ernlund A, Gadi A, Schneider RJ. mTORC1/2 inhibition re-sensitizes platinum-resistant ovarian cancer by disrupting selective translation of DNA damage and survival mRNAs. Oncotarget. 2018;9:33064–76.
98. Koppenhafer SL, Goss KL, Terry WW, Gordon DJ. mTORC1/2 and protein translation regulate levels of CHK1 and the sensitivity to CHK1 inhibitors in Ewing sarcoma cells. Mol Cancer Ther. 2018;17:2676–88.
99. Nam HY, Han MW, Chang HW, Lee YS, Lee M, Lee HJ, et al. Radioresistant cancer cells can be conditioned to enter senescence by mTOR inhibition. Cancer Res. 2013;73:4267–77.
100. Hayman TJ, Wahba A, Rath BH, Bae H, Kramp T, Shankavaram UT, et al. The ATP-competitive mTOR inhibitor INK128 enhances in vitro and in vivo radiosensitivity of pancreatic carcinoma cells. Clin Cancer Res. 2014;20:110–9.
101. Zhang Q, Shi C, Han L, Jain N, Roberts KG, Ma H, et al. Inhibition of mTORC1/C2 signaling improves anti-leukemia efficacy of JAK/STAT blockade in CRLF2 rearranged and/or JAK driven Philadelphia chromosome-like acute B-cell lymphoblastic leukemia. Oncotarget. 2018;9:8027–41.
102. Guichard SM, Curwen J, Bihani T, D'Cruz CM, Yates JW, Grondine M, et al. AZD2014, an inhibitor of mTORC1 and mTORC2, is highly effective in ER+

breast cancer when administered using intermittent or continuous schedules. Mol Cancer Ther. 2015;14:2508–18.
103. Jordan NJ, Dutkowski CM, Barrow D, Mottram HJ, Hutcheson IR, Nicholson RI, et al. Impact of dual mTORC1/2 mTOR kinase inhibitor AZD8055 on acquired endocrine resistance in breast cancer in vitro. Breast Cancer Res. 2014;16:R12.
104. Wong Te Fong AC. Thavasu P, Gagrica S, Swales KE, Leach MO, Cosulich SC, et al. Evaluation of the combination of the dual m-TORC1/2 inhibitor vistusertib (AZD2014) and paclitaxel in ovarian cancer models. Oncotarget. 2017;8:113874–84.
105. Kahn J, Hayman TJ, Jamal M, Rath BH, Kramp T, Camphausen K, et al. The mTORC1/mTORC2 inhibitor AZD2014 enhances the radiosensitivity of glioblastoma stem-like cells. Neuro Oncol. 2014;16:29–37.
106. Hall CP, Reynolds CP, Kang MH. Modulation of glucocorticoid resistance in pediatric T-cell acute lymphoblastic leukemia by increasing BIM expression with the PI3K/mTOR inhibitor BEZ235. Clin Cancer Res. 2016;22:621–32.
107. Gazi M, Moharram SA, Marhäll A, Kazi JU. The dual specificity PI3K/mTOR inhibitor PKI-587 displays efficacy against T-cell acute lymphoblastic leukemia (T-ALL). Cancer Lett. 2017;392:9–16.
108. Chiarini F, Evangelisti C, McCubrey JA, Martelli AM. Current treatment strategies for inhibiting mTOR in cancer. Trends Pharmacol Sci. 2015;36:124–35.
109. Fròsina G, Profumo A, Marubbi D, Marcello D, Ravetti JL, Daga A. ATR kinase inhibitors NVP-BEZ235 and AZD6738 effectively penetrate the brain after systemic administration. Radiat Oncol. 2018;13:76.
110. Yu Z, Xie G, Zhou G, Cheng Y, Zhang G, Yao G, et al. NVP-BEZ235, a novel dual PI3K-mTOR inhibitor displays anti-glioma activity and reduces chemoresistance to temozolomide in human glioma cells. Cancer Lett. 2015;367:58–68.
111. Chen D, Lin X, Zhang C, Liu Z, Chen Z, Li Z, et al. Dual PI3K/mTOR inhibitor BEZ235 as a promising therapeutic strategy against paclitaxel-resistant gastric cancer via targeting PI3K/Akt/mTOR pathway. Cell Death Dis. 2018;9:123.
112. Zaidi AH, Kosovec JE, Matsui D, Omstead AN, Raj M, Rao RR, et al. PI3K/mTOR dual inhibitor, LY3023414, demonstrates potent antitumor efficacy against esophageal adenocarcinoma in a rat model. Ann Surg. 2017;266:91–8.
113. Smith MC, Mader MM, Cook JA, Iversen P, Ajamie R, Perkins E, et al. Characterization of LY3023414, a novel PI3K/mTOR dual inhibitor eliciting transient target modulation to impede tumor growth. Mol Cancer Ther. 2016;15:2344–56.
114. Yu P, Laird AD, Du X, Wu J, Won KA, Yamaguchi K, et al. Characterization of the activity of the PI3K/mTOR inhibitor XL765 (SAR245409) in tumor models with diverse genetic alterations affecting the PI3K pathway. Mol Cancer Ther. 2014;13:1078–91.
115. Inaba K, Oda K, Ikeda Y, Sone K, Miyasaka A, Kashiyama T, et al. Antitumor activity of a combination of dual PI3K/mTOR inhibitor SAR245409 and selective MEK1/2 inhibitor pimasertib in endometrial carcinomas. Gynecol Oncol. 2015;138:323–31.
116. Beaufils F, Cmiljanovic N, Cmiljanovic V, Bohnacker T, Melone A, Marone R, et al. 5-(4,6-Dimorpholino-1,3,5-triazin-2-yl)-4-(trifluoromethyl)pyridin-2-amine (PQR309), a potent, brain-penetrant, orally bioavailable, pan-class I PI3K/mTOR inhibitor as clinical candidate in oncology. J Med Chem. 2017;60:7524–38.
117. Tarantelli C, Gaudio E, Arribas AJ, Kwee I, Hillmann P, Rinaldi A, et al. PQR309 is a novel dual PI3K/mTOR inhibitor with preclinical antitumor activity in lymphomas as a single agent and in combination therapy. Clin Cancer Res. 2018;24:120–9.
118. Brandt C, Hillmann P, Noack A, Römermann K, Öhler LA, Rageot D, et al. The novel, catalytic mTORC1/2 inhibitor PQR620 and the PI3K/mTORC1/2 inhibitor PQR530 effectively cross the blood-brain barrier and increase seizure threshold in a mouse model of chronic epilepsy. Neuropharmacology. 2018;140:107–20.
119. Venkatesan AM, Dehnhardt CM, Delos Santos E, Chen Z, Dos Santos O, Ayral-Kaloustian S, et al. Bis(morpholino-1,3,5-triazine) derivatives: potent adenosine 5′-triphosphate competitive phosphatidylinositol-3-kinase/mammalian target of rapamycin inhibitors: discovery of compound 26 (PKI-587), a highly efficacious dual inhibitor. J Med Chem. 2010;53:2636–45.
120. Tasian SK, Teachey DT, Li Y, Shen F, Harvey RC, Chen IM, et al. Potent efficacy of combined PI3K/mTOR and JAK or ABL inhibition in murine xenograft models of Ph-like acute lymphoblastic leukemia. Blood. 2017;129:177–87.
121. Leiker AJ, DeGraff W, Choudhuri R, Sowers AL, Thetford A, Cook JA, et al. Radiation enhancement of head and neck squamous cell carcinoma by the dual PI3K/mTOR inhibitor PF-05212384. Clin Cancer Res. 2015;21:2792–801.
122. Liu T, Sun Q, Li Q, Yang H, Zhang Y, Wang R, et al. Dual PI3K/mTOR inhibitors, GSK2126458 and PKI-587, suppress tumor progression and increase radiosensitivity in nasopharyngeal carcinoma. Mol Cancer Ther. 2015;14:429–39.
123. D'Amato V, Rosa R, D'Amato C, Formisano L, Marciano R, Nappi L, et al. The dual PI3K/mTOR inhibitor PKI-587 enhances sensitivity to cetuximab in EGFR-resistant human head and neck cancer models. Br J Cancer. 2014;110:2887–95.
124. Knight SD, Adams ND, Burgess JL, Chaudhari AM, Darcy MG, Donatelli CA, et al. Discovery of GSK2126458, a highly potent inhibitor of PI3K and the mammalian target of rapamycin. ACS Med Chem Lett. 2010;1:39–43.
125. McKinnon T, Venier R, Yohe M, Sindiri S, Gryder BE, Shern JF, et al. Functional screening of FGFR4-driven tumorigenesis identifies PI3K/mTOR inhibition as a therapeutic strategy in rhabdomyosarcoma. Oncogene. 2018;37:2630–44.
126. Zhou J, Toh SH, Chan ZL, Quah JY, Chooi JY, Tan TZ, et al. A loss-of-function genetic screening reveals synergistic targeting of AKT/mTORand WTN/β-catenin pathways for treatment of AML with high PRL-3 phosphatase. J Hematol Oncol. 2018;11:36.
127. Fan Q, Aksoy O, Wong RA, Ilkhanizadeh S, Novotny CJ, Gustafson WC, et al. A kinase inhibitor targeted to mTORC1 drives regression in glioblastoma. Cancer Cell. 2017;31:424–35.
128. Oricchio E, Katanayeva N, Donaldson MC, Sungalee S, Pasion JP, Béguelin W, et al. Genetic and epigenetic inactivation of SESTRIN1 controls mTORC1 and response to EZH2 inhibition in follicular lymphoma. Sci Transl Med. 2017;9:eaak9969.
129. Hua H, Zhang H, Kong Q, Wang J, Jiang Y. Complex roles of the old drug aspirin in cancer chemoprevention and therapy. Med Res Rev. 2019;39:114–45.
130. Boyle KA, Van Wickle J, Hill RB, Marchese A, Kalyanaraman B, Dwinell MB. Mitochondria-targeted drugs stimulate mitophagy and abrogate colon cancer cell proliferation. J Biol Chem. 2018;293:14891–904.
131. Ling S, Xie H, Yang F, Shan Q, Dai H, Zhuo J, et al. Metformin potentiates the effect of arsenic trioxide suppressing intrahepatic cholangiocarcinoma: roles of p38 MAPK, ERK3, and mTORC1. J Hematol Oncol. 2017;10:59.
132. Lin F, de Gooijer MC, Hanekamp D, Chandrasekaran G, Buil LC, Thota N, et al. PI3K-mTOR pathway inhibition exhibits efficacy against high-grade glioma in clinically relevant mouse models. Clin Cancer Res. 2017;23:1286–98.
133. Hurvitz SA, Kalous O, Conklin D, Desai AJ, Dering J, Anderson L, et al. In vitro activity of the mTOR inhibitor everolimus, in a large panel of breast cancer cell lines and analysis for predictors of response. Breast Cancer Res Treat. 2015;149:669–80.
134. Tang SC, Sparidans RW, Cheung KL, Fukami T, Durmus S, Wagenaar E, et al. P-glycoprotein, CYP3A, and plasma carboxylesterase determine brain and blood disposition of the mTOR Inhibitor everolimus (Afinitor) in mice. Clin Cancer Res. 2014;20:3133–45.
135. Wu CP, Murakami M, Hsiao SH, Chou AW, Li YQ, Huang YH, et al. Overexpression of ATP-binding cassette subfamily G member 2 confers resistance to phosphatidylinositol 3-kinase inhibitor PF-4989216 in cancer cells. Mol Pharm. 2017;14:2368–77.
136. Becker CM, Oberoi RK, McFarren SJ, Muldoon DM, Pafundi DH, Pokorny JL, et al. Decreased affinity for efflux transporters increases brain penetrance and molecular targeting of a PI3K/mTOR inhibitor in a mouse model of glioblastoma. Neuro Oncol. 2015;17:1210–9.
137. Begicevic RR, Falasca M. ABC transporters in cancer stem cells: beyond chemoresistance. Int J Mol Sci. 2017;18:e2362.
138. Katsuno Y, Meyer DS, Zhang Z, Shokat KM, Akhurst RJ, Miyazono K, et al. Chronic TGF-β exposure drives stabilized EMT, tumor stemness, and cancer drug resistance with vulnerability to bitopic mTOR inhibition. Sci Signal. 2019;12:eaau8544.
139. Francipane MG, Lagasse E. Therapeutic potential of mTOR inhibitors for targeting cancer stem cells. Br J Clin Pharmacol. 2016;82:1180–8.
140. Karthik GM, Ma R, Lövrot J, Kis LL, Lindh C, Blomquist L, et al. mTOR inhibitors counteract tamoxifen-induced activation of breast cancer stem cells. Cancer Lett. 2015;367:76–87.
141. de Graffenried LA, Friedrichs WE, Russell DH, Donzis EJ, Middleton AK, Silva JM, et al. Inhibition of mTOR activity restores tamoxifen response in breast cancer cells with aberrant Akt Activity. Clin Cancer Res. 2004;10:8059–67.

142. Kolev VN, Wright QG, Vidal CM, Ring JE, Shapiro IM, Ricono J, et al. PI3K/mTOR dual inhibitor VS-5584 preferentially targets cancer stem cells. Cancer Res. 2015;75:446–55.
143. Francipane MG, Lagasse E. Selective targeting of human colon cancer stem-like cells by the mTOR inhibitor Torin-1. Oncotarget. 2013;4:1948–62.
144. Fourneaux B, Bourdon A, Dadone B, Lucchesi C, Daigle SR, Richard E, et al. Identifying and targeting cancer stem cells in leiomyosarcoma: prognostic impact and role to overcome secondary resistance to PI3K/mTOR inhibition. J Hematol Oncol. 2019;12:11.
145. Tan J, Li Z, Lee PL, Guan P, Aau MY, Lee ST, et al. PDK1 signaling toward PLK1-MYC activation confers oncogenic transformation, tumor-initiating cell activation, and resistance to mTOR-targeted therapy. Cancer Discov. 2013;3:1156–71.
146. Venkatesan S, Hoogstraat M, Caljouw E, Pierson T, Spoor JK, Zeneyedpour L, et al. TP53 mutated glioblastoma stem-like cell cultures are sensitive to dual mTORC1/2 inhibition while resistance in TP53 wild type cultures can be overcome by combined inhibition of mTORC1/2 and Bcl-2. Oncotarget. 2016;7:58435–44.
147. Bhola NE, Jansen VM, Koch JP, Li H, Formisano L, Williams JA, et al. Treatment of triple-negative breast cancer with TORC1/2 inhibitors sustains a drug-resistant and Notch-dependent cancer stem cell population. Cancer Res. 2016;76:440–52.
148. Belmont PJ, Jiang P, McKee TD, Xie T, Isaacson J, Baryla NE, et al. Resistance to dual blockade of the kinases PI3K and mTOR in KRAS-mutant colorectal cancer models results in combined sensitivity to inhibition of the receptor tyrosine kinase EGFR. Sci Signal. 2014;7:ra107.
149. Merrick WC. Cap-dependent and cap-independent translation in eukaryotic systems. Gene. 2004;332:1–11.
150. Shatsky IN, Terenin IM, Smirnova VV, Andreev DE. Cap-independent translation: what's in a name? Trends Biochem Sci. 2018;43:882–95.
151. Ducker GS, Atreya CE, Simko JP, Hom YK, Matli MR, Benes CH, et al. Incomplete inhibition of phosphorylation of 4E-BP1 as a mechanism of primary resistance to ATP-competitive mTOR inhibitors. Oncogene. 2014;33:1590–600.
152. Wang J, Ye Q, Cao Y, Guo Y, Huang X, Mi W, et al. Snail determines the therapeutic response to mTOR kinase inhibitors by transcriptional repression of 4E-BP1. Nat Commun. 2017;8:2207.
153. Robichaud N, del Rincon SV, Huor B, Alain T, Petruccelli LA, Hearnden J, et al. Phosphorylation of eIF4E promotes EMT and metastasis via translational control of SNAIL and MMP-3. Oncogene. 2015;34:2032–42.
154. Cope CL, Gilley R, Balmanno K, Sale MJ, Howarth KD, Hampson M, et al. Adaptation to mTOR kinase inhibitors by amplification of eIF4E to maintain cap-dependent translation. J Cell Sci. 2014;127:788–800.
155. D'Abronzo LS, Bose S, Crapuchettes ME, Beggs RE, Vinall RL, Tepper CG, et al. The androgen receptor is a negative regulator of eIF4E phosphorylation at S209: implications for the use of mTOR inhibitors in advanced prostate cancer. Oncogene. 2017;36:6359–73.
156. Wang X, Yue P, Chan CB, Ye K, Ueda T, Watanabe-Fukunaga R, et al. Inhibition of mammalian target of rapamycin induces phosphatidylinositol 3-kinase-dependent and Mnk-mediated eukaryotic translation initiation factor 4E phosphorylation. Mol Cell Biol. 2007;27:7405–13.
157. Wen Q, Wang W, Luo J, Chu S, Chen L, Xu L, et al. CGP57380 enhances efficacy of RAD001 in non-small cell lung cancer through abrogating mTOR inhibition-induced phosphorylation of eIF4E and activating mitochondrial apoptotic pathway. Oncotarget. 2016;7:27787–801.
158. Shuda M, Velásquez C, Cheng E, Cordek DG, Kwun HJ, Chang Y, et al. CDK1 substitutes for mTOR kinase to activate mitotic cap-dependent protein translation. Proc Natl Acad Sci U S A. 2015;112:5875–82.
159. Choi SH, Martinez TF, Kim S, Donaldson C, Shokhirev MN, Saghatelian A, et al. CDK12 phosphorylates 4E-BP1 to enable mTORC1-dependent translation and mitotic genome stability. Genes Dev. 2019;33:418–35.
160. Shin S, Wolgamott L, Tcherkezian J, Vallabhapurapu S, Yu Y, Roux PP, et al. Glycogen synthase kinase-3β positively regulates protein synthesis and cell proliferation through the regulation of translation initiation factor 4E-binding protein 1. Oncogene. 2014;33:1690–9.
161. Grabiner BC, Nardi V, Birsoy K, Possemato R, Shen K, Sinha S, et al. A diverse array of cancer-associated MTOR mutations are hyperactivating and can predict rapamycin sensitivity. Cancer Discov. 2014;4:554–63.
162. Hassan B, Akcakanat A, Sangai T, Evans KW, Adkins F, Eterovic AK, et al. Catalytic mTOR inhibitors can overcome intrinsic and acquired resistance to allosteric mTOR inhibitors. Oncotarget. 2014;5:8544–57.
163. Lawrence MS, Stojanov P, Mermel CH, Robinson JT, Garraway LA, Golub TR, et al. Discovery and saturation analysis of cancer genes across 21 tumour types. Nature. 2014;505:495–501.
164. Donnella HJ, Webber JT, Levin RS, Camarda R, Momcilovic O, Bayani N, et al. Kinome rewiring reveals AURKA limits PI3K-pathway inhibitor efficacy in breast cancer. Nat Chem Biol. 2018;14:768–77.
165. Balaji S, Ahmed M, Lorence E, Yan F, Nomie K, Wang M. NF-κB signaling and its relevance to the treatment of mantle cell lymphoma. J Hematol Oncol. 2018;11:83.
166. Janku F, Wheler JJ, Naing A, Falchook GS, Hong DS, Stepanek VM, et al. PIK3CA mutation H1047R is associated with response to PI3K/AKT/mTOR signaling pathway inhibitors in early-phase clinical trials. Cancer Res. 2013;73:276–84.
167. Meric-Bernstam F, Akcakanat A, Chen H, Do KA, Sangai T, Adkins F, et al. PIK3CA/PTEN mutations and Akt activation as markers of sensitivity to allosteric mTOR inhibitors. Clin Cancer Res. 2012;18:1777–89.
168. Citi V, Del Re M, Martelli A, Calderone V, Breschi MC, Danesi R. Phosphorylation of AKT and ERK1/2 and mutations of PIK3CA and PTEN are predictive of breast cancer cell sensitivity to everolimus in vitro. Cancer Chemother Pharmacol. 2018;81:745-54.
169. Di Nicolantonio F, Arena S, Tabernero J, Grosso S, Molinari F, Macarulla T, et al. Deregulation of the PI3K and KRAS signaling pathways in human cancer cells determines their response to everolimus. J Clin Invest. 2010;120:2858–66.
170. Mohan S, Vander Broek R, Shah S, Eytan DF, Pierce ML, Carlson SG, et al. MEK inhibitor PD-0325901 overcomes resistance to PI3K/mTOR inhibitor PF-5212384 and potentiates antitumor effects in human head and neck squamous cell carcinoma. Clin Cancer Res. 2015;21:3946–56.
171. Xu DQ, Toyoda H, Qi L, Morimoto M, Hanaki R, Iwamoto S, et al. Induction of MEK/ERK activity by AZD8055 confers acquired resistance in neuroblastoma. Biochem Biophys Res Commun. 2018;499:425–32.
172. Olmez I, Brenneman B, Xiao A, Serbulea V, Benamar M, Zhang Y, et al. Combined CDK4/6 and mTOR inhibition is synergistic against glioblastoma via multiple mechanisms. Clin Cancer Res. 2017;23:6958–68.
173. Song X, Liu X, Wang H, Wang J, Qiao Y, Cigliano A, et al. Combined CDK4/6 and pan-mTOR inhibition is synergistic against intrahepatic cholangiocarcinoma. Clin Cancer Res. 2019;25:403–13.
174. Hai J, Liu S, Bufe L, Do K, Chen T, Wang X, et al. Synergy of WEE1 and mTOR inhibition in mutant KRAS-driven lung cancers. Clin Cancer Res. 2017;23:6993–7005.
175. Fujishita T, Kojima Y, Kajino-Sakamoto R, Taketo MM, Aoki M. Tumor microenvironment confers mTOR inhibitor resistance in invasive intestinal adenocarcinoma. Oncogene. 2017;36:6480–9.
176. Park YL, Kim HP, Cho YW, Min DW, Cheon SK, Lim YJ, et al. Activation of WNT/β-catenin signaling results in resistance to a dual PI3K/mTOR inhibitor in colorectal cancer cells harboring PIK3CA mutations. Int J Cancer. 2019;144:389–401.
177. Momcilovic M, Bailey ST, Lee JT, Fishbein MC, Braas D, Go J, et al. The GSK3 signaling axis regulates adaptive glutamine metabolism in lung squamous cell carcinoma. Cancer Cell. 2018;33:905–21.
178. Tanaka K, Sasayama T, Irino Y, Takata K, Nagashima H, Satoh N, et al. Compensatory glutamine metabolism promotes glioblastoma resistance to mTOR inhibitor treatment. J Clin Invest. 2015;125:1591–602.
179. Makinoshima H, Umemura S, Suzuki A, Nakanishi H, Maruyama A, Udagawa H, et al. Metabolic determinants of sensitivity to phosphatidylinositol 3-kinase pathway inhibitor in small-cell lung carcinoma. Cancer Res. 2018;78:2179–90.
180. Ben-Sahra I, Hoxhaj G, Ricoult SJH, Asara JM, Manning BD. mTORC1 induces purine synthesis through control of the mitochondrial tetrahydrofolate cycle. Science. 2016;351:728–33.
181. Robitaille AM, Christen S, Shimobayashi M, Cornu M, Fava LL, Moes S, et al. Quantitative phosphoproteomics reveal mTORC1 activates de novo pyrimidine synthesis. Science. 2013;339:1320–3.
182. Ben-Sahra I, Howell JJ, Asara JM, Manning BD. Stimulation of de novo pyrimidine synthesis by growth signaling through mTOR and S6K1. Science. 2013;339:1323–8.
183. Cao J, Huang W. Compensatory increase of transglutaminase 2 is responsible for resistance to mTOR inhibitor treatment. PLOS One. 2016;11:e0149388.
184. Mehta K, Kumar A, Kim HI. Transglutaminase 2: a multi-tasking protein in the complex circuitry of inflammation and cancer. Biochem Pharmacol. 2010;80:1921–9.

185. Park D, Choi SS, Ha KS. Transglutaminase 2: a multi-functional protein in multiple subcellular compartments. Amino Acids. 2010;39:619–31.
186. Morita M, Prudent J, Basu K, Goyon V, Katsumura S, Hulea L, et al. mTOR controls mitochondrial dynamics and cell survival via MTFP1. Mol Cell. 2017;67:922–35.
187. Lew S, Chamberlain RS. Risk of metabolic complications in patients with solid tumors treated with mTOR inhibitors: meta-analysis. Anticancer Res. 2016;36:1711–8.
188. Johnston PB, Pinter-Brown LC, Warsi G, White K, Ramchandren R. Phase 2 study of everolimus for relapsed or refractory classical Hodgkin lymphoma. Exp Hematol Oncol. 2018;7:12.
189. Pearson AD, Federico SM, Aerts I, Hargrave DR, DuBois SG, Iannone R, et al. A phase 1 study of oral ridaforolimus in pediatric patients with advanced solid tumors. Oncotarget. 2016;7:84736–47.
190. Mita MM, Mita AC, Chu QS, Rowinsky EK, Fetterly GJ, Goldston M, et al. Phase I trial of the novel mammalian target of rapamycin inhibitor deforolimus (AP23573; MK-8669) administered intravenously daily for 5 days every 2 weeks to patients with advanced malignancies. J Clin Oncol. 2008;26:361–7.
191. Graham L, Banda K, Torres A, Carver BS, Chen Y, Pisano K, et al. A phase II study of the dual mTOR inhibitor MLN0128 in patients with metastatic castration resistant prostate cancer. Invest New Drugs. 2018;36:458–67.
192. Antonuzzo L, Del Re M, Barucca V, Spada F, Meoni G, Restante G, et al. Critical focus on mechanisms of resistance and toxicity of m-TOR inhibitors in pancreatic neuroendocrine tumors. Cancer Treat Rev. 2017;57:28–35.
193. Carlo MI, Molina AM, Lakhman Y, Patil S, Woo K, DeLuca J, et al. A phase Ib study of BEZ235, a dual inhibitor of phosphatidylinositol 3-kinase (PI3K) and mammalian target of rapamycin (mTOR), in patients with advanced renal cell carcinoma. Oncologist. 2016;21:787–8.
194. Powles T, Lackner MR, Oudard S, Escudier B, Ralph C, Brown JE, et al. Randomized open-label phase II trial of apitolisib (GDC-0980), a novel inhibitor of the PI3K/mammalian target of rapamycin pathway, versus everolimus in patients with metastatic renal cell carcinoma. J Clin Oncol. 2016;34:1660–8.
195. Grilley-Olson JE, Bedard PL, Fasolo A, Cornfeld M, Cartee L, Razak AR, et al. A phase Ib dose-escalation study of the MEK inhibitor trametinib in combination with the PI3K/mTOR inhibitor GSK2126458 in patients with advanced solid tumors. Invest New Drugs. 2016;34:740–9.
196. Britten CD, Adjei AA, Millham R, Houk BE, Borzillo G, Pierce K, et al. Phase I study of PF-04691502, a small-molecule, oral, dual inhibitor of PI3K and mTOR, in patients with advanced cancer. Invest New Drugs. 2014;32:510–7.
197. Yao JC, Shah MH, Ito T, Bohas CL, Wolin EM, Van Cutsem E, et al. Everolimus for advanced pancreatic neuroendocrine tumors. N Engl J Med. 2011;364:514–23.
198. Wahl M, Chang SM, Phillips JJ, Molinaro AM, Costello JF, Mazor T, et al. Probing the phosphatidylinositol 3-kinase/mammalian target of rapamycin pathway in gliomas: a phase 2 study of everolimus for recurrent adult low-grade gliomas. Cancer. 2017;123:4631–9.
199. Lau DK, Tay RY, Yeung YH, Chionh F, Mooi J, Murone C, et al. Phase II study of everolimus (RAD001) monotherapy as first-line treatment in advanced biliary tract cancer with biomarker exploration: the RADiChol Study. Br J Cancer. 2018;118:966–71.
200. Schneider TC, de Wit D, Links TP, van Erp NP, van der Hoeven JJ, Gelderblom H, et al. Everolimus in patients with advanced follicular-derived thyroid cancer: results of a phase II clinical trial. J Clin Endocrinol Metab. 2017;102:698–707.
201. Colombo N, McMeekin DS, Schwartz PE, Sessa C, Gehrig PA, Holloway R, et al. Ridaforolimus as a single agent in advanced endometrial cancer: results of a single-arm, phase 2 trial. Br J Cancer. 2013;108:1021–6.
202. Brown JR, Hamadani M, Hayslip J, Janssens A, Wagner-Johnston N, Ottmann O, et al. Voxtalisib (XL765) in patients with relapsed or refractory non-Hodgkin lymphoma or chronic lymphocytic leukaemia: an open-label, phase 2 trial. Lancet Haematol. 2018;5:e170–80.
203. Powles T, Wheater M, Din O, Geldart T, Boleti E, Stockdale A, et al. A randomised phase 2 study of AZD2014 versus everolimus in patients with VEGF-refractory metastatic clear cell renal cancer. Eur Urol. 2016;69:450–6.
204. Del Campo JM, Birrer M, Davis C, Fujiwara K, Gollerkeri A, Gore M, et al. A randomized phase II non-comparative study of PF-04691502 and gedatolisib (PF-05212384) in patients with recurrent endometrial cancer. Gynecol Oncol. 2016;142:62–9.
205. Zhou J, Toh SH, Chan ZL, Quah JY, Chooi JY, Tan TZ, et al. A loss-of-function genetic screening reveals synergistic targeting of AKT/mTOR and WTN/β-catenin pathways for treatment of AML with high PRL-3 phosphatase. J Hematol Oncol. 2018;11:36.
206. Yardley DA, Noguchi S, Pritchard KI, Burris HA 3rd, Baselga J, Gnant M, et al. Everolimus plus exemestane in postmenopausal patients with HR(+) breast cancer: BOLERO-2 final progression-free survival analysis. Adv Ther. 2013;30:870–84.
207. Piccart M, Hortobagyi GN, Campone M, Pritchard KI, Lebrun F, Ito Y, et al. Everolimus plus exemestane for hormone-receptor-positive, human epidermal growth factor receptor-2-negative advanced breast cancer: overall survival results from BOLERO-2†. Ann Oncol. 2014;25:2357–62.
208. O'Shaughnessy J, Thaddeus Beck J, Royce M. Everolimus-based combination therapies for HR+, HER2- metastatic breast cancer. Cancer Treat Rev. 2018;69:204–14.
209. Colon-Otero G, Weroha SJ, Foster NR, Haluska P, Hou X, Wahner-Hendrickson AE, et al. Phase 2 trial of everolimus and letrozole in relapsed estrogen receptor-positive high-grade ovarian cancers. Gynecol Oncol. 2017;146:64–8.
210. Hurvitz SA, Dalenc F, Campone M, O'Regan RM, Tjan-Heijnen VC, Gligorov J, et al. A phase 2 study of everolimus combined with trastuzumab and paclitaxel in patients with HER2-overexpressing advanced breast cancer that progressed during prior trastuzumab and taxane therapy. Breast Cancer Res Treat. 2013;141:437–46.
211. Singh J, Novik Y, Stein S, Volm M, Meyers M, Smith J, et al. Phase 2 trial of everolimus and carboplatin combination in patients with triple negative metastatic breast cancer. Breast Cancer Res. 2014;16:R32.
212. Barnes JA, Jacobsen E, Feng Y, Freedman A, Hochberg EP, LaCasce AS, et al. Everolimus in combination with rituximab induces complete responses in heavily pretreated diffuse large B-cell lymphoma. Haematologica. 2013;98:615–9.
213. Johnston PB, LaPlant B, McPhail E, Habermann TM, Inwards DJ, Micallef IN, et al. Everolimus combined with R-CHOP-21 for new, untreated, diffuse large B-cell lymphoma (NCCTG 1085 [Alliance]): safety and efficacy results of a phase 1 and feasibility trial. Lancet Haematol. 2016;3:e309–16.
214. Basu B, Krebs MG, Sundar R, Wilson RH, Spicer J, Jones R, et al. Vistusertib (dual m-TORC1/2 inhibitor) in combination with paclitaxel in patients with high-grade serous ovarian and squamous non-small-cell lung cancer. Ann Oncol. 2018;29:1918–25.
215. Schwartz GK, Tap WD, Qin LX, Livingston MB, Undevia SD, Chmielowski B, et al. Cixutumumab and temsirolimus for patients with bone and soft-tissue sarcoma: a multicentre, open-label, phase 2 trial. Lancet Oncol. 2013;14:371–82.
216. Naing A, Kurzrock R, Burger A, Gupta S, Lei X, Busaidy N, et al. Phase I trial of cixutumumab combined with temsirolimus in patients with advanced cancer. Clin Cancer Res. 2011;17:6052–60.
217. Harzstark AL, Small EJ, Weinberg VK, Sun J, Ryan CJ, Lin AM, et al. A phase 1 study of everolimus and sorafenib for metastatic clear cell renal cell carcinoma. Cancer. 2011;117:4194–200.
218. Grignani G, Palmerini E, Ferraresi V, D'Ambrosio L, Bertulli R, Asaftei SD, et al. Sorafenib and everolimus for patients with unresectable high-grade osteosarcoma progressing after standard treatment: a non-randomised phase 2 clinical trial. Lancet Oncol. 2015;16:98–107.
219. Schiff D, Jaeckle KA, Anderson SK, Galanis E, Giannini C, Buckner JC, et al. Phase 1/2 trial of temsirolimus and sorafenib in the treatment of patients with recurrent glioblastoma: North Central Cancer Treatment Group Study/Alliance N0572. Cancer. 2018;124:1455–63.
220. Rathkopf DE, Larson SM, Anand A, Morris MJ, Slovin SF, Shaffer DR, et al. Everolimus combined with gefitinib in patients with metastatic castration-resistant prostate cancer: phase 1/2 results and signaling pathway implications. Cancer. 2015;121:3853–61.
221. Davis ID, Long A, Yip S, Espinoza D, Thompson JF, Kichenadasse G, et al. EVERSUN: a phase 2 trial of alternating sunitinib and everolimus as first-line therapy for advanced renal cell carcinoma. Ann Oncol. 2015;26:1118–23.
222. Knox JJ, Barrios CH, Kim TM, Cosgriff T, Srimuninnimit V, Pittman K, et al. Final overall survival analysis for the phase II RECORD-3 study of first-line everolimus followed by sunitinib versus first-line sunitinib followed by everolimus in metastatic RCC. Ann Oncol. 2017;28:1339–45.
223. Stacchiotti S, Morosi C, Lo Vullo S, Casale A, Palassini E, Frezza AM, et al. Imatinib and everolimus in patients with progressing advanced chordoma: a phase 2 clinical study. Cancer. 2018;124:4056–63.
224. Schram AM, Gandhi L, Mita MM, Damstrup L, Campana F, Hidalgo M, et al. A phase Ib dose-escalation and expansion study of the oral MEK inhibitor

pimasertib and PI3K/MTOR inhibitor voxtalisib in patients with advanced solid tumours. Br J Cancer. 2018;119:1471–6.
225. Xu H, Jiao Y, Qin S, Zhao W, Chu Q, Wu K. Organoid technology in disease modelling, drug development, personalized treatment and regeneration medicine. Exp Hematol Oncol. 2018;7:30.
226. Xu H, Lyu X, Yi M, Zhao W, Song Y, Wu K. Organoid technology and applications in cancer research. J Hematol Oncol. 2018;11:116.
227. Chen Z, Huang W, Tian T, Zang W, Wang J, Liu Z, et al. Characterization and validation of potential therapeutic targets based on the molecular signature of patient-derived xenografts in gastric cancer. J Hematol Oncol. 2018;11:20.
228. Frenel JS, Carreira S, Goodall J, Roda D, Perez-Lopez R, Tunariu N, et al. Serial next-generation sequencing of circulating cell-free DNA evaluating tumor clone response to molecularly targeted drug administration. Clin Cancer Res. 2015;21:4586–96.
229. Yang M, Topaloglu U, Petty WJ, Pagni M, Foley KL, Grant SC, et al. Circulating mutational portrait of cancer: manifestation of aggressive clonal events in both early and late stages. J Hematol Oncol. 2017;10:100.
230. Rossi G, Ignatiadis M. Promises and pitfalls of using liquid biopsy for precision medicine. Cancer Res. 2019. https://doi.org/10.1158/0008-5472.

Publisher's Note

frontiers in Immunology

MINI REVIEW
published: 27 March 2018
doi: 10.3389/fimmu.2018.00578

mTOR at the Transmitting and Receiving Ends in Tumor Immunity

Yakir Guri[1], Thierry M. Nordmann[2] and Jason Roszik[3,4]**

[1] Biozentrum, University of Basel, Basel, Switzerland, [2] Department of Dermatology, University Hospital Zurich, Zurich, Switzerland, [3] Department of Melanoma Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, United States, [4] Department of Genomic Medicine, The University of Texas MD Anderson Cancer Center, Houston, TX, United States

Cancer is a complex disease and a leading cause of death worldwide. Immunity is critical for cancer control. Cancer cells exhibit high mutational rates and therefore altered self or neo-antigens, eliciting an immune response to promote tumor eradication. Failure to mount a proper immune response leads to cancer progression. mTOR signaling controls cellular metabolism, immune cell differentiation, and effector function. Deregulated mTOR signaling in cancer cells modulates the tumor microenvironment, thereby affecting tumor immunity and possibly promoting carcinogenesis.

Keywords: signaling, tumorigenesis, metabolism, immunity, immunotherapy, rapamycin

OPEN ACCESS

Edited by:
Sherven Sharma, VA Greater Los Angeles Healthcare System (VHA), United States

Reviewed by:
Kawaljit Kaur, University of California, Los Angeles, United States
Amedeo Amedei, Università degli Studi di Firenze, Italy

****Correspondence:***
Yakir Guri
yakir.guri@unibas.ch;
Jason Roszik
jroszik@mdanderson.org

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *14 December 2017*
Accepted: *07 March 2018*
Published: *27 March 2018*

Citation:
Guri Y, Nordmann TM and Roszik J (2018) mTOR at the Transmitting and Receiving Ends in Tumor Immunity. Front. Immunol. 9:578. doi: 10.3389/fimmu.2018.00578

INTRODUCTION

Tumor bulk is a mass containing heterogeneous cell populations including malignant cancer cells, non-malignant cells, and supporting stroma (1). In addition to tumor cells, non-malignant cells and the supporting stroma play a dynamic and possibly tumor promoting role (2). Non-malignant cells in the tumor microenvironment include cells of the lymphoid and myeloid immune system (3). The supporting stroma is largely composed of cancer-associated fibroblasts (CAFs), vascular and lymphatic endothelial cells, and pericytes. Cells within the tumor "communicate" by secretion of various factors to the tumor microenvironment, including matrix remodeling enzymes, cytokines, chemokines, growth factors, and metabolites (4, 5). This interplay between malignant, non-malignant, and stromal cells has functional consequences on tumor progression.

Target Of Rapamycin (TOR) is an evolutionarily conserved serine/threonine protein kinase. TOR controls cellular metabolism and growth and functions in two complexes: TOR Complex 1 (TORC1) and TORC2 (6, 7) (**Figure 1**). Mammalian TORC1 (mTORC1) comprises mTOR, mammalian lethal with sec-13 protein 8 (mLST8), and regulatory-associated protein of mammalian target of rapamycin (RAPTOR). mTORC1 is activated by growth factors, nutrients (amino acids), and cellular energy (8, 9), and is allosterically inhibited by rapamycin (10). Various growth factors regulate mTORC1 *via* a heterotrimeric tuberous sclerosis complex (TSC) complex. Growth factors bind receptor tyrosine kinases (RTKs) and activate Phosphatidylinositol-4,5-Bisphosphate 3-Kinase (PI3K), which generates Phosphatidylinositol-3,4,5-Trisphosphate (PIP3) (11). PI3K activity is counteracted by the tumor suppressor, phosphatase, and Tensin Homolog Deleted on Chromosome 10 (PTEN). mTORC1 promotes anabolic processes, such as protein and nucleotide synthesis and inhibits catabolic processes, such as autophagy (12–14). mTORC2 contains mTOR, mLST8, mammalian stress-activated map kinase-interacting protein 1 (mSIN1), and Rapamycin-Insensitive Companion of mTOR (RICTOR), and is activated by growth factors in association with ribosomes (15) (**Figure 1**). mTORC1 and mTORC2 are frequently activated in human cancers and, as discussed below, reported to modulate the tumor microenvironment or respond to its changes.

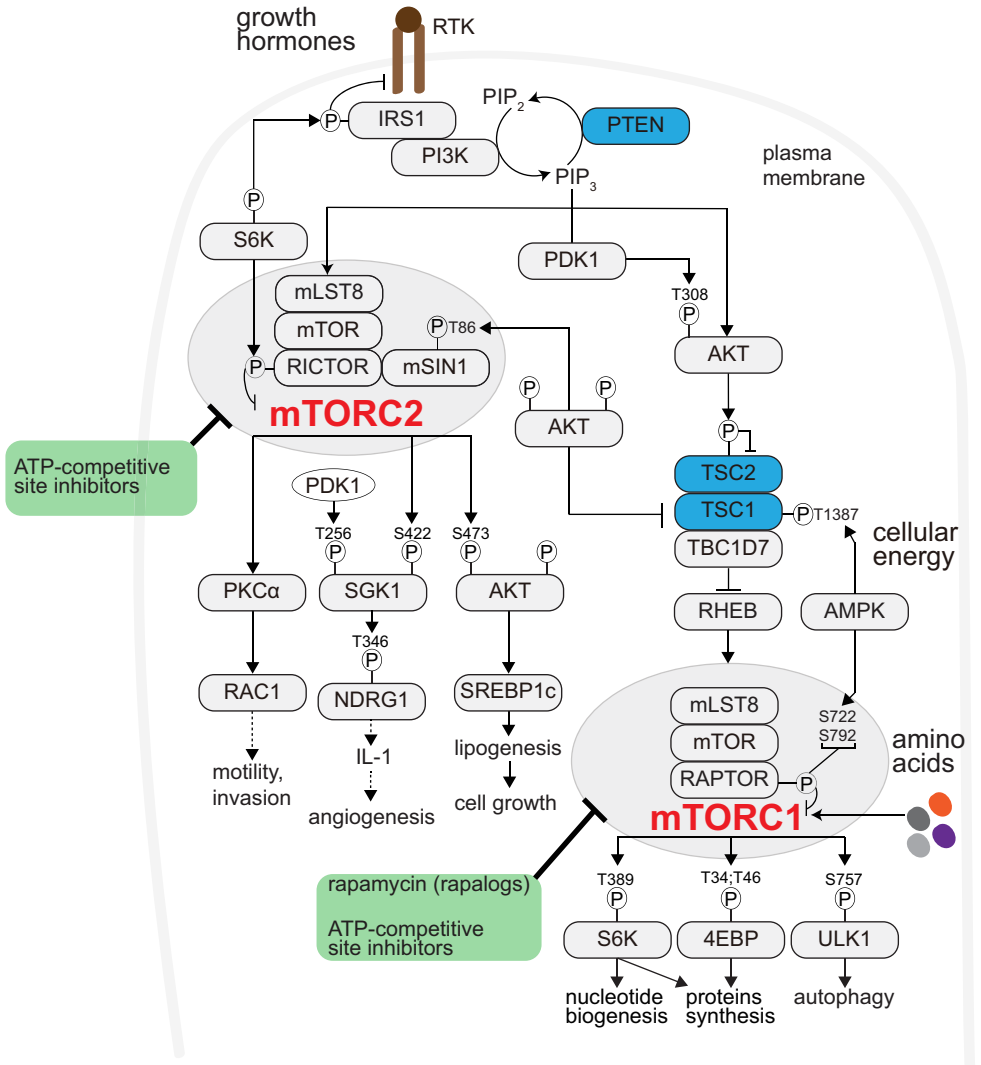


FIGURE 1 | mTOR signaling promotes anabolism. Receptor Tyrosine Kinases (RTKs)- Phosphatidyl-Inositol-4,5-bisphosphate 3-Kinase (PI3K) activated by growth factor (like insulin). PI3K generates phosphatidylinositol-3,4,5-trisphosphate (PIP3) from the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PIP2). Phosphatase and Tensin Homolog Deleted on Chromosome 10 (PTEN) counteracts PI3K activity (restoring PIP3 to PIP2). PIP3 recruits to the plasma membrane and activates phosphoinositide-dependent kinase 1 (PDK1) and AKT. PDK1 phosphorylates and activates AKT (pAKT-Thr308). pAKT-Thr308 phosphorylates and inhibits the TSC complex. The TSC complex, composed of tuberous sclerosis complex 1 (TSC1) and TSC2 and TRE2-BUB2-CDC16 domain family member 7 (TBC1D7), activates the lysosomal RAS homolog enriched in brain (RHEB). RHEB interacts with and activates mTORC1. mTORC1 comprises mTOR, mammalian lethal with sec-13 protein 8 (mLST8), and regulatory-associated protein of mammalian target of rapamycin (RAPTOR). mTORC1 can also be activated by nutrients (such as amino acids). Cellular energy status also regulates mTORC1 through AMPK-mediated TSC or RAPTOR phosphorylation. mTORC1 promotes anabolism, among others, through ribosomal protein S6 kinase (S6K), eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4EBP1), and blocks cellular catabolism through Unc-51-like kinase 1 (ULK1). Through S6K-mediated IRS1 phosphorylation, mTORC1 negatively regulates mTORC2-AKT signaling. Rapamycin and its analogs (so-called rapalogues) acutely inhibit mTORC1 allosterically. The ATP-site competitive inhibitor(s) potently block both mTORC1 and mTORC2 signaling. mTORC2 is also activated by RTKs, and consists of mTOR, mLST8, mammalian stress-activated map kinase-interacting protein 1 (mSIN1), and rapamycin-insensitive companion of mTOR (RICTOR). mTORC2 regulates the AGC kinase family members AKT, serum/glucocorticoid-regulated kinase (SGK), and protein kinase C (PKC). Prolonged rapamycin administration may block mTORC2 activity.

CANCER CELL-INTRINSIC mTOR ACTIVATION MODULATING THE TUMOR MICROENVIRONMENT

Oncogenic mutations drive tumorigenesis by activating various growth controlling signaling pathways (16). The PI3K–mTOR–AKT signaling pathway is activated in the majority of tumors, due to upstream oncogenic mutation(s). Alternatively, parallel growth controlling (oncogenic) pathways, such as the MEK–ERK, may also activate PI3K–mTOR–AKT signaling (12). Either way, PI3K–mTOR–AKT activation promotes cell growth and proliferation (**Figure 1**). In addition to the cell-intrinsic growth-promoting effect, PI3K–mTOR–AKT activation appears to alter the tumor microenvironment.

T LYMPHOCYTES

T cells play a critical role in adaptive and innate immunity. Antigen recognition and adaptive immunity involves, among others, CD4 + and CD8 + T cells. While tumor eradication is largely mediated by cytotoxic CD8 + T lymphocytes (CTL), CD4 + T cells are critical in regulating and propagating the immune response, hence referred to as T helper cells (Th) (17). In solid tumors, the extent of T-cell infiltration is an important prognostic determinate. Increased CD4 + and CD8 + T-cell levels are associated with an improved clinical outcome (18). In colorectal tumors, increased density of T cells (i.e., Th1 adaptive immunity) correlated with reduced tumor recurrence, and provided a better prognostic tool than conventional histopathological methods (19). Conversely, tumors with a higher density of immune-suppressive cells (such T regulatory cells, as discussed below) exhibit a worse prognosis, in colorectal (19) and other tumor types (20). Thus, adaptive immunity plays a critical role in tumor progression and prognosis.

Various cytokines and chemokines attract immune cells to the site of inflammation (21). In addition to cytokines and chemokines, also metabolites in the tumor microenvironment (some of which are secreted by cancer cells) activate immune cells (22). Non-Alcoholic Fatty Liver Disease (NAFLD) is a metabolic disorder and a risk factor for hepatocellular carcinoma (HCC) (23). In NAFLD, increased linoleic acid levels disrupt adaptive immunity, specifically by depleting CD4 + T cells, which in turn promotes HCC (24). These data indicate that a metabolite accumulating in the tumor microenvironment may affect neighboring T cells, disturb their function, and promote cancer. It is not fully understood what regulates linoleic acid accumulation, but hepatic fatty acid (FA) synthesis (including linoleic acid) is controlled by mTORC2 (25). Importantly, constitutively active hepatic mTORC2 signaling is oncogenic and promotes HCC (26), and is particularly important in case of NAFLD to HCC transition (27). Thus, it is likely that mTORC2-mediated FA (and perhaps lipid) synthesis in cancer cells modulates immunity.

mTORC2 mediates various cellular processes *via* AGC kinase family members AKT, serum/glucocorticoid-regulated kinase (SGK), and protein kinase C (PKC) (28, 29) (**Figure 1**). In a mammary gland tumor model, *Rictor* deletion disrupted secondary mammary ductal branching, cell motility, and survival. This effect was mediated by PKCα-Rac1, but not AKT (30), suggesting an AKT-independent role of mTORC2 in motility and metastasis. mTORC2 phosphorylates and activates AKT (pAKT-Ser473). Melanoma with increased pAKT-Ser473 correlated with reduced T-cell infiltration, possibly due to increased secretion of inhibitory cytokines by cancer cells, and exhibit resistance to immune checkpoint inhibitors (31). The mTORC2 target SGK is frequently expressed in tumors (32). In gastric tumors, increased expression of the SGK1 target, NDRG1, is suggested to stimulate IL-1 expression and promote angiogenesis (33). Taken together, these data suggest that increased PI3K–mTORC2–AKT signaling in cancer cells may affect T cells and thereby tumorigenesis. It is possible that other immune cells in the tumor microenvironment are also modulated by PI3K–mTORC2–AKT, as described further below.

REGULATORY T CELLS (Tregs)

Regulatory T cells suppress inflammation and are detrimental in tumor immunity. Genetic and pharmacological (rapamycin) abrogation of mTOR signaling induce Treg expansion *via* Foxp3 expression (34, 35). Furthermore, Treg-specific conditional TSC deletion in mice (constitutively active mTORC1) propelled Treg differentiation and a strong effector-like phenotype, reversed by S6K1 knockdown (36), suggesting that mTORC1 is an important checkpoint in Treg homeostasis.

Programmed Death 1 (PD-1) and Cytotoxic T-Lymphocyte-associated Antigen 4 (CTLA-4) immune checkpoints negatively regulate T-cell immune function. Immune suppression in the tumor microenvironment through PD-1 or CTLA-4 occurs in various tumors, and immune checkpoint inhibitors (anti-PD-1, anti-PD-L1, or anti-CTLA-4) amplify antitumor T-cell response (37). The surface protein PD-L1 is widely expressed in various tumors. PD-L1 binds to either the T-cell-expressed PD-1 or CD80 receptors thereby inhibiting their effector responses. PD-L1 and PD-1 interaction induces differentiation of naïve CD4 + T cells into Tregs, leading to an immune suppressive environment. In addition to inhibiting T-cell effector function, cancer cell-intrinsic PD-1 expression may promote tumor growth (38). Thus, PD-1 axis has a twofold effect in tumorigenesis: first by inhibiting cancer cell clearance by T cells, and second, promoting cancer cell growth. In a lung carcinoma mouse model, mTORC1 increased PD-L1 expression, allowing cancer cells to escape killing by immune cells (39, 40). Within the tumor, PD-L1 seems to be enriched in Tumor Initiating Cells (TICs) (also referred to as Cancer Stem Cells) (41–43). TICs are tumor cells with self-renewal capacity and considered to be more resistant to targeted cancer therapies. In syngeneic ovarian mouse model experiments, PD-L1 appeared to control the expression of canonical "stemness" genes, such as *Oct4* and *Nanog* (44, 45). PD-L1 expression correlated with mTOR activation in human lung adenocarcinomas and squamous cell carcinomas (39), suggesting that oncogenic AKT-mTOR activation promotes immune escape through PD-L1 upregulation. Furthermore, anti-PD-1 therapy inhibited human melanoma xenograft growth and reduced S6 phosphorylation, suggesting that PD-1 in tumor cells activates mTORC1. Importantly, cells expressing high levels of PD-L1 appear to be more sensitive to the mTORC1 inhibitor rapamycin, further suggesting that some of the PD-L1 growth-controlling mechanisms are *via* mTOR signaling. Collectively, these data suggest a functional relationship between mTOR signaling, PD-L1 expression, and resistance to targeted therapies (i.e., TICs). However, the mechanism(s) by which mTORC1 signaling regulates PD-L1 expression remains to be elucidated. We note that in addition to Treg and Th1, other T-cell subsets, such as Th17, may be involved in cancer immune response.

TUMOR-ASSOCIATED MACROPHAGES (TAMs)

Tumor-associated macrophages originate from expansion of tissue-resident macrophages or are recruited to tumor site (by chemotactic factors), and are present at multiple stages of tumor

progression (2). Macrophages are not a homogenous population and can be subdivided into M1 and M2. M1 macrophages produce Th1 cytokines, promoting phagocyte-dependent inflammation and thereby an antitumor response. M2 macrophages enforce antibody response, but inhibit several phagocytic functions, therefore seemingly enabling a growth-tolerant tumor microenvironment. TAMs predominantly exhibit M2 phenotypes, therefore considered tumor-promoting. Several factors can promote polarization of TAMs to M2 during cancer progression, including IL-4, IL-10, TGF-β, and M-CSF (46). TAMs promote tumorigenesis by modulating lymph- and angiogenesis (47), but more recently, TAMs were shown to express PD-1. The presence of TAM expressing PD-1 steadily increases with cancer progression and results in an overall reduction in cancer cell phagocytosis (48). Because macrophages activation and function is, at least in part, controlled by PI3K–mTOR–AKT (49), it would be valuable to examine whether the observed reduction in phagocytosis is related to mTOR signaling. Furthermore, mTOR regulates macrophage polarization (50), and M1 and M2 macrophages exhibit dependency on distinct metabolic pathways. While M1 macrophages upregulate glycolysis and lipogenesis, M2 macrophages upregulate beta-oxidation. This is important because metabolic shifts are coupled to macrophage function (51, 52). For instance, IL-4 activate AKT and thereby inducing M2 gene transcription, possibly *via* ACLY expression and regulation of histone acetylation (53), indicating that mTOR signaling couple metabolic inputs to modulate immune response. Moreover, PI3K–AKT appears to recruit immune-suppressive monocytes to tumors *via* monocyte chemoattractant protein-1 (MCP-1) expression, in a mechanism that potentially involves TGFβ1 (54). MCP1 plays a similar role in other tumors (55), but whether PI3K–AKT induced MCP1 expression can be generalized to other tumors remains to be investigated. mTORC2 appears to be particularly important for differentiation of M2 macrophages (as opposed to M1), as not only monocytes recruitment but also monocyte polarization is involved in tumor progression (56); therefore, mTORC2 plays a dual immunosuppressive role.

Antigen-presenting cells (APCs), especially dendritic cells (DCs), are crucial in mounting antitumor immune response (57). Indeed, abrogation of mTORC2 signaling in the professional APCs, DCs, led to enhanced tumor eradication possibly *via* engagement of CTLs (58). Rapamycin administration augmented the expression of costimulatory molecules and enhanced DC life span, *via* modulation of glucose metabolism (59). These data suggest that mTOR signaling in APC cells imposes an immunosuppressive environment.

MYELOID-DERIVED SUPPRESSOR CELLS (MDSCs)

Myeloid-derived suppressor cells are a heterogeneous population defined as CD11b + Gr1 + cells. Based on Ly6G and Ly6C expression, MDSCs can be further classified as granulocytic or monocytic subsets, respectively. Both CD11b + Ly6G + and CD11b + Ly6C + cells play immunosuppressive roles. The allosteric mTORC1 inhibitor, rapamycin, inhibits MDSC accumulation in tumors and skin allografts (60). In breast cancer, accumulation of MDSCs in tumors occurred *via* G-CSF. Rapamycin administration or *Raptor* deletion (a core-component of mTORC1) reduced G-CSF levels (61), suggesting that mTORC1 in tumor cells attracts MDSCs by upregulating G-CSF. Increased G-CSF levels also correlated with elevated mTOR activity in human tumors. Interestingly, there is correlation between presence of TICs, elevated mTORC1 signaling, and G-CSF production. Moreover, rapamycin administration leads to reduced TIC levels (61). These data suggest that mTOR activity in a subset of cells within the tumor mass (i.e., intra-tumoral heterogeneity) mediates MDSC accumulation.

OTHER CELLS OF THE TUMOR MICROENVIRONMENT: CAFs

Fibroblasts are not only involved in the deposition of stromal extra-cellular matrix (ECM) but also in the secretion of growth factors. CAFs seem to play a role in cancer progression and initiation, particularly in stroma-rich tumors like pancreatic cancers (62, 63). In pancreatic tumors, CAFs are also involved in resistance to anticancer drugs (64). Interleukin-6 (IL-6) is linked to resistance-to-cancer drug therapies (65), possibly *via* its downstream effector pSTAT3 (66). In pancreatic CAFs, the somatostatin receptor sst1 inhibits mTOR-mediated IL-6 protein synthesis, thereby counteracting mTOR/IL-6-driven resistance to anticancer drugs (67). How mTOR regulates IL-6 expression in stromal cells remains to be investigated, but this mechanism seems to involve the quintessential mTORC1 target, 4E-BP1 (67). In lung carcinoma, paracrine IGF-II secretion by CAFs activated insulin growth factor receptor 1 (IGF1R) signaling in cancer cells, possibly activating a TICs (stemness)-like phenotype (68). Conversely, in irradiated tumors, IGF-II secreted from CAFs appears to block mTORC1 signaling in neighboring cancer cells. mTORC1 inhibition allowed autophagy initiation and thereby tumor regrowth (69). It seems counterintuitive that mTOR inhibition allows tumor growth, but possibly under stress or nutrient-poor conditions autophagy initiation provide the required nutrients. Nevertheless, this hypothesis needs to be examined in other cancer models. Yet, liver specific *Raptor* knockout mice (abrogated mTORC1 signaling) developed more HCC when challenged with the hepato-carcinogen diethyl-nitrosamine, as compared with wild-type mice (70). These data suggest that "too low" mTORC1 activity may also be oncogenic. Taken together, it is likely that the response to drug therapies is not only dependent on stromal cells and their secretome but also on the conditions in which therapies are given.

mTOR ON THE RECEIVING END OF CANCER IMMUNITY

mTOR signaling is also on the receiving end of cues coming from the tumor microenvironment. For example, non-tumorigenic (stromal) cells of the tumor microenvironment secrete MCP1 to activate the mTOR pathway in neighboring breast cancer cells (71).

Moreover, metabolic activation of natural killer (NK) cells is dependent on IL-15 stimulation to prompt intracellular mTOR signaling (72). NK cells are suggested to play a pivotal role in cancer control and are increased in metastatic melanoma (73). Conversely, TGF-β represses mTOR signaling, both in mice and humans, to inhibit NK cell activation (74), suggesting an mTOR-dependent immune suppressive role for TGF-β in tumor microenvironment. Additionally, genetic activation of mTORC1 (mutated TSC) causes impairment of NK cell development (75). Notably, mTOR also regulates Th1 and Th2 differentiation; and while mTORC1 is distinctly critical for Th1 and Th17 differentiation, mTORC2 seems to promote Th2 differentiation (76). Furthermore, mTORC1 regulates CD8 + T-cell effector function (77), thereby allowing better clearance of tumor cells. Although mTORC2 seems to be dispensable for the effector function of CD8 + T cells, it is critical for generation of CD8 + memory cells (77). Further studies are required to examine how extracellular signals affect mTOR in T cells; nonetheless, the data demonstrate that mTOR signaling differentially regulates T cells.

CLINICAL IMPLICATIONS

Various mTOR inhibitors are in ongoing clinical trials and the FDA-approved rapalog everolimus is used in various cancer cell types (10). Because mTOR signaling plays a key role in cancer and immune cell function (78), it is possible that some of the anticancer effect of mTOR inhibitors is *via* immune modulation (**Figure 2**). Indeed, rapamycin is clinically used for prevention of renal graft rejection and is traditionally considered as a "pure" immunosuppressant, possibly by blocking T-cell activation. However, as discussed above, mTOR seems to play a more complex role in immunity. Under certain conditions, mTOR inhibition poses an immune-activating function, such as induction of memory CD8 + T cell (77) that may in turn increase the

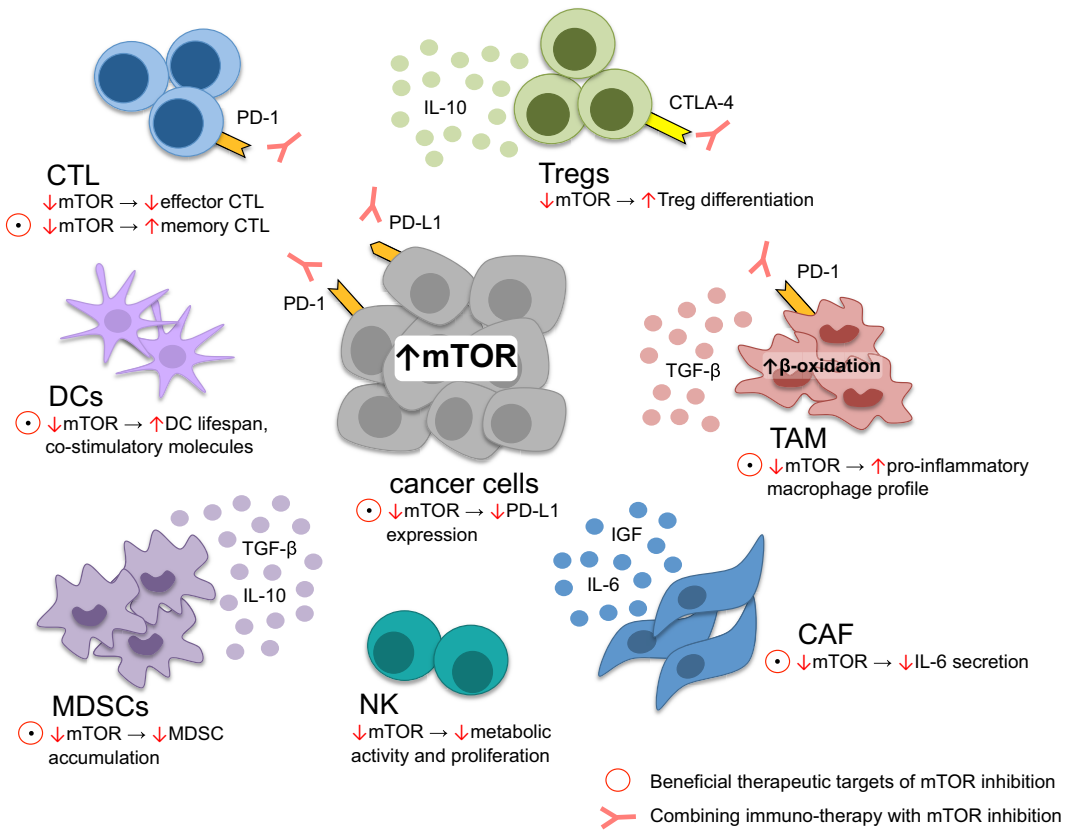


FIGURE 2 | Tumor microenvironment modulation through mTOR. mTOR inhibition induces memory cytotoxic CD8 + T lymphocytes (CTL) formation while reducing effector CTL function, critical for cellular antitumor response. In dendritic cells, lifespan and the expression of costimulatory molecules is increased upon mTOR suppression, leading to improved foreign-antigen recognition. On the other hand, metabolic NK-cell function, essential for antitumor response, is diminished upon mTOR inhibition. Myeloid derived stem cells (MDSC), regulatory T cells (Tregs), tumor-associated macrophages (TAM), and cancer-associated fibroblasts (CAF) contribute to tumor immune-evasion and tumor growth. The immune-suppressive environment generated through MDSC is limited through mTOR blockage by restraining MDSC accumulation. Similarly, anti-inflammatory TAM may be skewed toward a more pro-inflammatory profile upon mTOR inhibition. CAF secrete various cytokines promoting tumor growth and therapy resistance, counteracted by mTOR blockage. In contrast, Tregs are preferentially differentiated upon mTOR downregulation. Within the majority of cancer cells, the PI3K–mTOR–AKT pathway is upregulated, driving PD-L1 expression maintaining an immune-suppressive state within the tumor microenvironment: a process that may be interrupted through mTOR inhibition. However, not all therapeutic targets of mTOR inhibition seem to be beneficial, such as reducing effector CTL function and T-Reg differentiation. Accordingly, rationale exists to combine anti-PD-1/PD-L1 or anti-CTLA-4 and mTOR inhibitors, alleviating reduced CTL effector function and Treg differentiation.

durability of antitumor effector T-cell function. It is also likely that ATP kinase mTOR inhibitors, blocking robustly mTORC1, and mTORC2 signaling (79) act on cancer cells, as well as on the tumor microenvironment.

While checkpoint and mTOR inhibitors have revolutionized cancer treatment, as monotherapies these drugs seem to be insufficient to fully block cancer progression. Oncogenic PI3K–mTOR–AKT pathway reduces T-cell tumor infiltration and causes inferior outcome after PD-1 inhibition (31), providing a rationale for the design of combination therapies of mTOR and immune checkpoint inhibitors, as recently shown for HCC (80) (**Figure 2**). Nonetheless, the specific oncogenic mechanism downstream of mTOR remains unknown. Understanding these pathways is critical for the rational design of selective inhibitors. The combination of checkpoint and mTOR inhibitors might be limited by its side effects because: (i) PI3K–mTOR–AKT signaling plays a critical role in physiological cell homeostasis, (ii) rapamycin administration reduces the effector CD8 + T-cell function (that are otherwise required for execution of anticancer effect), and (iii) possibly relieve negative feedback loops that may induce compensatory pathway activation.

CONCLUSION

Collectively, the above suggests that mTOR signaling has both tumor-intrinsic and tumor-extrinsic (i.e., tumor microenvironment) activities. mTOR-kinase quickly responds to stimuli in the tumor microenvironment and executes various (possibly opposing) effects on immune cells. Thus, a prime challenge is to dissect the role of mTOR in the different cell types in the tumor microenvironment and to assess the overall "net effect" of mTOR blockade.

AUTHOR CONTRIBUTIONS

YG wrote the original draft. YG, TMN, and JR wrote and approved the final version of the review.

ACKNOWLEDGMENTS

We thank Dale Braunschweig for helpful suggestions on our manuscript. This work was supported by generous philanthropic contributions to The University of Texas MD Anderson Moon Shots Program, the Swiss National Science Foundation and the Swiss Cancer Research foundation (KFS).

REFERENCES

1. Allen E, Mieville P, Warren CM, Saghafinia S, Li L, Peng MW, et al. Metabolic symbiosis enables adaptive resistance to anti-angiogenic therapy that is dependent on mTOR signaling. *Cell Rep* (2016) 15:1144–60. doi:10.1016/j.celrep.2016.04.029
2. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell* (2012) 21:309–22. doi:10.1016/j.ccr.2012.02.022
3. Gajewski TF, Schreiber H, Fu YX. Innate and adaptive immune cells in the tumor microenvironment. *Nat Immunol* (2013) 14:1014–22. doi:10.1038/ni.2703
4. Wendler F, Favicchio R, Simon T, Alifrangis C, Stebbing J, Giamas G. Extracellular vesicles swarm the cancer microenvironment: from tumor-stroma communication to drug intervention. *Oncogene* (2017) 36:877–84. doi:10.1038/onc.2016.253
5. Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. *Nat Med* (2013) 19:1423–37. doi:10.1038/nm.3394
6. Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. *Cell* (2006) 124:471–84. doi:10.1016/j.cell.2006.01.016
7. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell* (2012) 149:274–93. doi:10.1016/j.cell.2012.03.017
8. Shimobayashi M, Hall MN. Multiple amino acid sensing inputs to mTORC1. *Cell Res* (2016) 26:7–20. doi:10.1038/cr.2015.146
9. Dibble CC, Manning BD. Signal integration by mTORC1 coordinates nutrient input with biosynthetic output. *Nat Cell Biol* (2013) 15:555–64. doi:10.1038/ncb2763
10. Benjamin D, Colombi M, Moroni C, Hall MN. Rapamycin passes the torch: a new generation of mTOR inhibitors. *Nat Rev Drug Discov* (2011) 10:868–80. doi:10.1038/nrd3531
11. Dibble CC, Cantley LC. Regulation of mTORC1 by PI3K signaling. *Trends Cell Biol* (2015) 25:545–55. doi:10.1016/j.tcb.2015.06.002
12. Guri Y, Hall MN. mTOR signaling confers resistance to targeted cancer drugs. *Trends Cancer* (2016) 2:688–97. doi:10.1016/j.trecan.2016.10.006
13. Mamane Y, Petroulakis E, LeBacquer O, Sonenberg N. mTOR, translation initiation and cancer. *Oncogene* (2006) 25:6416–22. doi:10.1038/sj.onc.1209888
14. Ma XM, Blenis J. Molecular mechanisms of mTOR-mediated translational control. *Nat Rev Mol Cell Biol* (2009) 10:307–18. doi:10.1038/nrm2672
15. Zinzalla V, Stracka D, Oppliger W, Hall MN. Activation of mTORC2 by association with the ribosome. *Cell* (2011) 144:757–68. doi:10.1016/j.cell.2011.02.014
16. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* (2011) 144:646–74. doi:10.1016/j.cell.2011.02.013
17. Disis ML. Immune regulation of cancer. *J Clin Oncol* (2010) 28:4531–8. doi:10.1200/JCO.2009.27.2146
18. Fridman WH, Pages F, Sautes-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer* (2012) 12:298–306. doi:10.1038/nrc3245
19. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* (2006) 313:1960–4. doi:10.1126/science.1129139
20. Kitano Y, Okabe H, Yamashita YI, Nakagawa S, Saito Y, Umezaki N, et al. Tumour-infiltrating inflammatory and immune cells in patients with extrahepatic cholangiocarcinoma. *Br J Cancer* (2018) 118(2):171–80. doi:10.1038/bjc.2017.401
21. Restifo NP, Dudley ME, Rosenberg SA. Adoptive immunotherapy for cancer: harnessing the T cell response. *Nat Rev Immunol* (2012) 12:269–81. doi:10.1038/nri3191
22. Bantug GR, Galluzzi L, Kroemer G, Hess C. The spectrum of T cell metabolism in health and disease. *Nat Rev Immunol* (2018) 18(1):19–34. doi:10.1038/nri.2017.99
23. Michelotti GA, Machado MV, Diehl AM. NAFLD, NASH and liver cancer. *Nat Rev Gastroenterol Hepatol* (2013) 10:656–65. doi:10.1038/nrgastro.2013.183
24. Ma C, Kesarwala AH, Eggert T, Medina-Echeverz J, Kleiner DE, Jin P, et al. NAFLD causes selective CD4(+) T lymphocyte loss and promotes hepatocarcinogenesis. *Nature* (2016) 531:253–7. doi:10.1038/nature16969
25. Hagiwara A, Cornu M, Cybulski N, Polak P, Betz C, Trapani F, et al. Hepatic mTORC2 activates glycolysis and lipogenesis through Akt, glucokinase, and SREBP1c. *Cell Metab* (2012) 15:725–38. doi:10.1016/j.cmet.2012.03.015
26. Guertin DA, Stevens DM, Thoreen CC, Burds AA, Kalaany NY, Moffat J, et al. Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1. *Dev Cell* (2006) 11:859–71. doi:10.1016/j.devcel.2006.10.007

27. Guri Y, Colombi M, Dazert E, Hindupur SK, Roszik J, Moes S, et al. mTORC2 promotes tumorigenesis via lipid synthesis. *Cancer Cell* (2017) 32:807–23.e12. doi:10.1016/j.ccell.2017.11.011
28. Cybulski N, Polak P, Auwerx J, Ruegg MA, Hall MN. mTOR complex 2 in adipose tissue negatively controls whole-body growth. *Proc Natl Acad Sci U S A* (2009) 106:9902–7. doi:10.1073/pnas.0811321106
29. Sparks CA, Guertin DA. Targeting mTOR: prospects for mTOR complex 2 inhibitors in cancer therapy. *Oncogene* (2010) 29:3733–44. doi:10.1038/onc.2010.139
30. Morrison MM, Young CD, Wang S, Sobolik T, Sanchez VM, Hicks DJ, et al. mTOR directs breast morphogenesis through the PKC-alpha-Rac1 signaling axis. *PLoS Genet* (2015) 11:e1005291. doi:10.1371/journal.pgen.1005291
31. Peng W, Chen JQ, Liu C, Malu S, Creasy C, Tetzlaff MT, et al. Loss of PTEN promotes resistance to T cell-mediated immunotherapy. *Cancer Discov* (2016) 6:202–16. doi:10.1158/2159-8290.CD-15-0283
32. Sommer EM, Dry H, Cross D, Guichard S, Davies BR, Alessi DR. Elevated SGK1 predicts resistance of breast cancer cells to Akt inhibitors. *Biochem J* (2013) 452:499–508. doi:10.1042/BJ20130342
33. Murakami Y, Watari K, Shibata T, Uba M, Ureshino H, Kawahara A, et al. N-myc downstream-regulated gene 1 promotes tumor inflammatory angiogenesis through JNK activation and autocrine loop of interleukin-1alpha by human gastric cancer cells. *J Biol Chem* (2013) 288:25025–37. doi:10.1074/jbc.M113.472068
34. Battaglia M, Stabilini A, Migliavacca B, Horejs-Hoeck J, Kaupper T, Roncarolo MG. Rapamycin promotes expansion of functional CD4+ CD25+FOXP3+ regulatory T cells of both healthy subjects and type 1 diabetic patients. *J Immunol* (2006) 177:8338–47. doi:10.4049/jimmunol.177.12.8338
35. Delgoffe GM, Kole TP, Zheng Y, Zarek PE, Matthews KL, Xiao B, et al. The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. *Immunity* (2009) 30:832–44. doi:10.1016/j.immuni.2009.04.014
36. Park Y, Jin HS, Lopez J, Elly C, Kim G, Murai M, et al. TSC1 regulates the balance between effector and regulatory T cells. *J Clin Invest* (2013) 123:5165–78. doi:10.1172/JCI69751
37. Anderson KG, Stromnes IM, Greenberg PD. Obstacles posed by the tumor microenvironment to T cell activity: a case for synergistic therapies. *Cancer Cell* (2017) 31:311–25. doi:10.1016/j.ccell.2017.02.008
38. Kleffel S, Posch C, Barthel SR, Mueller H, Schlapbach C, Guenova E, et al. Melanoma cell-intrinsic PD-1 receptor functions promote tumor growth. *Cell* (2015) 162:1242–56. doi:10.1016/j.cell.2015.08.052
39. Lastwika KJ, Wilson W III, Li QK, Norris J, Xu H, Ghazarian SR, et al. Control of PD-L1 expression by oncogenic activation of the AKT-mTOR pathway in non-small cell lung cancer. *Cancer Res* (2016) 76:227–38. doi:10.1158/0008-5472.CAN-14-3362
40. Clark CA, Gupta HB, Sareddy G, Pandeswara S, Lao S, Yuan B, et al. Tumor-intrinsic PD-L1 signals regulate cell growth, pathogenesis, and autophagy in ovarian cancer and melanoma. *Cancer Res* (2016) 76:6964–74. doi:10.1158/0008-5472.CAN-16-0258
41. Yao Y, Tao R, Wang X, Wang Y, Mao Y, Zhou LF. B7-H1 is correlated with malignancy-grade gliomas but is not expressed exclusively on tumor stem-like cells. *Neuro Oncol* (2009) 11:757–66. doi:10.1215/15228517-2009-014
42. Zhi Y, Mou Z, Chen J, He Y, Dong H, Fu X, et al. B7H1 expression and epithelial-to-mesenchymal transition phenotypes on colorectal cancer stem-like cells. *PLoS One* (2015) 10:e0135528. doi:10.1371/journal.pone.0135528
43. Lee Y, Shin JH, Longmire M, Wang H, Kohrt HE, Chang HY, et al. CD44+ cells in head and neck squamous cell carcinoma suppress T-cell-mediated immunity by selective constitutive and inducible expression of PD-L1. *Clin Cancer Res* (2016) 22:3571–81. doi:10.1158/1078-0432.CCR-15-2665
44. Clark CA, Gupta HB, Curiel TJ. Tumor cell-intrinsic CD274/PD-L1: a novel metabolic balancing act with clinical potential. *Autophagy* (2017) 13:987–8. doi:10.1080/15548627.2017.1280223
45. Gupta HB, Clark CA, Yuan B, Sareddy G, Pandeswara S, Padron AS, et al. Tumor cell-intrinsic PD-L1 promotes tumor-initiating cell generation and functions in melanoma and ovarian cancer. *Signal Transduct Target Ther* (2016) 1. doi:10.1038/sigtrans.2016.30
46. Laoui D, Movahedi K, Van Overmeire E, Van den Bossche J, Schouppe E, Mommer C, et al. Tumor-associated macrophages in breast cancer: distinct subsets, distinct functions. *Int J Dev Biol* (2011) 55:861–7. doi:10.1387/ijdb.113371dl
47. Riabov V, Gudima A, Wang N, Mickley A, Orekhov A, Kzhyshkowska J. Role of tumor associated macrophages in tumor angiogenesis and lymphangiogenesis. *Front Physiol* (2014) 5:75. doi:10.3389/fphys.2014.00075
48. Gordon SR, Maute RL, Dulken BW, Hutter G, George BM, McCracken MN, et al. PD-1 expression by tumour-associated macrophages inhibits phagocytosis and tumour immunity. *Nature* (2017) 545:495–9. doi:10.1038/nature22396
49. Covarrubias AJ, Aksoylar HI, Horng T. Control of macrophage metabolism and activation by mTOR and Akt signaling. *Semin Immunol* (2015) 27:286–96. doi:10.1016/j.smim.2015.08.001
50. Zhu L, Yang T, Li L, Sun L, Hou Y, Hu X, et al. TSC1 controls macrophage polarization to prevent inflammatory disease. *Nat Commun* (2014) 5:4696. doi:10.1038/ncomms5696
51. Huang SC, Smith AM, Everts B, Colonna M, Pearce EL, Schilling JD, et al. Metabolic reprogramming mediated by the mTORC2-IRF4 signaling axis is essential for macrophage alternative activation. *Immunity* (2016) 45:817–30. doi:10.1016/j.immuni.2016.09.016
52. Jha AK, Huang SC, Sergushichev A, Lampropoulou V, Ivanova Y, Loginicheva E, et al. Network integration of parallel metabolic and transcriptional data reveals metabolic modules that regulate macrophage polarization. *Immunity* (2015) 42:419–30. doi:10.1016/j.immuni.2015.02.005
53. Covarrubias AJ, Aksoylar HI, Yu J, Snyder NW, Worth AJ, Iyer SS, et al. Akt-mTORC1 signaling regulates Acly to integrate metabolic input to control of macrophage activation. *Elife* (2016) 5. doi:10.7554/eLife.11612
54. Diaz-Valdes N, Basagoiti M, Dotor J, Aranda F, Monreal I, Riezu-Boj JI, et al. Induction of monocyte chemoattractant protein-1 and interleukin-10 by TGFbeta1 in melanoma enhances tumor infiltration and immunosuppression. *Cancer Res* (2011) 71:812–21. doi:10.1158/0008-5472.CAN-10-2698
55. Furukawa S, Soeda S, Kiko Y, Suzuki O, Hashimoto Y, Watanabe T, et al. MCP-1 promotes invasion and adhesion of human ovarian cancer cells. *Anticancer Res* (2013) 33:4785–90.
56. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol* (2002) 23:549–55. doi:10.1016/S1471-4906(02)02302-5
57. Martin K, Schreiner J, Zippelius A. Modulation of APC function and anti-tumor immunity by anti-cancer drugs. *Front Immunol* (2015) 6:501. doi:10.3389/fimmu.2015.00501
58. Raich-Regue D, Fabian KP, Watson AR, Fecek RJ, Storkus WJ, Thomson AW. Intratumoral delivery of mTORC2-deficient dendritic cells inhibits B16 melanoma growth by promoting CD8(+) effector T cell responses. *Oncoimmunology* (2016) 5:e1146841. doi:10.1080/2162402X.2016.1146841
59. Amiel E, Everts B, Freitas TC, King IL, Curtis JD, Pearce EL, et al. Inhibition of mechanistic target of rapamycin promotes dendritic cell activation and enhances therapeutic autologous vaccination in mice. *J Immunol* (2012) 189:2151–8. doi:10.4049/jimmunol.1103741
60. Wu T, Zhao Y, Wang H, Li Y, Shao L, Wang R, et al. mTOR masters monocytic myeloid-derived suppressor cells in mice with allografts or tumors. *Sci Rep* (2016) 6:20250. doi:10.1038/srep20250
61. Welte T, Kim IS, Tian L, Gao X, Wang H, Li J, et al. Oncogenic mTOR signalling recruits myeloid-derived suppressor cells to promote tumour initiation. *Nat Cell Biol* (2016) 18:632–44. doi:10.1038/ncb3355
62. Erez N, Truitt M, Olson P, Arron ST, Hanahan D. Cancer-associated fibroblasts are activated in incipient neoplasia to orchestrate tumor-promoting inflammation in an NF-kappaB-dependent manner. *Cancer Cell* (2010) 17:135–47. doi:10.1016/j.ccr.2009.12.041
63. Feig C, Gopinathan A, Neesse A, Chan DS, Cook N, Tuveson DA. The pancreas cancer microenvironment. *Clin Cancer Res* (2012) 18:4266–76. doi:10.1158/1078-0432.CCR-11-3114
64. Erkan M. Understanding the stroma of pancreatic cancer: co-evolution of the microenvironment with epithelial carcinogenesis. *J Pathol* (2013) 231:4–7. doi:10.1002/path.4213
65. Kumari N, Dwarakanath BS, Das A, Bhatt AN. Role of interleukin-6 in cancer progression and therapeutic resistance. *Tumour Biol* (2016) 37:11553–72. doi:10.1007/s13277-016-5098-7
66. Grivennikov S, Karin E, Terzic J, Mucida D, Yu GY, Vallabhapurapu S, et al. IL-6 and Stat3 are required for survival of intestinal epithelial cells and

development of colitis-associated cancer. *Cancer Cell* (2009) 15:103–13. doi:10.1016/j.ccr.2009.01.001
67. Duluc C, Moatassim-Billah S, Chalabi-Dchar M, Perraud A, Samain R, Breibach F, et al. Pharmacological targeting of the protein synthesis mTOR/4E-BP1 pathway in cancer-associated fibroblasts abrogates pancreatic tumour chemoresistance. *EMBO Mol Med* (2015) 7:735–53. doi:10.15252/emmm.201404346
68. Chen WJ, Ho CC, Chang YL, Chen HY, Lin CA, Ling TY, et al. Cancer-associated fibroblasts regulate the plasticity of lung cancer stemness via paracrine signalling. *Nat Commun* (2014) 5:3472. doi:10.1038/ncomms4472
69. Wang Y, Gan G, Wang B, Wu J, Cao Y, Zhu D, et al. Cancer-associated fibroblasts promote irradiated cancer cell recovery through autophagy. *EBioMedicine* (2017) 17:45–56. doi:10.1016/j.ebiom.2017.02.019
70. Umemura A, Park EJ, Taniguchi K, Lee JH, Shalapour S, Valasek MA, et al. Liver damage, inflammation, and enhanced tumorigenesis after persistent mTORC1 inhibition. *Cell Metab* (2014) 20:133–44. doi:10.1016/j.cmet.2014.05.001
71. Riverso M, Kortenkamp A, Silva E. Non-tumorigenic epithelial cells secrete MCP-1 and other cytokines that promote cell division in breast cancer cells by activating ERalpha via PI3K/Akt/mTOR signaling. *Int J Biochem Cell Biol* (2014) 53:281–94. doi:10.1016/j.biocel.2014.05.023
72. Marcais A, Cherfils-Vicini J, Viant C, Degouve S, Viel S, Fenis A, et al. The metabolic checkpoint kinase mTOR is essential for IL-15 signaling during the development and activation of NK cells. *Nat Immunol* (2014) 15:749–57. doi:10.1038/ni.2936
73. Riaz N, Havel JJ, Makarov V, Desrichard A, Urba WJ, Sims JS, et al. Tumor and microenvironment evolution during immunotherapy with nivolumab. *Cell* (2017) 171:934–49.e15. doi:10.1016/j.cell.2017.09.028
74. Viel S, Marcais A, Guimaraes FS-F, Loftus R, Rabilloud J, Grau M, et al. TGF-beta inhibits the activation and functions of NK cells by repressing the mTOR pathway. *Sci Signal* (2016) 9:ra19. doi:10.1126/scisignal.aad1884
75. Yang M, Chen S, Du J, He J, Wang Y, Li Z, et al. NK cell development requires Tsc1-dependent negative regulation of IL-15-triggered mTORC1 activation. *Nat Commun* (2016) 7:12730. doi:10.1038/ncomms12730
76. Delgoffe GM, Pollizzi KN, Waickman AT, Heikamp E, Meyers DJ, Horton MR, et al. The kinase mTOR regulates the differentiation of helper T cells through the selective activation of signaling by mTORC1 and mTORC2. *Nat Immunol* (2011) 12:295–303. doi:10.1038/ni.2005
77. Pollizzi KN, Patel CH, Sun IH, Oh MH, Waickman AT, Wen J, et al. mTORC1 and mTORC2 selectively regulate CD8(+) T cell differentiation. *J Clin Invest* (2015) 125:2090–108. doi:10.1172/JCI77746
78. O'Donnell JS, Massi D, Teng MWL, Mandala M. PI3K-AKT-mTOR inhibition in cancer immunotherapy, redux. *Semin Cancer Biol* (2018) 48:91–103. doi:10.1016/j.semcancer.2017.04.015
79. Rodrik-Outmezguine VS, Okaniwa M, Yao Z, Novotny CJ, McWhirter C, Banaji A, et al. Overcoming mTOR resistance mutations with a new-generation mTOR inhibitor. *Nature* (2016) 534:272–6. doi:10.1038/nature17963
80. Li H, Li X, Liu S, Guo L, Zhang B, Zhang J, et al. Programmed cell death-1 (PD-1) checkpoint blockade in combination with a mammalian target of rapamycin inhibitor restrains hepatocellular carcinoma growth induced by hepatoma cell-intrinsic PD-1. *Hepatology* (2017) 66:1920–33. doi:10.1002/hep.29360

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

nutrients

MDPI

Article

A Comprehensive Assessment of Apigenin as an Antiproliferative, Proapoptotic, Antiangiogenic and Immunomodulatory Phytocompound

Alexandra Ghiţu [1], Anja Schwiebs [2], Heinfried H. Radeke [2], Stefana Avram [1], Istvan Zupko [3], Andrea Bor [3], Ioana Zinuca Pavel [1,*], Cristina Adriana Dehelean [4], Camelia Oprean [5,6], Florina Bojin [6,7], Claudia Farcas [8], Codruta Soica [9], Oana Duicu [10] and Corina Danciu [1]

1 Department of Pharmacognosy, "Victor Babeş" University of Medicine and Pharmacy, 2, Eftimie Murgu Square, 300041 Timişoara, Romania; ghitu.alexandra@gmail.com (A.G.); stefana.avram@umft.ro (S.A.); corina.danciu@umft.ro (C.D.)

2 Pharmazentrum Frankfurt/ZAFES, Institute of General Pharmacology and Toxicology, Clinic of the Goethe University, D-60590 Frankfurt/Main, Germany; schwiebs@med.uni-frankfurt.de (A.S.); radeke@em.uni-frankfurt.de (H.H.R.)

3 Department of Pharmacodynamics and Biopharmacy, University of Szeged, Eötvös u. 6, H-6720 Szeged, Hungary; zupko@pharm.u-szeged.hu (I.Z.); andrea.bor@pharm.u-szeged.hu (A.B.)

4 Department of Toxicology, "Victor Babeş" University of Medicine and Pharmacy, 2, Eftimie Murgu Square, 300041 Timişoara, Romania; cadehelean@umft.ro

5 Department of Drug Analysis, Chemistry of the Environment and Food, "Victor Babeş" University of Medicine and Pharmacy, 2, Eftimie Murgu Square, 300041 Timişoara, Romania; camelia.oprean@umft.ro

6 OncoGen Centre, "Pius Brînzeu" Emergency County Hospital, 156, Liviu Rebreanu Boulevard, 300736 Timişoara, Romania; florinabojin@umft.ro

7 Department of Functional Sciences, "Victor Babeş" University of Medicine and Pharmacy, 2, Eftimie Murgu Square, 300041 Timişoara, Romania

8 Department of Pharmaceutical Physics, "Victor Babeş" University of Medicine and Pharmacy, 2, Eftimie Murgu Square, 300041 Timişoara, Romania; farcas.claudia@umft.ro

9 Department of Pharmaceutical Chemistry, "Victor Babeş" University of Medicine and Pharmacy, 2, Eftimie Murgu Square, 300041 Timişoara, Romania; codrutasoica@umft.ro

10 Department of Functional Sciences—Pathophysiology, "Victor Babeş" University of Medicine and Pharmacy, 2, Eftimie Murgu Square, 300041 Timişoara, Romania; oanaduicu@umft.ro

* Correspondence: ioanaz.pavel@umft.ro; Tel.: +40-762-659-981

Received: 12 February 2019; Accepted: 12 April 2019; Published: 16 April 2019

Abstract: Apigenin (4′,5,7-trihydroxyflavone) (Api) is an important component of the human diet, being distributed in a wide number of fruits, vegetables and herbs with the most important sources being represented by chamomile, celery, celeriac and parsley. This study was designed for a comprehensive evaluation of Api as an antiproliferative, proapoptotic, antiangiogenic and immunomodulatory phytocompound. In the set experimental conditions, Api presents antiproliferative activity against the A375 human melanoma cell line, a G2/M arrest of the cell cycle and cytotoxic events as revealed by the lactate dehydrogenase release. Caspase 3 activity was inversely proportional to the Api tested doses, namely 30 μM and 60 μM. Phenomena of early apoptosis, late apoptosis and necrosis following incubation with Api were detected by Annexin V-PI double staining. The flavone interfered with the mitochondrial respiration by modulating both glycolytic and mitochondrial pathways for ATP production. The metabolic activity of human dendritic cells (DCs) under LPS-activation was clearly attenuated by stimulation with high concentrations of Api. Il-6 and IL-10 secretion was almost completely blocked while TNF alpha secretion was reduced by about 60%. Api elicited antiangiogenic properties in a dose-dependent manner. Both concentrations of Api influenced tumour cell growth and migration, inducing a limited tumour area inside the application ring, associated with a low number of capillaries.

Keywords: apigenin; A375 human melanoma cell line; proliferation; apoptosis; mitochondrial bioenergetics and glycolysis; angiogenesis

1. Introduction

Natural products, either in the form of total extracts or purified active compounds, have been demonstrated to play a vital role in the current management of different types of cancer, with directions being pulled toward both treatment and prevention. As proven by current cancer therapy, an increased number of anticancer drugs used in the clinic are based on natural products obtained from different sources (plants, animals, microorganisms) [1]. Due to the fact that the biodiversity of our planet has not been fully exploited, many specialised research institutions, including the National Cancer Institute, have allocated important funds and brilliant minds in order to use what mother nature provided to fight the biggest challenge of the 21st century medicine: cancer.

Important studies in the field have shown that the incidence of melanoma has been growing especially in countries with light skin population [2]. Although melanoma accounts for only around 1% of the types of skin cancers, it is the most dangerous form, as it is responsible for most death cases. Moreover, the number of Americans diagnosed with skin cancer at a certain point in their lives in the last thirty years is estimated to be higher than the number of all other cancers summed up [3]. The standard models of evolution include: (a) benign naevi; (b) dysplastic naevi; (c) melanoma in situ; and (d) invasive melanoma [4]. The number of papers on PubMed that have the word "melanoma" in their title exceeds 120,000, thus indicating that melanoma represents a continuing hot topic that is being approached by an impressive number of different therapeutic strategies.

With respect to the main classes of natural compounds, flavonoids have been intensively studied as natural compounds with chemo-preventive properties against different types of cancer due to their biological activities which include antiproliferative and proapoptotic effects [5]. Apigenin (4′,5,7-trihydroxyflavone) (Api) is a natural compound belonging to the flavone subclass of flavonoids. The aglycone is part of the chemical composition of some glycosides, the main representatives being apigetrin, vitexin, isovitexin, apiin [6]. The flavone is an important component of the human diet, being distributed in a large category of fruits, vegetables and herbs, with the most important sources being represented by chamomile, celery, celeriac and parsley [7]. Other frequently used nutraceutics rich in this flavonol include oranges, grapefruit, garlic, and propolis [8]. Venigalla et al. established that in the case of ligulate flowers of chamomile, apigenin represents around 68% of the total flavonoids [9]. Apigenin has been described by numerous in vitro and in vivo studies in the field, using various cancer cell lines as a natural compound with chemo-preventive activity and tumour growth inhibition potential [10–12]. The relationship between cancer and inflammation is very well defined in the scholarly literature [13]. Following a complex study, Perrott et al. assigned apigenin some anti-inflammatory properties [14]. A current comprehensive review describes other biological properties of apigenin as follows: prevention of cardiovascular diseases due to different causes (atherosclerosis, hypertension, cardiac hypertrophy, induced heart injury), protective effect on the liver, on the respiratory system, on the endocrine system, on central nervous system, on bones and joins [8].

A recent paper has reported that the flavone exhibited antiproliferative, anti-invasive and proapoptotic properties in vitro against two human cancer cell lines, namely A375 and C8161 [15]. Following the same train of thought, a study conducted by Caltagirone et al. confirmed apigenin and quercetin as active compounds presenting the potential to inhibit melanoma onset and metastatic spreading in a murine model of melanoma designed by the injection of B16-BL6 cells into C57BL/6N mice [16]. This approach was investigated in more detail by the group of Piantelli et al., who assigned the anti-metastatic effect to a mechanism that involves impairing tumour cell endothelium interactions [17].

Using B16-F10 cell injection into syngeneic mice, Cao et al. proposed an additional mechanism, namely the inhibition of the STAT3 signalling pathway [18].

The aim of this study was to assess the antiproliferative, proapoptotic, antiangiogenic properties, the modulation of mitochondrial respiratory chain and the glycolysis by this flavone against A375 human melanoma cells, as well as to analyse its immunomodulatory effect in human dendritic cells.

2. Materials and Methods

Apigenin ≥99% (HPLC) (CAS Number 520-36-5) (Api) was acquired from Sigma-Aldrich, Steinheim, Germany.

2.1. Cell Culture and Preparation

The human melanoma (A375) cell line (ECACC; Sigma Aldrich origin Japan stored UK) was grown into Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL, Invitrogen, Carlsbad, CA, USA) supplemented with 1% penicillin/streptomycin mixture (Pen/Strep, 10,000 IU/mL; PromoCell, Heidelberg, Germany) and 10% foetal calf serum (FCS; PromoCell, Heidelberg, Germany). At 80–90% confluence, the cells were passaged following treatment with EDTA (5 mM).

Human dendritic cells were differentiated from isolated PBMCs by buffy coats, as described previously [19]. Briefly, Ficoll (GE Healthcare, Uppsala, Sweden) was used for density centrifugation.

Several (2×10^8) cells per well of isolated, PBMCs were plated, and the supernatant was discarded upon 2 h of plastic adherence. Afterwards, RPMI 1640 GlutaMax medium (Thermo Fisher Scientific, Boston, MA, USA) was used for cell differentiation. The medium was supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin with 10% FCS, 10 mM HEPES (Sigma-Aldrich, Steinheim, Germany), 1 mM sodium pyruvate and 50 µM 2-β-ME (Thermo Fisher Scientific, Boston, MA, USA) supplemented with 40 ng/mL recombinant human GM-CSF (Peprotech, NJ, USA) and human IL4 (Peprotech, NJ, USA); the medium was renewed after 4 days.

Differentiated cells were collected by cell scraping and transferred to 6-well plates for further experiments or to tissue treated 8-well chambered cover slides (Ibidi, Martinsried, Germany) for fluorescence microscopy staining. The supernatants were tested for TNF-alpha IL-10 and IL-6 (R&D Systems, Wiesbaden, Germany) by ELISAs according to the manufacturer's protocol.

2.2. Cell Viability Assays

The antiproliferative property of Api was determined by means of MTT (Sigma-Aldrich, Budapest, Hungary) assay against A375 human melanoma cells. Experiments were carried out as described previously [20]. Briefly, cells were seeded into 96-well plates (5000 cells/well) and treated with different concentrations of Api (0.3–60.0 µM) under standard conditions (37 °C, 5% CO_2). After 72 h of incubation, 5 mg/mL MTT solution was added and the microplates were incubated for an additional 4 h. The resulting formazan crystals were dissolved in dimethyl sulfoxide and the absorbance was determined at 545 nm with an ELISA reader (Awareness Technology, Palm City, FL, USA). Cisplatin, a clinically used anticancer agent was applied as a reference agent. Sigmoidal concentration–response curves were fitted to the determined results and IC_{50} values were calculated by means of GraphPad Prism (GraphPad Software, San Diego, CA, USA).

For cells viability assessment, the XTT assay (Thermo Fischer Scientific) was used according to the manufacturer on human dendritic cells. Briefly, the final XTT solution was put on wells with cells or medium only as a control. After 45 min incubation time at 37 °C and 5% CO_2, aliquots of the cells were assessed in flat 96-well plates (Greiner, Frickenhausen, Germany) at 460 and normalised to 650 nm. Previously, cells had been stimulated with the vehicle or with Api, in the presence or absence of LPS for 24 h and 48 h at the indicated concentrations. After XTT assay, the cell number was obtained and normalised to 10^4 cells.

2.3. Cell Cycle Analysis by Flow Cytometry

To describe cell cycle distribution, the DNA content of the cells was determined by flow cytometry. A375 cells were plated into 6-well plates (300,000–400,000 cells/well) and pre-incubated for 24 h. Then the cells were washed twice with cold phosphate-buffered saline (PBS), trypsinised and centrifuged (1500 rpm, 10 min). Cells were washed and fixed in 1 mL of cold ice 70% ethanol for 30 min. Samples were treated with dye solution containing RNAse A (0.02 mg/mL), propidium iodide (0.1 mg/mL), Triton-X (0.003 mL/mL) and sodium citrate (1.0 mg/mL) in distilled water and the mixtures were kept in the dark for one hour at room temperature. DNA content of the cells was analysed by a Partec CyFlow flow cytometer (Partec GmbH, Münster, Germany). In each sample, 20,000 cells were assessed, and the proportion of the cells in the different cell cycle phases (subG1, G1, S and G2/M) were calculated using ModFit (Verity Software House, Topsham, ME, USA).

2.4. Anti-Migratory Potential—Scratch Assay Method

For the assessment of the regressive effect of Api on the invasion capacity of the human melanoma-A375 cell line, the scratch assay test was performed. Several 2×10^5 cells/well were seeded onto 12-well culture plates until 90% confluence was reached. After that, the attached cells were scratched following the diameter of the well using a sterile pipette tip. The detached cells and cellular debris were removed by gently washing the wells with PBS. Furthermore, the cells were stimulated with Api at two different concentrations 30 μM and 60 μM. Wells were captured on images at 0 h and 24 h, in order to compare the cell growth of the stimulated vs. control (no stimulation) cells in early stages and at consistent times. Each well was marked below with a line, to improve identification of the same imaging area. Images were taken with Olympus IX73 inverted microscope provided with DP74 camera (Olympus, Tokyo, Japan) and cellSense Dimension software was used for analysing the cell growth. To quantify the migratory ability of the cells, the wound closure percentage was calculated as previously described [21].

2.5. Determination of In Situ Caspase Activity

Caspase-3 is one of the key players in the apoptotic machinery. To determine the effects of Api on the activity of caspase-3, a colorimetric assay (Sigma-Aldrich, Budapest, Hungary) was performed. Ten and 12 million cells were plated in tissue culture flasks for control and treatment condition, respectively. After 24 h of pre-incubation, the cells were treated with Api (30 uM or 60 uM) for 48 h. Then cells were sampled, counted, centrifuged, washed with PBS and re-suspended in kit lysis buffer (10^7 cells/100 μL), and incubated on ice for 20 min. The lysate was centrifuged, and the protein concentration of the supernatant was determined (Pierce Biotechnology, Rockford, IL, USA). According to the manufacturer's protocol, 5.0 μL portions of treated and control lysates were incubated with 10 μL selective substrate of the enzyme (acetyl-Asp-Glu-Val-Asp-*p*-nitroaniline) in a final volume of 100 μL buffer. After a night of incubation at cell culture conditions, the absorbance of *p*-nitroaniline was measured at 405 nm with an ELISA reader. The treatment-related change in the caspase activity was expressed as fold increase.

2.6. Annexin V—Propidium Iodide Assay

Into 6-well plates (Greiner bio-one), 5×10^5 cells/well were seeded and allowed to attach to the bottom of the plate overnight. After 24 h, the cultured medium (DMEM) was thrown and a fresh one containing Api at the highest concentration (60 μM) was added. Starting from a stock solution of 10 mM Api in DMSO, successive dilutions into the medium were performed in order to obtain the final concentration of the tested compounds. As a control sample, untreated cells were used; cells incubated with DMSO were used as solvent control. After 72 h, the cells were trypsinised and analysed for the apoptotic effect of the tested compounds using flow cytometry. Annexin V-FITC mixed with propidium iodide (PI) kit (Invitrogen, ThermoFisher, Vienna, Austria) was used for cell death flow cytometric

studies (apoptosis) according to the manufacturer's manual. In brief, 2–5 × 10^5 cells were washed two times in 1 × Annexin V binding buffer, and then centrifuged at 1,500 RPM for 5 min, re-suspended in the binding buffer and incubated with 5 µL of Annexin V-FITC in the dark for 15 min. After the cells were washed with 200 µL of the specific binding buffer and then centrifuged, the cell pellet obtained was re-suspended in a volume of 190 µL binding buffer, and immediately prior to analysis by flow cytometry 10 µL of PI solution was added.

2.7. Assessment of Cytotoxicity via LDH Released

Lactate dehydrogenase (LDH) assay kit (No 88954, Thermo Fisher Scientific, Boston, MA, USA) was employed to determine the cytotoxic effect of Api and DMSO at concentration of 30µM and 60 µM on the human melanoma A375 cell line. This technique is based on the cytosolic enzyme released (LDH) into the media which can be further quantified by an enzymatic reaction, leading to formazan production. The level of formazan is directly proportional to LDH leakage into the media, which is an indicative of the cytotoxic effect.

Briefly, 5000 cells/well in 200 µM specific media were cultured in 96-well plate and allowed to attach overnight. The next day, the cells were treated with Api and DMSO at concentration of 30 µM and 60 µM and incubated for 72 h. After this step, the LDH released into the media was transferred into a new 96-well plate, followed by an addition of reaction mixture. The plate was incubated at room temperature, protected from light; stop solution was added after 30 min. The concentration of formazan is measured at 490 nm and 680 nm wavelengths via spectrophotometry with a microplate reader (xMarkTMMicroplate, Serial No. 10578, Biorad, Japan).

2.8. Fluorescence Microscopy

Tissue-treated 8-well chambered cover slides (Ibidi, Martinsried, Germany) were used for dendritic cells growth. The cells were cultured and fixed as previously described [19]. DAPI (4′,6-Diamidine-2′-phenylindole dihydrochloride) solution (Roche Diagnostics, Mannheim, Germany) and phalloidin Alexa Flour 488 solution (Thermo Fisher) were applied for 1 h. A Zeiss LSM510 Meta system equipped with an inverted Observer Z1 microscope and a Plan-Apochromat 63 ×/1.4 oil immersion objective (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) were used for confocal laser scanning microscopy.

2.9. Extracellular Flux (XF) Analysis

A375 cells (2 × 10^4 cells/well) were seeded in Seahorse 24-well cell culture plates and allowed to attach overnight. On the second day, the cells were stimulated with two concentrations of Api (30 and 60 µM) or with DMSO; the control group is represented by untreated cells, incubated only with cell culture medium. Background correction wells (wells that were not seeded with cells) were included in the assay, in order to normalise the data to background plate noise. Cells were incubated at 37 °C and 5% CO_2 with the samples for 72 h.

The cells were divided into five groups: control—untreated cells; cells treated with DMSO; and cells treated with Api 30 µM and Api 60 µM, respectively. The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using the Seahorse XFe24 (Seahorse Agilent) extracellular flux analyser, as previously described [22]. Three injections were performed in order to change cells metabolism, namely: oligomycin (1 µg/mL)—a complex V inhibitor; FCCP (3 µM)—a classic uncoupling agent, and antimycin A+Rotenone (2.5 µM + 2 µM)—inhibitors of complex I and III, respectively [23]. The OCR parameters recorded in the analysis were as follows: (i) the basal respiration (before oligomycin addition); (ii) the leak state (after oligomycin addition)—the amount of O_2 consumption needed to sustain the proton gradient; (iii) the maximal respiration (after FCCP addition)—respiration in the presence of a classic uncoupler of oxidative phosphorylation; (iv) the ATP turnover (the difference between the basal respiration and the leak state)—the amount of O_2 consumption used for ATP production; (v) the reserve capacity (the difference between the maximal

and the basal respiration). The reserve capacity is a fundamental parameter of cellular bioenergetics, and shows the capacity to respond to an increased energy demand [24]. OCR was reported in units of pmoles/minute and ECAR in mpH/minute.

2.10. Chorioallantoic Membrane Assay (CAM)

The CAM assay involves the use of fertilised hen (Gallus gallus domesticus) eggs. We implemented a slightly modified technique developed by Ribatti et al. [25]. Briefly, the general method includes egg disinfection with 70% ethanol prior to incubation at controlled 37 °C and 50% humidity. On the third day of incubation, noted as the embryonic day of development (EDD 3), 3–4 mL of albumen was removed, followed by cutting and resealing a window on the upper side of the eggs on EDD 4. In ovo macroscopic assessment was performed in daytime by means of a stereomicroscope (Discovery 8 Stereomicroscope, Zeiss). For further morphometric analysis, significant images were registered on a daily basis, using the Axio CAM 105 colour, Zeiss digital camera and processed by Zeiss ZEN software, ImageJ and GIMP.

The morphometric evaluation of the angiogenic reaction can be assessed using different approaches, namely semi-quantitative scales [26] or equations [27–29]. In this study, macroscopic images were used in order to count the number of blood vessels (BV) intersecting the inoculation ring. Angiogenesis inhibition (AI) can be expressed in percentages using the following equation:

$$\text{AI (\%)} = (1 - \text{No BVtest/No BVcontrol}) \times 100. \quad (1)$$

2.11. Normal Angiogenesis Assessment on the Chorioallantoic Membrane

Firstly, we assessed the effects induced by Api on the normal developing CAM. This type of assay is indicative for the tolerability of Api on normal tissues, but also stands for the predictability of its implications on highly angiogenic blood vessels (between EDD 7–10). Starting on EDD 7 (day 0, 0 h) three test groups of samples were designed: (a) Api in 30 μM (API 30) concentration; (b) Api in 60 μM (API 60) concentration; (c) DMSO 1% as solvent control (1% DMSO *v*/*v* in double distilled water). All samples in volumes of 5 μL/egg were applied directly inside a plastic ring placed on top of the CAM. The assessment was carried out for 48 h, representing a medium-term tolerability assessment.

2.12. Tumour Angiogenesis Assessment on the Chorioallantoic Membrane

The assessment of Api in an in vivo melanoma model using the CAM assay requires the inoculation of the melanoma cells on top of the developing membrane on EDD 10 (day 0, 0 h). A375 melanoma cells were cultured according to the above described protocol and subsequently inoculated onto the CAMs [30]. Briefly, after detaching the cells from the culture plate by trypsinisation, they were cleansed and re-suspended in the culture medium until reaching the final concentration of 10^5/5 μL. On the 10th day of incubation, 5 μL of the melanoma cell suspension was inoculated inside a plastic ring previously placed on the CAM. All specimens were inoculated with 5 μl of A375 melanoma cells and were divided in three test groups: (a) Api in 30 μM concentration; (b) Api in 60 μM concentration; (c) DMSO 1% as solvent control. Each test solution was applied in volumes of 5 μl and was repeated daily for 96 h, until EDD 14. Relevant images were captured every day in vivo, and on the final day of the experiment, after detaching the fine membranes of the tested specimens, ex vivo images were also taken. The same type of angiogenesis analysis as described for the normal tested CAMs was performed for the melanoma treated specimens.

2.13. Statistics

The Prism software package (Graph Pad Prism 5.0 for Windows) was used for data collection and presentation. The data ranged from three to five separate experiments is presented as the mean ± SD. An unpaired Student's *t*-test, one-way ANOVA or two-way ANOVA followed by a Bonferroni post-test or Newman-Keuls post-test were used to determine the significant differences between the

various experimental and control groups. A paired Student's *t*-test was used to determine significant differences in all experiments concerning dendritic cells. *, **, ***, **** indicate $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively, compared to the control group or otherwise-indicated groups.

3. Results

3.1. Cell Growth Inhibition

As can be observed in Figure 1, in the range of tested concentrations, Api presents substantial antiproliferative effect against A375 human melanoma cell line starting from the 30 μM concentration. The calculated IC_{50} is 33.02 μM.

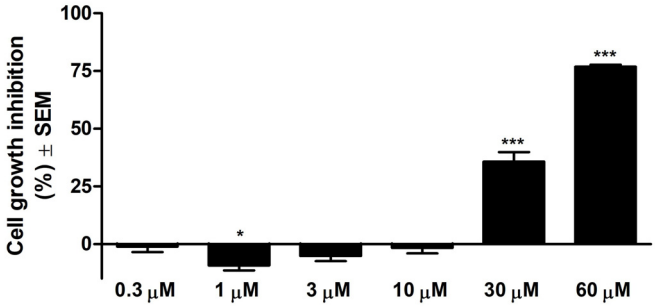


Figure 1. Cell growth inhibition (%) ± SEM against A375 human melanoma cells after 72 h of incubation with Api. * $p < 0.05$; *** $p < 0.001$.

3.2. Api Effects on Cell Cycle Phases

To have a complete picture of the antiproliferative effect, the concentrations that led to this kind of event, namely 30 μM and 60 μM Api, were used to analyse the effect on the phases of the cell cycle. Results showed that in the case of both concentrations, Api induced a G2/M arrest by increasing the percentage of A375 cells in this phase of the cell cycle from 18.946 ± 1.91% (control) to 33.423 ± 0.15% (30 μM Api) and 33.653 ± 0.96% (60 μM Api). Results are described in Figure 2.

3.3. Antiproliferative Effect of Api

As shown in Figure 3, both concentrations of Api (30 μM and 60 μM) manifested a significant inhibitory effect on the migratory capacity of human melanoma A375 cells, when compared to the migratory ability of control cells. Human melanoma A375 cells displayed wound width modifications of only 38 μm, from 575.05 μm to 537.59 μm, after treatment with Api 60 μM and from 550.84 μm to 418.90 μm after stimulation with Api 30 μM in a 24 h timeframe. The wound healing rate induced after Api treatment at 30 μM and 60 μM concentrations was 23% and 6.84%, respectively. This means that the inhibitory rate expressed by Api (30, 60 μM) on cell migration was 77% and 93.16%, respectively. However, the control cells (no stimulation) displayed a good wound healing rate above 77% after 24 h.

In addition, it can be observed that cells showed apoptotic characteristics by changing their shape and morphology, followed by cell detachment after 24 h, after stimulation with Api at 60 μM concentration.

The aforementioned data show that in the set experimental conditions Api exhibits antiproliferative potential.

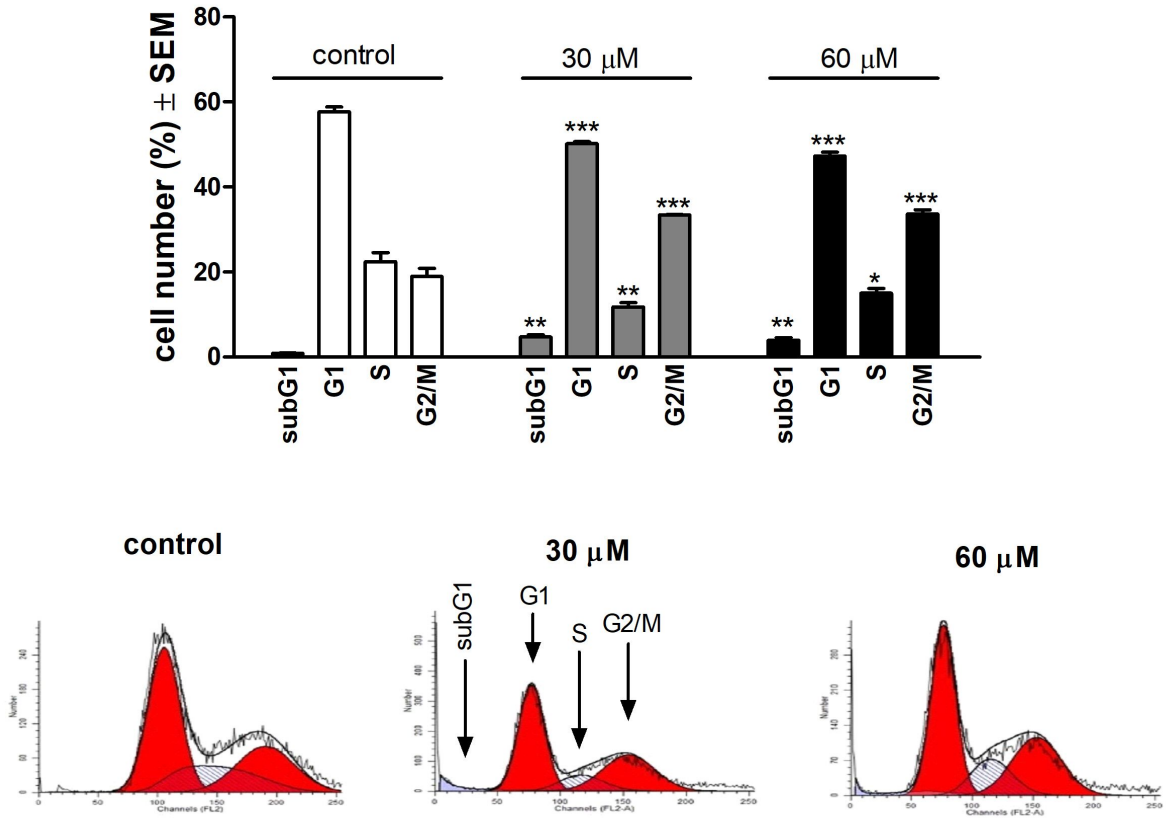


Figure 2. Upper panel: effects of API on the A375 human melanoma cell cycle after incubation for 24 h. Results are mean values ± SEM from three measurements. *, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ as compared with the control cells, respectively. Lower panel: representative histograms.

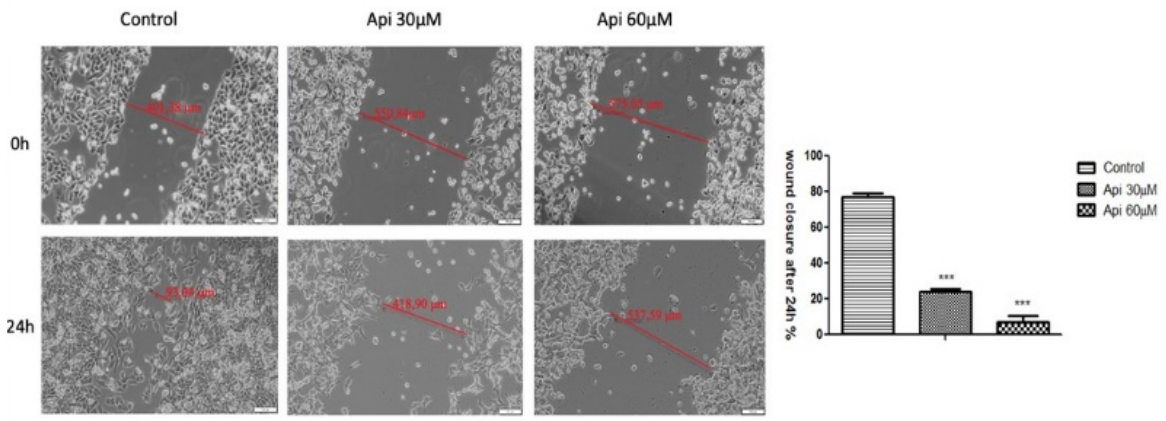


Figure 3. Antiproliferative effect of Api on the human melanoma A375-cell line, after stimulation with Api at 30 μM and 60 μM concentrations. Images were taken by bright field microscopy at 10× magnification. Scale bars represent 100 μm. The bar graphs are expressed as percentage of wound closure after 24 h compared to the initial surface. The data represent the mean values ± SD of three independent experiments. One-way ANOVA analysis was performed to determine the statistical differences followed by Tukey post-test (*** $p < 0.001$ vs. control—no stimulation).

3.4. Caspase 3 Activity

A new set of experiments was conducted in order to gain insights about potential proapoptotic and/or cytotoxic effect. In this type of experiment, the Hormesis phenomena can be observed to be characterised by a biphasic response. An increased activity of protein caspase 3 following 72 h of incubation with Api 30 μM was observed. Interestingly, Api 60 μM did not enhance caspase 3

activity, presumably because of a cytotoxic effect at this concentration/incubation time (Figure 4). Also, the expression of caspase 2 and p53 proteins was analysed by immunocytochemistry, but none of these proteins were expressed following incubation with the highest tested concentration of Api (Supplementary Figure S1).

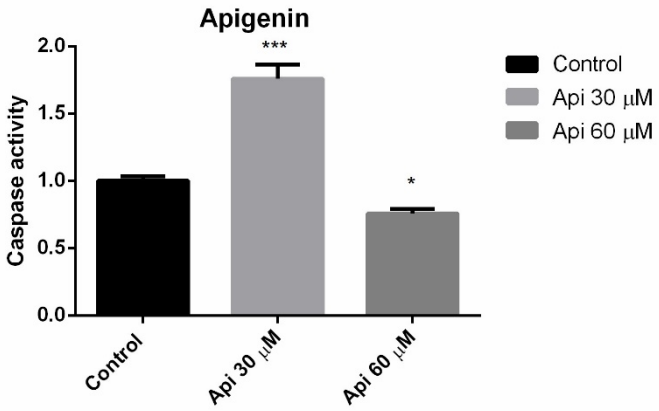


Figure 4. Caspase 3 activity in A375 human melanoma cells after 72 h of incubation with Api. * $p < 0.05$; *** $p < 0.001$.

3.5. Annexin V-PI

This approach was followed by the Annexin-PI double staining, a consecrated assay that makes it possible to get information regarding phenomena of early apoptosis, late apoptosis and necrosis. Api at the tested concentrations (30 μM, 60 μM) induced both early and late apoptosis phenomena, as well as necrosis. As can be seen from Figure 5, where the means of three different experiments are represented, Api 30 μM caused preponderantly early apoptotic events (8.5 ± 1.8% vs. 86.25% ± 1.8 with respect to Control), while Api 60 μM (12.25 ± 2.9% vs. 77.5 ± 3.2% with respect to Control) induced predominantly late apoptotic events.

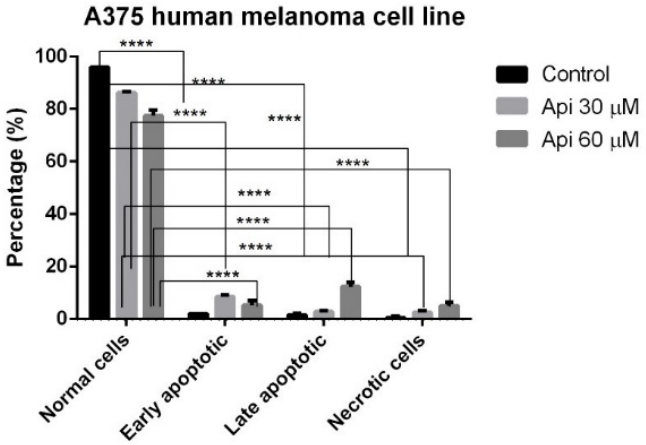


Figure 5. Annexin-PI staining in A375 human melanoma cells incubated with Api 30 μM and Api 60 μM, showing the populations of normal, early apoptotic, late apoptotic and necrotic cells. **** $p < 0.0001$.

3.6. LDH Assay

To gain more information regarding the cytotoxic potential of Api at the selected concentrations, lactate dehydrogenase assay was performed. After 72 h, a significant difference was observed in the release of lactate dehydrogenase between Api 30 μM (cytotoxicity rate of 20.75%) and DMSO (cytotoxicity rate of 1.12%). However, increment of Api concentration at 60 μM did not increase its

cytotoxic effect on the human melanoma A375 cell line, showing a cytotoxic effect of almost 19%. Results are presented in Figure 6.

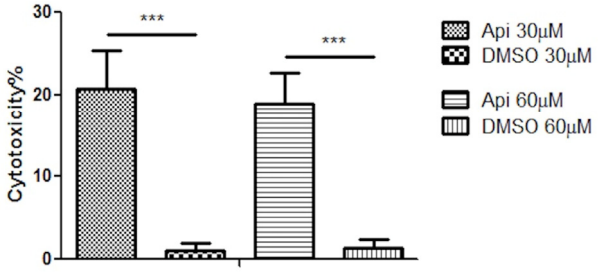


Figure 6. The cytotoxic effect of Api and DMSO at 30 μM and 60 μM concentrations on the human melanoma A375 cell line after 72 h exposure time. *** $p < 0.001$.

3.7. Bioenergetic Profile of A375 Human Melanoma Cells

Within this study we also assessed the Api effect on cellular bioenergetics in A375 human melanoma cells. The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured at 72 h post-treatment of A375 human melanoma cells with two concentrations of Api (30 and 60 μM) using the Seahorse XFe24 (Seahorse Agilent) extracellular flux analyser. Results describing the bioenergetic profile of A375 human melanoma cells at 72 h post-stimulation are presented in Figure 7.

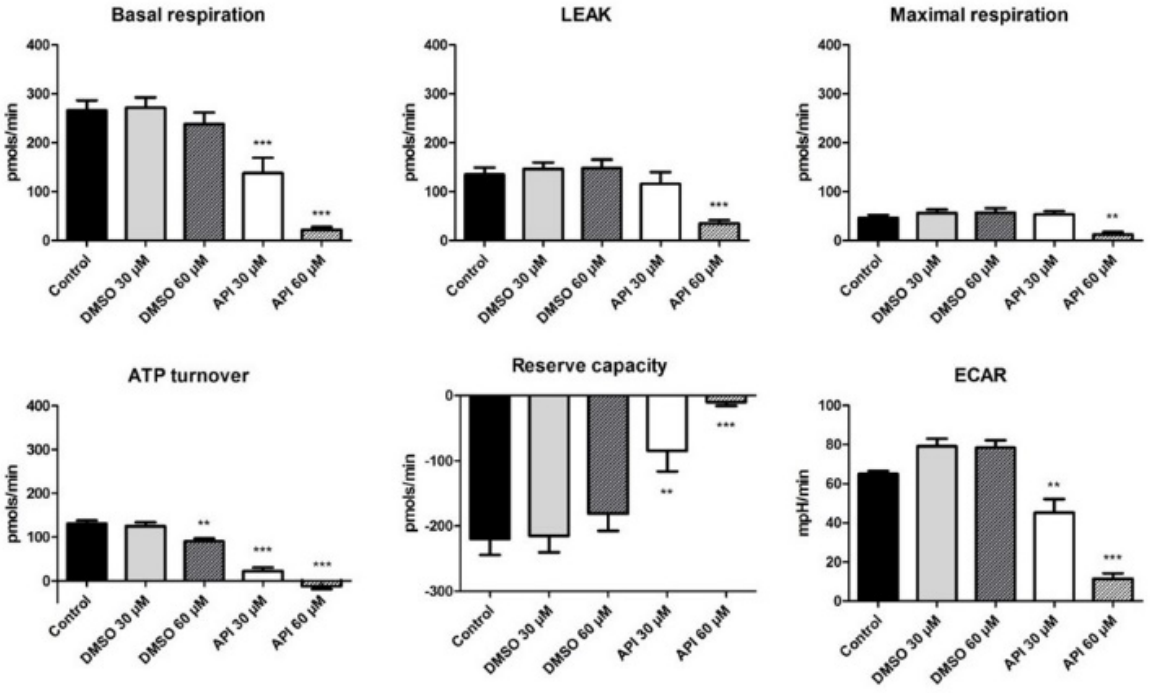


Figure 7. The effect of Api (30 and 60 μM) on A375 human melanoma cells (72 h treatment) with respect to OCR and ECAR parameters. ** $p < 0.01$; *** $p < 0.001$.

We observed that in the basal respiration state, the cells are in the unchallenged state. Treatment with Api induced a significant dose-dependent decrease in the baseline rates (basal respiration for Api 30 μM → 137.9 ± 31.4 pmols/min vs. Control → 266.07 ± 20.8 pmols/min; Api 60 μM → 22.2 ± 5.5 pmols/min vs. Control). In the leakage state, there is a decrease in the OCR due to blockage of ATP production following oligomycin injection. At the higher employed dose—60 μM Api—a decrease in the proton leak state (34.9 ± 7.1 pmols/min vs. Control → 135.01 ± 14.6 pmols/min) and in the maximal respiration rate (12.4 ± 5.3 pmols/min vs. Control → 46.5 ± 5.9 pmols/min) was elicited.

After adding the respiratory chain uncoupling agent (FCCP), there is normally an increase in the O_2 consumption due to the uncoupling mechanism. In our case, the maximal respiration was lower than the basal respiration, because in the case of tumour cells, there is a shift to a glycolytic state. The maximal respiration for Api 30 µM is 53.3 ± 6.9 pmols/min, whereas for Api 60 µM a value of 12.4 ± 5.3 pmols/min was recorded. Furthermore, the ATP turnover also displayed a decrease after stimulation with Api. This effect was recorded for Api in both tested doses and also in the case of DMSO, at the higher tested dose, 60 µM (ATP turnover for Api 30 µM was -12.65 ± 6.5 pmols/min vs. Control 131.05 ± 7.1 pmols/min and for Api 60 µM it was 22.1 ± 8.3 pmols/min vs. Control).

As previously mentioned, the reserve capacity represents the difference between the maximal and the basal respiration, and due to the fact that the first recorded lower values than the basal respiration, we obtained negative values. Our data indicate that treatment with Api decreased A375 tumour cells reserve capacity considerably, which proves that it is more difficult for treated cancer cells to respond to stress than untreated cells or cells treated with DMSO (Reserve capacity for Api 30 µM → −9.8 ± 5.8 pmols/min vs. Control → −219.5 ± 24.5 pmols/min and for Api 60 µM → −84.6 ± 31.4 pmols/min vs. Control).

In the DMSO groups, there is a slight increase in ECAR, whereas in the Api groups, there is a significant dose-dependent decrease of ECAR, indicating that the compound also induced an impairment in cellular glycolytic activity (Api 30 µM → 11.4 ± 2.8 mpH/min vs. Control → 65.04 ± 1.5 mpH/min and for Api 60 µM → 45.1 ± 7 mpH/min vs. Control). Our results indicate a significant alteration of the bioenergetic profile in A375 human melanoma cells treated with 60 µM Api, an effect that might emphasise its beneficial properties against tumour cells.

3.8. Immunomodulatory Effects of Api

To test the immunomodulatory effects of Api, primary peripheral blood mononuclear cells (PBMCs) were isolated from human blood, differentiated into dendritic cells (DCs), and stimulated with corresponding amounts of Api. Cell expansion of human dendritic cells after 24 h with vehicle, Api in different concentrations, or DMSO in the absence (naïve) or presence of LPS, as well as representative transmitted light microscopic images of Api-stimulated human dendritic cells after 24 h in the absence (native) or presence of LPS, are presented in Figure 8a,b.

As expected, vehicle- and DMSO-treated cells expanded within 24 h of lipopolysaccharide (LPS) activation (Figure 8a). This cell expansion was completely abrogated by parallel stimulation of 30 and 60 µM Api, while low-dose Api (1µM) had no effect compared to control. Transmitted light microscopic images highlight the strong effect of Api on cell morphology (Figure 8b). The cells acquired a round shape upon high-dose Api stimulation. Moreover, LPS stimulation failed to provoke the typical development of dendrites as seen in the control cells or at the low dose of Api.

3.9. XTT Assay for Metabolic Activity

The metabolic activity of human DCs under LPS-activation was clearly impaired by stimulation with high concentrations of Api with 24 h and even stronger within 48 h (Figure 9a). Confocal microscopy performed 48 h after stimulation revealed substantial cell damage with 60 µM Api stimulation, while control cells appeared normal (Figure 9b, upper panel). Furthermore, LPS activation led to a typical aggregation of DCs to cell-clusters under control conditions, which was not present under a high dose of Api stimulation (lower panel).

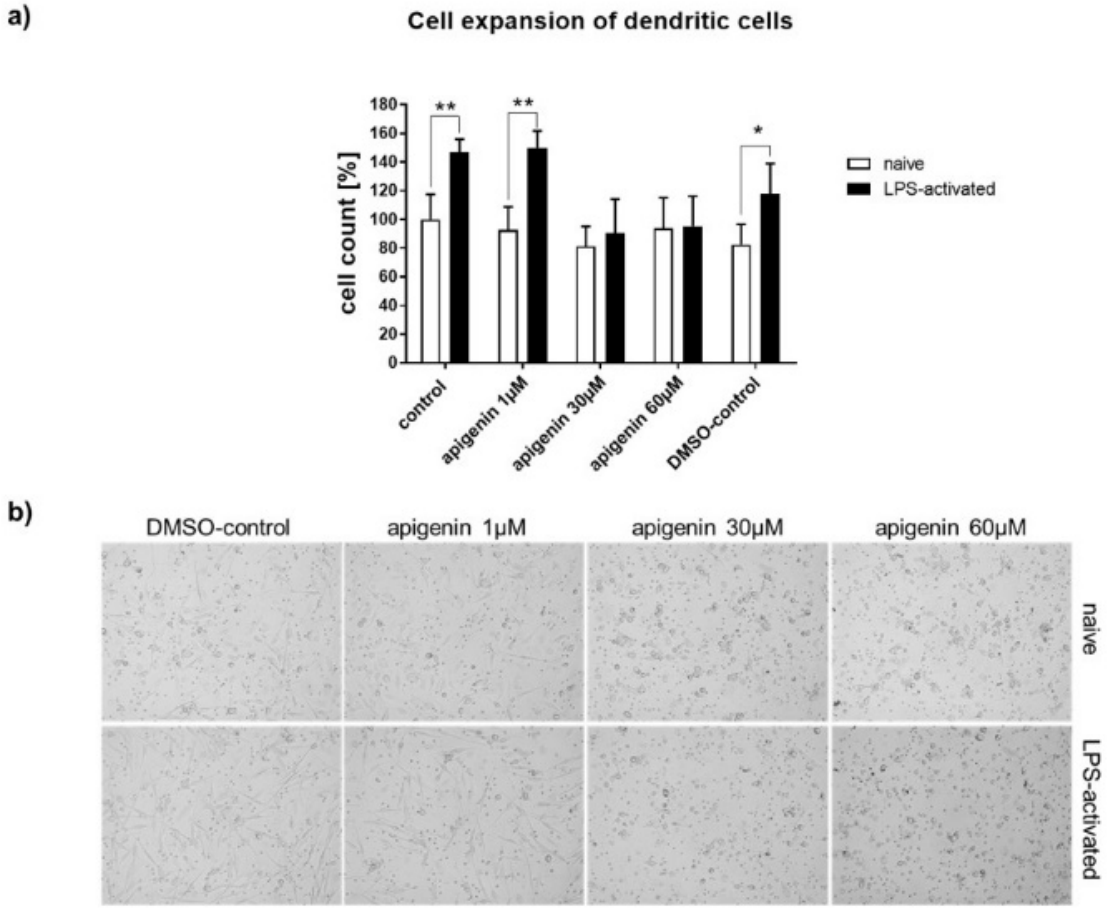


Figure 8. (**a**) Cell expansion of human dendritic cells after 24h with vehicle, Api in different concentrations or DMSO in the absence (native) or presence of LPS. (**b**) Representative transmitted light microscopic images of Api stimulated human dendritic cells after 24 h in the absence (native) or presence of LPS (magnification 20×). * $p < 0.05$; ** $p < 0.01$.

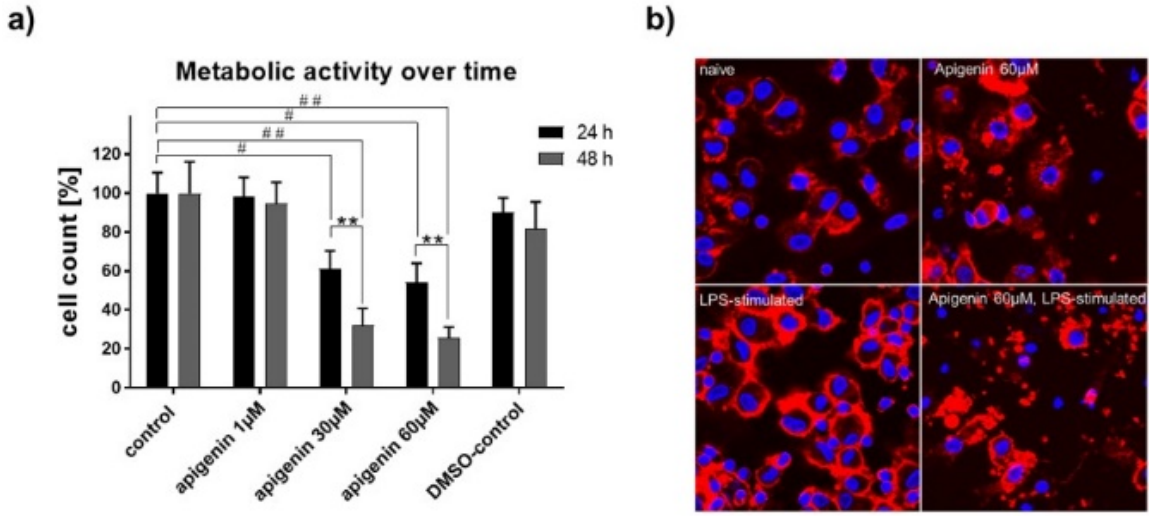


Figure 9. (**a**) XTT assay for metabolic activity of human dendritic cells stimulated with vehicle, Api in different concentrations or DMSO in the presence of LPS for 24 h and 48 h. (**b**) Representative confocal microscopic images of human dendritic cells treated with vehicle or 60 µM Api in absence (upper panel) or presence (lower panel) of LPS for 48 h (magnification 63x). Data are expressed as mean ± standard deviation (SD); significant differences are indicated as ** $p < 0.01$; $^{\#\#}$ $p < 0.01$, $^{\#}$ $p < 0.05$; $n = 4$.

3.10. Cytokine Quantification

Cytokine secretion was analysed in order to see if the reduced cell activity of LPS-stimulated cells had functional consequences. Even though DMSO led to a significant increase in IL-6 and TNF-alpha secretion, possibly because of some induced increase in the membrane permeability, the cytokine secretion was very strongly blocked by higher concentrations of Api (Figure 10). IL-6 and IL-10 secretion was almost completely blocked by 30 and 60 µM stimulation with Api and TNF alpha secretion was reduced by about 60%. The low dose of Api had no effect compared to the control secretion in all cytokines.

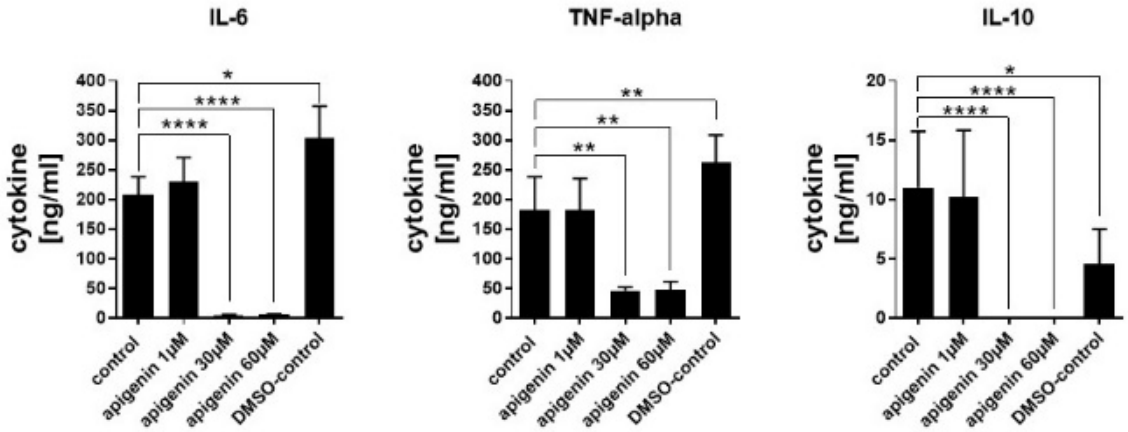


Figure 10. Quantification of cytokines in the supernatant of human dendritic cells stimulated with vehicle, Api in different concentrations or DMSO for 24 h in presence of LPS. Data are expressed as mean ± standard deviation (SD), significant differences are indicated as * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$; $n = 3$.

3.11. Chorioallantoic Membrane Assay

Using the in vivo chick chorioallantoic membrane assay, we investigated in ovo the tolerability and potential influence of Api on the normal and tumoural angiogenic process, next to the effect produced directly on the development of A375 melanoma cells. The assessment of Api in 30 and 60 µM concentrations was performed compared to the solvent control, diluted DMSO.

Firstly, we used the normal angiogenesis type of assay in order to assess the biocompatibility and tolerability. Survival rates of the embryos were similar for both concentrations of Api and the solvent control. However, some differences were observed concerning the inflammatory and irritation responses or the development of the CAM. Api in 60 µM concentration induced an irritation and fibrotic process that involved a higher number of capillaries surrounding the lesioned area. By testing the compound during a time-frame characterised by a rapid mitotic index and growth of endothelial cells, we were also able to assess the influence of Api on such a process. Interestingly, the low concentration showed a more important effect of reducing the number of capillaries inside the application area. Api at 60 µM induced a reduced inhibition of the angiogenesis, compared to Api at 30 µM, but still higher than the control (Figure 11).

When tested on the tumour model in the CAM assay, using A375 melanoma cells (Figure 12), the influence of Api on tumour cells was also assessed, next to the influence of the compound on the tumour angiogenic process. Both concentrations of Api influenced tumour cell growth and migration, inducing a limited tumour area inside the application ring, associated with a low number of capillaries. Still, the migration of melanoma cells was not totally inhibited, areas of cells were observed outside the ring and spokes wheel pattern of vessels converging on the tumour cells.

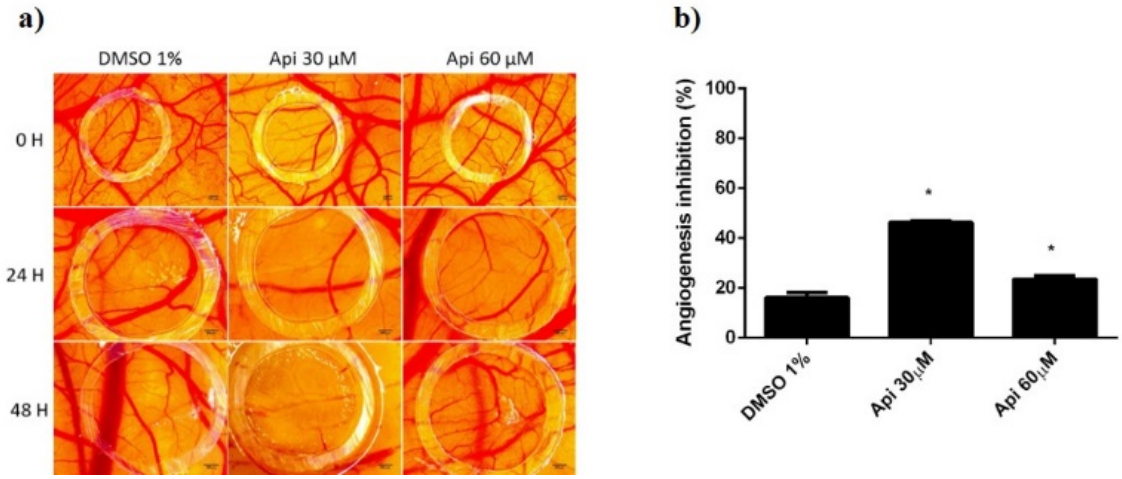


Figure 11. Normal CAMs treated with Api: (**a**) Stereomicroscopic in vivo images of the areas treated with Api 30 and 60 μM and with DMSO 1% as solvent control: initially—0 h, after 24 h, and after 48 h; (**b**) the angiogenic inhibition % at 48 h for Api 30 μM and 60 μM compared to DMSO 1%. * $p < 0.05$.

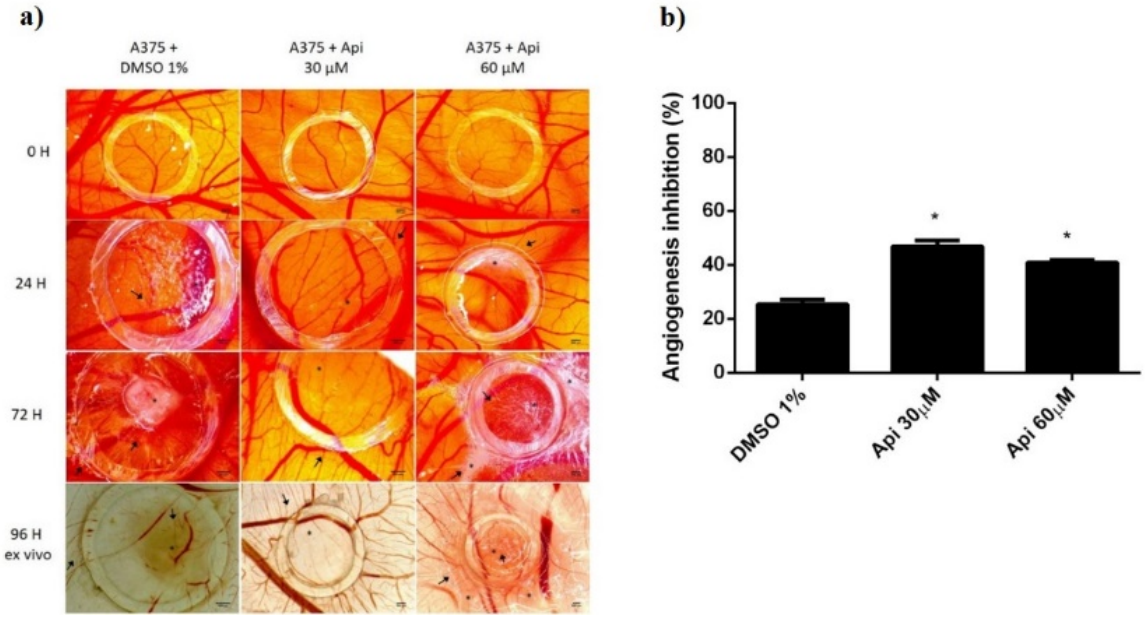


Figure 12. A375 melanoma cells on CAMs treated with Api: (**a**) Stereomicroscopic in vivo images of the areas previously inoculated with melanoma cells and treated with Api 30 and 60 μM and with DMSO 1% as solvent control; initially—0 h, after 24 h, after 72 h, and after 96 h, ex vivo, after membranes biopsies were obtained; (**b**) the angiogenic inhibition % in A375 melanoma cells environment, at 48 h, for Api 30 μM and 60 μM compared to DMSO 1%. * $p < 0.05$.

Differences noticed consist of the effect induced by the two concentrations of Api. A reduced number of cells with minimal aggregation pattern were observed for the low concentration of Api (30 μM). The number of vessels inside the ring was also reduced, with capillaries showing irregularities. The higher concentration (30 μM) also showed a limited growth of tumour cells inside the ring, with reduced number of interconnected capillaries, though; the vascularisation was less inhibited compared to the low concentration, possibly owing to the irritation potential that was observed in the normal setting of CAM assay. Still, the differences between the two concentrations are reduced in the tumour angiogenesis model, and both are significantly more active in angiogenesis inhibitors compared to the control.

4. Discussion

In this study, we have demonstrated that Api is an antiproliferative and proapoptotic agent against the A375 human melanoma cell line, leading to an IC_{50} of 33.02 μM for the tested dose ranges (0.3–60 μM). The same conclusion was drawn by the group of Zhao et al. [15]. In their approach, in order to assess the antiproliferative potential, two melanoma cell lines were used, namely A375 and C8161, and concentrations in the interval of 40–240 μM with check points at 24 h, 48 h, 72 h and 96 h. Api inhibited proliferation in a dose-dependent manner, as well as in a time-dependent manner. Moreover, Api inhibited migration at the 40 and 80 μM concentrations after 24 h of exposure, but the effect was abolished at 100 μM. Relative to the invasion, Api inhibited this process at 40 μM concentrations after 72 h of exposure, but again the effect was abolished at 80 μM. Using a higher concentration than in our study, namely 100 μM, they also demonstrated that Api causes a G2/M arrest in the two selected melanoma cell lines. They also detected apoptotic events after 24 h of exposure at 40 and 80 μM [15]. The ability of this flavone to arrest the cell cycle in the G2/M phase in the case of epidermoid carcinoma A431 cells was postulated by Chan et al. [31]. Hasnat et al., using A375 and A2058 human melanoma cell lines, showed that Api in 50 μM concentration and after a period of incubation of 24 h significantly decreased the number and viability and altered the morphology of selected cells [32]. As discussed in the results section of our study, we have demonstrated that Api at 30 μM concentration and after a period of 72 h induced caspase 3 activation; however, when 60 μM was applied, the concentration of caspase 3 decreased as compared to control, presumably because of a cytotoxic effect at this concentration and timeframe. The assessment of the cytotoxicity was also performed by quantifying the amount of LDH, a cytosolic enzyme which is released by the cells undergoing necrosis [33,34]. However, the sensitivity of this technique is questionable when cells are exposed to compounds that induce cell cycle arrest [35]. Due to the fact that cells no longer express proliferative properties, the amount of LDH that can be released from the cells will be quite low, thus undermining the sample-induced cytotoxic effect. This phenomenon was also observed in our case for the A375 human melanoma cells stimulated with Api 60 μM. A more intense cytotoxic effect manifested by Api at 60 μM compared to the effect induced by Api at 30 μM cannot be disputed, as shown by the images performed during LDH assessment (Figure S2); yet, under these conditions, the LDH technique yielded diminished cytotoxicity results. Using the Western blot analysis, Zhao et al. showed that after 24 h of incubation, Api 100 μM augmented the expression of cleaved caspase-3 in the case of A375 and C8161 human melanoma cells [15]. In an in vivo study, Caltagirone et al. demonstrated that this flavonoid administrated intraperitoneally in mice bearing a murine model of melanoma by employing B16-BL6 cells led to a dose-dependent delay of tumour growth and decreased the number of B16-BL6 colonies in the lungs. Moreover, they showed that the phytocompound is not toxic and is able to potentiate the activity of cisplatin [16]. Along the same line of thought, Cao et al. published that the dietary flavonoid suppressed metastasis in mice bearing B16F10 lung tumours and also inhibited invasion and migration in both human and murine melanoma cell lines. A possible mechanism was assigned to the inhibition of the STAT3 signalling pathway [18]. Chao et al. showed that in the case of different human uveal melanoma cell lines, Api inhibits expression and secretion of VEGF by a mechanism that involves suppression of ERK1/2 and PI3K/Akt pathways [36]. Das et al. used Api obtained from an ethanoic extract of the plant *Lycopodium clavatum* (LC) and assessed the anticancer potential using both the A375 human melanoma cell line and the A549 human lung cancer cell line in the 20–250 μg/mL dose range interval. Their study also confirmed the in vitro antiproliferative potential against the melanoma cell line and proposed it as a mechanism for apoptosis DNA interaction, damage and mitochondrial dysfunction by the direct activity on mitochondrial oxidative phosphorylation system [37].

In the last decade, several studies have reported that Api can directly target mitochondria in tumour cell lines, revealing the activation of the mitochondrial apoptotic pathway; Api is associated with DNA fragmentation, production of reactive oxygen species, mitochondrial membrane depolarisation, release of cytochrome c and up-regulation of Bax, caspase 3, 9 and PARF [38–40]. Furthermore, it

has been shown that Api mediates mitochondrial dysfunction in melanoma cells, namely disrupting the oxidative phosphorylation system of the A375 melanoma cell line [37]. However, the authors did not indicate whether there is impairment in the glycolytic state after Api treatment. Usually, the ECAR increase as a consequence of the cells effort to generate ATP in order to maintain their energy balance [41]. As previously mentioned, our data showed a significant alteration in the A375 human melanoma cells bioenergetic profile after the treatment with Api, especially at the higher tested dose, 60 μM, an effect that might be correlated with its beneficial effects against melanoma. To the best of our knowledge, this is the first approach that presents the effects of Api on both mitochondrial respiration and glycolysis on A375 human melanoma cells.

It is very well known that the immune system plays a crucial role with respect to development as well as resolution of malignant lesions [42]. The main 'effectors' for the activation of the adaptive immune system are the dendritic cells (DCs). These sentinels of the immune system have the ability to cross-present exogenous antigens to T lymphocytes [43]. On human dendritic cells, pure Api completely blocked normal LPS-mediated cell activation. Moreover, the high dose of Api even reduced typical metabolic activity of DCs. This effect was strongly reflected in a complete abrogation of the cytokine secretion of IL-6 and IL-10. We have previously shown that natural sources of Api, e.g., chamomile extracts containing about 34.103μg/100 μg extract of apigenin glucoside, or 1.388 μg/100 μg extract of apigenin, also had an effect on dendritic cell activation, although this effect was very minor compared to the pure, high dose of Api [20]. Also, the secretion of the mentioned cytokines was not reduced by chamomile extracts. Obviously, a strong concentration-dependent correlation of Api and its DC immunomodulatory effect is highlighted in the former and the current study. High doses of Api are potentially able to reduce inflammatory responses to a high extend. While Api reduces the survival of cancer cells, as indicated in this study on human melanoma cells, the effect of Api as a potential immune response suppressor has to be taken into account when considering Api as an anti-cancer agent.

Recently, Api has been studied intensively, and anticancer effects have been documented, with a possible efficacy for limiting cancer progression. Angiogenesis mediated anticancer activity is being reported in several studies, on various cancer types (e.g., lung cancer, prostate cancer, skin cancer, neuroblastoma, breast cancer) by modulating different pathways [44]. The effect was described for lung and colon cancer cells on the chorioallantoic membrane and was correlated with the decrease in the HIF-1 and VEGF expression at 20 μM concentration [45]. Our study indicated a better effect on angiogenesis inhibition in normal conditions and in the presence of melanoma cells, at 30 μM concentration, but not at 60 μM, a concentration which induced irritation phenomena on the CAM. Shankar et al. showed the effect on melanoma lung metastasis by impairing the interactions between tumour cells and the endothelial cells [44]. Little data is available on the effects of Api in vivo using the chorioallantoic membrane assay in the case of human melanoma.

5. Conclusions

The comprehensive assessment of Api against the A375 human melanoma cancer cell line shows that under the set experimental conditions, the flavone presents an anticancer mechanism that involves inhibition of proliferation, induction of apoptosis, modulation of bioenergetics profile and inhibition of angiogenesis. However, under the aforementioned parameters, Api does not show any immune-stimulatory properties.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/11/4/858/s1, Figure S1: Expression of caspase 2 and p53 proteins after stimulation with Api 60 μM vs. Control, Figure S2: Morphological aspects of human melanoma A375 cells exposed to Api at concentrations of 30 μM and 60 μM during LDH assessment. Images were taken 72 h post-treatment.

Author Contributions: A.G. performed part of the in vitro experiments, analysed the data and drafted the work; A.S. performed and investigated the dendritic cells experiments; H.H.R. conceived, visualised and validated the dendritic cells experiments; S.A. performed, investigated, visualised and validated the angiogenesis experiments; I.Z., A.B., C.O., F.B., C.F. conceived, performed, visualised, analysed the data and validated the in vitro experiments;

I.Z.P. conceived, performed and investigated the mitochondrial respiration experiments; O.D. visualised and validated the mitochondrial respiration experiments; C.A.D., C.S. reviewed and edited the manuscript, C.D. reviewed and supervised the work.

Funding: This research was financed by "Victor Babeş" University of Medicine and Pharmacy, 2, Eftimie Murgu Square, 300041 Timişoara, Romania; A.S. and H.H.R. were financed by a DFG-SFB1039 grant.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Nobili, S.; Lippi, D.; Witort, E.; Donnini, M.; Bausi, L.; Mini, E.; Capaccioli, S. Natural Compounds for Cancer Treatment and Prevention. *Pharmacol. Res.* **2009**, *59*, 365–378. [CrossRef] [PubMed]
2. Hallberg, Ö.; Johansson, O. Increasing Melanoma—Too Many Skin Cell Damages or Too Few Repairs? *Cancers* **2013**, *5*, 184–204. [CrossRef] [PubMed]
3. HHS. Available online: https://www.hhs.gov/ (accessed on 1 February 2019).
4. Shain, A.H.; Bastian, B.C. From Melanocytes to Melanomas. *Nat. Rev. Cancer* **2016**, *16*, 345–358. [CrossRef]
5. Chahar, M.K.; Sharma, N.; Dobhal, M.P.; Joshi, Y.C. Flavonoids: A Versatile Source of Anticancer Drugs. *Pharmacogn. Rev.* **2011**, *5*, 1–12. [CrossRef] [PubMed]
6. Choi, E.J.; Kim, G.H. Apigenin Induces Apoptosis through a Mitochondria/Caspase-Pathway in Human Breast Cancer MDA-MB-453 Cells. *J. Clin. Biochem. Nutr.* **2009**, *44*, 260–265. [CrossRef]
7. Moga, M.A.; Dimienescu, O.G.; Arvatescu, C.A.; Mironescu, A.; Dracea, L.; Ples, L. The Role of Natural Polyphenols in the Prevention and Treatment of Cervical Cancer-An Overview. *Molecules* **2016**, *21*, 1055. [CrossRef]
8. Zhou, X.; Wang, F.; Zhou, R.; Song, X.; Xie, M. Apigenin: A Current Review on its beneficial Biological Activities. *J. Food Biochem.* **2017**, *41*, e12376. [CrossRef]
9. Venigalla, M.; Gyengesi, E.; Münch, G. Curcumin and Apigenin—Novel and Promising Therapeutics Against Chronic Neuroinflammation in Alzheimer's Disease. *Neural Regen. Res.* **2015**, *10*, 1181–1185. [CrossRef] [PubMed]
10. Sharma, H.; Kanwal, R.; Bhaskaran, N.; Gupta, S. Plant Flavone Apigenin Binds to Nucleic Acid Bases and Reduces Oxidative DNA Damage in Prostate Epithelial Cells. *PLoS ONE* **2014**, *9*, e91588. [CrossRef]
11. Yan, X.; Qi, M.; Li, P.; Zhan, Y.; Shao, H. Apigenin in Cancer Therapy: Anti-cancer Effects and Mechanisms of Action. *Cell Biosci.* **2017**, *7*, 50. [CrossRef]
12. Madunić, J.; Madunić, I.V.; Gajski, G.; Popić, J.; Garaj-Vrhovac, V. Apigenin: A Dietary Flavonoid with Diverse Anticancer Properties. *Cancer Lett.* **2018**, *413*, 11–22. [CrossRef]
13. Rakoff-Nahoum, S. Why Cancer and Inflammation? *Yale J. Biol. Med.* **2006**, *79*, 123–130.
14. Perrott, K.M.; Wiley, C.D.; Desprez, P.Y.; Campisi, J. Apigenin Suppresses the Senescence-Associated Secretory Phenotype and Paracrine Effects on Breast Cancer Cells. *GeroScience* **2017**, *39*, 161–173. [CrossRef]
15. Zhao, G.; Han, X.; Cheng, W.; Ni, J.; Zhang, Y.; Lin, J.; Song, Z. Apigenin Inhibits Proliferation and Invasion, and Induces Apoptosis and Cell Cycle Arrest in Human Melanoma Cells. *Oncol. Rep.* **2017**, *37*, 2277–2285. [CrossRef] [PubMed]
16. Caltagirone, S.; Rossi, C.; Poggi, A.; Ranelletti, F.O.; Natali, P.G.; Brunetti, M.; Aiello, F.B.; Piantelli, M. Flavonoids Apigenin and Quercetin Inhibit Melanoma Growth and Metastatic Potential. *Int. J. Cancer* **2000**, *87*, 595–600. [CrossRef]
17. Piantelli, M.; Rossi, C.; Iezzi, M.; La Sorda, R.; Iacobelli, S.; Alberti, S.; Natali, P.G. Flavonoids Inhibit Melanoma Lung Metastasis by Impairing Tumour Cells Endothelium Interactions. *J. Cell Physiol.* **2006**, *207*, 23–29. [CrossRef] [PubMed]
18. Cao, H.H.; Chu, J.H.; Kwan, H.Y.; Su, T.; Yu, H.; Cheng, C.Y.; Fu, X.Q.; Guo, H.; Li, T.; Tse, A.K.; et al. Inhibition of the STAT3 signaling pathway contributes to Apigenin-Mediated Anti-Metastatic Effect in Melanoma. *Sci. Rep.* **2016**, *6*, 21731. [CrossRef] [PubMed]
19. Schwiebs, A.; Thomas, D.; Kleuser, B.; Pfeilschifter, J.M.; Radeke, H.H. Nuclear Translocation of SGPP-1 and Decrease of SGPL-1 Activity Contribute to Sphingolipid Rheostat Regulation of Inflammatory Dendritic Cells. *Mediat. Inflamm.* **2017**, *2017*, 5187368. [CrossRef]

20. Danciu, C.; Zupko, I.; Bor, A.; Schwiebs, A.; Radeke, H.; Hancianu, M.; Cioanca, O.; Alexa, E.; Oprean, C.; Bojin, F.; et al. Botanical Therapeutics: Phytochemical Screening and Biological Assessment of Chamomile, Parsley and Celery Extracts against A375 Human Melanoma and Dendritic Cells. *Int. J. Mol. Sci.* **2018**, *19*, 3624. [CrossRef]
21. Danciu, C.; Muntean, D.; Alexa, E.; Farcas, C.; Oprean, C.; Zupko, I.; Bor, A.; Minda, D.; Proks, M.; Buda, V.; et al. Phytochemical Characterization and Evaluation of the Antimicrobial, Antiproliferative and Proapoptotic Potential of Ephedra Alata Decne. Hydroalcoholic Extract against the MCF-7 Breast Cancer Cell Line. *Molecules* **2018**, *24*, 13. [CrossRef]
22. Duicu, O.M.; Scurtu, I.; Popescu, R.; Sturza, A.; Coricovac, D.; Dănilă, M.D.; Privistirescu, A.; Muntean, D.M. Assessment of the Effects of Methylene Blue on Cellular Bioenergetics in H9c2 Cells. *Rev. Chim.* **2015**, *66*, 519–522.
23. Zuo, Y.H.; Han, Q.B.; Dong, G.T.; Yue, R.Q.; Ren, X.C.; Liu, J.X.; Liu, L.; Luo, P.; Zhou, H. Panax ginseng Polysaccharide Protected H9c2 Cardiomyocyte from Hypoxia/Reoxygenation Injury through Regulating Mitochondrial Metabolism and RISK Pathway. *Front. Physiol.* **2018**, *9*, 699. [CrossRef] [PubMed]
24. Yadava, N.; Nicholls, D.G. Spare Respiratory Capacity Rather than Oxidative Stress Regulates Glutamate Excitotoxicity after Partial Respiratory Inhibition of Mitochondrial Complex I with Rotenone. *J. Neurosci.* **2007**, *27*, 7310–7317. [CrossRef]
25. Ribatti, D.; Vacca, A.; Roncali, L.; Dammacco, F. The Chick Embryo Chorioallantoic Membrane as a Model for In Vivo Research on Anti-Angiogenesis. *Curr. Pharm. Biotechnol.* **2000**, *1*, 73–82. [CrossRef] [PubMed]
26. Ribatti, D. The Chick Embryo Chorioallantoic Membrane in the Study of Tumour Angiogenesis. *Rom. J. Morphol. Embryol.* **2008**, *49*, 131–135. [PubMed]
27. Demir, R.; Peros, G.; Hohenberger, W. Definition of the "Drug-Angiogenic-Activity-Index" that Allows the Quantification of the Positive and Negative Angiogenic Active Drugs: A Study Based on the Chorioallantoic Membrane Model. *Pathol. Oncol. Res.* **2011**, *17*, 309–313. [CrossRef]
28. Özcetin, A.; Aigner, A.; Bakowsky, U. A Chorioallantoic Membrane Model for the Determination of Antiangiogenic Effects of Imatinib. *Eur. J. Pharm. Biopharm.* **2013**, *85*, 711–715. [CrossRef]
29. Zhong, L.; Guo, X.N.; Zhang, X.H.; Sun, Q.M.; Tong, L.J.; Wu, Z.X.; Luo, X.M.; Jiang, H.L.; Nan, F.J.; Zhang, X.W.; et al. TKI-31 Inhibits Angiogenesis by Combined Suppression Signaling Pathway of VEGFR2 and PDGFRbeta. *Cancer Biol. Ther.* **2006**, *5*, 323–330. [CrossRef]
30. Avram, S.; Coricovac, D.E.; Pavel, I.Z.; Pinzaru, I.; Ghiulai, R.; Baderca, F.; Soica, C.; Muntean, D.; Branisteanu, D.E.; Spandidos, D.A.; et al. Standardization of A375 human melanoma models on Chicken Embryo Chorioallantoic Membrane and Balb/c Nude Mice. *Oncol. Rep.* **2017**, *38*, 89–99. [CrossRef] [PubMed]
31. Chan, L.P.; Chou, T.H.; Ding, H.Y.; Chen, P.R.; Chiang, F.Y.; Kuo, P.L.; Liang, C.H. Apigenin Induces Apoptosis via Tumour Necrosis Factor Receptor- and Bcl-2-Mediated Pathway and Enhances Susceptibility of Head and Neck Squamous Cell Carcinoma to 5-Fluorouracil and Cisplatin. *Biochim. Biophys. Acta* **2012**, *1820*, 1081–1091. [CrossRef]
32. Hasnat, M.A.; Pervin, M.; Lim, J.H.; Lim, B.O. Apigenin Attenuates Melanoma Cell Migration by Inducing Anoikis through Integrin and Focal Adhesion Kinase Inhibition. *Molecules* **2015**, *20*, 21157–21166. [CrossRef] [PubMed]
33. Chan, F.; Moriwaki, K.; De Rosa, M. Detection of Necrosis by Release of Lactate Dehydrogenase Activity. In *Immune Homeostasis: Methods in Molecular Biology (Methods and Protocols)*; Snow, A., Lenardo, M., Eds.; Humana Press: Totowa, NJ, USA, 2013; Volume 979, pp. 65–70.
34. Renz, A.; Berdel, W.E.; Kreuter, M.; Belka, C.; Schulze-Osthoff, K.; Los, M. Rapid Extracellular Release of Cytochrome c Is Specific for Apoptosis and Marks Cell Death In Vivo. *Blood* **2001**, *98*, 1542–1548. [CrossRef] [PubMed]
35. Smith, S.M.; Wunder, M.B.; Norris, D.A.; Shellman, Y.G. A Simple Protocol for Using a LDH-Based Cytotoxicity Assay to Assess the Effects of Death and Growth Inhibition at the Same Time. *PLoS ONE* **2011**, *6*, e26908. [CrossRef] [PubMed]
36. Chao, S.C.; Huang, S.C.; Hu, D.N.; Lin, H.Y. Subtoxic Levels of Apigenin Inhibit Expression and Secretion of VEGF by Uveal Melanoma Cells via Suppression of ERK1/2 and PI3K/Akt Pathways. *Evid.-Based Complement Altern. Med.* **2013**, *2013*, 817674. [CrossRef] [PubMed]

37. Das, S.; Das, J.; Samadder, A.; Boujedaini, N.; Khuda-Bukhsh, A.R. Apigenin-Induced Apoptosis in A375 and A549 Cells through Selective Action and Dysfunction of Mitochondria. *Exp. Biol. Med.* **2012**, *237*, 1433–1448. [CrossRef] [PubMed]
38. Chen, V.; Staub, R.E.; Baggett, S.; Chimmani, R.; Tagliaferri, M.; Cohen, I.; Shtivelman, E. Identification and Analysis of the Active Phytochemicals from the anti-Cancer Botanical Extract Bezielle. *PLoS ONE* **2012**, *7*, e30107. [CrossRef] [PubMed]
39. Seydi, E.; Rasekh, H.R.; Salimi, A.; Mohsenifar, Z.; Pourahmad, J. Selective Toxicity of Apigenin on Cancerous Hepatocytes by Directly Targeting their Mitochondria. *Anticancer Agents Med. Chem.* **2016**, *16*, 1576–1586. [CrossRef]
40. Salmani, J.M.M.; Zhang, X.P.; Jacob, J.A.; Chen, B.A. Apigenin's anticancer properties And Molecular Mechanisms of Action: Recent Advances and Future Prospectives. *Chin. J. Nat. Med.* **2017**, *15*, 321–329. [CrossRef]
41. Nicholls, D.G.; Darley-Usmar, V.M.; Wu, M.; Jensen, P.B.; Rogers, G.W.; Ferrick, D.A. Bioenergetic Profile Experiment using C2C12 Myoblast Cells. *J. Vis. Exp.* **2010**, *46*, e2511. [CrossRef]
42. Nicholson, L.B. The Immune System. *Essays Biochem.* **2016**, *60*, 275–301. [CrossRef]
43. Spel, L.; Boelens, J.J.; Nierkens, S.; Boes, M. Antitumor Immune Responses Mediated by Dendritic Cells: How Signals Derived from Dying Cancer Cells Drive Antigen Cross-presentation. *Oncoimmunology* **2013**, *2*, e26403. [CrossRef] [PubMed]
44. Shankar, E.; Goel, A.; Gupta, K.; Gupta, S. Plant Flavone Apigenin: An Emerging Anticancer Agent. *Curr. Pharmacol. Rep.* **2017**, *3*, 423–446. [CrossRef] [PubMed]
45. Fang, J.; Zhou, Q.; Liu, L.Z.; Xia, C.; Hu, X.; Shi, X.; Jiang, B.H. Apigenin Inhibits Tumour Angiogenesis through Decreasing HIF-1alpha and VEGF Expression. *Carcinogenesis* **2007**, *28*, 858–864. [CrossRef] [PubMed]

SCIENTIFIC REPORTS

OPEN

Curcumin decreases Warburg effect in cancer cells by down-regulating pyruvate kinase M2 via mTOR-HIF1α inhibition

Received: 9 November 2017
Accepted: 18 April 2018
Published online: 29 May 2018

Farid Ahmad Siddiqui[1], Gopinath Prakasam[2], Shilpi Chattopadhyay[3], Asad Ur Rehman[1,4], Rayees Ahmad Padder[1], Mohammad Afaque Ansari[1], Rasha Irshad[1], Kailash Mangalhara[2], Rameshwar N. K. Bamezai[2], Mohammad Husain[1], Syed Mansoor Ali[1] & Mohammad Askandar Iqbal[1]

Warburg effect is an emerging hallmark of cancer cells with pyruvate kinase M2 (PKM2) as its key regulator. Curcumin is an extensively-studied anti-cancer compound, however, its role in affecting cancer metabolism remains poorly understood. Herein, we show that curcumin inhibits glucose uptake and lactate production (Warburg effect) in a variety of cancer cell lines by down-regulating PKM2 expression, via inhibition of mTOR-HIF1α axis. Stable PKM2 silencing revealed that PKM2 is required for Warburg effect and proliferation of cancer cells. PKM2 over-expression abrogated the effects of curcumin, demonstrating that inhibition of Warburg effect by curcumin is PKM2-mediated. High PKM2 expression correlated strongly with poor overall survival in cancer, suggesting the requirement of PKM2 in cancer progression. The study unravels novel PKM2-mediated inhibitory effect of curcumin on metabolic capacities of cancer cells. To the best of our knowledge, this is the first study linking curcumin with PKM2-driven cancer glycolysis, thus, providing new perspectives into the mechanism of its anticancer activity.

Metabolic priorities of cancer cells differ remarkably from normal cells, thus providing a new therapeutic window. Metabolic reprogramming in cancer cells support their growth, survival, proliferation and maintenance[1]. In 1920's, Otto Warburg observed that tumor cells produce large quantities of lactate even when sufficient oxygen is present, a phenomenon referred to as *Warburg effect* or *aerobic glycolysis*[2]. Warburg effect is characterized by high glucose uptake and lactate release and is now considered as a hallmark of nearly all tumors[3]. This metabolic adaptation benefits cancer cells in surviving through hypoxic conditions, commonly found in tumors, and to support their anabolic requirements[4,5]. Tendency of cancer cells to take-up large quantities of glucose is exploited in clinical detection of tumors by 18fluorodeoxyglucose-positron emission tomography (FDG-PET) scan[6]. Hyper-activating mutations in growth signaling are known to induce expression of enzymes that are vital for cancer metabolism[7]. Interest in studying cancer metabolism has been rekindled recently with a burst in number of publications in last decade[8]. Now, metabolism of cancer cells is considered a therapeutic hotspot for dietary and pharmacologic interventions[9–11].

Extensive studies have shown that pyruvate kinase M2 (PKM2) is one of the critical regulator of Warburg effect[12,13]. Accordingly, PKM2 is highly expressed in proliferating cells like tumor and embryonic cells[14]. PKM2 is one of the four isoform of pyruvate kinase- PKL, PKR, PKM1 and PKM2[15]. Switch towards PKM2 isoform is a pre-requisite for aerobic glycolysis to take place[16]. Oncogenic transcription factor c-Myc has been demonstrated to control the mutually exclusive alternative splicing of PKM mRNA in favor of PKM2[17]. PKM2 expression has been used as a tumor marker[18–21]. We and others have previously reported that PKM2 is associated

[1]Department of Biotechnology, Faculty of Natural Sciences, Jamia Millia Islamia (A Central University), Jamia Nagar, New Delhi, 110025, India. [2]School of Life Sciences, Jawaharlal Nehru University, New Mehrauli Road, New Delhi, 110067, India. [3]Department of Toxicology, School of Chemical and Life Sciences, Jamia Hamdard (Deemed University), New Delhi, 110062, India. [4]Department of Biosciences, Faculty of Natural Sciences, Jamia Millia Islamia (A Central University), Jamia Nagar, New Delhi, 110025, India. Correspondence and requests for materials should be addressed to M.A.I. (email: miqbal2@jmi.ac.in)

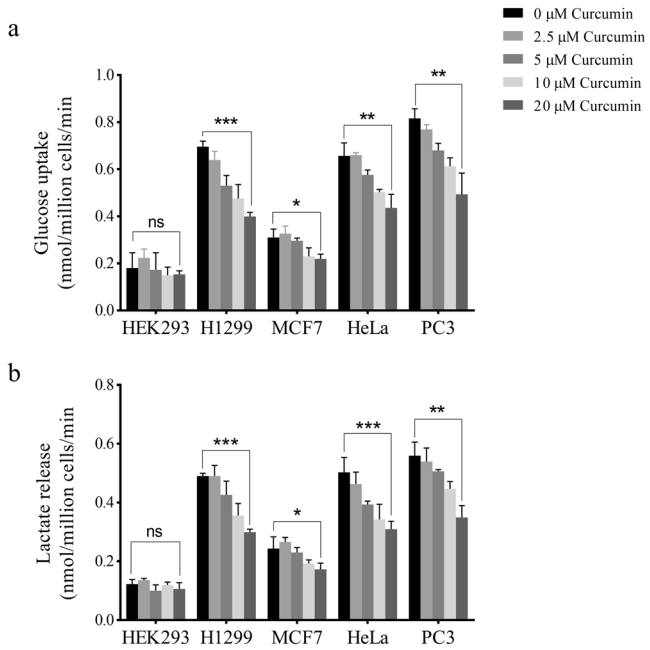


Figure 1. Dose-dependent effect of curcumin on Warburg effect. High glucose uptake and lactate production, also referred to as Warburg effect, is a hallmark feature of cancer cells (see text) needed to support proliferation of cancer cells. Glucose uptake (**a**) and lactate release (**b**) by H1299, MCF-7, HeLa and PC3 cells was reduced significantly upon curcumin treatment compared with HEK293 where the change in glucose and lactate was not significant. Different doses of curcumin (2.5, 5, 10 and 20 μM) for 24 hours were used for treatment purpose. Maximal decrease in Warburg effect was observed at 20 μM. Error bars represent mean ± SD.

with tumor metabolism and growth[22,23]. Several studies have suggested PKM2 as a therapeutic target for cancer treatment[24,25]. Therefore, it is pertinent to evaluate drugs that could suppress PKM2 expression, thus, inhibiting cancer metabolism.

Curcumin (diferuloylmethane) is a well-known phytopolyphenolic compound isolated from rhizome of the plant *Curcuma longa* (Zingiberaceae)[26]. Curcumin is considered as a valuable medicinal plant in Indian systems of medicine. Numerous studies have shown the anti-cancer properties of curcumin in a wide variety of cell lines and animals[27–33]. The major features of carcinogenesis have been shown to be inhibited by curcumin[34]. Several mechanisms for anti-cancer activities of curcumin have been proposed, including, induction of apoptosis[34], p53 stabilization[35], mTOR[33], Wnt[36], Notch[37], PI3K[38], signaling inhibition, AMPK activation[39], cell cycle inhibition[40], inhibition of oncogenes[41], inactivation of NF-kB[42], metastasis inhibition[43], angiogenesis inhibition[44], miRNA regulation[45], DNA damage and repair[46]. However, the effect of curcumin on cancer metabolism, an emerging hallmark of cancer, remains unknown.

Here, we investigated the effect of curcumin on cancer metabolism and report novel PKM2-mediated inhibitory effects of curcumin on Warburg effect. Our results identify a new anti-cancer mechanism of curcumin and endorse its therapeutic relevance in inhibiting cancer.

Results

Curcumin inhibits Warburg effect in cancer cells. The effect of curcumin on Warburg effect was studied by measuring the rate of glucose uptake and lactate production in cancer cell lines- lung (H1299), breast (MCF-7), cervical (HeLa) and prostate (PC3) and human embryonic kidney (HEK) 293 cells, taken as control. Sub-toxic concentrations of 0–20 μM curcumin for 24 hours were used for the study. Significant inhibition in glucose uptake and lactate release was observed across the four cell lines, however, no appreciable decrease in Warburg effect was observed in HEK 293 cells (Fig. 1a,b). Dose-dependent decrease in Warburg effect started at 2.5 μM with maximal decrease at 20 μM curcumin.

Curcumin down-regulates PKM2 via inhibition of mTOR-HIF1α axis. To understand the decrease in glucose consumption and lactate production by curcumin-treated cell lines, we studied the status of PKM2, a critical regulator of Warburg effect. Since maximal decrease in Warburg effect was observed at 20 μM curcumin, we used this concentration to study the effect of curcumin on PKM2 status in H1299, MCF-7, HeLa and PC3 cell lines. Curcumin treatment substantially reduced PKM2 mRNA and protein as assessed by qRT-PCR and immunoblotting (Fig. 2a,b and Supplementary Figure S2). Further, in an attempt to elucidate the mechanism responsible for PKM2 down-regulation, we studied the mTOR/HIF1α pathway inhibition upon curcumin treatment. mTOR is frequently hyper-activated in various cancers[47] and curcumin has been shown to inhibit mTOR signaling[48]. HIF1α is a known transcriptional activator of PKM2[13,49]. Upon curcumin treatment, decreased PKM2 expression coincided with decreased Threonine 389 (T389) phosphorylation of p70S6 kinase and decreased

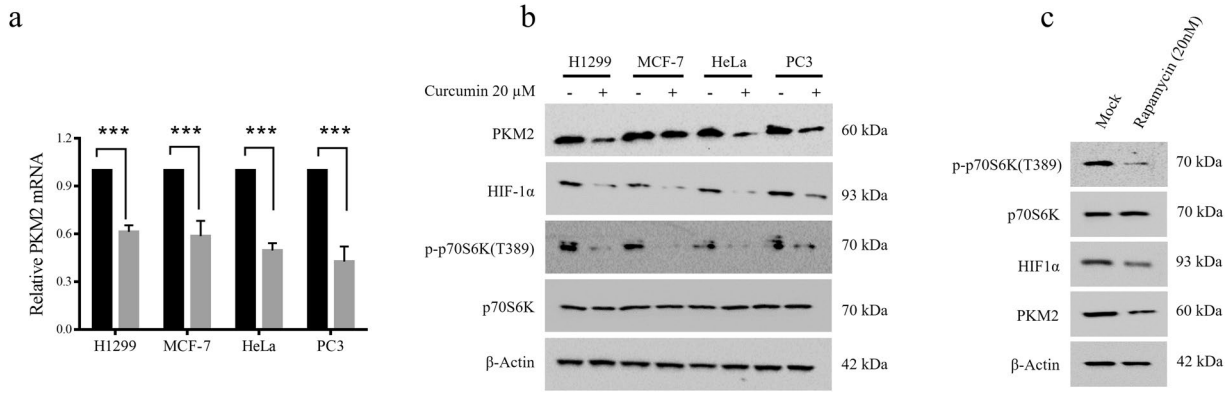


Figure 2. PKM2 expression is inhibited by curcumin through mTOR/HIF1α axis. (**a**) Substantial reduction in PKM2 mRNA in H1299, MCF-7, HeLa and PC3 cells treated with 20 μM for 24 hours. (**b**) Immunoblot of H1299, MCF-7, HeLa and PC3 cells showing coincided reduction in PKM2, HIF1α and phosphorylated-p70S6K (T389), upon curcumin treatment. (**c**) Treatment with standard mTOR inhibitor-rapamycin also showed similar expression pattern. Results suggested that curcumin inhibited PKM2 expression via inhibition of mTOR and HIF1α. Error bars in PKM2 mRNA graph represent mean ± SD.

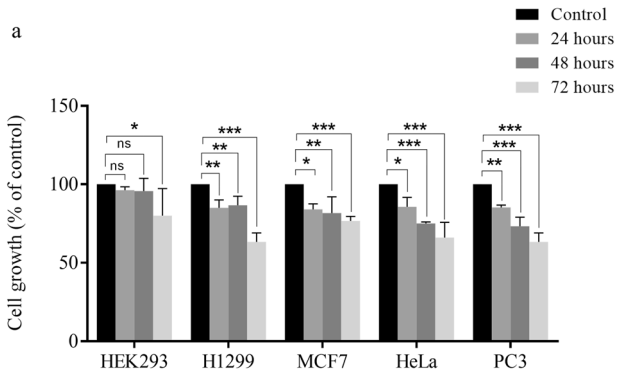


Figure 3. Decreased cell viability upon curcumin treatment. (**a**) Curcumin treatment decreased viability of H1299, MCF-7, HeLa and PC3 cells over the time course of 24, 48 and 72 hours. Decrease in viability of four cancer cell lines is significantly higher compared to control HEK293 cells. Maximum decrease in viability of all studied cell lines was observed at 72 hours. Error bars represent mean ± SD.

HIF1α protein, suggesting that curcumin down regulated PKM2 by inhibiting the mTOR/HIF1α signaling. In addition, inhibition of PKM2 expression by rapamycin (a well-known mTOR inhibitor), further validated that curcumin decreased PKM2 via inhibition of mTOR/HIF1α signaling (Fig. 2c). GLUT1 and HKII mRNA were also found to be decreased upon curcumin treatment, suggesting the contribution of these enzymes, in addition to PKM2, in inhibition of Warburg effect upon curcumin treatment (Supplementary Figure S1).

Curcumin decreases viability of cancer cells. To analyze if inhibition of PKM2 and cancer metabolism by curcumin contributed to reduction in viability, growth of HEK293, H1299, MCF-7, HeLa and PC3 cells was assessed over the period of 24–72 hours in presence of 20 μM curcumin. Decreasing trend of viability was observed in a time dependent manner (Fig. 3a). Maximum decrease in viability of all cell lines was observed at 72 hours, although; drop in viability started at 24 hours. However, no significant decrease in viability was observed in control HEK 293 cells.

PKM2 silencing decreases Warburg effect and cell viability. We conjectured that the decrease in Warburg effect upon curcumin treatment is, at least in part, due to down-regulation of PKM2 expression. To this end, PKM2 was stably silenced in H1299 cells using shRNA approach. Knock-down efficiency was checked by Western blotting (Fig. 4a). Thereafter, glucose consumption and lactate release were measured. shPKM2 transfected H1299 cells exhibited significant reduction in consumption of glucose and lactate production, indicating that PKM2 is crucial for Warburg effect (Fig. 4b,c). These results validated that curcumin inhibited aerobic glycolysis by down-regulating PKM2 expression. In addition to Warburg effect, PKM2 silencing also reduced viability of H1299 cells (Fig. 4d), further suggesting that PKM2-driven Warburg effect is essential for survival of cancer cells.

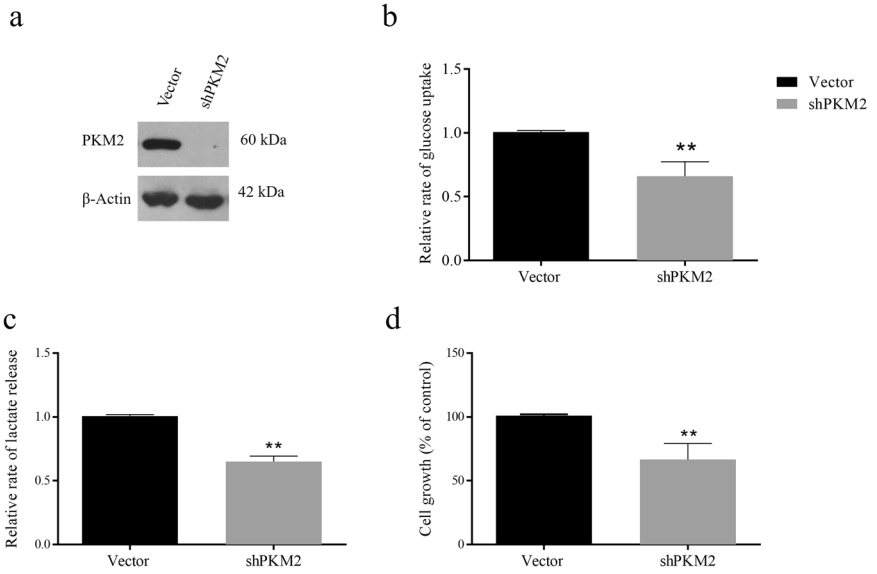


Figure 4. Knock-down of PKM2 reduces glucose uptake, lactate production and cell viability. PKM2 was stably silenced in H1299 cells using lentiviral shRNA as described in methods. (**a**) Confirmation of PKM2 knockdown by Western blotting, (**b**) drop in glucose uptake, (**c**) lactate production and (**d**) cell viability was observed. Error bars in PKM2 mRNA graph represent mean ± SD.

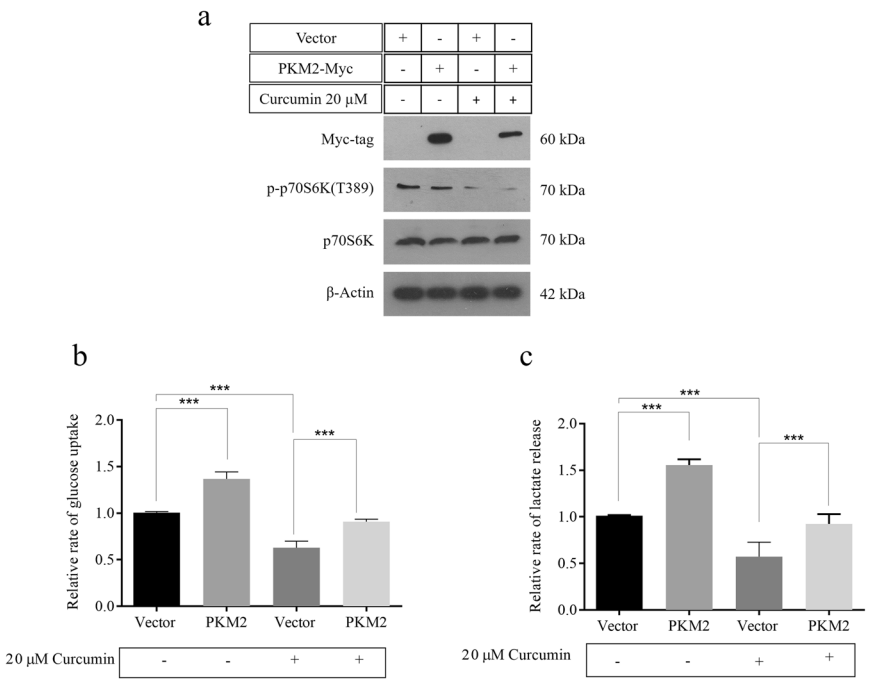


Figure 5. PKM2 over-expression reversed the effects of curcumin on cancer glycolysis. (**a**) Immunoblot confirming myc-PKM2 over-expression in H1299 cells continually exposed to 20 μM curcumin. Nearly diminished p-p70SK (T389) in both vector and PKM2 transfected H1299 cells confirmed mTOR inhibition by curcumin. Increased glucose uptake (**b**) and lactate release (**c**) in H1299 cells over-expressing PKM2, compared to vector transfected, in absence and presence of curcumin.

Over-expressing PKM2 reverses the effect of curcumin on metabolism of cancer cells. In order to further validate that inhibitory effects of curcumin on cancer glycolysis are PKM2-mediated, PKM2 was transiently over-expressed in H1299 cells in absence and presence of 20 μM curcumin. H1299 cells were either transfected with vector control or myc-PKM2. Confirmation of transfection was done by immunoblotting using anti-myc antibodies (Fig. 5a). Curcumin activity was measured by checking the inhibition of p70S6 kinase T389 phosphorylation. Expectedly, PKM2 over-expression resulted in augmented Warburg effect even in continuous presence of 20 μM curcumin (Fig. 5b,c). Results demonstrated the ectopic PKM2 expression repressed the effects

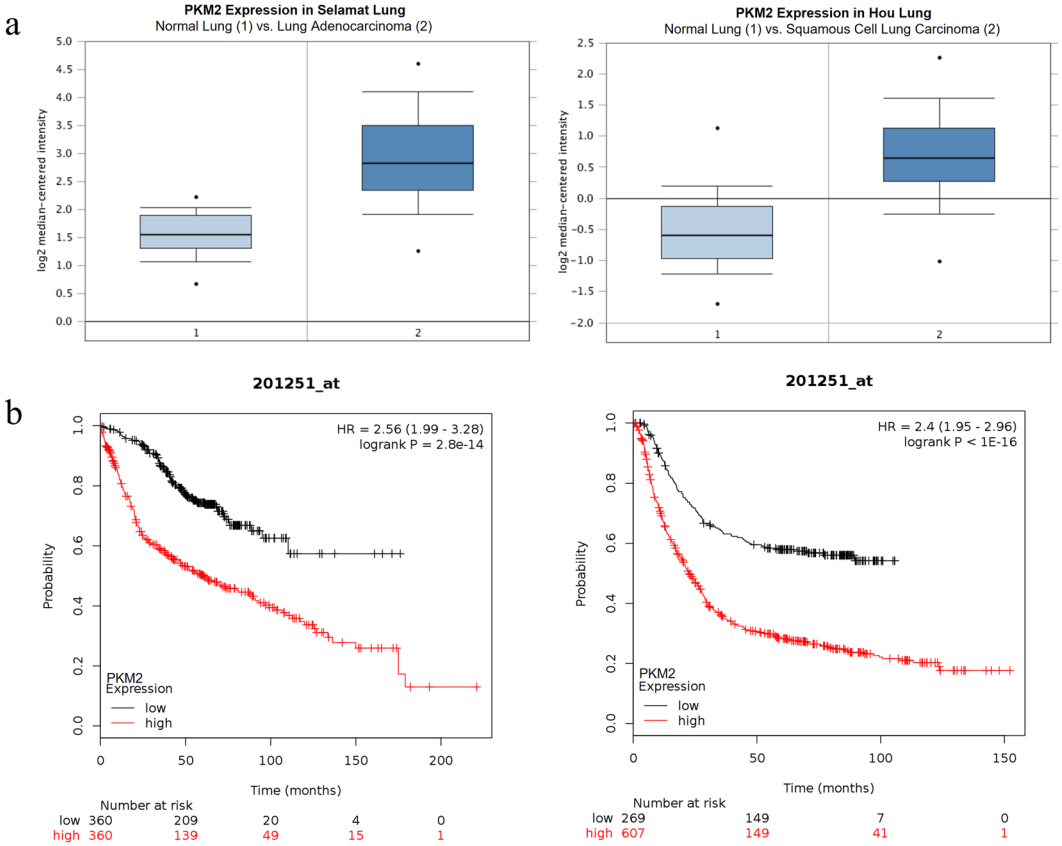


Figure 6. PKM2 expression is high in cancer and correlates with poor overall survival. (**a**) Box plots from Oncomine representing the higher PKM2 expression in lung adenocarcinoma and squamous cell lung carcinoma, compared to normal lung. (**b**) Kaplan-Meier overall survival (OS) curve of patients with lung cancer expressing low and high PKM2 mRNA. (PKM2 low expression group, n = 360; PKM2 high expression group, n = 360) and Kaplan-Meier overall survival (OS) curve of patients with gastric cancer expressing low and high PKM2 mRNA. (PKM2 low expression group, n = 269; PKM2 high expression group, n = 607).

of curcumin on glucose uptake and lactate release, substantiating that PKM2 is a target of curcumin. In absence of curcumin, PKM2 transfected cells compared with vector showed the expected increase in glucose uptake and lactate production. Consistent with Fig. 1, treated vector control showed decreased glucose and lactate compared with untreated vector control.

PKM2 expression is higher in cancer and associated with poor overall survival. To investigate the expression levels of PKM2 in tumour tissues, Oncomine database was utilised. In Selamat lung cancer study comparing expression of PKM2 in normal lung (n = 58) with the lung adenocarcinoma tissue (n = 58), PKM2 expression was found to be 2.5-fold higher in lung adenocarcinoma compared to normal lung (Fig. 6a)[50]. Similarly, Hou dataset also revealed 2.3-fold increase in PKM2 expression in squamous cell lung carcinoma as compared with normal lung (n = 65) (Fig. 6a)[51]. Further, we performed Kaplan-Meier analysis using online portal (www.kmplot.com) to correlate PKM2 expression with overall survival of cancer patients. Higher PKM2 expression correlated strongly with poor overall survival in lung and gastric cancer patients (Fig. 6b)[52,53]. These results suggest that higher PKM2 expression is associated with cancer and may represent a useful prognostic marker.

Discussion

Metabolic transformation in cancer cells has gained enormous attention in recent past for its immense potential as viable therapeutic target. Owing to the metabolic vulnerabilities of cancer cells, several drugs that target cancer metabolism are under clinical trials. Just as with any chemotherapeutic drug, drugs that target metabolism of cancer cells may have side-effects that could deteriorate patient's health and life-quality. On the contrary, natural plant based compounds have relatively less toxicity and side-effects. Therefore, there is an urgent need to screen more natural compounds for their inhibitory effects on the emerging hallmarks of cancer, like metabolism. Till date, very few phytochemicals have been shown to target cancer metabolism. Cleary, there is a need to screen more natural compounds for their negative effects on cancer metabolism. Metabolic enzymes could be therapeutic hotspots, provided they are key regulators of metabolic pathways operating in cancer cells. Large body of evidence suggests that PKM2 is the key regulator of cancer metabolism, thus, targeting PKM2 to inhibit cancer metabolism should be a viable anti-cancer strategy.

Curcumin has been well-studied as a inhibitor of a variety of cancer features; however, its effects on cancer metabolism remained un-elucidated. We demonstrated that curcumin inhibits cancer metabolism in a PKM2-dependent manner. Results presented here unravel a new dimension of anti-cancer mechanisms of curcumin. Results from this work, along with the already known literature on curcumin, suggest that curcumin inhibits most, if not all, hallmarks of cancer, making it a powerful plant-based anti-cancer compound. However, there are several caveats associated with the stability and bioavailability of curcumin, thus, hindering its application in clinical setting[54]. Owing to the unstable chemical nature of curcumin, it is known to degrade within few hours in culture media, thus, making its bioavailability abysmal[55]. Nonetheless, numerous studies have demonstrated the anti-cancer properties of curcumin[56]. This raises the possibility of role of the degradation products of curcumin in contributing to its onco-pharmacological effects[57].

The effect of curcumin on Warburg effect (Fig. 1) was found to be consistent across all four cancer cell lines studied, highlighting the spectrum of curcumin as an anti-cancer drug. Besides, these results also hint at the near-universal requirement of Warburg effect by solid cancers for their growth and survival. Interestingly, curcumin-induced inhibition of metabolism was evident at a low 2.5 μM concentration, demonstrating the sensitivity of cancer cells to curcumin and dependency on aerobic glycolysis. Notably, the negligible effects of curcumin on glycolysis and viability of epithelial HEK 293 cells suggested that the observed inhibition of Warburg effect by curcumin is specific to cancer cells. The decrease in glucose consumption and lactate production impedes the growth of cancer cells because glycolytic flux provides for anabolic synthesis in cancer cells to produce macromolecules for daughter cancer cells. Furthermore, intermediates of glycolysis acts as precursors or intermediates of cross-talking anabolic pathway like pentose phosphate pathway. Besides, high rates of glycolysis in cancer also serve the purpose of rapid ATP production, as in case of muscle during heavy exercise. Therefore, it is fathomable that high glycolytic rate is a life-line of dividing cancer cells. The decrease in PKM2 expression upon curcumin treatment explains the observed decrease in aerobic glycolysis in cell lines treated with curcumin. Since PKM2 acts a regulator of glycolytic flux[12], through its protein and pyruvate kinase activity, changes in its expression are expected to cause changes in glycolytic flux. Accordingly, silencing of PKM2 suppressed the Warburg effect and viability (Fig. 3). Moreover, higher PKM2 expression in cancer tissues and statistically significant association with poor overall survival of cancer patients, strongly suggested the role of PKM2 in cancer (Fig. 6).

mTOR is an important growth regulator and is usually very active in cancer cells due to mutations in regulatory pathways or the mTOR itself[58,59]. The role of mTOR in protein synthesis makes it indispensable for the survival of cancer cells. The regulation of HIF1α and PKM2 expression by mTOR highlights the importance of mTOR signaling in regulation of cancer metabolism (Fig. 2). mTOR is a tuner of metabolism because latter provides raw-material for protein synthesis i.e. amino acids and is known to regulate HIF1α and PKM2 expression[23,60,61]. Therefore, inhibition of mTOR is expected to inhibit PKM2 and glycolysis, which otherwise support anabolic metabolism in cancer[62]. Inhibition of Warburg effect results in decreased anabolism and therefore the viability of cancer cells is negatively affected by curcumin (Fig. 3) and silencing of PKM2 (Fig. 4) as all these factors converge to suppress macromolecular synthesis. The role of HIF1α in controlling the expression of PKM2 is an outcome of hypoxic conditions that exists in tumor cells. PKM2 can drive glycolysis even in absence of oxygen, by promoting the conversion of pyruvate to lactate through a largely unknown mechanism, thus, induction of PKM2 by HIF1α is an adaptation necessary for tumor growth. Abrogation of inhibitory effects of curcumin on aerobic glycolysis in PKM2 over-expressing cells not only suggested that effects were mediated by PKM2 but also highlighted the importance of PKM2 for cancer cell metabolism (Fig. 5).

Although curcumin has been shown to exert anti-cancer effects through a variety of mechanisms, it is important to identify mechanisms that could be targeted without harming normal cells. Metabolic rewiring in cancer cells is one such mechanism. The work presented here represents a new anti-cancer mechanism of curcumin and pin-points the enzyme responsible for mediating the anti-cancer effects of curcumin. Since bioavailability of curcumin is a challenge faced in clinical setting; stable analogues of curcumin should be tested for their ability to mimic inhibitory effects of curcumin on cancer metabolism.

In summary, this study unravels a new anti-cancer role of curcumin and invites further research into exploiting curcumin and its analogues for successful clinical inhibition of metabolic addictions in cancer cells.

Materials and Methods

Cell culture and drug treatment. HEK293, H1299, MCF-7, HeLa and PC3 cell lines were procured and maintained as described[63]. Briefly, cell lines were grown in monolayer and passaged routinely 2–3 times a week. Cell lines were maintained either in DMEM or RPMI with 10% FBS (Gibco) and 1% penicillin/streptomycin (Sigma) at 37°C and 5% CO_2 in a humidified incubator. 2 mM Curcumin (Sigma, MO, USA) and 200 μM rapamycin (Sigma, MO, USA) stock solutions were prepared using DMSO and stored at −80°C, until further use. Prior to drug treatment, cells were allowed to grow for 24 hours followed by treatment with either DMSO (mock control) or curcumin for 24–72 hours.

Metabolic assays. For glucose and lactate: Spent media was collected, centrifuged to remove any cell debris and deproteinized using 5% trichloroacetic acid (TCA) and glucose and lactate were assayed using colorimetric kits (BioVision, USA) as per the manufacturer's instructions. Standard curves were prepared and background corrections were done. All measurements were normalized to cell numbers. Rate of glucose uptake and lactate production were measured and expressed as nmol/million cells/minute.

Western blotting and qRT-PCR. Whole cell lysate was prepared in modified RIPA buffer containing 50 mM Tris-Cl pH 7.2, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100 with added 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), protease and phosphatse inhibitor cocktail (Geno Biosciences, USA). Lysates were then centrifuged and supernatant containing protein was quantified

using BCA (bicinchoninic acid) kit (Thermo Scientific, USA) as per the manufacturer protocol. Equal amount of protein was loaded and separated by SDS-PAGE, transferred to nitrocellulose membrane (mdi, USA) and probed with relevant primary antibodies. Membrane was incubated with appropriate secondary antibody for 1 hour at room temperature and proteins were detected using Luminata Forte (Millipore, USA). Densitometry analysis was carried out using ImageJ software to examine the relative gene expression change following normalizing with β-actin. Primary antibodies used were: anti-PKM2, anti-HIF1α, anti-myc, anti-p-p70S6K (T389), anti-p70S6K and anti-β-actin (Cell signaling Technology, USA). For qRT-PCR: total RNA was extracted from cell lines using TRI reagent (Sigma, MO, USA) followed by reverse transcription to cDNA using Superscript® III reverse transcriptase kit (Life technologies, USA). BIORAD CFX96 Touch™ real-time machine was used for RT-PCR analysis using SYBR green PCR master mix (Applied Biosystems). Comparative C_T method ($2^{-\Delta\Delta CT}$) was used for calculation of relative gene expression. Actin was used as endogenous control. Primers used for qRT-PCR assays are as follows: PKM2 (exon 10); forward 5′-TGCAATTATTTGAGGAACTCC-3′, reverse 5′-CACTGCAG CACTTGAAGGAG-3′. HKII; forward 5′- CCAACCTTAGGCTTGCCATT -3′, reverse 5′- CTTGGACATGGGAT GGGGTG-3′. GLUT 1; forward 5′- CTTTGTGGCCTTCTTTGAAGT-3′, reverse 5′- CCACACAGTTGCTCCA CAT-3′. ACTIN; forward 5′- ACTCTTCCAGCCTTCCTTC-3′, reverse 5′′- ATCTCCTTCTGCATCCTGTC-3′.

Stable shRNA silencing and transient transfections. For stable knockdown of PKM2: the lentiviral particles were generated as described[63]. Briefly, HEK293T cells were transfected with transfer vector (LKO.1) containing shRNAs, plus packaging vectors- psPAX and pMD2.G, using Lipofectamine® 3000 (ThermoFisher Scientific, USA). Forty eight hours post-transfection, viral particles were harvested and used to infect the H1299 cells. Selection of infected cells was done in DMEM containing 2 μg/ml puromycin over the period of 2 weeks. For PKM2 over-expression: pcDNA-PKM2-myc or pcDNA-myc were transfected using Lipofectamine® 3000 reagent (ThermoFisher Scientific, USA), as per the manufacturer's instructions.

MTT assay for cell viability. Cell viability was assessed using MTT (3-(4, 5-Dimethylthiazol-2yl)-2, 5-diphenyl tetrozolium bromide) (Sigma). Exponentially growing cells were seeded in 96 well plate with the density of 10,000 cells per well. Subsequent to the cell adherence to the surface of culture plate (after approximately 12 hours), cells were treated with 20 μM curcumin. Cell viability was measured every 24-hour interval (24 to 72 hours) by washing the cells and replacing with fresh medium containing 20 μl of MTT (5 mg/ml in PBS) to each well. The plates were incubated for three hours in dark. The formazon crystals developed were solubilized with 100 μl of DMSO and the plate was kept in dark for another 5–10 min. Absorbance was measured using a microplate reader (Molecular Device) at 570 nm. Cells were seeded in triplicates for each group and the experiment was independently repeated thrice.

Statistical analysis. Data were represented as mean with SD. Student's t-test or one-way or two-way ANOVA with multiple comparisons was used to calculate statistical significance. $P < 0.05$ was considered to be statistically significant. Statistical significance is represented as: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

References

1. DeBerardinis, R. J. & Chandel, N. S. Fundamentals of cancer metabolism. *Science advances* **2**, e1600200, https://doi.org/10.1126/sciadv.1600200 (2016).
2. Warburg, O., Posener, K. & Negelein, E. Über den Stoffwechsel der Carcinomzelle. *Biochem. Zeitschr* **152**, 309–344, https://doi.org/10.1016/j.tibs.2015.12.001 (1924).
3. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646–674, https://doi.org/10.1016/j.cell.2011.02.013 (2011).
4. Gatenby, R. A. & Gillies, R. J. Why do cancers have high aerobic glycolysis? *Nature reviews. Cancer* **4**, 891–899, https://doi.org/10.1038/nrc1478 (2004).
5. Iqbal, M. A., Gupta, V., Gopinath, P., Mazurek, S. & Bamezai, R. N. Pyruvate kinase M2 and cancer: an updated assessment. *FEBS letters* **588**, 2685–2692, https://doi.org/10.1016/j.febslet.2014.04.011 (2014).
6. Vander Heiden, M. G., Cantley, L. C. & Thompson, C. B. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* **324**, 1029–1033, https://doi.org/10.1126/science.1160809 (2009).
7. Nagarajan, A., Malvi, P. & Wajapeyee, N. Oncogene-directed alterations in cancer cell metabolism. *Trends in cancer* **2**, 365–377, https://doi.org/10.1016/j.trecan.2016.06.002 (2016).
8. Liberti, M. V. & Locasale, J. W. The Warburg Effect: How Does it Benefit Cancer Cells? *Trends in biochemical sciences* **41**, 211–218, https://doi.org/10.1016/j.tibs.2015.12.001 (2016).
9. Tennant, D. A., Duran, R. V. & Gottlieb, E. Targeting metabolic transformation for cancer therapy. *Nature reviews. Cancer* **10**, 267–277, https://doi.org/10.1038/nrc2817 (2010).
10. Borriello, A. & Della Ragione, F. The new anticancer era: Tumor metabolism targeting. *Cell Cycle* **16**, 310–311, https://doi.org/10.1080/15384101.2016.1271635 (2017).
11. Vernieri, C. *et al*. Targeting Cancer Metabolism: Dietary and Pharmacologic Interventions. *Cancer discovery* **6**, 1315–1333, https://doi.org/10.1158/2159-8290.CD-16-0615 (2016).
12. Chaneton, B. & Gottlieb, E. Rocking cell metabolism: revised functions of the key glycolytic regulator PKM2 in cancer. *Trends in biochemical sciences* **37**, 309–316, https://doi.org/10.1016/j.tibs.2012.04.003 (2012).
13. Prakasam, G., Iqbal, M. A., Bamezai, R. N. K. & Mazurek, S. Posttranslational Modifications of Pyruvate Kinase M2: Tweaks that BenefitCancer. *Frontiers in Oncology* **8**, https://doi.org/10.3389/fonc.2018.00022 (2018).
14. Gupta, V. & Bamezai, R. N. Human pyruvate kinase M2: a multifunctional protein. *Protein science: a publication of the Protein Society* **19**, 2031–2044, https://doi.org/10.1002/pro.505 (2010).
15. Prakasam, G., Iqbal, M. A., Gupta, V., Kumar, B. & Bamezai, R. N. K. In *Encyclopedia of Snaling Molecules* (ed Sangdun Choi) 1–11 (Springer New York, 2017).
16. Christofk, H. R. *et al*. The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. *Nature* **452**, 230–233, https://doi.org/10.1038/nature06734 (2008).
17. David, C. J., Chen, M., Assanah, M., Canoll, P. & Manley, J. L. HnRNP proteins controlled by c-Myc deregulate pyruvate kinase mRNA splicing in cancer. *Nature* **463**, 364–368, https://doi.org/10.1038/nature08697 (2010).

18. Ahmed, A. S. *et al*. M2-PK as a novel marker in ovarian cancer. A prospective cohort study. *European journal of gynaecological oncology* **28**, 83–88 (2007).
19. Ewald, N. *et al*. Pyruvate kinase M2 (tumor M2-PK) as a screening tool for colorectal cancer (CRC). A review of current published data. *Zeitschrift fur Gastroenterologie* **43**, 1313–1317, https://doi.org/10.1055/s-2005-858657 (2005).
20. Hardt, P. D. & Ewald, N. Tumor M2 pyruvate kinase: a tumor marker and its clinical application in gastrointestinal malignancy. *Expert review of molecular diagnostics* **8**, 579–585, https://doi.org/10.1586/14737159.8.5.579 (2008).
21. Hardt, P. D. *et al*. Faecal tumour M2 pyruvate kinase: a new, sensitive screening tool for colorectal cancer. *British journal of cancer* **91**, 980–984, https://doi.org/10.1038/sj.bjc.6602033 (2004).
22. Iqbal, M. A. *et al*. Missense mutations in pyruvate kinase M2 promote cancer metabolism, oxidative endurance, anchorage independence, and tumor growth in a dominant negative manner. *The Journal of biological chemistry* **289**, 8098–8105, https://doi.org/10.1074/jbc.M113.515742 (2014).
23. Sun, Q. *et al*. Mammalian target of rapamycin up-regulation of pyruvate kinase isoenzyme type M2 is critical for aerobic glycolysis and tumor growth. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 4129–4134, https://doi.org/10.1073/pnas.1014769108 (2011).
24. Chen, J. *et al*. Shikonin and its analogs inhibit cancer cell glycolysis by targeting tumor pyruvate kinase-M2. *Oncogene* **30**, 4297–4306, https://doi.org/10.1038/onc.2011.137 (2011).
25. Shi, H. S. *et al*. Silencing of pkm2 increases the efficacy of docetaxel in human lung cancer xenografts in mice. *Cancer science* **101**, 1447–1453, https://doi.org/10.1111/j.1349-7006.2010.01562.x (2010).
26. Noorafshan, A. & Ashkani-Esfahani, S. A review of therapeutic effects of curcumin. *Current pharmaceutical design* **19**, 2032–2046 (2013).
27. Ramasamy, T. S., Ayob, A. Z., Myint, H. H., Thiagarajah, S. & Amini, F. Targeting colorectal cancer stem cells using curcumin and curcumin analogues: insights into the mechanism of the therapeutic efficacy. *Cancer cell international* **15**, 96, https://doi.org/10.1186/s12935-015-0241-x (2015).
28. Amin, A. R. *et al*. Curcumin induces apoptosis of upper aerodigestive tract cancer cells by targeting multiple pathways. *PloS one* **10**, e0124218, https://doi.org/10.1371/journal.pone.0124218 (2015).
29. Radhakrishna Pillai, G., Srivastava, A. S., Hassanein, T. I., Chauhan, D. P. & Carrier, E. Induction of apoptosis in human lung cancer cells by curcumin. *Cancer letters* **208**, 163–170, https://doi.org/10.1016/j.canlet.2004.01.008 (2004).
30. Hartojo, W. *et al*. Curcumin promotes apoptosis, increases chemosensitivity, and inhibits nuclear factor kappaB in esophageal adenocarcinoma. *Translational oncology* **3**, 99–108 (2010).
31. Zhang, H. *et al*. Curcumin Promotes Cell Cycle Arrest and Inhibits Survival of Human Renal Cancer Cells by Negative Modulation of the PI3K/AKT Signaling Pathway. *Cell biochemistry and biophysics* **73**, 681–686, https://doi.org/10.1007/s12013-015-0694-5 (2015).
32. Li, F., Chen, X., Xu, B. & Zhou, H. Curcumin induces p53-independent necrosis in H1299 cells via a mitochondria-associated pathway. *Molecular medicine reports* **12**, 7806–7814, https://doi.org/10.3892/mmr.2015.4395 (2015).
33. Jiao, D. *et al*. Curcumin inhibited HGF-induced EMT and angiogenesis through regulating c-Met dependent PI3K/Akt/mTOR signaling pathways in lung cancer. *Molecular therapy oncolytics* **3**, 16018, https://doi.org/10.1038/mto.2016.18 (2016).
34. Beevers, C. S. *et al*. Curcumin disrupts the Mammalian target of rapamycin-raptor complex. *Cancer research* **69**, 1000–1008, https://doi.org/10.1158/0008-5472.CAN-08-2367 (2009).
35. Li, W. *et al*. A preliminary study of the effect of curcumin on the expression of p53 protein in a human multiple myeloma cell line. *Oncology letters* **9**, 1719–1724, https://doi.org/10.3892/ol.2015.2946 (2015).
36. He, M. *et al*. Curcumin suppresses cell proliferation through inhibition of the Wnt/beta-catenin signaling pathway in medulloblastoma. *Oncology reports* **32**, 173–180, https://doi.org/10.3892/or.2014.3206 (2014).
37. Yang, J. *et al*. Curcumin inhibits the survival and metastasis of prostate cancer cells via the Notch-1 signaling pathway. *APMIS: acta pathologica, microbiologica, et immunologica Scandinavica* **125**, 134–140, https://doi.org/10.1111/apm.12650 (2017).
38. Tian, B. *et al*. Curcumin inhibits urothelial tumor development by suppressing IGF2 and IGF2-mediated PI3K/AKT/mTOR signaling pathway. *Journal of drug targeting*, 1–11, https://doi.org/10.1080/1061186X.2017.1306535 (2017).
39. Kang, O. H. *et al*. Curcumin decreases oleic acid-induced lipid accumulation via AMPK phosphorylation in hepatocarcinoma cells. *European review for medical and pharmacological sciences* **17**, 2578–2586 (2013).
40. Hu, A. *et al*. Curcumin induces G2/M cell cycle arrest and apoptosis of head and neck squamous cell carcinoma *in vitro* and *in vivo* through ATM/Chk2/p53-dependent pathway. *Oncotarget*, https://doi.org/10.18632/oncotarget.17096 (2017).
41. Lin, J. K. Suppression of protein kinase C and nuclear oncogene expression as possible action mechanisms of cancer chemoprevention by Curcumin. *Archives of pharmacal research* **27**, 683–692 (2004).
42. Singh, S. & Aggarwal, B. B. Activation of transcription factor NF-kappa B is suppressed by curcumin (diferuloylmethane) [corrected]. *The Journal of biological chemistry* **270**, 24995–25000 (1995).
43. Liao, H., Wang, Z., Deng, Z., Ren, H. & Li, X. Curcumin inhibits lung cancer invasion and metastasis by attenuating GLUT1/MT1-MMP/MMP2 pathway. *International journal of clinical and experimental medicine* **8**, 8948–8957 (2015).
44. Bhandarkar, S. S. & Arbiser, J. L. Curcumin as an inhibitor of angiogenesis. *Advances in experimental medicine and biology* **595**, 185–195, https://doi.org/10.1007/978-0-387-46401-5_7 (2007).
45. Chen, J., Xu, T. & Chen, C. The critical roles of miR-21 in anti-cancer effects of curcumin. *Annals of translational medicine* **3**, 330, https://doi.org/10.3978/j.issn.2305-5839.2015.09.20 (2015).
46. Ji, Z. Targeting DNA damage and repair by curcumin. *Breast cancer: basic and clinical research* **4**, 1–3 (2010).
47. Chen, Y., Wei, H., Liu, F. & Guan, J. L. Hyperactivation of mammalian target of rapamycin complex 1 (mTORC1) promotes breast cancer progression through enhancing glucose starvation-induced autophagy and Akt signaling. *The Journal of biological chemistry* **289**, 1164–1173, https://doi.org/10.1074/jbc.M113.526335 (2014).
48. Huang, S. Inhibition of PI3K/Akt/mTOR signaling by natural products. *Anti-cancer agents in medicinal chemistry* **13**, 967–970 (2013).
49. Iqbal, M. A. *et al*. Insulin enhances metabolic capacities of cancer cells by dual regulation of glycolytic enzyme pyruvate kinase M2. *Molecular cancer* **12**, 72, https://doi.org/10.1186/1476-4598-12-72 (2013).
50. Selamat, S. A. *et al*. Genome-scale analysis of DNA methylation in lung adenocarcinoma and integration with mRNA expression. *Genome research* **22**, 1197–1211, https://doi.org/10.1101/gr.132662.111 (2012).
51. Hou, J. *et al*. Gene expression-based classification of non-small cell lung carcinomas and survival prediction. *PloS one* **5**, e10312, https://doi.org/10.1371/journal.pone.0010312 (2010).
52. Gyorffy, B., Surowiak, P., Budczies, J. & Lanczky, A. Online survival analysis software to assess the prognostic value of biomarkers using transcriptomic data in non-small-cell lung cancer. *PloS one* **8**, e82241, https://doi.org/10.1371/journal.pone.0082241 (2013).
53. Szasz, A. M. *et al*. Cross-validation of survival associated biomarkers in gastric cancer using transcriptomic data of 1,065 patients. *Oncotarget* **7**, 49322–49333, https://doi.org/10.18632/oncotarget.10337 (2016).
54. Shen, L., Liu, C. C., An, C. Y. & Ji, H. F. How does curcumin work with poor bioavailability? Clues from experimental and theoretical studies. *Scientific reports* **6**, 20872, https://doi.org/10.1038/srep20872 (2016).
55. Nelson, K. M. *et al*. The Essential Medicinal Chemistry of Curcumin. *Journal of medicinal chemistry* **60**, 1620–1637, https://doi.org/10.1021/acs.jmedchem.6b00975 (2017).

56. Heger, M., van Golen, R. F., Broekgaarden, M. & Michel, M. C. The molecular basis for the pharmacokinetics and pharmacodynamics of curcumin and its metabolites in relation to cancer. *Pharmacological reviews* **66**, 222–307, https://doi.org/10.1124/pr.110.004044 (2014).
57. Shen, L. & Ji, H. F. The pharmacology of curcumin: is it the degradation products? *Trends in molecular medicine* **18**, 138–144, https://doi.org/10.1016/j.molmed.2012.01.004 (2012).
58. Grabiner, B. C. *et al*. A diverse array of cancer-associated MTOR mutations are hyperactivating and can predict rapamycin sensitivity. *Cancer discovery* **4**, 554–563, https://doi.org/10.1158/2159-8290.CD-13-0929 (2014).
59. Yuan, T. L. & Cantley, L. C. PI3K pathway alterations in cancer: variations on a theme. *Oncogene* **27**, 5497–5510, https://doi.org/10.1038/onc.2008.245 (2008).
60. Land, S. C. & Tee, A. R. Hypoxia-inducible factor 1alpha is regulated by the mammalian target of rapamycin (mTOR) via an mTOR signaling motif. *The Journal of biological chemistry* **282**, 20534–20543, https://doi.org/10.1074/jbc.M611782200 (2007).
61. Hudson, C. C. *et al*. Regulation of hypoxia-inducible factor 1alpha expression and function by the mammalian target of rapamycin. *Molecular and cellular biology* **22**, 7004–7014 (2002).
62. Laplante, M. & Sabatini, D. M. mTOR signaling in growth control and disease. *Cell* **149**, 274–293, https://doi.org/10.1016/j.cell.2012.03.017 (2012).
63. Prakasam, G. *et al*. Pyruvate kinase M knockdown-induced signaling via AMP-activated protein kinase promotes mitochondrial biogenesis, autophagy, and cancer cell survival. *The Journal of biological chemistry* **292**, 15561–15576, https://doi.org/10.1074/jbc.M117.791343 (2017).

Acknowledgements

Department of Science and Technology (DST), Govt. of India, for DST-INSPIRE faculty award and research grant (DST/INSPIRE/04/2015/000556) to MAI.

Author Contributions

Data acquisition: F.A.S., P.G., S.C., A.U.R., R.A.P., M.A.A., R.I., K.M.; Data analysis and interpretation: M.A.I., F.A.S., P.G.; Wrote the manuscript: M.A.I.; Contributed reagents/analytical tools: M.A.I., R.N.K.B., M.H., S.M.A.; Conception, design and supervision of study: M.A.I. All authors contributed to manuscript revision.

Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-25524-3.

Competing Interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Published in final edited form as:

Curr Pharmacol Rep. 2017 December ; 3(6): 423–446. doi:10.1007/s40495-017-0113-2.

Plant flavone apigenin: An emerging anticancer agent

Eswar Shankar[a,b], **Aditi Goel**[c], **Karishma Gupta**[a,b], and **Sanjay Gupta**[*,a,b,d,e,f]

[a]Department of Urology, The James and Eilleen Dicke Laboratory, Case Western Reserve University, Cleveland, OH 44106, USA

[b]Department of Urology, The Urology Institute, University Hospitals Cleveland Medical Center, Cleveland, OH 44106, USA

[c]Department of Biology, School of Undergraduate Studies, Case Western Reserve University, Cleveland, OH 44106, USA

[d]Department of Nutrition, Case Western Reserve University, Cleveland, OH 44106, USA

[e]Division of General Medical Sciences, Case Comprehensive Cancer Center, Cleveland, OH 44106, USA

[f]Department of Urology, Louis Stokes Cleveland Veterans Affairs Medical Center, Cleveland, OH 44106, USA

Abstract

Research in cancer chemoprevention provides convincing evidence that increased intake of vegetables and fruits may reduce the risk of several human malignancies. Phytochemicals present therein provide beneficial anti-inflammatory and antioxidant properties that serve to improve the cellular microenvironment. Compounds known as flavonoids categorized anthocyanidins, flavonols, flavanones, flavonols, flavones, and isoflavones have shown considerable promise as chemopreventive agents. Apigenin (4′, 5, 7-trihydroxyflavone), a major plant flavone, possessing antioxidant, anti-inflammatory, and anticancer properties affecting several molecular and cellular targets used to treat various human diseases. Epidemiologic and case-control studies have suggested apigenin reduces the risk of certain cancers. Studies demonstrate that apigenin retain potent therapeutic properties alone and/or increases the efficacy of several chemotherapeutic drugs in combination on a variety of human cancers. Apigenin's anticancer effects could also be due to its differential effects in causing minimal toxicity to normal cells with delayed plasma clearance and slow decomposition in liver increasing the systemic bioavailability in pharmacokinetic studies. Here we discuss the anticancer role of apigenin highlighting its potential activity as a chemopreventive and therapeutic agent. We also highlight the current caveats that preclude apigenin for its use in the human trials.

[*]**Correspondence to:** Sanjay Gupta, Ph.D. sanjay.gupta@case.edu, Department of Urology, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, Ohio 44106, Phone: (216) 368-6162, Fax: (216) 368-0213.

Conflict of Interest

The authors have no competing interest.

Keywords

cancer chemoprevention; dietary agents; plant flavonoids; health effects; nanoparticle; polyphenols

1.0 Introduction

Cancer is the nemesis in today's global vicissitudes despite progress, increase in life expectancy and rapidly increasing population. There will be an estimated 1,688,780 new cancer cases diagnosed and 600,920 cancer-related deaths occur in the United States in the year 2017 [1]. Global projections of new cancer cases are expected to increase from 16.8 million in 2017 to 21.7 million by 2030 due to increased prevalence and distribution of risk factors [2]. Despite recent advancements under development in the area of chemotherapeutic agents and other modalities to treat cancer, the side effects and chemoresistance obstruct recovery and survival. These shortcomings have led to changes in strategy that aim to reduce the incidence and burden of cancer through the development of agents to prevent, reverse, or delay the carcinogenic process [3]. The goal of cancer chemoprevention is to identify and design new compounds and determine their molecular targets [4]. Epidemiological research helps recognize natural dietary substances associated with reduced cancer mortality and incidence and clarifies variations in dietary patterns and lifestyle practices across a wide geographical range.

Diet is a major lifestyle factor that affects health; poor diets lead to chronic diseases and cancer. The World Cancer Research Fund (WCRF) and the American Institute for Cancer Research (AICR), recommend increased consumption of vegetables, fruits, and grains to help decrease the possibility of cancer development and progression [5]. Foods are not only a source of macronutrients (proteins, fats, and carbohydrates) and micronutrients (vitamins and minerals); they also contain various amounts of non-nutritional molecules called phytochemicals, which have beneficial health effects [6]. The most extensively studied groups of phytochemicals include the polyphenols that are present in plants, grains, fruits, and vegetables. Flavonoids are natural polyphenols produced by plants that can be subcategorized into anthocyanidins, flavanols, flavanones, flavonols, flavones, and isoflavones [7]. The beneficial properties attributed to plant flavonoids include antioxidant, anti-inflammatory, and anti-carcinogenic effects.

Several epidemiological studies have highlighted the benefits of a healthy diet and the number of phytochemicals consumed around the globe including a population-based analysis of the average intake of five flavonoids viz. quercetin, kaempferol, myricetin, luteolin, and apigenin among 7 countries [8–13]. These contents varies from 6 mg/day in Finland to 64 mg/day in Japan with intermediary intake in the United States (13 mg/day), Italy (27 mg/day), and the Netherlands (33 mg/day) [8]. Aherne and O'Brien (2002) assessed the number of flavonoids consumed within Europe and found lower rates of consumption among the Hungarian population as opposed to the Dutch (23 mg/day), Danish (28 mg/day), and Finnish (55 mg/day) populations [9]. A comparative study conducted on the flavonoid consumption between continents has revealed the lowest intake (1–9 mg/day) in the South African diet while the highest intake was from the Scandinavian diet (75–81

mg/day) [10]. The dietary sources for flavones and flavonols vary between various countries; for example, sources include teas in Japan (95%) and the Netherlands (65%) and beer and red wine in Italy (46%). Finland consumes 100% of flavonols and flavones from vegetables, whereas 80% of the dietary source in the United States is from fruits and vegetables. In the Australian sub-continent, tea provides a primary source of dietary flavonoids, especially flavan-3-ols (75%), with rates of consumption as high as 351mg/person/day [11]. A comparison between phenol-rich and phenol-depleted diets showed that a diet rich in flavonoids decreases oxidative stress markers in blood including antioxidant vitamins in plasma, erythrocyte superoxide dismutase (SOD), and diminution in lymphocyte DNA damage, endorsing the benefits of flavonoids and their effects on human health [12]. Apigenin is a major component of the Mediterranean diet associated with lower rates of numerous human diseases, including cancer [13]. In addition to other plant flavones, apigenin is an important compound found in fruit- and vegetable-rich diets. In this review, we discuss the anticancer properties of apigenin and the required efforts to strengthen its role as a chemopreventive and chemotherapeutic agent.

2.0 Sources of apigenin

The name apigenin is derived from the Apium genus in the Apiaceae (the celery, carrot, or parsley family, also known as Umbelliferae). Apigenin naturally occurs as a 4′, 5, 7-trihydroxyflavone. Structurally, the compound possesses hydroxyl groups at positions C-5 and C-7 of the A-ring and C-4′ of the B-ring. Apigenin is a yellow crystalline powder that is insoluble in water and soluble in dimethyl sulfoxide and hot ethanol. It has the molecular formula $C_{15}H_{10}O_5$, and its molecular weight is 270.24 Da. Biapigenin, an apigenin dimer, isolated from the buds and flowers of *Hypericum perforatum* exhibits neuroprotective effects. Naturally, apigenin exists as apigenin-7-*O*-glucoside and various acylated derivatives. Apigenin is found as a single ingredient in chamomile tea, obtained from the dried flowers of *Matricaria chamomilla*, an annual herb native to Western Asia and Europe; naturalized in Australia, Britain, and the United States. Drinks prepared from chamomile contains 0.8% to 1.2% apigenin and essential oils possessing aromatic, flavoring, and coloring properties. Apigenin is also a component of red wine and beer. Apigenin is abundant in a variety of natural sources, including fruits and vegetables. The best sources of apigenin are parsley, chamomile, celery, vine-spinach, artichokes, and oregano, and the richest sources are in the dried forms [14, 15]. Dried parsley has been reported to have the maximum quantity of apigenin, at 45,035 μg/g. Additional sources of apigenin are dried flower of chamomile, containing 3,000 to 5,000 μg/g; celery seeds, containing 786.5 μg/g; and vine spinach and Chinese celery, containing 622 μg/g and 240.2 μg/g [16].

3.0 Apigenin in human health and disease

Extensive studies performed in patients and healthy individuals have shown apigenin to possess anticancer properties. In the past, apigenin was documented as a chemopreventive agent with significant efforts in studying its efficacy in preventing cancer progression. A study conducted by Nielsen et al. [17], apigenin was systematically absorbed in subjects consuming parley-rich diets; subsequently, higher levels of erythrocyte glutathione reductase and superoxide dismutase was observed. However, erythrocyte catalase and glutathione

peroxidase levels remained unchanged. Flavonoids have been shown to induce reductions in the plasma levels of low-density lipoproteins, inhibit platelet aggregation, and reduce cell proliferation [18–21]. A cross-sectional study in Japanese women demonstrate an inverse relationship between total flavonoid intake and total cholesterol and low-density lipoprotein concentration in the plasma [22]. Janssen et al. [23] evaluated the impact of daily dietary consumption of apigenin (84 ± 6 mg from parsley) and quercetin (377 ± 10 mmol from onions) on platelet aggregation and other hematologic measures in a seven-day study of eighteen healthy men and women. No significant alterations in collagen- or ADP-induced platelet numbers, factor VII, plasminogen and plasminogen activator inhibitor-1 activity, or fibrinogen concentrations were observed. These studies highlight the inherent properties of apigenin and help categorize it as a beneficial compound with health-promoting and disease-preventing properties.

Apigenin containing dietary source have found profound application in treating several ailments. The presence of apigenin in passion flower makes it useful to treat asthma, neuralgia, shingles, intransigent insomnia and Parkinson's disease [15]. Similarly high apigenin content in chamomile makes it a good antiphlogistic, antispasmodic, and antibacterial agent. For centuries chamomile tea has been used as a folk medicine remedy. Consumption of 3–4 cups of chamomile tea per day has been used to relieve indigestion or calming gastritis and as skincare product reducing cutaneous inflammation and dermatological problems [24]. Studies providing objective evidence of the beneficial properties of apigenin on human health are quite limited; however, some epidemiologic and clinical studies substantiate that apigenin is useful in counteracting coronary artery disease, gastrointestinal irritation, dermatological disorders, in alleviating labor pain, and in providing antidepressant, calming and relaxing effects. Also, several studies reveal that the anticancer properties of apigenin are mediated through responses to oxidative stress DNA damage, inhibition of angiogenesis and inflammation, suppression of cell growth, and induction of apoptosis and autophagy [16]. At the molecular level, apigenin remains a well-known inhibitor of several protein-tyrosine and serine- kinases including MAPK, PI3K-Akt, Src kinase, casein kinase 2, cell cycle regulated kinases, JAK kinases affecting various signaling pathways including IGF-growth axis, NF-κB, Stat-3, p53, etc. Other putative targets of apigenin include heat shock proteins [15, 25], telomerase [26], fatty acid synthase [27], matrix metalloproteinases [28], and aryl hydrocarbon receptor HER2/neu [29], all of which have relevance to the initiation of several human malignancies. A list of potential targets of apigenin in different human cancers is shown in Table 1.

4.0 Epidemiological studies on apigenin in cancer

Epidemiologic and case-control studies have shown an inverse relationship between gastric, colorectal, breast, ovarian, and endometrial cancers and non-Hodgkin's lymphoma and intake of total flavonoids, flavonoid subgroups, or individual flavonoids [15]. The Zutphen study followed a cohort of 878 for 25 years following the intake of 5 flavonoids: apigenin, kaempferol, luteolin, myricetin, and quercetin and found reduced incidence of mortality from various cancers. These findings were encouraging in demonstrating that high flavonoids source from vegetables and fruits intake reduced the risk of cancer [30]. A large cohort of 9,959 Finnish men demonstrate stronger support for the protective effect of

flavonoids against lung cancer and other malignancies followed from 1967 to 1991 [31]. Another Zutphen study examined the relationship between five common dietary flavonoid intake on ovarian cancer. The study established that dietary supplementation of these flavonoids decreased ovarian cancer risk [32]. A multi-centric case-control study conducted between 1992 and 1999 in Italy further confirmed the inverse relationship between the consumption of common flavonoids and ovarian cancer risk by utilizing data from 1,031 cases with histologically confirmed epithelial ovarian cancer and 2,411 control cases [33]. Another case-control study from Italy conducted between 1991 and 1994 exhibited an inverse association between flavone intake and risk of breast cancer on 2,569 women with histologically confirmed breast cancer and 2,588 controls cases [34]. A study analyzing a group of five flavonoids (quercetin, kaempferol, myricetin, apigenin, and luteolin) in combined food samples, performed on 738 elderly patients, a median age of 65–84 years, and without a history of cancer. The subjects followed for five years and the study concluded that a high intake of flavonoids from fruits and vegetables was associated with a reduced risk of cancer [35]. To establish a dietary baseline and dietary history, the study, including 10,054 men, showed that males with higher myricetin intakes have a lower prostate cancer risk [36]. Conclusions drawn from other studies also support similar findings where flavonoid consumption was associated with decreased risk of sporadic colorectal cancer including reduction in disease recurrence [37]. Gates et al. [38] studied the association between apigenin, kaempferol, luteolin, myricetin and quercetin, and ovarian cancer risk in 1,141 patients with ovarian cancer and 1,183 frequency-matched control subjects. The study showed no clear relationship between the rates of ovarian cancer and the total ingestion of the five flavonoid compounds. An association between flavonoid intake and ovarian cancer risk among women with and without a history of tubal ligation revealed a stronger association with apigenin consumption and ovarian cancer without a history of tubal ligation [39]. Also, for women with no history of tubal ligation, apigenin consumption (quintiles four and five) showed a significant decrease in ovarian cancer risk [39]. Thus, analyses of individual flavonoids revealed that only apigenin intake was associated with reduced cancer risk (with significance), suggesting its strong role as an anticancer agent.

5.0 Effect of apigenin in various human cancers

5.1 Breast cancer

Apigenin has been shown to possess anti-proliferative effects on breast cancer cell lines exhibiting varying levels of HER2/neu [40, 41]. HER2/neu belongs to the family of human epidermal growth factor receptor (HER/EGFR/ERBB). Amplification or overexpression of HER2/neu plays an important role in the development and progression of breast cancer with aggressive behavior [40]. The anti-proliferative effects of apigenin was significantly higher in breast cancer cells over-expressing HER2/neu but was much less efficacious in restricting the growth of cell lines expressing HER2/neu at basal levels [40]. Apigenin induces apoptosis in a dose- and time-dependent fashion in breast cancer cells over-expressing HER2/neu [41]. Phosphatidylinositol 3-kinase (PI3K) and Akt/PKB play a significant role in inhibition of apoptosis in cells over-expressing HER2/neu. Apigenin interferes in the cell survival pathway by inhibiting Akt function by directly blocking PI3K activity [42]. Moreover, apigenin administration led to the depletion of HER2/neu protein in vivo,

consequently inhibiting its auto phosphorylation and transphosphorylation. Additional studies demonstrate apigenin-mediated apoptosis in HER2/neu-expressing breast cancer cells resulted in decrease expression of HER2/neu protein and, subsequently, suppressing the signaling cascade of the HER2/HER3-PI3K/Akt cell survival pathway. Apigenin exposure to breast cancer cells resulted in apoptosis through the release of cytochrome c with the rapid increase of DNA fragmentation factor (GADD)-45. Apigenin treatment in breast cancer cells also results in decreased expression of cyclin D1, D3, and cdk4 and increased quantities of p27 protein [41]. Furthermore, groups have also uncovered the role of apigenin as an agent that induces autophagy during apoptosis [16]. Apigenin-induced autophagy in aggressive breast cancer cells simultaneously caused apoptosis suggesting the involvement of processes inhibiting proliferation and inducing cell death [43]. Low-dose apigenin has the potential to slow or prevent breast cancer progression [44]. Apigenin exerts its toxic effects on breast cancer cells while causing minimal damage to healthy cells, thereby indicating its selectivity in inhibiting tumor possession [45]. In triple-negative breast cancer cells, apigenin induces apoptosis by inhibiting the PI3K/Akt pathway thereby increasing FOXO3a expression that may attenuate cell migration and invasion-inducing apoptosis [46].

Protein kinase C (PKC)-activating phorbol ester (PMA) and peptide hormones prevent apoptosis through the stimulation of PI3K and MAPK pathways [42]. MCF-7 breast cancer cells treated with PMA resulted in the suppression of TNFα-induced apoptosis [42]. Other effects of apigenin on cell survival pathways relates to its suppression of PMA-induced AP-1 activity, thereby supporting its role as an anti-tumor agent. Apigenin treatment inhibited tumor cell invasion in estrogen-insensitive MDA-MB231 breast cancer cell line in a dose-dependent manner [47]. In breast carcinoma cells, apigenin prevents growth and induces G2/M arrest through its downstream effects on ERK MAP kinase and cyclin-CDK regulators [48]. Apigenin exerts inhibitory effects on breast cancer MCF-7 cells, expressing tumor suppressors' wild-type p53 and retinoblastoma (Rb) and expressing mutant p53 and no Rb in MDA-MB-468 cells. Apigenin caused G2/M phase cell cycle arrest which correlated with a marked decrease in CDK1 and cyclin B1 expression and reduction in CDK1 kinase activity. Apigenin exposure resulted in reduced levels of cyclin D1 and A, CDK4, and the inhibition of Rb-phosphorylation; however, cyclin E, CDK2, and CDK6 levels were unaffected. Moreover, apigenin treatment resulted in activation of ERK MAP kinase phosphorylation in MDA-MB-468 cells [48]. Wang & Kurzer [49, 50] analyzed the relationship between apigenin and DNA synthesis on estrogen-dependent MCF-7 cells exposed to estradiol (E3), epidermal growth factor, insulin, or tamoxifen and observed that apigenin inhibits E2-induced DNA synthesis in a variable and concentration-dependent manner. Collins-Burow et al. [51] assessed estrogenic and anti-estrogenic effects on flavonoids in the MCF-7 cells and demonstrated that apigenin exhibits anti-estrogenic activity facilitated through ER binding-dependent and independent- mechanisms [51]. The anti-estrogenic effect demonstrated by apigenin is important in the regulation of breast cancer cell proliferation.

Zhang et al. [52] studied the effects of several plant flavonoids used alone or in combinations of breast cancer resistance protein (BCRP) and found that the combination at equimolar concentrations was highly effective in inhibiting BCRP. Additionally, Stroheker et al. [53] examined the endocrine disruption capabilities of bisphenol derivatives on apigenin

and other plant flavonoids, and the impact on ER(+) MCF-7 and AR(+) and GR(+) MDA-MB453 human breast carcinoma cells. The results suggest that these compounds exert a biphasic effect, acting as partial androgen receptor (AR) agonists at low levels and as GR agonists at high concentrations [53]. Apigenin and genistein have demonstrated anti-proliferative activity on MCF-7 and T47D cells (ERα-positive), but not the ERα-negative MDA-MB-435 cell line [54]. Therefore, the estrogenicity of the phytochemicals contributes to the stimulation of cellular proliferation or reduction in aromatase activity, suggesting that these flavonoids must be used with caution [55]. In HER2-overexpressing breast cancer cells, apigenin-exerted anti-proliferative activity by inhibiting STAT3 signaling, suggesting its potential as a preventive agent [56]. In addition, apigenin also down-regulated STAT3 target genes MMP-2, MMP-9, VEGF and Twist1, which are involved in cell migration and invasion of breast cancer cells [56–58]. Combination treatment of apigenin with chrysin in triple negative breast cancer cells caused marked decrease in cell motility as a consequence of the inhibition of matrix metalloproteinases (MMP)-2 and MMP-9, leading to apoptosis [59].

Brusselmans et al. [60] demonstrated that plant flavonoids induce apoptosis in prostate and breast carcinoma cells due to their ability to inhibit fatty acid synthase, an enzyme that catalyzes long chain fatty acid synthesis and is over-expressed in several cancer cells. This study provided indication that six plant flavonoids, including apigenin, inhibit cancer cell growth and survival due to their effects on fatty acid synthesis [60]. Identification of new target protein interactions through phage display, coupled with second generation sequencing reported in breast cancer cells, discovered three main pathways affected by apigenin, including GTPase activation, membrane transport, and mRNA metabolism/alternative splicing. Apigenin binds to the glycine-rich domain of hnRNPA2, thereby inhibiting its dimerization, a mechanism that is essential for RNA binding. hnRNPA2 is involved in mRNA metabolism and splicing associated with various malignancies. The interaction between apigenin and hnRNPA2 explains that apigenin exerts anti-carcinogenic activity by increasing apoptosis together with the use of chemotherapeutic drugs [61]. Also, studies suggest that combining apigenin with other flavones such as genistein inhibited tumor growth by complexing with Cu (II), thereby significantly enhancing the antitumor properties [62]. In triple-negative breast cancer cells, apigenin results in epigenetic changes by inducing histone H3 acetylation, thereby decreasing cyclin A and cyclin B and increasing p21/Waf1/Cip1 levels [63]. A sub-cytotoxic concentration of apigenin inhibits IFN-γ-induced PD-L1 expression in MDA-MB-468 and 4T1 breast cancer cells, associated with reduced phosphorylation of STAT1, transiently at Tyr701 and persistently at Ser727. However, apigenin exposure did not affect constitutive PD-L1 expression in triple-negative MDA-MB-231 BC cells but was more susceptible to T-cell-mediated antitumor immune responses [64].

5.2 Colon cancer

Wang et al. [65] were the first to report the effect of apigenin on cell growth in various human colon cancer cell lines [65]. Apigenin exposure to colon cancer cells demonstrated anti-proliferative effects, followed by reversible G2/M phase arrest in the cell cycle, and led to decreased levels of cyclin B1 and p34 (cdc2) protein levels. Wang et al. [66] further

analyzed the effects of seven analogs of apigenin on cell viability and cell cycle in human colon cancer cell lines. The results of this study suggest that five of the seven apigenin analogs caused induction of cell-cycle arrest and their combination at small doses synergistically protect against colorectal cancer by collectively inhibiting cell-cycle progression. Apigenin has been shown to stabilize the tumor suppressor p53 in normal cells. In fact, cancer preventive effects of apigenin may be due to its modifying effects on tumor suppressor p53 [67]. Apigenin exposure to two p53-mutant HT-29 and MG63 cancer cell lines leads to growth inhibition and G2/M cell cycle arrest, accompanied by increase in p21/Waf1 expression in a dose- and time-dependent manner [68]. The result indicate the possibility of p53-independent pathway responsible for apigenin-mediated growth inhibition through induction in p21/Waf1 expression in p53-mutant cells.

Farah et al. [69] demonstrated apigenin- and 5,6-dichloro-ribifuranosylbenzimidazole-(DRB)-induced sensitization of HCT-116 and HT-29 colon cancer cells to TNFα-mediated apoptosis. Apigenin and DRB inhibited casein kinase (CK)-2 expression in these cells resulting in a synergistic diminution in cell survival after exposure to TNFα [69]. VanDross et al. [70] highlighted that modulation of the MAPK cascade, may in part, be responsible for the chemopreventive activity of apigenin. Induction of ERK and p38 kinase phosphorylation was noted after apigenin exposure, which was dose-dependent; with little effect on the phosphorylation of c-Jun amino-terminal kinase (JNK). In the azoxymethane (AOM)-induced CF-1 mice, apigenin treatment resulted in the inhibition of formation of aberrant crypt foci and ornithine decarboxylase (ODC), a rate-limiting enzyme of the polyamine synthesis pathway, along with mutant Adenomatous Polyposis Coli (APC) gene [71].

Svehlikova et al. [72] demonstrated the relationship between apigenin and sulforaphane in the induction of UDP-glucuronosyltransferase 1-1 (UGT1A1) and glutathione S-transferase A1 (GSTA1) and the phase II detoxifying enzymes in human colorectal adenocarcinoma CaCo-2 cells. Apigenin induces UGT1A1 transcription but not GSTA1, whereas sulforaphane-induced both UGT1A1 and GSTA1 transcription in a time- and dose-dependent fashion. Therefore, apigenin and sulforaphane synergistically induce UGT1A1 mRNA expression but not GSTA1. The results suggest that diverse signal transduction pathways might regulate the expression of detoxification enzymes [73]. Al-Fayez et al. [74] determined that apigenin exhibits more potency than quercetin or tricin in downregulating inducible COX-2 levels in HCEC cells. Apigenin also contributes to TRAIL-mediated cell death by activating the DR5 death receptor in colon cancer cells [75]. TRAIL, a member of the TNF family, triggers apoptosis in cancer cells via interacting with death receptor 4 (DR4) and death receptor 5 (DR5) leading to the formation of the death-inducing signaling complex (DISC) with a subsequent binding of caspase-8. Recruitment of caspase-8 to the DISC activates its proteolytic properties initiating terminal caspase-3 activation, promoting the cleavage of death substrates and thereby inducing apoptosis. Further investigation shown that apigenin inhibits ODC activity and the formation of aberrant crypt foci (ACF) but fails to inhibit adenoma formation in the Min mouse, showing promise as a chemopreventive agent [74]. Studies demonstrate that apigenin mediates apoptosis and G2M cell cycle arrest in colon cancer cells involving APC tumor suppressor [76]. Feeding rats with azoxymethane, an inducer of colon cancer, with a diet of 97% pure apigenin isolated from Citrus aurantium L. protected the animals from colon cancer, reducing the incidence of ACF

significantly [77]. One of the mechanisms of action of apigenin in colon cancer cells to induce apoptosis suggests the involvement of PKCδ that activates p21 and growth/differentiation factor 15 (NAG-1), followed by apoptosis and inhibition cell proliferation in a p53-independent manner [78]. The same study also exhibited that apigenin could activate p53 by phosphorylating it at Ser-37 and Ser-15 via the ataxia telangiectasia mutated (ATM) pathway to potentiate apoptosis. Turketekin et al. [79] reported that apigenin-induced cell cycle arrest and apoptosis in p53 mutant colon cancer cells. Similar studies also show that apigenin possesses autophagy-inducing effects in HCT116 colon cancer cells, and combined treatment with inhibitor 3-methyladenine (3-MA), an autophagy inhibitor, potentiates apoptosis [80]. CD26, a multifunctional cell-surface protein, in humans, is encoded by the DPPIV gene. The substrates of CD26/DPPIV are proline (or alanine)-containing peptides that binds with enzyme adenosine deaminase and appears to work as tumor suppressor inhibiting pathways involved in tumor metastasis. CD26/DPPIV is down-regulated in various cancers including colorectal carcinoma. Apigenin substantially upregulates CD26/DPPIV on human colorectal cancer HT-29 and HRT-18 cells to inhibit metastasis [81]. Synergistic interaction between apigenin and ABT-263, a BH3 mimetic inhibitor of the Bcl-2 family, was more potent than apigenin or ABT-263 alone in inhibiting tumor growth in xenograft tumors of colon cancer cells [82].

Wang et al. [83] demonstrated that apigenin at 20 mg/kg caused the antitumor activity of human colorectal carcinoma (CRC) SW480 xenografts implanted in nude mice. In an orthotopic CRC model, apigenin at a dose of 10 mg/kg inhibited tumor growth and metastasis to the liver and lungs. Apigenin treatment up-regulated transgelin, suppressed MMP-9 expression by attenuating phosphorylation of Akt at Ser473 and in particular Thr308 to prevent cell proliferation and migration [84].

5.3 Gastric cancer

Wu et al. [85] examined the effect of apigenin on gastric cancer by utilizing human gastric carcinoma SGC-7901 cells. Apigenin treatment resulted in dose-dependent cell growth inhibition, clone formation via induction of apoptosis [85]. In *Helicobacter pylori*-infected gastric adenocarcinoma cells, apigenin treatment effectively inhibited NF-κB activation, scavenged free radicals, and stimulated MUC-2 secretion. Its effect on the NF-κB pathway impacted relevant inflammatory factors such as cyclooxygenase (COX)-2, intercellular adhesion molecule (ICAM)-1, reactive oxygen species (ROS), interleukin (IL)-6, and IL-8. Apigenin exhibits higher potential for the prevention of H. pylori-induced gastric epithelial inflammation [86]. Apigenin remarkably inhibited *H. pylori*-induced atrophic gastritis and gastric cancer progression in eight-week-old Mongolian gerbils. The study also reports apigenin to possess potent anti-gastric cancer activity [86]. Apigenin treatment of HGC-27 and SGC-7901 gastric cancer cells resulted in the inhibition of proliferation followed by mitochondrial depolarization resulting in apoptosis [87].

5.4 Liver Cancer

The 7-hydroxyl group present in plant flavonoids is a putative inhibitor of the human P-form phenol sulfotransferase, which plays an important role in drugs metabolism. A major function of the P-form phenol sulfotransferase is to inactivate and rapid eliminate sulfuric

acid ester conjugates or facilitate the formation of conjugates possessing higher pharmacological activity [88]. Studies demonstrate that addition of a prenyl group to apigenin increases the hydrophobicity, thereby enhancing its pharmacological and biochemical properties. C8-prenylation of apigenin augments the cytotoxic effects and induces apoptosis in H4IIE hepatoma cells without modifying anti-oxidative properties [89]. Yee et al. [90] demonstrated that treatment of human hepatocellular carcinoma HepG2 cells with apigenin and luteolin resulted in inhibition of cell growth, cell cycle arrest, and downregulation of CDK4 expression, along with induction of p53 and p21 [90]. In Wistar albino rats, Jeyabal et al. [91] found apigenin to exert protective effects against N-nitroso-diethylamine-induced and phenobarbital promoted hepatocarcinogenesis. Two weeks of 25 mg/kg apigenin supplementation led to protection against the oxidative stress and DNA damage caused by exposure to the carcinogen [91]. Anti-proliferative effects of apigenin in HepG cells revealed the activation of p53 mediated the induction of p21, causing G2M cell cycle arrest [90]. Further studies revealed apigenin-induced apoptosis in hepatoma tumor cells by utilizing ROS generated through the activation of the NADPH oxidase [92]. The inhibitory role of apigenin in hepatoma cell growth is reported to be mediated by alterations in gene expression profiles confirmed through cDNA microarrays [93]. Apigenin significantly sensitized doxorubicin-resistant BEL-7402 (BEL-7402/ADM) cells to doxorubicin (ADM) and increased the intracellular concentration of ADM by reducing Nrf2-mediated genes through the downregulation of the PI3K/Akt pathway. Apigenin and ADM co-treatment resulted in reduced proliferation, tumor growth inhibition, and apoptosis induction, compared to ADM treatment alone. These results underline the role of apigenin as an adjuvant to overcome chemoresistance [95]. In hepatocellular carcinoma tumors, apigenin enhanced the cytotoxicity of fluorouracil (5-FU), overcoming resistance by inhibiting ROS-mediated drug resistance, leading to mitochondrial depolarization and apoptosis [96]. Cell-based assays demonstrated apigenin as a potent inhibitor of human 26S proteasome. Apigenin treatment led to increase in the ubiquitination of endogenous proteins and inhibition of chymotrypsin-like, trypsin-like, and caspase-like activities of the human 26S proteasome. Apigenin also enhanced the phosphorylation of signal transducer and activator of transcription proteins (STAT1 and STAT2) and promoted the endogenous IFNα-regulated gene expression preventing the ubiquitination of type I interferon receptor 1 (IFNAR1) [97].

5.5 Lung cancer

Liu et al. [98] determined whether apigenin had a dose-dependent inverse relationship between proliferation of A549 lung cancer cells and transcriptional activation of the vascular endothelial growth factor (VEGF). VEGF is the most important growth factor that provides the tissue surrounding the tumor with nutrients for vascular permeability. Apigenin acts on the HIF-1 binding site, which decreases HIF-1α, but not the HIF-1β subunit, thereby inhibiting VEGF. Apigenin further acts to inhibit AKT and p70S6K1 activation, factors that play a role in mediating VEGF transcriptional activation. Additionally, apigenin at 15 μM treatment in mice carrying A549 lung cancer xenografts reduced the tumor volume by partially inhibiting the HIF-1α-vascular endothelial growth factor pathway and subsequent suppression of angiogenesis and cell proliferation [98]. Lung cancer SQ-5 cells exposed to apigenin exhibited greater radiosensitivity and apoptosis compared to cells without apigenin

exposure [99]. Moreover, apigenin was found to inhibit angiogenesis, as suggested by decreased HIF-1α and VEGF expression in cancer cells after exposure in nude mice with implanted lung tumors. Another study by Engelmann et al. [100] demonstrated the effects of apigenin treatment in experimental Lewis lung carcinomas (LLC), C-6 gliomas and DHDK 12 colonic cancers *in vivo*. Mice with tumors received an apigenin dose of 50 mg/kg/day in three different galenical formulations over 12 days in 8-h intervals. Although LLC, C-6, and DHDK 12 and endothelial cells demonstrated high sensitivity to apigenin in cell culture with marked growth suppression at concentrations beyond 30 μg/ml; however, no *in vivo* response was evident [100]. Mice on B57BL/6N background implanted with B16-BL6 tumors exhibited a decreased quantity of tumor cells adhered to lung vessels after treatment with apigenin and quercetin in a single dose [101]. Apigenin seems to be a promising radio-sensitizer for use in human lung carcinomas. Apigenin sensitized SQ-5 spheroids (cell aggregates growing in a three-dimensional structure that simulate growth and microenvironment conditions of *in vivo* tumors) to radiation [99]. In lung cancer cells, apigenin treatment caused dysfunction of mitochondria leading to Bax activation, cytochrome c release, AIF, and Endo G, resulting in caspase-mediated apoptosis [102, 103]. Similar studies by Das et al. [104] reveal that apigenin treatment in lung cancer cells caused DNA interaction, damage, and mitochondrial dysfunction either by direct or indirect action on mitochondrial oxidative phosphorylation system.

Bruno et al. [105] demonstrate that apigenin upregulates leptin receptors to cause apoptosis in lung cancer cells while co-treatment with leptin inhibited cell proliferation. Synergistic administration of curcumin and apigenin may be beneficial for further development as cost-effective anticancer drug combination. Combined treatment with these agents being applied to lung cancer cells induced apoptosis and blocked cell cycle progression at the G2/M phase. Co-administration of apigenin and curcumin, exhibited strong depolymerizing effects on interphase microtubules and inhibited reassembly of cold depolymerized microtubules. This outcome suggests that these agents bind to tubulin at diverse locations [106]. Apigenin exposure NSCLC lung cancer cell resulted in inhibition of proliferation and downregulation of Axl expression, with subsequent alterations in p21 and XIAP expression [107]. Apigenin induces apoptosis and slows cell growth through metabolic and oxidative stress as a consequence of the down-regulation of glucose transporter 1 (GLUT1). Such action leads to a decreased glucose utilization in lung cancer cells. On the contrary, the activation of pentose phosphate pathway-mediated NADPH reversed the effects of apigenin by ectopic GLUT1 overexpression and galactose supplementation. The combined treatment of apigenin with a glutaminase inhibitor, compound 968, sensitized lung cancer cells and caused severe metabolic stress [108]. A small concentration of apigenin synergistically induced cell apoptosis through multiple targets that included caspases and NF-κB pathways in NSCLC cell lines in combination with tumor necrosis factor related apoptosis-inducing ligands (TRAIL). These studies suggest that apigenin possesses substantial therapeutic value for use in conjunction with TRAIL against lung cancer cells [109].

5.6 Pancreatic cancer

Pancreatic cancer remains one of the most deadly forms of human cancer with poor prognoses in spite of attempts to resection and adjuvant therapy. Studies with apigenin in

combination with cell cycle inhibitor flavopiridol have shown to inhibit pancreatic tumor growth through suppression of cyclin B-associated cdc2 activity and G2/M arrest [110]. Apigenin administered in combination with gemcitabine enhanced anti-tumor efficacy through suppression of Akt and NF-κB activity and apoptosis induction in human pancreatic cancer MiaPaca-2 and AsPC-1 cells and pancreatic tumors from nude mice [111]. In a study conducted by Strouch et al. [112], co-treatment with apigenin and gemcitabine, led to cell cycle arrest, down-regulation of p-Akt, and induction of apoptosis in pancreatic cancer cells. Individually, apigenin regressed pancreatic tumors by inhibiting the key members of the NF-κB pathway [113]. In both hypoxic and normoxic conditions, apigenin inhibited GLUT-1, HIF-1α, and VEGF at mRNA and protein levels in pancreatic cancer cells. The study suggests that apigenin has a potential to be developed as a future chemopreventive agent [114]. King et al. [115] demonstrated that treatment of pancreatic cells with apigenin enhanced the acetylation of p53 at Lysine382 causing increased nuclear translocation increasing its DNA binding. In this study, six weeks treatment of orthotopically implanted nude mouse model of human pancreatic cancer with apigenin in diet at 0.2% significantly caused antitumor activity. Furthermore, apigenin increased the functions of mutant p53 in pancreatic cell lines [115]. Apigenin inhibited the tobacco-derived carcinogen-mediated cell proliferation and migration involving the β-AR and its downstream signals FAK and ERK activation [116]. Another study suggests apigenin causing inhibition of GSK-3β and NF-κB pathway in pancreatic cancer cells to mediate apoptosis [117]. Restriction of the NF-κB pathway by apigenin has been shown to involve the inhibition of its upstream kinase IKKβ and overcome TNFα-induced NF-κB activity [113].

Pancreatic cancer cells evade immune destruction through the development of regulatory T cells (Tregs) that inhibit effector T cells through regulation of transcription factor Ikaros. The decrease in Ikaros expression causes a reduction in the CD4+ and CD8+ T cell expression, and an increase in CD4+ CD25+ Tregs in tumor-bearing mice. In pancreatic cancer cells, CK2 regulates Ikaros expression, and apigenin has shown to stabilize Ikaros by downregulating CK2 to central T cell homeostasis [118].

5.7 Prostate cancer

Knowles et al. [119] determined the effects of plant flavonoids including apigenin on the androgen-refractory human prostate cancer PC-3 cell line, and observed that apigenin exposure led to complete growth restriction of these cells. Studies have also investigated the effects of flavonoids on the activity and phosphotyrosine content of proline-directed protein kinase FA (PDPK FA), an oncogene utilizing human prostate cancer cells. Low doses treatment of human prostate cancer cells with quercetin, apigenin, and kaempferol for prolonged times resulted in tyrosine dephosphorylation and inactivation of oncogenic PDPK FA [120]. Furthermore, apigenin treatment to various human prostate cancer LNCaP, PC-3, and DU145 cells and in transformed human prostate epithelial PWR-1E cells resulted in decrease proliferation and induction of apoptosis [121]. In the study, the LNCaP and PWR-1E cells were more sensitive to apigenin-induced apoptosis than PC-3 and DU145 cells. Apigenin also causes caspase-dependent apoptosis in prostate cancer cells. Apigenin exposure resulted in increased ROS generation, loss of mitochondrial Bcl-2 expression, increases mitochondrial permeability, causing cytochrome C release, and cleavage of

caspase 3, 7, 8, and 9 with the concomitant cleavage of the inhibitor of apoptosis protein, cIAP-2 [121]. Over-expression of Bcl-2 in LNCaP B10 cells decreases the apoptotic effects of apigenin. Hessenauer et al. [122] determined that apigenin exposure inhibited CK2 activity in both hormone responsive LNCaP cells and hormone refractory PC-3 cells; however, only LNCaP cells exhibited apoptosis. [122].

Gupta et al. [123] evaluated the growth-inhibitory effects of apigenin on normal (NHPE), virally transformed (PZ-HPV-7) and prostate cancer (CA-HPV-10) cells. Apigenin exposure caused similar levels of mild growth inhibition in NHPE and PZ-HPV-7 cells. In contrast, a marked reduction in cell viability was noted in CA-HPV-10 cells. Gupta et al. [124] elucidated the molecular basis for the apigenin-induced growth restrictions of androgen-responsive LNCaP cells. Apigenin treatment resulted in decreased intracellular and secreted forms of PSA as well as AR protein expression. Apigenin exposure further resulted in G1 cell cycle arrest after, together with marked reduction in cyclin D1, D2, and E levels as well as CDK2, 4, and 6. Furthermore, there was a concomitant induction of Waf1/p21 (in p53 dependent manner) and Kip1/p27 after the apigenin treatment. Moreover, apigenin restricted the hyperphosphorylation of the pRb protein in LNCaP cells [124]. Shukla and Gupta [125] studied apigenin-mediated effects on androgen-insensitive DU145 cells harboring mutations in p53 and pRb. Apigenin treatment of DU145 cells, in both dose- and time-dependent fashion, inhibited growth and colony formation and resulted in G1 cell cycle arrest in these cells. Apigenin exposure further altered the Bax/Bcl2 ratio in favor of apoptosis through induction of apoptotic protease-activating factor-1 (Apaf-1). This cascade led to an upsurge in cleaved products of caspase-9, -3, and poly (ADP-ribose) polymerase (PARP). Treatment with apigenin caused downregulation in the nuclear expression of NF-κB/p65 and NF-κB/p50, associated with upregulation of cytosolic IκBα [125]. Additional investigations by Shukla & Gupta [126] analyzed the effectiveness of apigenin in moderating NF-κB expression, a ubiquitous transcription factor that regulates cell survival, apoptosis and immune functions. Apigenin treatment of PC-3 cells reduced DNA binding and nuclear levels of the NF-κB/p65 and NF-κB/p50 subunits with a simultaneous decrease in IκBα degradation, IκBα phosphorylation, and IKKα kinase activity. Moreover, apigenin was determined to reduce TNFα-induced NF-κB activation via the IκBα pathway, sensitizing cells to TNFα-induced apoptosis. Inhibition of NF-κB corresponded with reduced levels of NF-κB-dependent reporter gene, as well as NF-κB-regulated genes including Bcl2, cyclin D1, cyclooxygenase-2, matrix metalloproteinase 9, nitric oxide synthase-2, and VEGF. Additional evidence supported that apigenin-mediated reduction in cell proliferation, invasiveness, and decrease in tumor growth results from the downregulation of IKKα and downstream targets affecting NF-κB signaling pathways [126]. Shukla et al. [127] elucidated that apigenin directly binds to IKKα, inhibiting its kinase activity. Apigenin treatment restored IκBα expression, preventing its phosphorylation by upstream kinase IKK which undergoes proteasomal degradation [127]. Further assessment by Shukla et al. [128] examined the apigenin-induced 22Rv1 tumor growth inhibition subcutaneously implanted in athymic male nude mice. The results of this study suggested that apigenin restricted tumor growth via increased accumulation of human IGFBP-3 and apoptosis induction. Apigenin supplementation also led to low levels of serum IGF-I in tumor xenografts, signifying that the downstream effects of apigenin involve regulation of IGF-signaling in prostate cancer

[128]. In further studies, oral supplementation of apigenin resulted in increased expression of Waf1/p21, Kip1/p27, INK4a/p16, and INK4c/p18 along with decrease in expression of cyclins D1, D2, and E, in dose-dependent manner. A decrease in cyclin-dependent kinases (cdk) cdk2, cdk4, and cdk6; p-Rb (Ser780); increase in the binding of cyclin D1 toward Waf1/p21 and Kip1/p27; and decrease in the binding of cyclin E toward cdk2 were noted in tumor specimens [129]. Furthermore apigenin treatment resulted in G0-G1 cell cycle arrest, decreased total retinoblastoma (Rb) levels and p-Rb at Ser780 and Ser807/811 in a dose- and time-dependent manner in LNCaP and PC-3 cells. Apigenin was found to increase ERK1/2 and JNK1/2 phosphorylation, resulting in reduction of ELK-1 phosphorylation and c-FOS expression, thereby preventing cell survival. Also, apigenin exposure led to decreased levels of cell cycle regulatory proteins including cyclin D1, D2 and E and their regulatory partners CDK2, 4, and 6, with the loss of RNA polymerase II phosphorylation. The downstream effects of apigenin exposure reinforce its effectiveness in inhibiting transcription of integral proteins [130]. Shukla et al. [131] evaluated the effect of apigenin on TRansgenic Adenocarcinoma of Mouse Prostate (TRAMP) mice. Apigenin supplementation at doses of 20 and 50 mg/mouse/day, 6 days per week for 20 weeks, significantly decreased prostate cancer tumor volumes and complete elimination of metastases. Apigenin treatment resulted in higher levels of E-cadherin and reduced levels of nuclear β-catenin, c-Myc, and cyclin D1 in the prostates of TRAMP mice. These studies suggest that apigenin has ability to block β-catenin signaling, suppressing prostate carcinogenesis *in vivo* [131].

Our investigations of apigenin in cell culture and *in vivo* models suggest that apigenin exerts its anticancer effects by perturbing various pathways leading to inhibition of prostate cancer. Pandey et al. [132] demonstrated the role of apigenin as a histone deacetylase inhibitor. As such, apigenin acts on HDAC1 and HDAC3 and increases the global histone acetylation and the localized hyperacetylation of histone H3 on the p21/Waf1 promoter [132]. A study by Kanwal et al. [133] demonstrated that apigenin could also function as a dual epigenetic inhibitor having ability to alter the DNA methyltransferase and histone deacetylase activity. It does so by reversing both DNA methylation and the trimethylation of lysine 27 at the H3 histone in cultured cells and in an artificial in vitro system [133]. This study further supported an investigation on apigenin and its role as a chemopreventive agent.

Abnormal alterations to the insulin-like growth factor (IGF) pathway has been reported to stimulate prostate cancer progression, adaptation, and survival in a castrated environment and metastasis. A study by Shukla et al. [134] demonstrated that apigenin hindered cancer progression in TRAMP mice by altering IGF-I/IGFBP-3 signaling pathway with an effect on the inhibition of angiogenesis and metastasis. A reduction in the IGF-1 and increase in IGFBP-3 levels in the serum and the dorsolateral prostate was observed in apigenin-treated mice. Furthermore, apigenin intake resulted in marked inhibition of p-Akt, p-ERK1/2, VEGF, uPA, MMP-2 and MMP-9, corresponding with tumor growth and metastasis inhibition in TRAMP mice [134]. Further studies on apigenin in TRAMP mice suggested that reduction in PI3K/Akt signaling could also activate FoxO3a and its DNA binding ability, increasing BIM and p27/Kip1 protein expression, and ultimately resulting in cell cycle arrest and reduced proliferation and metastasis in prostate tumors [135]. Studies evaluating apigenin treatment alone or in combination with additional modalities such as radiation and chemotherapy suggest apigenin may be beneficial in managing advance-stage

prostate cancer. Combination of apigenin with cisplatin showed synergistic cytotoxic and anti-migration activity of CD44 positive prostate cancer stem cells. This treatment induced apoptosis through downregulation of Bcl-2, upregulation of Apaf-1, p21/Waf1 and p53 expression, and inhibition of PI3K/Akt and NF-κB signaling pathways [136]. Shukla et al. [137] demonstrated the ability of apigenin in inhibiting the IAP family of proteins, thereby making prostate cancer cells more susceptible to Bax-mediated apoptosis. Apigenin treatment to prostate cancer cells caused decrease in HDAC1 thereby altering acetylation status, resulting in increased acetylation of the lysine residues of Ku70, thereby releasing Bax from the complex facilitating apoptosis [137]. In further studies of apigenin-mediated apoptosis in prostate cancer cells, Sharma et al. [138] demonstrate preferential uptake and accumulation of apigenin in the nuclear matrix, binding it with the DNA to reduce oxidative DNA damage and apoptosis in prostate epithelial cells.

5.8 Skin cancer

Apigenin displayed beneficial effects in SKH-1 mice in preventing UVA/B-induced skin carcinogenesis [139]. Lepley et al. [140] demonstrated that apigenin, when applied topically, inhibits UV-mediated stimulation of ornithine decarboxylase activity, reduces tumor incidence, and increases tumor-free survival in mice. Apigenin also prevents UV-induced skin tumorigenesis by inhibiting cyclins and cyclin-dependent kinases driving cell cycle progression. [140]. In mouse keratinocytes, apigenin causes G2/M cell cycle arrest, accumulation of p53, and induction of p21/Waf1. Further, apigenin-mediated cell cycle arrest led to reduction in p34 (cdk2) kinase activity, independent of p21/Waf1 [141]. Apigenin halts the cell cycle at G0/G1 phase by inhibiting cdk2 kinase and inducing p21/Waf1 in human diploid fibroblasts. Li et al. [142] developed a short-term in vivo model to evaluate the efficacy of topical apigenin when applied to local skin lesions. This study noted that topical application of apigenin was capable of targeting local tissue [140]. Li et al. [143] further demonstrated the response of percutaneous absorption of apigenin using different vehicles both *in vivo* and *in vitro* models.

Recent observations suggest that apigenin suppresses UVB-induced increase in COX-2 protein and mRNA in mouse and human keratinocytes [144,145]. COX-2 is an enzyme that converts arachidonic acid to prostaglandins, and its overexpression leads to carcinogenesis. Caltagirone et al. [146] determined that the combined treatment of quercetin and apigenin in vivo inhibited B16-BL6 melanoma lung tumor metastasis. This action was attributed to the reduction of endothelial interaction in tumor cells [146]. Topical application of apigenin to mouse skin effectively reduces the incidence and size of skin tumors caused by UVB exposure inducing apoptosis via the intrinsic and extrinsic apoptotic pathways [147]. Exposure of apigenin and luteolin to human keratinocytes inhibited UVA-induced collagenolytic MMP-1 production through interference with Ca(2+)-dependent MAPKs and AP-1 signaling [148]. Apigenin modifies membrane fluidity by altering the motional freedom of polar head groups, thereby decreasing penetration of Pr3+ ions to the membrane. The structural and dynamic changes to the membrane caused by apigenin are crucial for tumor suppression, signal transduction pathways and cell cycle regulation [149]. The *in vivo* skin model developed by Byun et al. [150] supports that apigenin prevents UVB-induced ear edema development, COX-2 expression and Src kinase activity in SKH-1 hairless mice

[150]. These results indicate that apigenin prevents skin cancer by epigenetic modifications. Apigenin inhibits UVB-induced cutaneous angiogenesis through maintenance of the normal high levels of endogenous TSP1 attenuating neo-angiogenesis, proliferation and epidermal thickening in mice exposed to UVB irradiation [152]. The protective role of apigenin was further deciphered using non-melanoma skin cancer model where apigenin inhibited COX-2 that promotes proliferation and tumorigenesis. Thus, inhibition of COX-2 averts skin tumor development [153]. Aberrant activation of Akt/mTOR characterizes skin cancer development as a result of UVB radiation. Apigenin inhibited UVB-mediated mTOR activation in mouse skin and in mouse epidermal keratinocytes independent of Akt, and this led to autophagy [154]. Using 7,12-dimethyl benz[a]anthracene (DMBA)-induced experimental oral carcinogenesis golden hamsters buccal pouch model by painting 0.5% DMBA three times a week for 14 weeks, Silvan et al. [155] demonstrated that oral administration of 2.5 mg/kg apigenin reduced tumor volume causing inhibition of cell proliferation, apoptosis inflammation, and angiogenesis markers, and modulation of phase I and II detoxification cascades. In another study, topical application of apigenin in murine skin tumorigenesis initiated by DMBA and promoted by 12-O-tetradecanoylphorbol-13-acetate in SENCAR mice caused marked reduction of incidence, number of papillomas and carcinomas [156]. Mafuvadze et al. [157] demonstrated that treatment of 50 mg/kg apigenin to mice bearing BT-474 xenograft tumors exposed to medroxyprogesterone acetate resulted in the progression and development of xenograft tumors by inducing apoptosis, inhibiting cell proliferation, and reducing Her2/neu expression.

5.9 Cervical cancer

Zheng et al. [158] first reported that apigenin hinders the progression of human cervical carcinoma HeLa cells through apoptosis. Apigenin prevents cell growth, causes cell cycle arrest in the G1 phase, and prompts p53-dependent apoptosis associated with p21/Waf1 induction and upregulation of Fas/APO-1 and caspase-3. Apigenin exposure also resulted in downregulation of Bcl-2 protein [158]. Czyz et al. [159] established that apigenin interferes with cell proliferation and survival through gap junctional coupling. Apigenin treatment of HeLa cells (wild-type variant) and their connexin43 (Cx43) transfected counterparts caused marked inhibition of cell translocation. Apigenin, at low concentrations, did not demonstrate significant effects on cell proliferation whereas was effective on cell motility and invasiveness in HeLa Cx43 cells [159]. An extract containing parthenolide, camphor, luteolin, and apigenin prepared from the medicinal herb feverfew (*Tanacetum parthenium*) exhibited anti-proliferative activity against human cervical cancer SiHa cells [160]. A study by Liu et al. [161] demonstrated that in HeLa cells, casein kinase 2 (CK2) is a positive regulator in the self-renewal of cervical cancer stem-like cells. They also revealed that apigenin inhibits self-renewal capability through the downregulation of CK2α protein expression. These findings provide evidence for the potential benefits of apigenin as a CK2 inhibitor in the treatment of human cervical cancer by targeting cancer stem cells [161]. The consequence of apigenin on cell proliferation was less pronounced, especially at low concentrations, whereas the effect of apigenin on cell motility corresponded with a blunting of the invasive potential of HeLa Cx43 cells [159]. Another recent study with the medicinal herb feverfew (*Tanacetum parthenium*) extract containing parthenolide, camphor, luteolin, and apigenin, showed anti-proliferative activity against human cervical cancer SiHa cells

[160]. A study by Liu et al. [161] demonstrated that in HeLa cells, casein kinase 2 (CK2) is a positive regulator in the self-renewal of cervical cancer stem-like cells. They also revealed that apigenin inhibits self-renewal capability through the downregulation of CK2α protein expression. These findings provide evidence for the potential benefits of apigenin as a CK2 inhibitor in the treatment of human cervical cancer by targeting cancer stem cells [161]. Apigenin has been shown to exert a selective dose-dependent cytotoxic effect in cervical cancer cells inducing apoptosis as a result of changes in mitochondrial redox potential impairment and inhibition reductions in of cancer cell migration and invasion. These results show that apigenin had a strong and selective anti-tumor effect on cervical cancer cells immortalized by infected with human papilloma virus, especially HPV16 and HPV18. These results indicate that apigenin has potential to be developed as therapeutic agent for (HPV) 16, HPV 18, and HPV 16 and 18 indicating its potential to be a powerful candidate in developing therapeutic agent for all cervical cancer types [162]. Apigenin enhances the inhibitory effect of IFN-α on cell viability in HeLa cancer cells but did not exhibit an effect on cell proliferation and apoptosis in HeLa cancer cells [163]. These results support that additional preclinical and clinical studies are required for further validation of antitumor effects of apigenin applicable to cervical cancer.

5.10 Endometrial cancer

O'Toole et al. [164] performed array-based comparative genomic hybridization on treated endometrial cancer cells treated with phyto-estrogenic compounds agents including apigenin using array-based comparative genomic hybridization. The results of this study found that β-estradiol modified over 20% of the array genes involving involved in insulin metabolism compared to those after treatment with apigenin at similar treated with the same concentration of apigenin. Therefore, this evidence suggests that apigenin may could be beneficial to in the treatment of endometrial cancer.

5.11 Ovarian cancer

Studies in patients with ovarian cancer have shown that intake of apigenin is significantly associated with a lower risk [38, 39]. Fang et al. [165, 166] demonstrated that exposure of human ovarian cancer cells with apigenin decreases invasiveness through suppression of VEGF expression at the transcriptional level in these cells along with expression of HIF-1α via the PI3K/AKT/p70S6K1 and HDM2/p53 pathways [165–167]. Apigenin also contributes to the prevention of tube formation by endothelial cells *in vitro*. Additionally, in human ovarian carcinoma HO-8910PM cells, apigenin restricts MAPK and PI3K the activity of MAPK and PI3K [168]. Due to adenoviral toxicity, most of the patients become immunocompromised. Treatment of ovarian cancer cell lines with apigenin after adenoviral infection employing Ad5/3-Delta24 that targets the Rb pathway revealed the reduction of adenovirus replication and associated toxicity in *in vitro* cell culture and in vivo models. This suggested that apigenin was able to overcome toxicity due to adenovirus infection [169]. Further studies also show apigenin regulating the focal adhesion kinase (FAK) in A2780 ovarian cancer cells to attenuate migration and invasion, suggesting that targeting FAK may be a useful strategy for chemoprevention and/or chemotherapeutics of ovarian cancers [170]. Apigenin's effectiveness is further supported by studies that show its inhibitory effect on Id1 (inhibitor of differentiation or DNA binding protein 1) through

activating transcription factor 3 (ATF3) to retract proliferation and tumorigenesis of human ovarian cancer A2780 cells [171]. In another study, apigenin has shown effectiveness in overcoming chemo-resistance in doxorubicin (DOX) and etoposide (VP16) resistance in 2008/MRP1 ovarian carcinoma cells to DOX by altering the multidrug resistance protein 1 (MRP-1) [172]. The Hedgehog (Hh) signaling pathway plays a critical role in the stimulation of cancer stem cell growth and casein kinase 2 (CK2), a protein kinase frequently activated in cancers. SKOV3 derived SFCs express high levels of CK2α and glioma-associated oncogene 1 (Gli1) proteins. Apigenin inhibited the self-renewal capacity of SKOV3 sphere-forming cells (SFC) by downregulating Gli1 regulated by CK2α [173]. In orthotropic tumors induced using ovarian epithelial cancer cells, OVCAR-3 cells oral treatment of apigenin caused downregulation of MMP-9 mediated by the AKT/p70S6K1 pathway to inhibit tumor progression [174]. In taxol-resistant ovarian cancer cells, apigenin caused down regulation of TAM family of tyrosine kinase receptors and also caused inhibition of IL-6/STAT3 axis, thereby attenuating proliferation. This study suggests that apigenin has ability to overcome taxol resistance in ovarian cancer cells [175].

5.12 Hematologic cancer

Human leukemia cells were evaluated to determine the effects of apigenin treatment. Compared to other flavonoids, apigenin was more effective in leukemia cells in terms of inducing apoptosis [176]. Additional studies have demonstrated that a combination treatment of apigenin and quercetin inhibits topoisomerase-catalyzed DNA irregularities, which are often seen in leukemia cell DNA metabolism, especially in replication and transcription. Vargo et al. [177] determined that apigenin treatment provoked differential anti-proliferative and apoptotic response in monocytic and lymphocytic leukemia cell lines, attributed to protein kinase C delta induction [177]. Utilizing human leukemia cells, Chen et al. [178] examined several flavonoids including apigenin for their proteasome-inhibitory and apoptosis-inducing abilities. The results of this study suggest that apigenin and quercetin exhibit stronger potency in inhibiting chymotrypsin-like activity of purified 20S and 26S proteasome, as well as altering the ubiquitinated forms of two proteasome target proteins, Bax and IκBα, caspase-3 and poly ADP-ribose polymerase (PARP) in Jurkat T cells. Furthermore, the level of proteasome inhibition of these flavonoids corresponds with their potency in inducing apoptosis [178].

Wang et al. [176] demonstrated that human promyelocytic leukemia HL-60 cells underwent apoptosis after exposure to structurally related flavonoids, including apigenin, quercetin, myricetin, and kaempferol due to induction of caspase-3 and PARP cleavage. Consequently, exposure to flavonoids led to the mitochondrial transmembrane potential loss, with a spike in reactive oxygen species, and cytochrome c release into the cytosol with induction of procaspase-9. Apigenin ranked higher than other flavonoids in inducing apoptosis. In another study, apigenin-7-*O*-glucoside extracted from seven principal Tunisian olive varieties reduced the differential marker nitro blue tetrazolium in HL-60 cells [179]. In another study, Monasterio et al. [180] examined the apoptotic potential of twenty two flavonoids and related compounds in leukemic U937 cells. The results of this study revealed that apigenin and additional flavones induced apoptosis in U937 cells; however, isoflavones and flavonones were not as effective in provoking apoptosis.

Horvathova et al. [181] studied the protective effects of various plant flavonoids including apigenin on H2O2-induced DNA damage in murine leukemia L1210 cells. Apigenin at low doses was slightly effective in reducing the scope of DNA damage. Comparatively, at higher concentrations, apigenin exposure led to DNA single strand breaks, suggesting its potential role as a pro-oxidant [181]. In another study, Strick et al. [182] examined the ability of dietary bioflavonoids to cause MLL gene cleavage, which may lead to infant leukemia. Using primary progenitor hematopoietic cells from healthy newborns and adults, apigenin was shown to induce DNA cleavage by targeting topoisomerase II, an enzyme that alters DNA topology. However, it is uncertain whether or not this in-vitro study can be extrapolated to humans due to dose and bioavailability concerns [182]. Apigenin caused the apoptosis in human lymphoma B cells in vitro and prevented the reverted mutations with a high hindrance percentage. This suggests that it has anti-mutagenic properties [183]. In elucidating the mechanism of apoptosis as a result of apigenin treatment in leukemia cells, Gonzalez-Mejia et al. [184] revealed the phosphorylation of Hsp27 as an important event in causing cell death. Additionally, it was shown that apigenin treatment in a late phase involves the activation of p38 and PKCδ to modulate Hsp27, thus leading to apoptosis [184]. A study by Jayasoorya et al. [185] demonstrated that apigenin inhibits cell growth and diminishes telomerase activity in human-derived leukemia cells, ultimately leading to apoptosis. In addition to inducing apoptosis in leukemia cells, apigenin-7 glycoside has been shown to induce granulocyte differentiation that was confirmed by the presence of significant amounts of CD11b positive cells [186]. Evidence also suggests that PI3K/Akt and JNK kinases and their related pathways are potential targets for apigenin-induced apoptosis in leukemia cells [187]. Apigenin-induced expressions of α, β, and γ globin genes increased the expression of glycosporin as a marker for differentiation. The hydroxyl groups are likely to render apigenin effective for inducing cell differentiation [188].

5.13 Adrenal cortical cancer

Previous studies of adrenocortical cancers have established the existence of aberrations in numerous signaling pathways and enzymes, including aromatase, an enzyme involved in the conversion of androgens to estrogen. Sanderson et al. [189], utilizing human adenocortical cancer H295R cells, studied the effect of plant flavonoids on the catalytic and promoter specific expression of aromatase. Results revealed that plant flavonoids are potent aromatase inhibitors, an action related to increased intracellular concentrations of cAMP [189]. Ohno et al. [190] also evaluated the relationship between exposure of plant flavonoids and cortisol production in H295R cells, and determined that apigenin-exposed cells exhibited reduced cortisol production, and decrease in 3β-HSD II and P450c21 activity.

5.14 Thyroid cancer

Yin et al. [191] studied the effect of apigenin on various human thyroid carcinoma cell lines including UCLA NPA-87-1 (NPA) (papillary carcinoma), UCLA RO-82W-1 (WRO) (follicular carcinoma), and UCLA RO-81A-1 (ARO) (anaplastic carcinoma). Apigenin exposure to these cells resulted in inhibition of cell proliferation amongst other plant flavonoids [191]. Yin et al. [192] further illustrated the apigenin-induced inhibition of ARO cell proliferation was associated with the disruption of EGFR tyrosine auto-phosphorylation and its downstream effector MAPK phosphorylation [192]. Schroder-van der Elst et al. [193]

examined the effect of various flavonoid on iodide transport and growth utilizing human follicular thyroid FTC133 cancer cells, stably transfected with the human Na (+)/I (−) symporter (hNIS). Apigenin exposure to these cells prevented NIS mRNA expression, which may have therapeutic implications for the radioiodide treatment of thyroid carcinoma [193]. Apigenin induces apoptosis in ATC cells, mediated through c-Myc, with concomitant changes in p53 and p38 in FRO ATC cells. Apigenin, together with BRAFV600E inhibitor PLX4032, induced cytotoxicity, suppressing Akt in ATC cells which harbor BRAFV600E [194]. The apigenin-enhanced iodide influx rate is increased by Akt inhibition in thyroid cells under acute TSH stimulation and requires p38 MAPK activity. Treatment with apigenin increases radioiodide accumulation in thyroid cells expressing BRAFV600E and in primary cultured thyroid tumor cells from TRβ (PV/PV) mice. Thus, along with Akt inhibitors, apigenin can further enhance the efficacy of radioiodine therapy for thyroid cancer patients [195]. Apigenin-induced cell death has been shown to involve autophagy in papillary thyroid cancer, perhaps because of ROS stimulation, induction of DNA damage and G2/M phase cell cycle arrest [196]. Targeted radioiodine therapy, used in thyroid cancer, depends on thyrotropin-mediated selective stimulation of Na+/I− Symporter (NIS)-mediated radioactive iodide uptake (RAIU) by thyroid cells. Unfortunately, patients with advanced thyroid cancer do not benefit from radioiodine therapy due to reduced or absent NIS expression. Though PI3K inhibitors could induce RAIU by diminished iodide efflux rate where TGF-β, a secreted cytokine facilitates the growth of thyroid cancers, has been observed to reverse the effect. A combination treatment of apigenin with PI3K inhibitor GDC-0941 attenuated the effect of TGF-β increasing RAIU in both BRAFV600E and RET/PTC3 expressing cells [197].

5.15 Neuroblastoma

Torkin et al. [198] studied the downstream effect of apigenin on various neuroblastoma cell lines of human origin. Apigenin exposure to cells resulted in reduced colony-forming ability and survival, leading to induction of apoptosis which was accompanied by an increase in tumor suppressor p53 and its downstream targets including p21/Waf1 and Bax. Furthermore, apigenin demonstrated differential response by causing cell death and apoptosis of neuroblastoma cells expressing wild-type p53, but not mutant p53. Apigenin also augmented caspase-3 activity and PARP cleavage [198]. Neuroblastoma SH-SY5Y cells treated with apigenin led to induction of apoptosis, accompanied by higher levels of intracellular free [Ca(2+)] and shift in Bax:Bcl-2 ratio in favor of apoptosis, cytochrome c release, followed by activation caspase-9, calpain, caspase-3 and caspase-12 [199]. In neuroblastoma SK-N-DZ cells, a combination of the small molecule Bcl-2 inhibitor HA14-1 (HA) and apigenin worked synergistically to decrease cell viability and suppress the expression of angiogenic factors thereby activating extrinsic and intrinsic apoptotic pathways [200]. A combination treatment of apigenin with synthetic retinoid N-(4-hydroxyphenyl) retinamide (4-HPR) in serum-starved human malignant neuroblastoma cells suppressed autophagy and promoted apoptosis [201]. In malignant neuroblastoma cells, ectopic expression of Krüpple-like factor 4 (KLF4) in combination with apigenin treatment resulted in induction of apoptosis downregulating Bcl-2 expression and impairment of transcription and translation of MMP-2 and MMP-9 leading to prevention of tumor cell migration [202]. Apigenin treatment of malignant neuroblastoma cells led to sequential telomerase reverse transcriptase (hTERT)

knockdown, inhibited cell invasion and proliferation and induced apoptosis [203]. Further studies revealed that apigenin involved miR-138 more effectively than hTERT to induce apoptosis in malignant neuroblastoma cancer cell lines [204]. Similar studies using the oncogene N-Myc silencing in combination with apigenin prevented cell migration and decreased N-Myc driven survival, angiogenesis, and invasive factors. This activity suggests that using the said process is a promising strategy for controlling the growth of N-Myc amplified human malignant neuroblastoma cells [205]. Apigenin diminished insulin fibril-induced reactive oxygen species (ROS) production and lipid peroxidation in neuroblastoma cells. These effects led to increased catalase activity and alterations in intracellular glutathione levels, subsequently reducing nitric oxide production and NF-κB activity. Inhibition of NF-κB pathway caused a concomitant reduction in TNFα and IL-6 levels [206]. The suppressive nature of apigenin to inhibit ROS in human neuroblastoma SH-SY5Y cells has been shown to involve reduction in the oxidation of cellular glutathione and subsequent formation of malondialdehyde and carbonyls [207].

5.16 Bladder cancer

Using MEKK1 overexpression in bladder smooth muscle (SM) cell, Liu et al. [208] demonstrated the effect of apigenin in the phosphorylation of MAPKs, ERK, JNK and p38, which are the downstream molecules of MEKK1. Apigenin exposure at 50 μM to these cells significantly inhibited activation/phosphorylation of MAPKs and migration of SM cells induced by MEKK1 overexpression. Furthermore, apigenin also inhibited actin polymerization, which underlines muscle contraction and cell migration [208]. In another study, apigenin suppressed proliferation and inhibited the migration and invasion potential of T24 bladder cancer cells in a dose- and time-dependent manner, which was associated with induced G2/M phase cell cycle arrest and apoptosis through the involvement of PI3K/Akt pathway and Bcl-2 family proteins. In addition, apigenin increased caspase-3 activity and PARP cleavage, indicating that apigenin induced apoptosis in a caspase-dependent way [209]. Zhu et al. [210] demonstrated that apigenin treatment caused decrease proliferation and induction of apoptosis in human bladder T24 cells, associated with an increase in the phospho-p53, p53, p21, and p27 levels, and with a decrease in the cyclin A, cyclin B1, cyclin E, CDK2, Cdc2, and Cdc25C expression, thereby blocking cell cycle progression. In addition, apigenin increased the Bax, Bad, and Bak levels, but reduced the Bcl-xL, Bcl-2, and Mcl-1 levels, and subsequently triggered the mitochondrial apoptotic pathway through release of cytochrome c and activation of caspase-9, caspase-3, caspase-7, and PARP cleavage [210].

5.17 Mesothelioma

Malignant mesothelioma (MM) is a tumor arising from mesothelium. Masuelli et al. [211] demonstrated that apigenin in MM cells caused apoptosis and not autophagy by altering Bax/Bcl2 ratio. Apigenin also caused the activation of p53 and caspase-9. The authors further evidenced that apigenin inhibited Akt and NF-κB/p65 pathways because of MAPK attenuation in these cells. Additionally, tumors with MM cells implanted in C57BL/6 mice treated 20 mg/kg apigenin prolonged the survival time. These evidences suggests the anti-metastatic role of apigenin in a wide variety of *in vitro* and preclinical MM models.

5.18 Osteosacrcoma

The effect of apigenin on human osteosarcoma U2OS cells has been demonstrated. Apigenin significantly decrease cell viability, induced apoptosis through the activations of caspase-3, -8, -9, and BAX and promoted the release of AIF in U2OS cells. Furthermore, nude mice bearing U2OS xenograft tumors, 2 mg/kg apigenin every 3 day for 30 days inhibited tumor growth [212]. Bumke-Vogt et al. [213] demonstrated the impact of apigenin and luteolin on U2OS cells in rapid intracellular translocation of the forkhead box transcription factor O1 (FOXO1), an important mediator of insulin signal transduction. Treatment of human hepatoma HepG2 cells with apigenin and luteolin on the expression of the gluconeogenic enzymes viz. phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G6Pc), the lipogenic enzymes fatty-acid synthase (FASN) and acetyl-CoA-carboxylase (ACC) which were downregulated by both flavones with smaller effective dosages of apigenin than for luteolin. Furthermore, apigenin and luteolin reduced the expression of PKB/AKT-, PRAS40-, p70S6K-, and S6-phosphorylation was reduced by and luteolin but not that of the insulin-like growth factor receptor IGF-1R. Liu et al. [214] demonstrated that apigenin inhibited proliferation and reduced invasion in human U2OS and MG63 osteosarcoma cell lines through downregulation of the expression of β-catenin in these cells.

6.0 Combinational studies with apigenin

Apigenin has also shown to have a significant effect when combined with various chemotherapeutic agents where various cancer cells exhibit differential effects [215–222]. Studies with pancreatic cancer cells reveal that 24 h pretreatment with low concentrations of apigenin for 24 h followed by cisplatin (10 μM), 5-Flurouracil (50 μM), oxaliplatin (0.1 μM), gemcitabine hydrochloride (10 μM) for 36 h resulted in increased growth inhibition of pancreatic cancer cells, compared to co-treatment or individual treatments, where the effects were less than additive [215]. In human multiple myeloma U266 and RPMI 8226 cell lines, apigenin significantly decreased Hsp90 clients when combined with the Hsp90 inhibitor geldanamycin and the histone deacetylase inhibitor vorinostat to induce apoptosis [216]. Similarly combination of synthetic retinoid N-(4-hydroxyphenyl) retinamide (4-HPR) (0.5 μM) and apigenin (50 μM) in the serum-starved human malignant neuroblastoma cells inhibited autophagy inducing apoptosis [217]. The same group had earlier published that combination of apigenin with Bcl-2 inhibitor, small molecule HA14-1 (HA) induces apoptosis in human malignant neuroblastoma cells inhibiting angiogenic factors [218]. In human cervical epithelial carcinoma HeLa cells combination of apigenin and paclitaxel significantly increased inhibition of cell proliferation, suppressing the activity of SOD, inducing ROS accumulation leading to apoptosis by activation of caspase-2 [219]. In MiaPaCa-2 subcutaneous xenograft model of pancreatic cancer, combination treatment of gemcitabine (125 mg/kg) with apigenin (50 mg/kg) exhibited significant tumor growth inhibition, decrease in tumor volume and weight. This combination caused a signification decrease in the activation of Akt and inhibited the DNA binding of NF-κB/p65 in the nucleus [220]. Zhu et al. [221] demonstrated that apigenin inhibits expression of ABCB1, the ATP-binding cassette (ABC) transporter family, and resensitizes docetaxel-resistant prostate cancer cells to docetaxel treatment. In another study by Hu et al. [222] sub-toxic concentrations of apigenin (4 μmol/L) significantly enhanced the cytotoxicity of 5-FU (100

μg/mL) in hepatocellular carcinoma (HCC) cells through mitochondrial membrane potential (ΔΨm)-mediated apoptosis. Furthermore, *in vivo*, combined treatment with 20 mg/kg apigenin five times per week in 3 week protocol and 20 mg/kg 5-FU for 5 consecutive days significantly inhibited the growth of HCC xenograft tumors [222]. Gao et al. [95] demonstrated that apigenin significantly sensitizes doxorubicin-resistant BEL-7402 (BEL-7402/ADM) cells to doxorubicin (ADM) and increases intracellular concentration of ADM through downregulation of PI3K/Akt pathway, leading to a reduction of Nrf2-downstream genes. In BEL-7402 xenografts, apigenin and ADM cotreatment inhibited tumor growth, reduced cell proliferation and induced apoptosis more substantially when compared with ADM treatment alone.

7.0 Clinical trials with apigenin

Early clinical trial with apigenin was based on the epidemiologic studies demonstrating that dietary intake of flavonols and flavones was inversely associated with risk for cardiovascular disease. Janssen et al. [23] performed a study feeding 18 healthy volunteers with 220 g onions and 5 g dried parsley per day providing 114 mg quercetin and 84 mg apigenin respectively, or a placebo for 7 day each in a randomized crossover experiment to determine their effect on platelet aggregation. Although onion consumption raised mean plasma quercetin concentrations to 1.5 μmol/L; plasma apigenin could not be measured. No significant effects of onions or parsley were found on platelet aggregation, thromboxane B2 production, factor VII, or other hemostatic variables. In another study, Hoensch et al. [223] investigated the preventive effects of dietary flavonoid mixture composed of 20 mg apigenin and 20 mg epigallocatechin-3-gallate on recurrence risk on 31 patients with resected colorectal cancer or adenoma polypectomy and compared with matched control group consisting of 56 patients observed for 3–4 years by surveillance colonoscopy. No cancer recurrence was noted and only one adenoma developed in resected colon cancer group. In untreated control, 20% patients developed recurrence and 27% evolved adenomas, suggesting that continuous long-term supplementation of flavonoid mixture could reduce the recurrence of colon neoplasia. In another double-blind, placebo-controlled study, Amsterdam et al. [224] examined the antianxiety and antidepressant action of 220 mg oral chamomile (*Matricaria recutita*) extract standardized to 1.2% apigenin in participants with symptoms of comorbid anxiety and depression evaluated by the scores from the Hamilton Depression Rating (HAM-D) questionnaire among treatment groups. A significant reduction over time in total HAM-D scores for chamomile versus placebo in all participants was noted. Furthermore, a clinically meaningful but nonsignificant trend was noted for a greater reduction in total HAM-D scores for chamomile *versus* placebo in participants with current comorbid depression provided evidence that chamomile may provide clinically meaningful antidepressant activity. Choi et al. [225] demonstrated the clinical efficacy of apigenin on aged skin, using an apigenin-containing cream on forty women, aged over 30 years, through a randomized and double-blinded clinical trial with four weeks of treatment. Application of apigenin-containing cream increased dermal density and elasticity, and reduced fine wrinkle length along with improved skin evenness, moisture content and transepidermal water loss, compared to the placebo group. These results suggest that apigenin possess anti-aging

properties. Based on the published literature, no studies in humans have been conducted solely with apigenin with respect to solid cancer.

8.0 Major limitations of apigenin

Apigenin is unstable and is not very soluble in water or organic solvents. These properties restrict the use of apigenin in its pure forms. Naturally, apigenin is available in foods as glycoside and acylated derivatives, having higher solubility in water compared to the parent compound [226, 227]. The moiety conjugating apigenin helps determine its absorption and bioavailability, facilitating enzymatic cleavage by mammalian or microbial glucosidases [227]. Consequently, it seems likely that apigenin in the natural form bound to β-glycosides may provide better bioavailability. In the intestines, apigenin is extensively metabolized in a method involving both enteric and enterohepatic recycling [228, 229]. Apigenin rapidly metabolizes via UDP-glucuronosyltransferase UGT1A1 as glucoroside and sulfate conjugates that are more readily transported in the blood and excreted in bile or urine [230]. Oral intake of radio-labeled apigenin in a single dose by rats demonstrated 51% recovery of radioactivity in urine, 12% in feces, 1.2% in blood, 9.4% in the intestine, 1.2% in liver, 0.4% in the kidneys, and 24.8% in the rest of the body within 10 days. The radioactivity appeared in blood 24 h after oral apigenin intake. A relatively high elimination of apigenin with a half-time of 91.8 h was noted in the blood in kinetics study, compared to other dietary flavonoids [231]. These results endorse the fact that although the bioavailability of apigenin is limited, however the slow pharmacokinetics may be the reason for possible accumulation of this flavone in the peripheral tissues for its effective chemopreventive effects. In an attempt to achieve better functionality, nano-formulations of apigenin are under investigation in *in vitro* and *in vivo* models. Gold nanoparticles (AuNPs) as biosensors, in photothermal therapy and in imaging have gained popularity due to their possible applications in cancer treatment and in drug delivery [232]. The ability of apigenin to reduce Au3+ ions to form AuNPs is attributed to–OH and C=O groups present in apigenin that reduce Au3+ facilitating the stabilization of the AuNPs [233]. Studies on the synthesis of apigenin-AuNPs have revealed that Au3+ can be reduced by apigenin at a pH of 10 and at room temperature forming highly stable and spherical apigenin-AuNPs. These apigenin-AuNPs are biocompatible towards normal human epidermoid HaCat cells while inducing apoptosis of A431 and SiHa cells. The apigenin-AuNPs also exhibit decent antiangiogenic property. Hence, apigenin-AuNPs is a promising candidate for use in skin cancer treatment [234]. Further studies on nano-apigenin using poly (lactic-co-glycolide) (PLGA) was effective in oral and topical application for skin cancer, achieving a higher efficacy and potency with reduced toxicity [235]. Because of its reduced solubility in water and other lipid compounds, the oral bioavailability of apigenin remains relatively low. Formulations using carbon nanopowder solid dispersions for apigenin have proven to enhance its bioavailability. These apigenin nanoparticles showed low levels of toxicity in animals [236]. Apigenin-loaded lipid nanocapsules prepared with phase inversion method showed high efficacy in restricting breast and liver cancer growth [237]. Cochran et al. [238] demonstrate that a long-term releasing apigenin-based polymer and subsequent nanoparticle delivery system has ability to inhibit tumor cell adhesion through the suppression of endothelial cell adhesion molecule

expression. These studies show promise that nano-formulations of apigenin could become an effective drug-delivery system.

9.0 Conclusion and future directions

In this review, we aim to justify the role of apigenin as an anticancer agent. The fact that apigenin impacts numerous essential pathways and targets associated with cancer is well established. In fact, current research also underlines apigenin as an epigenetic modulator that could act as a dual DNA methyltransferase and histone methyltransferase inhibitor. Nano formulations of apigenin have shown to increase the bioavailability leading to its possible accumulation in tissues. Being the most available bioactive compound from various plants, vegetables, and fruits, dietary apigenin supplementation is highly recommended. Bioavailability of apigenin following oral administration in rats and mice have been reported. So far, this information in humans, including pharmacokinetic and pharmacodynamics profiles, is not available. Further research is necessary concerning the bioavailability and safety profile in humans. A generation of scientific evidence is necessary to confirm the beneficial effects of apigenin in cancer patients to establish its role in chemoprevention and therapy better. However, the currently available reports suggest that apigenin possess potential to be developed as a chemopreventive or chemotherapeutic agent in the future.

Acknowledgments

We sincerely apologize to those investigators whose work could not be cited due to space constraints. The original work from author's laboratory outlined in this review was supported by VA Merit Review 1I01BX002494; United States Public Health Service Grants RO1CA108512, R21CA193080 and R03CA186179 and Department of Defense grant W81XWH-15-1-0558 to SG.

Abbreviations

4-HPR	N-(4-hydroxyphenyl) retinamide
5-FU	Fluorouracil
ACF	Aberrant crypt foci
ADM	Doxorubicin
ADP	Adenosine di-phosphate
AICR	American Institute for Cancer Research
AOM	Azoxymethane
APC	Adenomatous polyposis coli
ATF3	Activating transcription factor 3
BCRP	Breast cancer resistance protein
CK2	Casein kinase 2

COX-2	Cyclooxygenase-2
DISC	Death-inducing signaling complex
DOX	Doxorubicin
DR4	Death receptor 4
EGFR	Epidermal growth factor receptor
ERK	Extracellular regulated kinse
FAK	Focal adhesion kinase
GADD45	DNA fragmentation factor-45
Gli1	Glioma-associated oncogene 1
GLUT1	Glucose transporter 1
GSTA1	Glutathione S-transferase A1
HDAC	Histone deacetylase
HIF-1α	Hypoxia-inducible factor 1-alpha
hTERT	Telomerase reverse transcriptase
ICAM-1	Intercellular adhesion molecule-1
IFNAR1	Type I interferon receptor 1
IFN-γ	Interferon gamma
IGF	Insulin-like growth factor
IL6	Interleukin-6
JAK	Janus kinase
JNK	c-Jun amino-terminal kinase
KLF4	Krüpple-like factor 4
MAPK	Mitogen-activated protein kinases
MMP	Matrix metalloproteinases
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-*kappa*B
NIS	Na+/I- symporter
NSCLC	Non-small cell lung cancer
ODC	Ornithine decarboxylase

PARP	Poly (ADP-ribose) polymerase
PD-L1	Programmed death-ligand 1
PDPK FA	Proline-directed protein kinase FA
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKC	Protein kinase C
PLGA	Poly-lactic-co-glycolide
PMA	Protein kinase C-activating phorbol ester
ROS	Reactive oxygen species
SOD	Superoxide dismutase
Stat	Signal transducer and activator of transcription
TGF-β	Transforming growth factor-beta
TRAIL	Tumor necrosis factor related apoptosis-inducing ligands
TRAMP	Transgenic adenocarcinoma of the mouse prostate
UGT1A1	UDP-glucuronosyltransferase 1-1
VEGF	Vascular endothelial growth factor
WCRF	World Cancer Research Fund

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin. 2016; 66:7–30. [PubMed: 26742998]
2. Fidler MM, Soerjomataram I, Bray F. A global view on cancer incidence and national levels of the human development index. Int J Cancer. 2016; 139:2436–2446. [PubMed: 27522007]
3. Lippman SM, Hong WK. Cancer prevention science and practice. Cancer Res. 2002; 62:5119–5125. [PubMed: 12234971]
4. Gupta S. Prostate cancer chemoprevention: current status and future prospects. Toxicol Appl Pharmacol. 2007; 224:369–376. [PubMed: 17189645]
5. Key TJ, Schatzkin A, Willett WC, Allen NE, Spencer EA, Travis RC. Diet, nutrition and the prevention of cancer. Public Health Nutr. 2004; 7:187–200. [PubMed: 14972060]
6. Newell-McGloughlin M. Nutritionally improved agricultural crops. Plant Physiol. 2008; 147:939–953. [PubMed: 18612071]
7. Manach C, Scalbert A, Morand C, Rémésy C, Jiménez L. Polyphenols: food sources and bioavailability. Am J Clin Nutr. 2004; 79:727–747. [PubMed: 15113710]
8. Hertog MG. Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. Arch Int Medicine. 1995; 155:381–386.
9. Aherne SA, O'Brien NM. Dietary flavonols: chemistry, food content, and metabolism. Nutrition. 2002; 18:75–81. [PubMed: 11827770]
10. de Vries JH, Janssen PL, Hollman PC, van Staveren WA, Katan MB. Consumption of quercetin and kaempferol in free-living subjects eating a variety of diets. Cancer Lett. 1997; 114:114–141.

11. Johannot L, Somerset SM. Age-related variations in flavonoid intake and sources in the Australian population. Public Health Nutrition. 2006; 9:1045–1054. [PubMed: 17125569]
12. Kim H-Y, Kim OH, Sung MK. Effects of Phenol-Depleted and Phenol-Rich diets on blood markers of Oxidative stress, and urinary excretion of Quercetin and Kaempferol in healthy volunteers. Journal of the American College of Nutrition. 2003; 22:217–223. [PubMed: 12805248]
13. Arango D, Morohashi K, Yilmaz A, Kuramochi K, Parihar A, Brahimaj B, Grotewold E, Doseff AI. Molecular basis for the action of a dietary flavonoid revealed by the comprehensive identification of apigenin human targets. Proc Natl Acad Sci USA. 2013; 110:E2153–62. [PubMed: 23697369]
14. McKay DL, Blumberg JB. A review of the bioactivity and potential health benefits of chamomile tea (Matricaria recutita L.). Phytother Res. 2006; 20:519–530. [PubMed: 16628544]
15. Shukla S, Gupta S. Apigenin: a promising molecule for cancer prevention. Pharm Res. 2010; 27:962–978. [PubMed: 20306120]
16. Sung B, Chung HY, Kim ND. Role of Apigenin in Cancer Prevention via the Induction of Apoptosis and Autophagy. J Cancer Prev. 2016; 21:216–226. [PubMed: 28053955]
17. Nielsen SE, Young JF, Daneshvar B, Lauridsen ST, Knuthsen P, Sandström B, Dragsted LO. Effect of parsley (Petroselinum crispum) intake on urinary apigenin excretion, blood antioxidant enzymes and biomarkers for oxidative stress in human subjects. British Journal of Nutrition. 1999; 81:447–455. [PubMed: 10615220]
18. Surh YJ. Cancer chemoprevention with dietary phytochemicals. Nature Rev Cancer. 2003; 3:768–780. [PubMed: 14570043]
19. Yang CS, Landau JM, Huang MT, Newmark HL. Inhibition of carcinogenesis by dietary polyphenolic compounds. Annual Review of Nutrition. 2001; 21:381–406.
20. O'Prey J, Brown J, Fleming J, Harrison PR. Effects of dietary flavonoids on major signal transduction pathways in human epithelial cells. Biochem Pharmacol. 2003; 66:2075–2088. [PubMed: 14609732]
21. Thiery-Vuillemin A, Nguyen T, Pivot X, Spano JP, Dufresnne A, Soria JC. Molecularly targeted agents: Their promise as cancer chemopreventive interventions. European J Cancer. 2005; 41:2003–2015. [PubMed: 16098739]
22. Arai Y, Watanabe S, Kimira M, Shimoi K, Mochizuki R, Kinae N. Dietary Intakes of Flavonols, Flavones and Isoflavones by Japanese women and the inverse correlation between Quercetin intake and plasma LDL cholesterol concentration. J Nutr. 2000; 130:2243–2250. [PubMed: 10958819]
23. Janssen K, Mensink R, Cox F, Harryvan J, Hovenier R, Hollman P, Katan M. Effects of the flavonoids quercetin and apigenin on hemostasis in healthy volunteers: Results from an in vitro and a dietary supplement study. Amer J Clin Nutr. 1998; 67:255–262. [PubMed: 9459373]
24. Graf J. Herbal anti-inflammatory agents for skin disease. Skin Therapy Lett. 2000; 5:3–5.
25. Osada M, Imaoka S, Funae Y. Apigenin suppresses the expression of VEGF, an important factor for angiogenesis, in endothelial cells via degradation of HIF-1alpha protein. FEBS Lett. 2004; 575:59–63. [PubMed: 15388333]
26. Menichincheri M, Ballinari D, Bargiotti A, Bonomini L, Ceccarelli W, D'Alessio R, Fretta A, Moll J, Polucci P, Soncini C, Tibolla M, Trosset JY, Vanotti E. Catecholic flavonoids acting as telomerase inhibitors. J Med Chem. 2004; 47:6466–6475. [PubMed: 15588081]
27. Brusselmans K, Vrolix R, Verhoeven G, Swinnen JV. Induction of cancer cell apoptosis by flavonoids is associated with their ability to inhibit fatty acid synthase activity. J Biol Chem. 2005; 280:5636–5645. [PubMed: 15533929]
28. Kim MH. Flavonoids inhibit VEGF/bFGF-induced angiogenesis in vitro by inhibiting the matrix-degrading proteases. J Cell Biochem. 2003; 89:529–538. [PubMed: 12761886]
29. Way TD, Kao MC, Lin JK. Apigenin induces apoptosis through proteasomal degradation of HER2/neu in HER2/neu-overexpressing breast cancer cells via the phosphatidylinositol 3-kinase/Akt-dependent pathway. J Biol Chem. 2004; 279:4479–4489. [PubMed: 14602723]
30. Heederik D, Kromhout H, Burema J, Biersteker K, Kromhout D. Occupational exposure and 25-year incidence rate of non-specific lung disease: the Zutphen Study. Int J Epidemiol. 1990; 19:945–952. [PubMed: 2084026]

31. Knekt P, Järvinen R, Seppänen R, Hellövaara M, Teppo L, Pukkala E, Aromaa A. Dietary flavonoids and the risk of lung cancer and other malignant neoplasms. Am J Epidemiol. 1997; 146:223–230. [PubMed: 9247006]
32. Hertog MG, Feskens EJ, Hollman PC, Katan MB, Kromhout D. Dietary flavonoids and cancer risk in the Zutphen Elderly Study. Nutr Cancer. 1994; 22:175–184. [PubMed: 14502846]
33. Rossi M, Negri E, Lagiou P, Talamini R, Dal Maso L, Montella M, Franceschi S, La Vecchia C. Flavonoids and ovarian cancer risk: A case-control study in Italy. Int J Cancer. 2008; 123:895–898. [PubMed: 18491402]
34. Bosetti C, Spertini L, Parpinel M, Gnagnarella P, Lagiou P, Negri E, Franceschi S, Montella M, Peterson J, Dwyer J, Giacosa A, La Vecchia C. Flavonoids and breast cancer risk in Italy. Cancer Epidemiol Biomarkers Prev. 2005; 14:805–808. [PubMed: 15824147]
35. Hertog MG, Feskens EJ, Hollman PC, Katan MB, Kromhout D. Dietary flavonoids and cancer risk in the Zutphen Elderly Study. Nutr Cancer. 1994; 22:175–84. [PubMed: 14502846]
36. Knekt P, Kumpulainen J, Järvinen R, Rissanen H, Heliövaara M, Reunanen A, Hakulinen T, Aromaa A. Flavonoid intake and risk of chronic diseases. Am J Clin Nutr. 2002; 76:560–568. [PubMed: 12198000]
37. Hoensch H, Groh B, Edler L, Kirch W. Prospective cohort comparison of flavonoid treatment in patients with resected colorectal cancer to prevent recurrence. World J Gastroenterol. 2008; 14:2187–2193. [PubMed: 18407592]
38. Gates MA, Tworoger SS, Hecht JL, De Vivo I, Rosner B, Hankinson SE. A prospective study of dietary flavonoid intake and incidence of epithelial ovarian cancer. Int J Cancer. 2007; 121:2225–2232. [PubMed: 17471564]
39. Gates MA, Vitonis AF, Tworoger SS, Rosner B, Titus-Ernstoff L, Hankinson SE, Cramer DW. Flavonoid intake and ovarian cancer risk in a population-based case-control study. Int J Cancer. 2009; 124:1918–1925. [PubMed: 19117058]
40. Way TD, Kao MC, Lin JK. Apigenin induces apoptosis through proteasomal degradation of HER2/neu in HER2/neu-overexpressing breast cancer cells via the phosphatidylinositol 3-kinase/Akt-dependent pathway. J Biol Chem. 2004; 279:4479–4489. [PubMed: 14602723]
41. Way TD, Kao MC, Lin JK. Degradation of HER2/neu by apigenin induces apoptosis through cytochrome c release and caspase-3 activation in HER2/neu-overexpressing breast cancer cells. FEBS Lett. 2005; 579:145–152. [PubMed: 15620704]
42. Weldon CB, McKee A, Collins-Burow BM, Melnik LI, Scandurro AB, McLachlan JA, Burow ME, Beckman BS. PKC-mediated survival signaling in breast carcinoma cells: a role for MEK1-AP1 signaling. Int J Oncol. 2005; 26:763–768. [PubMed: 15703835]
43. Cao X, Liu B, Cao W, Zhang W, Zhang F, Zhao H, Niu R. Autophagy inhibition enhances apigenin-induced apoptosis in human breast cancer cells. Chin J Cancer Res. 2013; 25:212–222. [PubMed: 23592903]
44. Harrison ME, Power Coombs MR, Delaney LM, Hoskin DW. Exposure of breast cancer cells to a subcytotoxic dose of apigenin causes growth inhibition, oxidative stress, and hypophosphorylation of Akt. Exp Mol Pathol. 2014; 97:211–217. [PubMed: 25019465]
45. Bai H, Jin H, Yang F, Zhu H, Cai J. Apigenin induced MCF-7 cell apoptosis-associated reactive oxygen species. Scanning. 2014; 36:622–631. [PubMed: 25327419]
46. Lin CH, Chang CY, Lee KR, Lin HJ, Chen TH, Wan L. Flavones inhibit breast cancer proliferation through the Akt/FOXO3a signaling pathway. BMC Cancer. 2015; 15:958. [PubMed: 26675309]
47. Lindenmeyer F, Li H, Menashi S, Soria C, Lu H. Apigenin acts on the tumor cell invasion process and regulates protease production. Nutr Cancer. 2001; 39:139–147. [PubMed: 11588896]
48. Yin F, Giuliano AE, Law RE, Van Herle AJ. Apigenin inhibits growth and induces G2/M arrest by modulating cyclin-cDK regulators and ERK MAP kinase activation in breast carcinoma cells. Anticancer Res. 2001; 21:413–420. [PubMed: 11299771]
49. Wang C, Kurzer MS. Phytoestrogen concentration determines effects on DNA synthesis in human breast cancer cells. Nutr Cancer. 1997; 28:236–247. [PubMed: 9343831]
50. Wang C, Kurzer MS. Effects of phytoestrogens on DNA synthesis in MCF-7 cells in the presence of estradiol or growth factors. Nutr Cancer. 1998; 31:90–100. [PubMed: 9770719]

51. Collins-Burow B, Burow M, Duong B, McLachlan J. Estrogenic and antiestrogenic activities of flavonoid phytochemicals through estrogen receptor binding-dependent and -independent mechanisms. Nutr Cancer. 2001; 38:229–44.
52. Zhang S, Yang X, Morris ME. Combined effects of multiple Flavonoids on breast cancer resistance protein (ABCG2)-mediated transport. Pharm Res. 2004; 21:1263–1273. [PubMed: 15290869]
53. Stroheker T, Picard K, Lhuguenot JC, Canivenc-Lavier MC, Chagnon MC. Steroid activities comparison of natural and food wrap compounds in human breast cancer cell lines. Food and Chem Toxicol. 2004; 42:887–897. [PubMed: 15110097]
54. Seo HS, DeNardo DG, Jacquot Y, Laïos I, Vidal DS, Zambrana CR, Brown PH. Stimulatory effect of genistein and apigenin on the growth of breast cancer cells correlates with their ability to activate ER alpha. Breast Cancer Res Treatment. 2006; 99:121–134.
55. van Meeuwen JA, Korthagen N, de Jong PC, Piersma AH, van den Berg M. (Anti)estrogenic effects of phytochemicals on human primary mammary fibroblasts, MCF-7 cells and their co-culture. Toxicol Appl Pharmacol. 2007; 221:372–383. [PubMed: 17482226]
56. Seo HS, Ku JM, Choi HS, Woo JK, Jang BH, Shin YC, Ko SG. Induction of Caspase-dependent Apoptosis by Apigenin by inhibiting STAT3 signaling in HER2-overexpressing MDA-MB-453 breast cancer cells. Anticancer Res. 2014; 34:2869–2882. [PubMed: 24922650]
57. Seo HS, Jo JK, Ku JM, Choi HS, Choi YK, Woo JK, Kim HI, Kang SY, Lee KM, Nam KW, Park N, Jang BH, Shin YC, Ko SG. Induction of caspase-dependent extrinsic apoptosis by apigenin through inhibition of signal transducer and activator of transcription 3 (STAT3) signalling in HER2-overexpressing BT-474 breast cancer cells. Biosci Rep. 2015; 35(6)
58. Scherbakov AM, Andreeva OE. Apigenin Inhibits Growth of Breast Cancer Cells: The Role of ERα and HER2/neu. Acta Naturae. 2015; 7:133–139. [PubMed: 26483970]
59. Huang C, Wei YX, Shen MC, Tu YH, Wang CC, Huang HC. Chrysin, Abundant in Morinda citrifolia Fruit Water-EtOAc Extracts, Combined with Apigenin Synergistically Induced Apoptosis and Inhibited Migration in Human Breast and Liver Cancer Cells. J Agric Food Chem. 2016; 64:4235–4245. [PubMed: 27137679]
60. Brusselmans K, Vrolix R, Verhoeven G, Swinnen JV. Induction of cancer cell apoptosis by flavonoids is associated with their ability to inhibit fatty acid synthase activity. J Biol Chem. 2005; 280:5636–5645. [PubMed: 15533929]
61. Arango D, Morohashi K, Yilmaz A, Kuramochi K, Parihar A, Brahimaj B, Grotewold E, Doseff AI. Molecular basis for the action of a dietary flavonoid revealed by the comprehensive identification of apigenin human targets. Proc Natl Acad Sci U S A. 2013; 110:E2153–2162. [PubMed: 23697369]
62. Spoerlein C, Mahal K, Schmidt H, Schobert R. Effects of chrysin, apigenin, genistein and their homoleptic copper(II) complexes on the growth and metastatic potential of cancer cells. J Inorg Biochem. 2013; 127:107–115. [PubMed: 23973682]
63. Tseng TH, Chien MH, Lin WL, Wen YC, Chow JM, Chen CK, Kuo TC, Lee WJ. Inhibition of MDA-MB-231 breast cancer cell proliferation and tumor growth by apigenin through induction of G2/M arrest and histone H3 acetylation-mediated p21(WAF1/CIP1) expression. Environ Toxicol. 2017; 32:434–444. [PubMed: 26872304]
64. Coombs MR, Harrison ME, Hoskin DW. Apigenin inhibits the inducible expression of programmed death ligand 1 by human and mouse mammary carcinoma cells. Cancer Lett. 2016; 380:424–433. [PubMed: 27378243]
65. Wang W, Heideman L, Chung CS, Pelling JC, Koehler KJ, Birt DF. Cell-Cycle arrest at G2/M and growth inhibition by Apigenin in human colon carcinoma cell lines. Molecular Carcinogenesis. 2000; 28:102–110. [PubMed: 10900467]
66. Wang W, VanAlstyne PC, Irons KA, Chen S, Stewart JW, Birt DF. Individual and interactive effects of Apigenin Analogs on G2/M cell-cycle arrest in human colon carcinoma cell lines. Nut Cancer. 2004; 48:106–114.
67. Plaumann B, Fritsche M, Rimpler H, Brandner G, Hess R. Flavonoids activate wild-type p53. Oncogene. 1996; 13:1605–1614. [PubMed: 8895505]

68. Takagaki N, Sowa Y, Oki T, Nakanishi R, Yogosawa S, Sakai T. Apigenin induces cell cycle arrest and p21/WAF1 expression in a p53-independent pathway. Int J Oncol. 2005; 26:1629–1637. [PubMed: 15870879]
69. Farah M, Parhar K, Moussavi M, Eivemark S, Salh B. 5, 6-Dichloro-ribifuranosylbenzimidazole- and apigenin-induced sensitization of colon cancer cells to TNF-α-mediated apoptosis. Amer J Physiol - Gastrointestinal and Liver Physiology. 2003; 285:G919–G928.
70. Van Dross R, Xue Y, Knudson A, Pelling JC. The chemopreventive bioflavonoid apigenin modulates signal transduction pathways in keratinocyte and colon carcinoma cell lines. J Nutr. 2003; 133:3800S–3804S. [PubMed: 14608117]
71. Au A, Li B, Wang W, Roy H, Koehler K, Birt D. Effect of dietary apigenin on colonic ornithine decarboxylase activity, aberrant crypt foci formation, and tumorigenesis in different experimental models. Nutr Cancer. 2006; 54:243–251. [PubMed: 16898869]
72. Svehliková V, Bennett RN, Mellon FA, Needs PW, Piacente S, Kroon PA, Bao Y. Isolation, identification and stability of acylated derivatives of apigenin 7-O-glucoside from chamomile (Chamomilla recutita [L.] Rauschert). Phytochemistry. 2004; 65:2323–2332. [PubMed: 15381003]
73. Svehlikova V, Wang S, Jakubikova J, Williamson G, Mithen R, Bao Y. Interactions between sulforaphane and apigenin in the induction of UGT1A1 and GSTA1 in CaCo-2 cells. Carcinogenesis. 2004; 25:1629–1637. [PubMed: 15090468]
74. Al-Fayez M, Cai H, Tunstall R, Steward WP, Gescher AJ. Differential modulation of cyclooxygenase-mediated prostaglandin production by the putative cancer chemopreventive flavonoids tricin, apigenin and quercetin. Cancer Chemother Pharmacol. 2006; 58:816–825. [PubMed: 16552572]
75. Horinaka M, Yoshida T, Shiraishi T, Nakata S, Wakada M, Sakai T. The dietary flavonoid apigenin sensitizes malignant tumor cells to tumor necrosis factor-related apoptosis-inducing ligand. Mol Cancer Ther. 2006; 5:945–951. [PubMed: 16648565]
76. Chung CS, Jiang Y, Cheng D, Birt DF. Impact of adenomatous polyposis coli (APC) tumor supressor gene in human colon cancer cell lines on cell cycle arrest by apigenin. Molecular Carcinogenesis. 2007; 46:773–782. [PubMed: 17620292]
77. Leonardi T, Vanamala J, Taddeo SS, Davidson LA, Murphy ME, Patil BS, Wang N, Carroll RJ, Chapkin RS, Lupton JR, Turner ND. Apigenin and naringenin suppress colon carcinogenesis through the aberrant crypt stage in azoxymethane-treated rats. Exp Biol Med. 2010; 235:710–717.
78. Zhong Y, Krisanapun C, Lee SH, Nualsanit T, Sams C, Peungvicha P, Baek SJ. Molecular targets of apigenin in colorectal cancer cells: involvement of p21, NAG-1 and p53. Eur J Cancer. 2010; 46:3365–3374. [PubMed: 20709524]
79. Turktekin M, Konac E, Onen HI, Alp E, Yilmaz A, Menevse S. Evaluation of the effects of the flavonoid apigenin on apoptotic pathway gene expression on the colon cancer cell line (HT29). J Med Food. 2011; 14:1107–1117. [PubMed: 21548803]
80. Lee Y, Sung B, Kang YJ, Kim DH, Jang JY, Hwang SY, Kim M, Lim HS, Yoon JH, Chung HY, Kim ND. Apigenin-induced apoptosis is enhanced by inhibition of autophagy formation in HCT116 human colon cancer cells. Int J Oncol. 2014; 44:1599–1606. [PubMed: 24626522]
81. Lefort EC, Blay J. The dietary flavonoid apigenin enhances the activities of the anti-metastatic protein CD26 on human colon carcinoma cells. Clin Exp Metastasis. 2011; 28:337–349. [PubMed: 21298326]
82. Shao H, Jing K, Mahmoud E, Huang H, Fang X, Yu C. Apigenin sensitizes colon cancer cells to antitumor activity of ABT-263. Mol Cancer Ther. 2013; 12:2640–2650. [PubMed: 24126433]
83. Wang QR, Yao XQ, Wen G, Fan Q, Li YJ, Fu XQ, Li CK, Sun XG. Apigenin suppresses the growth of colorectal cancer xenografts via phosphorylation and up-regulated FADD expression. Oncol Lett. 2011; 2:43–47. [PubMed: 22870126]
84. Chunhua L, Donglan L, Xiuqiong F, Lihua Z, Qin F, Yawei L, Liang Z, Ge W, Linlin J, Ping Z, Kun L, Xuegang S. Apigenin up-regulates transgelin and inhibits invasion and migration of colorectal cancer through decreased phosphorylation of AKT. J Nutr Biochem. 2013; 24:1766–75. [PubMed: 23773626]
85. Wu K, Yuan L, Xia W. Inhibitory effects of apigenin on the growth of gastric carcinoma SGC-7901 cells. World J Gastroenterol. 2005; 11:4461–4464. [PubMed: 16052672]

86. Wang YC. Medicinal plant activity on Helicobacter pylori related diseases. World J Gastroenterol. 2014; 20:10368–10382. [PubMed: 25132753]

87. Chen J, Chen J, Li Z, Liu C, Yin L. The apoptotic effect of apigenin on human gastric carcinoma cells through mitochondrial signal pathway. Tumour Biol. 2014; 35:7719–7726. [PubMed: 24805829]

88. Eaton EA, Walle UK, Lewis AJ, Hudson T, Wilson AA, Walle T. Flavonoids, potent inhibitors of the human p-form phenolsulfotransferase. Potential role in drug metabolism and chemoprevention. Drug Metabol Disp. 1996; 24:232–237.

89. Wätjen W, Weber N, Lou YJ, Wang ZQ, Chovolou Y, Kampkötter A, Kahl R, Proksch P. Prenylation enhances cytotoxicity of apigenin and liquiritigenin in rat H4IIE hepatoma and C6 glioma cells. Food Chem Toxicol. 2007; 45:119–124. [PubMed: 17045382]

90. Yee SB, Lee JH, Chung HY, Im KS, Bae SJ, Choi JS, Kim ND. Inhibitory effects of luteolin isolated fromixeris sonchifolia hance on the proliferation of hepg2 human hepatocellular carcinoma cells. Arch Pharm Res. 2003; 26:151–156. [PubMed: 12643593]

91. Jeyabal PVS, Syed MB, Venkataraman M, Sambandham JK, Sakthisekaran D. Apigenin inhibits oxidative stress-induced macromolecular damage inN-nitrosodiethylamine (NDEA)-induced hepatocellular carcinogenesis in Wistar albino rats. Molecular Carcinogenesis. 2005; 44:11–20. [PubMed: 15924350]

92. Chiang LC, Ng LT, Lin IC, Kuo PL, Lin CC. Anti-proliferative effect of apigenin and its apoptotic induction in human Hep G2 cells. Cancer Lett. 2006; 237:207–214. [PubMed: 16023288]

93. Choi SI, Jeong CS, Cho SY, Lee YS. Mechanism of apoptosis induced by apigenin in HepG2 human hepatoma cells: involvement of reactive oxygen species generated by NADPH oxidase. Arch Pharm Res. 2007; 30:1328–1335. [PubMed: 18038912]

94. Cai J, Zhao XL, Liu AW, Nian H, Zhang SH. Apigenin inhibits hepatoma cell growth through alteration of gene expression patterns. Phytomedicine. 2011; 18:366–373. [PubMed: 20850954]

95. Gao AM, Ke ZP, Wang JN, Yang JY, Chen SY, Chen H. Apigenin sensitizes doxorubicin-resistant hepatocellular carcinoma BEL-7402/ADM cells to doxorubicin via inhibiting PI3K/Akt/Nrf2 pathway. Carcinogenesis. 2013; 34:1806–1814. [PubMed: 23563091]

96. Hu XY, Liang JY, Guo XJ, Liu L, Guo YB. 5-Fluorouracil combined with apigenin enhances anticancer activity through mitochondrial membrane potential(ΔΨm)-mediated apoptosis in hepatocellular carcinoma. Clin. Exp. Pharmacol. Physiol. 2015; 42:146–153.

97. Li S, Yang LJ, Wang P, He YJ, Huang JM, Liu HW, Shen XF, Wang F. Dietary apigenin potentiates the inhibitory effect of interferon-α on cancer cell viability through inhibition of 26S proteasome-mediated interferon receptor degradation. Food Nutr Res. 2016; 60:31288. [PubMed: 27356910]

98. Liu LZ, Fang J, Zhou Q, Hu X, Shi X, Jiang BH. Apigenin inhibits expression of vascular endothelial growth factor and angiogenesis in human lung cancer cells: implication of chemoprevention of lung cancer. Mol Pharmacol. 2005; 68:635–43. [PubMed: 15947208]

99. Watanabe N, Hirayama R, Kubota N. The chemopreventive flavonoid apigenin confers radiosensitizing effect in human tumor cells grown as monolayers and spheroids. J Radiat Res. 2007; 48:45–50. [PubMed: 17132915]

100. Engelmann C, Blot E, Panis Y, Bauer S, Trochon V, Nagy HJ, Lu H, Soria C. Apigenin–strong cytostatic and anti-angiogenic action in vitro contrasted by lack of efficacy in vivo. Phytomedicine. 2002; 9:489–495. [PubMed: 12403156]

101. Piantelli M, Rossi C, Iezzi M, La Sorda R, Iacobelli S, Alberti S, Natali PG. Flavonoids inhibit melanoma lung metastasis by impairing tumor cells endothelium interactions. J Cell Physiol. 2006; 207:23–29. [PubMed: 16222712]

102. Lu HF, Chie YJ, Yang MS, Lee CS, Fu JJ, Yang JS, Tan TW, Wu SH, Ma YS, Ip SW, Chung JG. Apigenin induces caspase-dependent apoptosis in human lung cancer A549 cells through Bax- and Bcl-2-triggered mitochondrial pathway. Int J Oncol. 2010; 36:1477–1484. [PubMed: 20428772]

103. Lu HF, Chie YJ, Yang MS, Lu KW, Fu JJ, Yang JS, Chen HY, Hsia TC, Ma CY, Ip SW, Chung JG. Apigenin induces apoptosis in human lung cancer H460 cells through caspase- and mitochondria-dependent pathways. Hum Exp Toxicol. 2011; 30:1053–1061. [PubMed: 20937639]

104. Das S, Das J, Samadder A, Boujedaini N, Khuda-Bukhsh AR. Apigenin-induced apoptosis in A375 and A549 cells through selective action and dysfunction of mitochondria. Exp Biol Med. 2012; 237:1433–1448.

105. Bruno A, Siena L, Gerbino S, Ferraro M, Chanez P, Giammanco M, Gjomarkaj M, Pace E. Apigenin affects leptin/leptin receptor pathway and induces cell apoptosis in lung adenocarcinoma cell line. Eur J Cancer. 2011; 47:2042–2051. [PubMed: 21550230]

106. Choudhury D, Ganguli A, Dastidar DG, Acharya BR, Das A, Chakrabarti G. Apigenin shows synergistic anticancer activity with curcumin by binding at different sites of tubulin. Biochimie. 2013; 95:1297–1309. [PubMed: 23485682]

107. Kim KC, Choi EH, Lee C. Axl receptor tyrosine kinase is a novel target of apigenin for the inhibition of cell proliferation. Int J Mol Med. 2014; 34:592–598. [PubMed: 24926787]

108. Lee YM, Lee G, Oh TI, Kim BM, Shim DW, Lee KH, Kim YJ, Lim BO, Lim JH. Inhibition of glutamine utilization sensitizes lung cancer cells to apigenin-induced apoptosis resulting from metabolic and oxidative stress. Int J Oncol. 2016; 48:399–408. [PubMed: 26573871]

109. Chen M, Wang X, Zha D, Cai F, Zhang W, He Y, Huang Q, Zhuang H, Hua ZC. Apigenin potentiates TRAIL therapy of non-small cell lung cancer via upregulating DR4/DR5 expression in a p53-dependent manner. Sci Rep. 2016; 6:35468. [PubMed: 27752089]

110. Ujiki MB, Ding XZ, Salabat MR, Bentrem DJ, Golkar L, Milam B, Talamonti MS, Bell RH Jr, Iwamura T, Adrian TE. Apigenin inhibits pancreatic cancer cell through G2/M cell cycle arrest. Mol Cancer. 2006; 5:76. [PubMed: 17196098]

111. Lee S, Ryu J, Lee K, Woo S, Park J, Yoo J, Yoon Y. Enhanced anti-tumor effect of combination therapy with gemcitabine and apigenin in pancreatic cancer. Cancer Lett. 2008; 259:39–49. [PubMed: 17967505]

112. Strouch MJ, Milam BM, Melstrom LG, McGill JJ, Salabat MR, Ujiki MB, Ding XZ, Bentrem DJ. The flavonoid apigenin potentiates the growth inhibitory effects of gemcitabine and abrogates gemcitabine resistance in human pancreatic cancer cells. Pancreas. 2009; 38:409–415. [PubMed: 19142175]

113. Wu DG, Yu P, Li JW, Jiang P, Sun J, Wang HZ, Zhang LD, Wen MB, Bie P. Apigenin potentiates the growth inhibitory effects by IKK-β-mediated NF-κB activation in pancreatic cancer cells. Toxicol Lett. 2014; 224:157–164. [PubMed: 24148603]

114. Melstrom LG, Salabat MR, Ding XZ, Strouch MJ, Grippo PJ, Mirzoeva S, Pelling JC, Bentrem DJ. Apigenin down-regulates the hypoxia response genes: HIF-1α,GLUT-1, and VEGF in human pancreatic cancer cells. J Surg Res. 2011; 167:173–181. [PubMed: 21227456]

115. King JC, Lu QY, Li G, Moro A, Takahashi H, Chen M, Go VL, Reber HA, Eibl G, Hines OJ. Evidence for activation of mutated p53 by apigenin in human pancreatic cancer. Biochim Biophys Acta. 2012; 1823:593–604. [PubMed: 22227579]

116. Pham H, Chen M, Takahashi H, King J, Reber HA, Hines OJ, Pandol S, Eibl G. Apigenin inhibits NNK-induced focal adhesion kinase activation in pancreatic cancer cells. Pancreas. 2012; 41:1306–1315. [PubMed: 22889981]

117. Johnson JL, Gonzalez de Mejia E. Interactions between dietary flavonoids apigenin or luteolin and chemotherapeutic drugs to potentiate anti-proliferative effect on human pancreatic cancer cells, in vitro. Food Chem Toxicol. 2013; 60:83–91. [PubMed: 23871783]

118. Nelson N, Szekeres K, Iclozan C, Rivera IO, McGill A, Johnson G, Nwogu O, Ghansah T. Apigenin: Selective CK2 inhibitor increases Ikaros expression and improves T cell homeostasis and function in murine pancreatic cancer. PLoS One. 2017; 12:e0170197. [PubMed: 28152014]

119. Knowles LM, Zigrossi DA, Tauber RA, Hightower C, Milner JA. Flavonoids suppress androgen-independent human prostate tumor proliferation. Nutr Cancer. 2000; 38:116–122. [PubMed: 11341036]

120. Lee SC, Kuan CY, Yang CC, Yang SD. Bioflavonoids commonly and potently induce tyrosine dephosphorylation/inactivation of oncogenic proline-directed protein kinase FA in human prostate carcinoma cells. Anticancer Res. 1998; 18:1117–1121. [PubMed: 9615775]

121. Morrissey C, O'Neill A, Spengler B, Christoffel V, Fitzpatrick JM, Watson RW. Apigenin drives the production of reactive oxygen species and initiates a mitochondrial mediated cell death pathway in prostate epithelial cells. Prostate. 2005; 63:131–142. [PubMed: 15486995]

122. Hessenauer A, Montenarh M, Götz C. Inhibition of CK2 activity provokes different responses in hormone-sensitive and hormone-refractory prostate cancer cells. Int J Oncol. 2003; 22:1263–1270. [PubMed: 12738992]

123. Gupta S, Afaq F, Mukhtar H. Selective growth-inhibitory, cell-cycle deregulatory and apoptotic response of apigenin in normal versus human prostate carcinoma cells. Biochem Biophys Res Commun. 2001; 287:914–920. [PubMed: 11573952]

124. Gupta S, Afaq F, Mukhtar H. Involvement of nuclear factor-kappa B, Bax and Bcl-2 in induction of cell cycle arrest and apoptosis by apigenin in human prostate carcinoma cells. Oncogene. 2002; 21:3727–3738. [PubMed: 12032841]

125. Shukla S, Gupta S. Molecular mechanisms for apigenin-induced cell-cycle arrest and apoptosis of hormone refractory human prostate carcinoma DU145 cells. Mol Carcinog. 2004; 39:114–126. [PubMed: 14750216]

126. Shukla S, Gupta S. Suppression of constitutive and tumor necrosis factor alpha-induced nuclear factor (NF)-kappaB activation and induction of apoptosis by apigenin in human prostate carcinoma PC-3 cells: correlation with down-regulation of NF-kappaB-responsive genes. Clin Cancer Res. 2004; 10:3169–3178. [PubMed: 15131058]

127. Shukla S, Kanwal R, Shankar E, Datt M, Chance MR, Fu P, Gupta S. Apigenin blocks IKKα activation and suppresses prostate cancer progression. Oncotarget. 2015; 6:31216–31232. [PubMed: 26435478]

128. Shukla S, Mishra A, Fu P, MacLennan G, Resnick M, Gupta S. Up-regulation of insulin-like growth factor binding protein-3 by apigenin leads to growth inhibition and apoptosis of 22Rv1 xenograft in athymic nude mice. FASEB J. 2005; 19:2042–2044. [PubMed: 16230333]

129. Shukla S, Gupta S. Molecular targets for apigenin-induced cell cycle arrest and apoptosis in prostate cancer cell xenograft. Mol Cancer Ther. 2006; 5:843–852. [PubMed: 16648554]

130. Shukla S, Gupta S. Apigenin-induced cell cycle arrest is mediated by modulation of MAPK, PI3K-Akt, and loss of cyclin D1 associated retinoblastoma dephosphorylation in human prostate cancer cells. Cell Cycle. 2007; 6:1102–1114. [PubMed: 17457054]

131. Shukla S, MacLennan GT, Flask CA, Fu P, Mishra A, Resnick MI, Gupta S. Blockade of beta-Catenin signaling by plant Flavonoid Apigenin suppresses prostate Carcinogenesis in TRAMP mice. Cancer Res. 2007; 67:6925–6935. [PubMed: 17638904]

132. Pandey M, Kaur P, Shukla S, Abbas A, Fu P, Gupta S. Plant flavone apigenin inhibits HDAC and remodels chromatin to induce growth arrest and apoptosis in human prostate cancer cells: in vitro and in vivo study. Mol Carcinog. 2012; 51:952–962. [PubMed: 22006862]

133. Kanwal R, Datt M, Liu X, Gupta S. Dietary Flavones as Dual Inhibitors of DNA Methyltransferases and Histone Methyltransferases. PLoS One. 2016; 11:e0162956. [PubMed: 27658199]

134. Shukla S, MacLennan GT, Fu P, Gupta S. Apigenin attenuates insulin-like growth factor-I signaling in an autochthonous mouse prostate cancer model. Pharm Res. 2012; 29:1506–1517. [PubMed: 22139534]

135. Shukla S, Bhaskaran N, Babcook MA, Fu P, Maclennan GT, Gupta S. Apigenin inhibits prostate cancer progression in TRAMP mice via targeting PI3K/Akt/FoxO pathway. Carcinogenesis. 2014; 35:452–460. [PubMed: 24067903]

136. Erdogan S, Turkekul K, Serttas R, Erdogan Z. The natural flavonoid apigenin sensitizes human CD44(+) prostate cancer stem cells to cisplatin therapy. Biomed Pharmacother. 2017; 88:210–217. [PubMed: 28107698]

137. Shukla S, Fu P, Gupta S. Apigenin induces apoptosis by targeting inhibitor of apoptosis proteins and Ku70-Bax interaction in prostate cancer. Apoptosis. 2014; 19:883–894. [PubMed: 24563225]

138. Sharma H, Kanwal R, Bhaskaran N, Gupta S. Plant Flavone Apigenin binds to nucleic acid bases and reduces oxidative DNA damage in prostate epithelial cells. PLoS ONE. 2014; 9:e91588. [PubMed: 24614817]

139. Birt DF, Mitchell D, Gold B, Pour P, Pinch HC. Inhibition of ultraviolet light induced skin carcinogenesis in SKH-1 mice by apigenin, a plant flavonoid. Anticancer Res. 1997; 17:85–91. [PubMed: 9066634]

140. Lepley DM, Li B, Birt DF, Pelling JC. The chemopreventive flavonoid apigenin induces G2/M arrest in keratinocytes. Carcinogenesis. 1996; 17:2367–2375. [PubMed: 8968050]

141. McVean M, Xiao H, Isobe K, Pelling JC. Increase in wild-type p53 stability and transactivational activity by the chemopreventive agent apigenin in keratinocytes. Carcinogenesis. 2000; 21:633–639. [PubMed: 10753197]

142. Li B, Pinch H, Birt DF. Influence of vehicle, distant topical delivery, and biotransformation on the chemopreventive activity of apigenin, a plant flavonoid, in mouse skin. Pharm Res. 1996; 13:1530–1534. [PubMed: 8899846]

143. Li B, Birt DF. In vivo and in vitro percutaneous absorption of cancer preventive flavonoid apigenin in different vehicles in mouse skin. Pharm Res. 1996; 13:1710–1715. [PubMed: 8956339]

144. Tong X, Van Dross RT, Abu-Yousif A, Morrison AR, Pelling JC. Apigenin prevents UVB-Induced Cyclooxygenase 2 expression: Coupled mRNA stabilization and Translational inhibition. Mol Cell Biol. 2006; 27:283–296. [PubMed: 17074806]

145. Van Dross RT, Hong X, Essengue S, Fischer SM, Pelling JC. Modulation of UVB-induced and basal cyclooxygenase-2 (COX-2) expression by apigenin in mouse keratinocytes: role of USF transcription factors. Mol Carcinog. 2007; 46:303–314. [PubMed: 17186551]

146. Caltagirone S, Rossi C, Poggi A, Ranelletti FO, Natali PG, Brunetti M, Piantelli M. Flavonoids apigenin and quercetin inhibit melanoma growth and metastatic potential. Int J Cancer. 2000; 87:595–600. [PubMed: 10918203]

147. Abu-Yousif AO, Smith KA, Getsios S, Green KJ, Van Dross RT, Pelling JC. Enhancement of UVB-induced apoptosis by apigenin in human keratinocytes and organotypic keratinocyte cultures. Cancer Res. 2008; 68:3057–3065. [PubMed: 18413777]

148. Hwang YP, Oh KN, Yun HJ, Jeong HG. The flavonoids apigenin and luteolin suppress ultraviolet A-induced matrix metalloproteinase-1 expression via MAPKs and AP-1-dependent signaling in HaCaT cells. J Dermatol Sci. 2011; 61:23–31. [PubMed: 21112745]

149. Pawlikowska-Pawlęga B, Misiak LE, Zarzyka B, Paduch R, Gawron A, Gruszecki WI. FTIR, (1)H NMR and EPR spectroscopy studies on the interaction of flavone apigenin with dipalmitoylphosphatidylcholine liposomes. Biochim Biophys Acta. 2013; 1828:518–527. [PubMed: 23088916]

150. Byun S, Park J, Lee E, Lim S, Yu JG, Lee SJ, Chen H, Dong Z, Lee KW, Lee HJ. Src kinase is a direct target of apigenin against UVB-induced skin inflammation. Carcinogenesis. 2013; 34:397–405. [PubMed: 23161610]

151. Paredes-Gonzalez X, Fuentes F, Su ZY, Kong AN. Apigenin reactivates Nrf2 anti-oxidative stress signaling in mouse skin epidermal JB6 P+ cells through epigenetics modifications. AAPS J. 2014; 16:727–735. [PubMed: 24830944]

152. Tong X, Mirzoeva S, Veliceasa D, Bridgeman BB, Fitchev P, Cornwell ML, Crawford SE, Pelling JC, Volpert OV. Chemopreventive apigenin controls UVB-induced cutaneous proliferation and angiogenesis through HuR and thrombospondin-1. Oncotarget. 2014; 5:11413–11427. [PubMed: 25526033]

153. Kiraly AJ, Soliman E, Jenkins A, Van Dross RT. Apigenin inhibits COX-2, PGE2, and EP1 and also initiates terminal differentiation in the epidermis of tumor bearing mice. Prostaglandins Leukot Essent Fatty Acids. 2016; 104:44–53. [PubMed: 26802941]

154. Bridgeman BB, Wang P, Ye B, Pelling JC, Volpert OV, Tong X. Inhibition of mTOR by apigenin in UVB-irradiated keratinocytes: A new implication of skin cancer prevention. Cell Signal. 2016; 28:460–468. [PubMed: 26876613]

155. Silvan S, Manoharan S, Baskaran N, Anusuya C, Karthikeyan S, Prabhakar MM. Chemopreventive potential of apigenin in 7,12-dimethylbenz(a)anthracene induced experimental oral carcinogenesis. Eur J Pharmacol. 2011; 670:571–577. [PubMed: 21970806]

156. Wei H, Tye L, Bresnick E, Birt DF. Inhibitory effect of apigenin, a plant flavonoid, on epidermal ornithine decarboxylase and skin tumor promotion in mice. Cancer Res. 1990; 50:499–502. [PubMed: 2105157]

157. Mafuvadze B, Liang Y, Besch-Williford C, Zhang X, Hyder SM. Apigenin induces apoptosis and blocks growth of medroxyprogesterone acetate-dependent BT-474 xenograft tumors. Horm Cancer. 2012; 3:160–171. [PubMed: 22569706]

158. Zheng PW, Chiang LC, Lin CC. Apigenin induced apoptosis through p53-dependent pathway in human cervical carcinoma cells. Life Sci. 2005; 76:1367–1379. [PubMed: 15670616]

159. Czyz J, Madeja Z, Irmer U, Korohoda W, Hülser DF. Flavonoid apigenin inhibits motility and invasiveness of carcinoma cells in vitro. Int J Cancer. 2005; 114:12–18. [PubMed: 15523693]

160. Wu C, Chen F, Rushing JW, Wang X, Kim HJ, Huang G, He G. Antiproliferative activities of Parthenolide and Golden Feverfew extract against Three human cancer cell lines. J Med Food. 2006; 9:55–61. [PubMed: 16579729]

161. Liu J, Cao XC, Xiao Q, Quan MF. Apigenin inhibits HeLa sphere-forming cells through inactivation of casein kinase 2α. Mol Med Rep. 2015; 11:665–669. [PubMed: 25334018]

162. Souza RP, Bonfim-Mendonça PS, Gimenes F, Ratti BA, Kaplum V, Bruschi ML, Nakamura CV, Silva SO, Maria-Engler SS, Consolaro ME. Oxidative Stress Triggered by Apigenin Induces Apoptosis in a Comprehensive Panel of Human Cervical Cancer-Derived Cell Lines. Oxid Med Cell Longev. 2017:1512745. [PubMed: 28191273]

163. Li S, Yang LJ, Wang P, He YJ, Huang JM, Liu HW, Shen XF, Wang F. Dietary apigenin potentiates the inhibitory effect of interferon-α on cancer cell viability through inhibition of 26S proteasome-mediated interferon receptor degradation. Food Nutr Res. 2016; 60:31288. [PubMed: 27356910]

164. O'Toole SA, Sheppard BL, Sheils O, O'Leary JJ, Spengler B, Christoffel V. Analysis of DNA in Endometrial cancer cells treated with Phyto-Estrogenic compounds using comparative Genomic Hybridisation Microarrays. Planta Medica. 2005; 71:435–439. [PubMed: 15931582]

165. Fang J. Apigenin inhibits VEGF and HIF-1 expression via PI3K/AKT/p70S6K1 and HDM2/p53 pathways. FASEB Journal. 2005; 19:342–353. [PubMed: 15746177]

166. Fang J, Xia C, Cao Z, Zheng JZ, Reed E, Jiang BH. Apigenin inhibits VEGF and HIF-1 expression via PI3K/AKT/p70S6K1 and HDM2/p53 pathways. FASEB J. 2005; 19:342–353. [PubMed: 15746177]

167. Fang J, Zhou Q, Liu LZ, Xia C, Hu X, Shi X, Jiang BH. Apigenin inhibits tumor angiogenesis through decreasing HIF-1alpha and VEGF expression. Carcinogenesis. 2007; 28:858–856. [PubMed: 17071632]

168. Zhu F, Liu XG, Liang NC. Effect of emodin and apigenin on invasion of human ovarian carcinoma HO-8910PM cells in vitro. Ai Zheng. 2003; 22:358–362. [PubMed: 12703988]

169. Kanerva A, Raki M, Ranki T, Särkioja M, Koponen J, Desmond RA, Helin A, Stenman UH, Isoniemi H, Höckerstedt K, Ristimäki A, Hemminki A. Chlorpromazine and apigenin reduce adenovirus replication and decrease replication associated toxicity. J Gene Med. 2007; 9:3–9. [PubMed: 17149790]

170. Hu XW, Meng D, Fang J. Apigenin inhibited migration and invasion of human ovarian cancer A2780 cells through focal adhesion kinase. Carcinogenesis. 2008; 29:2369–2376. [PubMed: 18974065]

171. Li ZD, Hu XW, Wang YT, Fang J. Apigenin inhibits proliferation of ovarian cancer A2780 cells through Id1. FEBS Lett. 2009; 583:1999–2003. [PubMed: 19447105]

172. Wong IL, Chan KF, Tsang KH, Lam CY, Zhao Y, Chan TH, Chow LM. Modulation of multidrug resistance protein 1 (MRP1/ABCC1)-mediated multidrug resistance by bivalent apigenin homodimers and their derivatives. J Med Chem. 2009; 52:5311–5322. [PubMed: 19725578]

173. Tang AQ, Cao XC, Tian L, He L, Liu F. Apigenin inhibits the self-renewal capacity of human ovarian cancer SKOV3-derived sphere-forming cells. Mol Med Rep. 2015; 11:2221–2226. [PubMed: 25405327]

174. He J, Xu Q, Wang M, Li C, Qian X, Shi Z, Liu LZ, Jiang BH. Oral administration of apigenin inhibits metastasis through AKT/P70S6K1/MMP-9 pathway in orthotopic ovarian tumor model. Int J Mol Sci. 2012; 13:7271–7282. [PubMed: 22837693]

175. Suh YA, Jo SY, Lee HY, Lee C. Inhibition of IL-6/STAT3 axis and targeting Axl and Tyro3 receptor tyrosine kinases by apigenin circumvent taxol resistance in ovarian cancer cells. Int J Oncol. 2015; 46:1405–1411. [PubMed: 25544427]

176. Wang IK, Lin-Shiau SY, Lin JK. Induction of apoptosis by apigenin and related flavonoids through cytochrome c release and activation of caspase-9 and caspase-3 in leukaemia HL-60 cells. Eur J Cancer. 1999; 35:1517–1525. [PubMed: 10673981]

177. Vargo MA, Voss OH, Poustka F, Cardounel AJ, Grotewold E, Doseff AI. Apigenin-induced-apoptosis is mediated by the activation of PKCdelta and caspases in leukemia cells. Biochem Pharmacol. 2006; 72:681–92. [PubMed: 16844095]

178. Chen D, Daniel KG, Chen MS, Kuhn DJ, Landis-Piwowar KR, Dou QP. Dietary flavonoids as proteasome inhibitors and apoptosis inducers in human leukemia cells. Biochem Pharmacol. 2005; 69:1421–1432. [PubMed: 15857606]

179. Abaza L, Talorete TP, Yamada P, Kurita Y, Zarrouk M, Isoda H. Induction of growth inhibition and differentiation of human leukemia HL-60 cells by a Tunisian gerboui olive leaf extract. Biosci Biotechnol Biochem. 2007; 71:1306–1312. [PubMed: 17485840]

180. Monasterio A, Urdaci MC, Pinchuk IV, Lopez-Moratalla N, Martinez-Irujo J. Flavonoids induce apoptosis in human leukemia U937 cells through caspase- and caspase-calpain-dependent pathways. J Nutr Cancer. 2004; 50:90–100.

181. Horvathova K, Novotny L, Vachalkova A. The free radical scavenging activity of four flavonoids determined by the comet assay. Neoplasma. 2003; 50:291–295. [PubMed: 12937843]

182. Strick R, Strissel PL, Borgers S, Smith SL, Rowley JD. Dietary bioflavonoids induce cleavage in the MLL gene and may contribute to infant leukemia. Proc Natl Acad Sci USA. 2000; 97:4790–4795. [PubMed: 10758153]

183. Hashemi M, Nouri Long M, Entezari M, Nafisi S, Nowroozii H. Anti-mutagenic and pro-apoptotic effects of apigenin on human chronic lymphocytic leukemia cells. Acta Med Iran. 2010; 48:283–288. [PubMed: 21287458]

184. Gonzalez-Mejia ME, Voss OH, Murnan EJ, Doseff AI. Apigenin-induced apoptosis of leukemia cells is mediated by a bimodal and differentially regulated residue-specific phosphorylation of heat-shock protein-27. Cell Death Dis. 2010; 1:e64. [PubMed: 21364669]

185. Jayasooriya RG, Kang SH, Kang CH, Choi YH, Moon DO, Hyun JW, Chang WY, Kim GY. Apigenin decreases cell viability and telomerase activity in human leukemia cell lines. Food Chem Toxicol. 2012; 50:2605–2611. [PubMed: 22617349]

186. Nakazaki E, Tsolmon S, Han J, Isoda H. Proteomic study of granulocytic differentiation induced by apigenin 7-glucoside in human promyelocytic leukemia HL-60 cells. Eur J Nutr. 2013; 52:25–35. [PubMed: 22113421]

187. Budhraja A, Gao N, Zhang Z, Son YO, Cheng S, Wang X, Ding S, Hitron A, Chen G, Luo J, Shi X. Apigenin induces apoptosis in human leukemia cells and exhibits anti-leukemic activity in vivo. Mol Cancer Ther. 2012; 11:132–142. [PubMed: 22084167]

188. Isoda H, Motojima H, Onaga S, Samet I, Villareal MO, Han J. Analysis of the erythroid differentiation effect of flavonoid apigenin on K562 human chronic leukemia cells. Chem Biol Interact. 2014; 220:269–277. [PubMed: 25058688]

189. Sanderson JT, Hordijk J, Denison MS, Springsteel MF, Nantz MH, van den Berg M. Induction and inhibition of aromatase (CYP19) activity by natural and synthetic flavonoid compounds in H295R human adrenocortical carcinoma cells. Toxicol Sci. 2004; 82:70–79. [PubMed: 15319488]

190. Ohno S, Shinoda S, Toyoshima S, Nakazawa H, Makino T, Nakajin S. Effects of flavonoid phytochemicals on cortisol production and on activities of steroidogenic enzymes in human adrenocortical H295R cells. J Steroid Biochem Mol Biol. 1999; 80:355–363.

191. Yin F, Giuliano AE, Van Herle AJ. Signal pathways involved in apigenin inhibition of growth and induction of apoptosis of human anaplastic thyroid cancer cells (ARO). Anticancer Res. 1999; 19:4297–4303. [PubMed: 10628390]

192. Yin F, Giuliano AE, Law RE, Van Herle AJ. Apigenin inhibits growth and induces G2/M arrest by modulating cyclin-CDK regulators and ERK MAP kinase activation in breast carcinoma cells. Anticancer Res. 2001; 21:413–420. [PubMed: 11299771]

193. Schröder-van der Elst JP, van der Heide D, Romijn JA, Smit JW. Differential effects of natural flavonoids on growth and iodide content in a human Na*/I− symporter-transfected follicular thyroid carcinoma cell line. Eur J Endocrinol. 2004; 150:557–564. [PubMed: 15080787]

194. Kim SH, Kang JG, Kim CS, Ihm SH, Choi MG, Yoo HJ, Lee SJ. Apigenin induces c-Myc-mediated apoptosis in FRO anaplastic thyroid carcinoma cells. Mol Cell Endocrinol. 2013; 369:130–139. [PubMed: 23376608]

195. Lakshmanan A, Doseff AI, Ringel MD, Saji M, Rousset B, Zhang X, Jhiang SM. Apigenin in combination with Akt inhibition significantly enhances thyrotropin-stimulated radioiodide accumulation in thyroid cells. Thyroid. 2014; 24:878–887. [PubMed: 24400871]

196. Zhang L, Cheng X, Gao Y, Zheng J, Xu Q, Sun Y, Guan H, Yu H, Sun Z. Apigenin induces autophagic cell death in human papillary thyroid carcinoma BCPAP cells. Food Funct. 2015; 6:3464–3472. [PubMed: 26292725]

197. Lakshmanan A, Scarberry D, Green JA, Zhang X, Selmi-Ruby S, Jhiang SM. Modulation of thyroidal radioiodide uptake by oncological pipeline inhibitors and apigenin. Oncotarget. 2015; 6:31792–31804. [PubMed: 26397139]

198. Torkin R, Lavoie JF, Kaplan DR, Yeger H. Induction of caspase-dependent, p53-mediated apoptosis by apigenin in human neuroblastoma. Mol Cancer Ther. 2005; 4:1–11. [PubMed: 15657348]

199. Das A, Banik NL, Ray SK. Mechanism of apoptosis with the involvement of calpain and caspase cascades in human malignant neuroblastoma SH-SY5Y cells exposed to flavonoids. Int J Cancer. 2006; 119:2575–2585. [PubMed: 16988947]

200. Karmakar S, Davis KA, Choudhury SR, Deeconda A, Banik NL, Ray SK. Bcl-2 inhibitor and apigenin worked synergistically in human malignant neuroblastoma cell lines and increased apoptosis with activation of extrinsic and intrinsic pathways. Biochem Biophys Res Commun. 2009; 388:705–710. [PubMed: 19695221]

201. Mohan N, Banik NL, Ray SK. Combination of N-(4-hydroxyphenyl) retinamide and apigenin suppressed starvation-induced autophagy and promoted apoptosis in malignant neuroblastoma cells. Neurosci Lett. 2011; 502:24–29. [PubMed: 21801811]

202. Mohan N, Ai W, Chakrabarti M, Banik NL, Ray SK. KLF4 overexpression and apigenin treatment down regulated anti-apoptotic Bcl-2 proteins and matrix metalloproteinases to control growth of human malignant neuroblastoma SK-N-DZ and IMR-32 cells. Mol Oncol. 2013; 7:464–474. [PubMed: 23317647]

203. Chakrabarti M, Banik NL, Ray SK. Sequential hTERT knockdown and apigenin treatment inhibited invasion and proliferation and induced apoptosis in human malignant neuroblastoma SK-N-DZ and SK-N-BE2 cells. J Mol Neurosci. 2013; 51:187–198. [PubMed: 23417743]

204. Chakrabarti M, Banik NL, Ray SK. miR-138 overexpression is more powerful than hTERT knockdown to potentiate apigenin for apoptosis in neuroblastoma in vitro and in vivo. Exp Cell Res. 2013; 319:1575–1585. [PubMed: 23562653]

205. Hossain MM, Banik NL, Ray SK. N-Myc knockdown and apigenin treatment controlled growth of malignant neuroblastoma cells having N-Myc amplification. Gene. 2013; 529:27–36. [PubMed: 23941992]

206. Amini R, Yazdanparast R, Ghaffari SH. Apigenin modulates the expression levels of pro-inflammatory mediators to reduce the human insulin amyloid-induced oxidant damages in SK-N-MC cells. Hum Exp Toxicol. 2015; 34:642–653. [PubMed: 25304968]

207. Kim A, Nam YJ, Lee MS, Shin YK, Sohn DS, Lee CS. Apigenin Reduces Proteasome Inhibition-Induced Neuronal Apoptosis by Suppressing the Cell Death Process. Neurochem Res. 2016; 41:2969–2980. [PubMed: 27473386]

208. Liu Q, Chen X, Yang G, Min X, Deng M. Apigenin inhibits cell migration through MAPK pathways in human bladder smooth muscle cells. Biocell. 2011; 35:71–79. [PubMed: 22423483]

209. Zhu Y, Mao Y, Chen H, Lin Y, Hu Z, Wu J, Xu X, Xu X, Qin J, Xie L. Apigenin promotes apoptosis, inhibits invasion and induces cell cycle arrest of T24 human bladder cancer cells. Cancer Cell Int. 2013; 13:54. [PubMed: 23724790]

210. Shi MD, Shiao CK, Lee YC, Shih YW. Apigenin, a dietary flavonoid, inhibits proliferation of human bladder cancer T-24 cells via blocking cell cycle progression and inducing apoptosis. Cancer Cell Int. 2015; 15:33. [PubMed: 25859163]

211. Masuelli L, Benvenuto M, Mattera R, Di Stefano E, Zago E, Taffera G, Tresoldi I, Giganti MG, Frajese GV, Berardi G, Modesti A, Bei R. In Vitro and In Vivo Anti-tumoral Effects of the

Flavonoid Apigenin in Malignant Mesothelioma. Front Pharmacol. 2017; 8:373. [PubMed: 28674496]

212. Lin CC, Chuang YJ, Yu CC, Yang JS, Lu CC, Chiang JH, Lin JP, Tang NY, Huang AC, Chung JG. Apigenin induces apoptosis through mitochondrial dysfunction in U-2 OS human osteosarcoma cells and inhibits osteosarcoma xenograft tumor growth in vivo. J Agric Food Chem. 2012; 60:11395–11402. [PubMed: 23066961]
213. Bumke-Vogt C, Osterhoff MA, Borchert A, Guzman-Perez V, Sarem Z, Birkenfeld AL, Bähr V, Pfeiffer AF. The flavones apigenin and luteolin induce FOXO1 translocation but inhibit gluconeogenic and lipogenic gene expression in human cells. PLoS One. 2014; 9:e104321. [PubMed: 25136826]
214. Liu X, Li L, Lv L, Chen D, Shen L, Xie Z. Apigenin inhibits the proliferation and invasion of osteosarcoma cells by suppressing the Wnt/β-catenin signaling pathway. Oncol Rep. 2015; 34:1035–1041. [PubMed: 26035210]
215. Johnson JL, Gonzalez de Mejia E. Interactions between dietary flavonoids apigenin or luteolin and chemotherapeutic drugs to potentiate anti-proliferative effect on human pancreatic cancer cells, in vitro. Food Chem Toxicol. 2013; 60:83–91. [PubMed: 23871783]
216. Zhao M, Ma J, Zhu HY, Zhang XH, Du ZY, Xu YJ, Yu XD. Apigenin inhibits proliferation and induces apoptosis in human multiple myeloma cells through targeting the trinity of CK2, Cdc37 and Hsp90. Mol Cancer. 2011; 10:104. [PubMed: 21871133]
217. Mohan N, Banik NL, Ray SK. Combination of N-(4-hydroxyphenyl) retinamide and apigenin suppressed starvation-induced autophagy and promoted apoptosis in malignant neuroblastoma cells. Neurosci Lett. 2011; 502:24–29. [PubMed: 21801811]
218. Karmakar S, Davis KA, Choudhury SR, Deeconda A, Banik NL, Ray SK. Bcl-2 inhibitor and apigenin worked synergistically in human malignant neuroblastoma cell lines and increased apoptosis with activation of extrinsic and intrinsic pathways. Biochem Biophys Res Commun. 2009; 388:705–710. [PubMed: 19695221]
219. Xu Y, Xin Y, Diao Y, Lu C, Fu J, Luo L, Yin Z. Synergistic effects of apigenin and paclitaxel on apoptosis of cancer cells. PLoS One. 2011; 6(12):e29169. [PubMed: 22216199]
220. Lee SH, Ryu JK, Lee KY, Woo SM, Park JK, Yoo JW, Kim YT, Yoon YB. Enhanced anti-tumor effect of combination therapy with gemcitabine and apigenin in pancreatic cancer. Cancer Lett. 2008; 259:39–49. [PubMed: 17967505]
221. Zhu Y, Liu C, Nadiminty N, Lou W, Tummala R, Evans CP, Gao AC. Inhibition of ABCB1 expression overcomes acquired docetaxel resistance in prostate cancer. Mol Cancer Ther. 2013; 12:1829–1836. [PubMed: 23861346]
222. Hu XY, Liang JY, Guo XJ, Liu L, Guo YB. 5-Fluorouracil combined with apigenin enhances anticancer activity through mitochondrial membrane potential (ΔΨm)-mediated apoptosis in hepatocellular carcinoma. Clin Exp Pharmacol Physiol. 2015; 42:146–153. [PubMed: 25363523]
223. Hoensch H, Groh B, Edler L, Kirch W. Prospective cohort comparison of flavonoid treatment in patients with resected colorectal cancer to prevent recurrence. World J Gastroenterol. 2008; 14:2187–2193. [PubMed: 18407592]
224. Amsterdam JD, Shults J, Soeller I, Mao JJ, Rockwell K, Newberg AB. Chamomile (Matricaria recutita) may provide antidepressant activity in anxious, depressed humans: an exploratory study. Altern Ther Health Med. 2012; 18:44–49.
225. Choi S, Youn J, Kim K, Joo da H, Shin S, Lee J, Lee HK, An IS, Kwon S, Youn HJ, Ahn KJ, An S, Cha HJ. Apigenin inhibits UVA-induced cytotoxicity in vitro and prevents signs of skin aging in vivo. Int J Mol Med. 2016; 38:627–634. [PubMed: 27279007]
226. Hollman PC, Katan MB. Health effects and bioavailability of dietary flavonols. Free Radical Res. 1999; 31:S75–80. [PubMed: 10694044]
227. Ross JA, Kasum CM. Dietary flavonoids: bioavailability, metabolic effects, and safety. Annu Rev Nutr. 2002; 22:19–34. [PubMed: 12055336]
228. Chen J, Lin H, Hu M. Metabolism of flavonoids via enteric recycling: Role of intestinal disposition. J Pharmacol Exp Ther. 2003; 304:1228–1235. [PubMed: 12604700]
229. Chen J, Wang S, Jia X, Bajimaya S, Lin H, Tam V, Hu M. Disposition of flavonoids via recycling: Comparison of intestinal versus hepatic disposition. Drug Metabol Disp. 2005; 33:1777–1784.

230. Walle UK, Walle T. Induction of human UDP-Glucuronosyltransferase UGT1A1 by Flavonoids—Structural requirements. Drug Metabol Disp. 2002; 30:564–569.

231. Gradolatto A, Basly JP, Berges R, Teyssier C, Chagnon MC, Siess MH, Canivenc-Lavier MC. Pharmacokinetics and metabolism of apigenin in female and male rats after a single oral administration. Drug Metabol Dispos. 2005; 33:49–54.

232. Llevot A, Astruc D. Applications of vectorized gold nanoparticles to the diagnosis and therapy of cancer. Chem Soc Rev. 2012; 41:242–257. [PubMed: 21785769]

233. Guo C, Zhu Y, Weng Y, Wang S, Guan Y, Wei G, Yin Y, Xi M, Wen A. Therapeutic time window and underlying therapeutic mechanism of breviscapine injection against cerebral ischemia/reperfusion injury in rats. J Ethnopharmacol. 2014; 151:660–666. [PubMed: 24291152]

234. Rajendran, I., Dhandapani, H., Anantharaman, R., Rajaram, R. Apigenin mediated gold nanoparticle synthesis and their anti-cancer effect on human epidermoid carcinoma (A431) cells. 2015. https://www.researchgate.net/publication/277604036,10.1039/C5RA04303D

235. Das S, Das J, Samadder A, Paul A, Khuda-Bukhsh AR. Strategic formulation of apigenin-loaded PLGA nanoparticles for intracellular trafficking, DNA targeting and improved therapeutic effects in skin melanoma in vitro. Toxicol Lett. 2013; 223:124–138. [PubMed: 24070738]

236. Ding S, Zhang Z, Song J, Cheng X, Jiang J, Jia X. Enhanced bioavailability of apigenin via preparation of a carbon nanopowder solid dispersion. International journal of nanomedicine. 2014; 9:2327–33. [PubMed: 24872695]

237. Ding B, Chen H, Wang C, Zhai Y. Preparation and in vitro evaluation of apigenin loaded lipid nanocapsules. J Nanosci Nanotechnol. 2013; 13:6546–6552. [PubMed: 24245113]

238. Cochran DB, Gray LN, Anderson KW, Dziubla TD. Degradable poly(apigenin) polymer inhibits tumor cell adhesion to vascular endothelial cells. J Biomed Mater Res B Appl Biomater. 2016; 104:1438–1447. [PubMed: 26251070]

Table 1

Effect of apigenin on various molecular targets in human cancers

Human Cancers	Molecular Targets	Apigenin concentrations	Ref.
Breast cancer	PD-L1, ErbB2, Bax, Bcl2, p450 CYP1, CYP19, Caspases, p21/Waf1, p53, Aromatase, VEGF, ERK, JNK, PI3K-Akt, Foxo3a, COX-2, ERβ, CK2, PKC, MAPK, Cyclin D1 and HER2/neu, JAK-STAT3, NF-κB, AP-1, c-Fos, cyclinB1	0–100 μM	40–64
Colon Cancer	APC, ODC, TRAIL, COX-2, PGE-2, MMP, GST, UDP-glucuronosyltransferases (UGT)1A1, p34(cdc2), cdc25, caspases -3, -8, -9, Bax, Bcl2, p21/Waf1, mTOR, CD26, JNK, p38, Elk	0–200 μM	65–84
Gastric Cancer	NF-κB, cyclinD1, COX-2, VEGF, Bcl2, IκBα, ICAM-1, IL6, IL8, Bax	9–80 μmol/L	85–87
Liver cancer	NF-κB, Snail, PIG3, cytochrome c, Bax, Bcl2, caspases -3, -9, Nrf2, PI3K/Akt, cyclin-dependent kinases (cdk)	0–200 μmol/L	88–97
Lung Cancer	p21/Waf1, XIAP, uPAR, GLUT1, NADPH, Bax, Bcl2, caspases -3, -9, cytochrome c, leptin, leptin receptor, Bid, AIF, GRP78, GADD153, HIF-1α, VEGF, Akt and p70S6K1	In vitro: 0–160 μM In vivo:1 mg/kg/day	98–109
Pancreatic Cancer	NF-κB, IκBα, GSK3β, cyclinB1, IL17, IFNB1, p53, p21/Waf1, PUMA, HIF-1α, VEGF, GLUT1, Cdc6, cyclin A, cdc2, cdc25	0–100 μM	110–118
Prostate Cancer	FAK, Src, PTEN, IGF1R, IGFBP-3, IGF-1, GSK-3β, Akt, p21/Waf1, p27, VEGF, IKKα, NF-κB, CK2, IAPs, TRAIL, p53, caspases -3, -8, -9, HIF-1, FAK, β-Catenin, c-Myc, cyclinD1, cyclin-dependent kinases -2, -4, -6, MAPK, PI3K-Akt, ERβ, AP-1, Bax, Bcl2, Bcl-XL, 17β-hydroxysteroid oxidoreductase, E-cadherin, GLUT1, HDAC-1, -3, XIAP, Ku70, FoxO3a, ABCB1, FAK, Smad -2, -3, IGF-1, IGFBP3, ERK, H3 and H4 acetylation, BAD, 14–3–3β, p14ARF, MDM2, c-Myc, Rb, RNA polymerase	In vitro: 0–40 μM In vivo: 20 & 50 μg/mouse/day	119–138
Skin Cancer	PGE2, EP1, EP2, cytochrome c, Bax, Bcl2, caspase -3, -9, PARP, p21/Waf1, COX-2, PKC, STAT3, MMP-2, MMP-9, VEGF, Twist1	In vitro :0–100 μM In vivo: 150 mg/day	139–157
Cervical Cancer	CK2α, Bax, Bcl2, p53, p21, Fas/Apo-1, caspase3	0–75 μM	158–163
Endometrial Cancer	Id1, VEGF, p70S6K1, HIF1 and FAK	0–40 μmol/L	164
Ovarian Cancer	Axl, tyrosine receptor kinase, Akt, MMP-9, p70S6K1, Id1, ATF3, FAK, VEGF, HIF-1α, HDM2/p53, platelet aggregation, proteasome degradation -20S, -26S, Bax and IκBα	In vitro: 0–60 μM In vivo: 5–150 mg/kg/day	165–175
Hematologic cancer/Leukemia	P34(cdc2), p21/Waf1, p38, PKCδ, ATM, caspases, telomere activity (hTERT), c-Myc,	0–100 μM	176–188
Adrenal cortical cancer	aromatase, cytochrome p450	0–20 μM	189, 190
Thyroid cancer	Beclin-1, LC3, Cdc25c, c-Myc, p53, p38, EGFR, MAPK, ERK, MAPK	0–80 μM	191–197
Neuroblastoma	Bid, Bcl2, Bax, cytochrome c, caspase -3, -8, -9, PARP-1, p53, p21/Waf1	0–60 μM	198–207
Bladder cancer	p53, p21/Waf1, p27/Kip1, CyclinA, cyclinB1, cyclinE, CDK2, cdc2, cdc25, Bax, Bad, Bak, Bcl2, Bcl-XL, Mcl-1, cytochrome c, caspase -3, -7, -9, PARP cleavage, GSH	0–80 μM	208–210
Mesothelioma	Bax/Bcl-2 ratio, p53, caspase-9, Akt, NF-κB/p65, MAPK	In vitro: 6.25–100 μM In vivo: 20 mg/kg	211
Osteosarcoma	β-catenin, Bax, caspase -3, -8, -9, AIF	0–75 μM	212–214

AIF Apoptosis inducing factor, AP-1 Activator protein-1, APC Adenomatous polyposis coli, Bax bcl-2-like protein 4, Bcl2 B-cell lymphoma 2, BID BH3 interacting-domain death agonist, CK2 casein kinase 2, COX-2 Cyclooxygenase-2, CYP cytochrome p, ErbB2 Erythroblastic leukemia viral oncogene homolog 2, ERK Extracellular Signal-Regulated Kinase, ERβ Estrogen receptor beta, FAK Focal Adhesion Kinase, Foxo3a

Forkhead box O3, GADD Growth Arrest DNA Damage, GLUT1 Glucose transporter 1, GRP78 glucose-regulated protein78 kDa, GSK3β Glycogen synthase kinase 3, GST Glutathione S-transferases, H3 Histone H3, HDAC Histone deacetylases, HER2/neu Receptor tyrosine-protein kinase erbB-2, HIF-1α Hypoxia-inducible factor 1-alpha, ICAM-1 Intercellular Adhesion Molecule 1, Id1 Inhibitor of differentiation or DNA binding protein 1, IFNB1 Interferon beta 1, IGF-1 Insulin-like growth factor 1, IGF1R Insulin-like growth factor 1 receptor, IGFBP-3 Insulin-like growth factor binding protein 3, IKKα IκB kinase alpha, IL Interleukin, JAK-STAT3 Janus kinase/signal transducers and activators of transcription 3, JNK c-Jun N-terminal kinase, MAPK Mitogen-activated protein kinase, MDM2 Mouse double minute 2 homolog, MMP Matrix metalloproteinase, NADPH Nicotinamide adenine dinucleotide phosphate, NF-κB Nuclear factor-kappa B, Nrf2 Nuclear factor (erythroid-derived 2)-like 2, ODC Ornithine decarboxylase, PARP Poly ADP ribose polymerase, PD-L1 Programmed death-ligand 1, PGE2 Prostaglandin E2, PI3K Phosphatidylinositol-4,5-bisphosphate 3-kinase, PIG3 p53-inducible gene 3, PKC Protein Kinase C, PTEN Phosphatase and tensin homolog, PUMA p53 upregulated modulator of apoptosis, Rb Retinoblastoma, Src v-src sarcoma, TRAIL TNF-related apoptosis-inducing ligand; UDP-glucuronosyltransferases, uPAR urokinase-type plasminogen activator receptor, VEGF Vascular endothelial growth factor, XIAP X-linked inhibitor of apoptosis protein