



## Review

### Antimetastatic effects of Citrus-derived bioactive ingredients: Mechanistic insights

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**Abstract:** The growing complexity of metastasis has sparked tremendous interest in unraveling of the underlying mechanisms which play fundamental role in cancer progression and metastasis. Ground-breaking discoveries in metastasis research have greatly enhanced our understanding about intricate nature of metastasis. Bioactive chemicals obtained from citrus fruits have gained noteworthy appreciation because of significant cancer chemopreventive roles. Deregulated oncogenic signaling cascades play central role in metastasis. Emerging evidence has started to shed light on the metastasis inhibitory properties of naringin, naringenin, tangeretin, nobiletin, hesperidin and hesperetin in different cancer cell lines and xenografted mice. Wnt/ $\beta$ -catenin, TGF/SMAD and NOTCH signaling cascades have been shown to play lynchpin role in carcinogenesis and metastasis. There is emerging evidence related to pharmacological targeting of Wnt/ $\beta$ -catenin, TGF/SMAD and NOTCH by citrus-derived bioactive components. These findings are indeed encouraging and will enable researchers to gain further insights into pharmacological targeting of oncogenic pathways to inhibit and prevent metastasis.

**Key words:** Cancer; Apoptosis; Signaling; Xenografted mice.

## Introduction

Citrus is one of the largest cultivated fruit crops, with more than 157 million tons globally in 2019 (1). Citrus is a term used to refer to the Citrus genus, including pomelo, mandarin, lime, sweet and blood orange, tangerine, clementine, grapefruit, sour orange, citron, and lemon (2). These fruits and their by-products (peels and seeds) are rich sources of bioactive compounds, such as flavonoids, essential oils, coumarins, alkaloids, limonoids, phenol acids, and carotenoids (3).

Flavonoids are the leading bioactive compound group found in citrus, and they are subdivided into flavanones, flavonols, flavones, flavans, and anthocyanins (4). Flavanones are the predominant group of flavonoids considering the total content in citrus (95% of total flavonoids), and they may be found in aglycone or glucoside forms. Hesperetin and naringenin are the aglycone forms and may be glycosylated with rutinose and neohesperidose to form hesperidin and naringin (5). Tangeretin and nobiletin are polymethoxylated flavones almost exclusively found in citrus (6). They are presented in low concentrations, but they show biological and specific effects due to the presence of methoxyl groups, which results in low polarity and a planar structure, in-

creasing the membrane permeability and transport.

The citrus-derived flavonoids exhibit in vitro and in vivo health effects, including anti-inflammatory, anti-oxidant, anti-aging, anti-allergy, and anti-carcinogenic properties. Cancer is one of the primary causes of death worldwide. It is of paramount importance to develop anticancer drugs with high efficiency and/or find foods with compounds that could reduce cancer risk. Cancer cells can have uncontrolled proliferation, apoptosis resistance, and the capacity to produce new blood vessels and metastasis to other body parts. Metastasis occurs after metastasis-inducing proteins secretion, detachment of cells at the initial site, migration, adhesion, and invasion at a different site. Citrus-derived flavonoids may be considered antimetastatic compounds due to their inhibition of cell invasion and metastasis, low toxicity, recognized safety, and overall availability. Flavanones and polymethoxylated flavones derived from citrus have shown the most promising antimetastatic activities among the flavonoid compounds.

In this review, we have summarized cutting-edge research works which revolutionized our understanding about pharmaceutical targeting of oncogenic pathways by citrus-derived bioactive components. We begin with a brief overview of the central role of Wnt/ $\beta$ -catenin

and TGF/SMAD in carcinogenesis and metastasis. We also summarize scientifically verified evidence related to cancer chemopreventive role of naringin, naringenin, tangeretin, nobiletin, hesperidin and hesperetin, more specifically in the context of metastasis.

## Overview of TGF $\beta$ /SMAD, Wnt/ $\beta$ -catenin and NOTCH pathways

Deregulation of oncogenic pathways have been shown to promote metastasis. Conceivably, the modularity of sophisticated signaling networks is a major stumbling block in the standardization of therapies.

### TGF $\beta$ /SMAD pathway

TGF $\beta$  (Transforming growth factor- $\beta$ ) cascade initiates from plasma membrane to the nucleus through the co-operation of the type I and II serine/threonine kinase receptors and their characteristically unique downstream effectors. TGF $\beta$ 1 characteristically initiates transduction cascade at the cell membrane by binding to a functionally active hetero-tetrameric complex comprising of two transmembrane serine/threonine kinases, known as T $\beta$ RI and T $\beta$ RII. Stabilization of SMAD proteins is necessary to ensure the activation of TGF $\beta$ /SMAD pathway.

PDLIM5 (PDZ and LIM domain protein-5) promoted TGF $\beta$ -induced transduction cascade by enhancing the stability of SMAD3 in NSCLC (7). STUB1 is an E3 ubiquitin ligase and ubiquitinates different proteins. PDLIM5 interacted with SMAD3 and competitively blocked the interactions between STUB1 and SMAD3. Consequently, PDLIM5 efficiently protected SMAD3 from STUB1-induced proteasomal degradation. Pulmonary metastatic nodules were found to be reduced significantly in mice intravenously injected with PDLIM5-knockdown-lewis lung carcinoma cells (7).

It has also been convincingly revealed that LINC00941 (an oncogenic long non-coding RNA) physically interacted with SMAD4 and prevented  $\beta$ -TrCP-mediated SMAD4 degradation (8). LINC00941-mediated stabilization of SMAD4 resulted in the activation of TGF $\beta$ /SMAD2/3 signaling cascade. TGF $\beta$ 1 increased the invasive and migratory capacities of LINC00941-silenced cells, whereas the inhibition of TGF $\beta$ 1 receptor caused a notable reduction in the invasive and migratory features of LINC00941-overexpressing cancer cells (8).

UCHL1 (Ubiquitin C-Terminal Hydrolase L1) facilitated TGF $\beta$  signaling-mediated metastasis by stabilization of TGF $\beta$  receptor as well as SMAD2 from ubiquitination and proteasomal degradation (9). UCHL1-overexpressing-MDA-MB-231 cancer cells revealed considerably enhanced metastases in different organs in tumor bearing mice (9).

Overall, these findings suggested that post-translational modifications of SMAD proteins play important role in the determination of their fate. Next section mainly deals with regulation of metastasis by Wnt/ $\beta$ -catenin pathway.

### Wnt/ $\beta$ -catenin pathway

Discovery of Wnt/ $\beta$ -catenin pathway has opened the door to an ever-increasing understanding of cellular processes that are controlled or influenced by  $\beta$ -catenin.

Cancer-associated fibroblasts (CAFs) are a heterogeneous subpopulation of stromal cells and are prominent components of the microenvironment. We have witnessed a substantial expansion in research related to the biology of CAFs. These cells modulate cancer metastasis through remodeling of the extracellular matrix, secretion of growth factors, modulation of angiogenesis and drug resistance.  $\alpha$ -SMA positive CAFs were found to be abundant in the tumors derived from WNT4-overexpressing SW480 cells in tumor-bearing mice (10). Tumors developed from WNT4-overexpressing SW480 cells were larger in size in xenografted mice. WNT4 promoted the recruitment and activation of fibroblasts via  $\beta$ -catenin-dependent pathway (10).

DKK3 (DICKKOPF Homolog-3) is a WNT ligand activity antagonist and serves as an extracellular inhibitor of WNT/ $\beta$ -catenin pathway. Cantharidin, a monoterpene increased the expression of DKK3 and inhibited WNT/ $\beta$ -catenin pathway by repression of inhibitory phosphorylation of GSK-3 $\beta$  (11). DKK3 is directly targeted by oncogenic miRNAs. Cantharidin inhibited proliferation and metastasis of osteosarcoma cells via downregulation of miR-214-3p expression (11).

Activation of WNT signaling is antagonized by the SFRPs (secreted Frizzled-related proteins). miRNA-454-3p directly targeted DKK3 and SFRP1 and caused activation of WNT/ $\beta$ -catenin pathway (12). There was a prominent pulmonary metastasis in the mice transplanted with miRNA-454-3p-expressing-MCF-7 cells. Whereas, blockade of WNT/ $\beta$ -catenin signaling by inhibition of  $\beta$ -catenin severely impaired the miRNA-454-3p-mediated increase in the metastatic potential of cancer cells. Borders of miRNA-454-3p-expressing-MCF-7 primary tumors demonstrated spike-like morphological features that penetrated into the surrounding muscles (12).

WD repeat-containing protein 74 (WDR74) has been reported to stabilize  $\beta$ -catenin in lung cancer cells (13). There was a marked reduction in the tumor nodules or almost no tumor nodules in mice injected with WDR74-silenced PC9 cells. Moreover, there was a significant decline in the number of micro-metastatic lesions in mice injected with WDR74-silenced PC9 cells. Whereas, larger metastatic lesions were noted on the surface of lungs in mice injected with WDR74-overexpressing-PC9 cells (13).

It seems clear that stability and nuclear accumulation of  $\beta$ -catenin is necessary to activate or repress target gene networks.

### NOTCH pathway

Ligand-triggered activation sequentially induced proteolytic cleavages of the members of the NOTCH family of receptors. Proteolytic processing caused the release of NICD (Notch intracellular domain). Once released, NICD accumulates in the nucleus and works synchronously with the DNA-binding molecule CSL and co-activator protein MAML (Mastermind-like transcriptional co-activator-1) for the transcriptional regulation of target gene network.

NSD3, a histone methyltransferase is frequently overexpressed in wider variety of cancers. NSD3-induced methylation of H3K36 is critical for metastasis. ADAM10 and ADAM17 are well characterized and

have been shown to play a crucial role in the cleavage and activation of NOTCH receptors (14). In NSD3-overexpressing MCF7 cells, ADAM12 knockdown abolished NSD3-induced NICD1 accumulation. NSD3 was recruited to the promoter regions of NOTCH3, DLL4, ADAM12 and promoted di- and tri-methylation at H3K36 regions to transcriptionally upregulate the expression of these genes. Besides, histone H3 acetylation was increased, whereas, H3K27me3 levels were decreased at the regions. Dissociation of EZH2 and co-recruitment of p300 acetyltransferase and RNA-polymerase-II facilitated histone acetylation. Collectively, these findings provided evidence that long isoform of NSD3-long interacted with RNA-polymerase-II and EZH2 to effectively block EZH2-mediated H3K27me3. More importantly, multicomponent machinery consisting of NSD3, p300 acetyltransferase and RNA-polymerase-II formed a transcriptionally active complex at the promoter regions of NOTCH-related genes for H3K36me2/3-dependent expression of the target genes. In an orthotopic xenograft, NSD3-induced rapidly growing tumors were noted to be inhibited by treatment with  $\gamma$ -secretase inhibitors and mice harboring tumors derived from NSD3-overexpressing MDA-MB-231 cancer cells showed higher sensitivity to  $\gamma$ -secretase inhibitors. Importantly, injection of NSD3-overexpressing MDA-MB-231 cancer cells into mice showed that NSD3-induced pulmonary metastases of breast tumors were inhibited significantly by  $\gamma$ -secretase inhibitors (14).

Glycosyltransferase EGF domain-specific O-linked GlcNAc transferase (EOGT) has been shown to glycosylate NOTCH1 (15). SHCBP1 (SHC SH2 domain-binding protein-1) has been reported to interact with EOGT and enhanced the metastasizing potential of pancreatic cancer cells. NICD levels were found to be elevated in SHCBP1- and EOGT-overexpressing cancer cells. EOGT and SHCBP1 potently increased O-GlcNAc glycosylation of NOTCH1. Overexpression of EOGT/SHCBP1 inhibited E-cadherin expression. DLL4 significantly enhanced NICD enrichment at the promoter region of E-cadherin but knockdown of SHCBP1/EOGT severely interfered with NICD-mediated transcriptional downregulation of E-cadherin. Mice injected with SHCBP1-overexpressing-BxPC-3 demonstrated significant increase in the lung metastasis (15).

Under physiological conditions, negative regulators restrain hyperactivation of NOTCH pathway. For instance, CDK8 (Cyclin dependent kinase 8) can be recruited to the NICD-RBP-J $\kappa$  complex to phosphorylate NICD (16). Subsequently, FBXW7, an E3 ligase recognized phosphorylated NICD and promoted ubiquitin-dependent degradation. Tight binding of RFC4 (DNA replication factor) to NICD1 competitively abrogated CDK8/FBXW7-mediated phosphorylation and polyubiquitination of NICD1. Mutant NICD-expressing-NSCLC cells generated excessive pulmonary metastases when they were intravenously injected and formed palpable tumors even when cells were subcutaneously inoculated. More importantly, RFC4 silencing failed to inhibit metastasizing ability of NICD1-mutant NSCLC cells (16).

Notably, percentage and number of pulmonary metastases in mice inoculated with HEY1-silenced-143B

cells were reported to be significantly reduced (17). Furthermore, the area of lung metastasis in the animal models inoculated with HEY1-silenced-143B cells was also significantly smaller in size (17).

After an overview of oncogenic cell signaling pathways, we will provide a summary of citrus-derived pharmacologically active molecules having significant properties to inhibit or prevent metastasis.

## Citrus-derived bioactive ingredients

### *Naringin*

Naringin has a molecular weight of 580.5. Naringin has been shown to effectively inhibit metastasis.

The trans-differentiation of epithelial cells into mesenchymal cells, a complex process classically known as epithelial-to-mesenchymal transition (EMT) has critical role in cancer progression. Importantly, this molecular switch in EMT is regulated by specific transcriptional factors, including SNAIL, ZEB (Zinc-finger E-box-binding Homeobox) and basic helix-loop-helix transcription factors. Naringin dose dependently reduced the levels of ZEB1 in osteosarcoma cells (18). Naringin also reduced the levels of matrix metalloproteinase (MMP2). Naringin significantly prevented lung degeneration and reduced the incidence of pulmonary metastatic nodules in mice implanted with MG63 cells (18).

Naringin concentration-dependently inhibited glucose metabolism of melanoma A375 cells. Furthermore, naringin significantly reduced the phosphorylation of c-Src (19).

Naringin reduced the protein levels and enzymatic activities of MMP2 and MMP9 (20). Additionally, naringin efficiently reduced phosphorylation of ERK (extracellular signal-regulated kinase), c-Jun N-terminal kinase and p38 MAPK. Collectively, naringin blocked MAPK signaling cascade including ERK, JNK and p38 (20).

Naringin dose-dependently decreased invasion and migratory potential of U87 MG cells (21). Naringin suppressed the levels of MMP2 and MMP9. Naringin also targeted FAK by blocking the phosphorylation of 397<sup>th</sup> tyrosine of FAK (21). Collectively, these findings indicated that naringin inhibited the growth and metastasis of U87 MG partially by inhibition of FAK signaling cascades.

miRNAs inhibit the functionality of protein-coding transcripts, that results in alterations in multiple aspects of molecular mechanisms. Oncogenic signaling cascades can be regulated either through single microRNAs or interconnected regulatory networks controlled by multiple microRNAs that participate in reciprocal feedback interactions with the targets that they regulate. VCAM1 (Vascular cell adhesion molecule-1) of the immunoglobulin superfamily has been reported to be interconnected with metastasis (22). miR-126 directly targeted VCAM1 in different cancers. Naringin stimulated the expression of miR-126 and enhanced miR-126-mediated targeting of VCAM1 in chondrosarcoma cells (22).

Levels of p-AKT, p-mTOR, VCAM1 and NF- $\kappa$ B were noted to be reduced in miR-126-overexpressing non-small cell lung cancer cells (23). More importantly, naringin-mediated anticancer effects were found to be

more pronounced in miR-126-overexpressing cancer cells (23).

Collectively, these findings suggested that naringenin pleiotropically modulated oncogenic proteins and EMT-associated proteins for inhibition of metastasis.

### Naringenin

Naringenin is an efficient and medicinally significant citrus-derived product. In this section, we will summarize metastasis-inhibitory role of naringenin via inactivation of TGF $\beta$ /SMAD and Wnt/ $\beta$ -catenin pathways. We have also discussed how circular RNAs play fundamental role in metastasis and warrant further research mainly in context of naringenin-mediated inhibition of metastasis.

Mouse TGF $\beta$ 1 was lentivirally transduced into mouse breast carcinoma (4T1-Luc2) cells and then transformant cells (4T1/TGF $\beta$ 1) were inoculated into Balb/c mice (24). There was an extensive pulmonary metastasis derived from 4T1/TGF $\beta$ 1 tumors. However, administration of naringenin or 1D11 (TGF $\beta$ 1 blocking antibody) caused significant blockade of pulmonary metastasis for both 4T1/TGF $\beta$ 1 tumors as well as 4T1/RFP tumors and increased survival of the mice. Importantly, mice inoculated with TGF $\beta$ 1-expressing 4T1 cancer cells had notably higher systemic immunosuppression. Furthermore, these mice represented a higher proportion of myeloid-derived suppressor cells and regulatory T cells and a lower fraction of activated T cells and IFN $\gamma$  expression in CD8 $^+$  T cells. Naringenin reduced TGF $\beta$ 1 secretion from the cells thus leading to an intracellular accumulation of TGF $\beta$ 1. Naringenin blocked TGF $\beta$ 1 trafficking from the trans-Golgi network by suppression of PKC activity and reduced TGF $\beta$ 1 secretion from breast cancer cells (24).

TGF $\beta$ /SMAD3 signaling transcriptionally controlled the expression of TIMP2 (tissue inhibitors of metalloproteinase-2) and MMP2 (25). Studies had shown that phosphorylated-SMAD3 transcriptionally upregulated MMP2 and simultaneously repressed the expression of TIMP2 (Fig. 1). Treatment with asiatic acid upregulated SMAD7, whereas naringenin inhibited SMAD3 activation. Development of metastatic nodules on liver, intestine and lung were noticed in mice inoculated with melanoma and lung carcinoma cells. However, naringenin or asiatic acid significantly inhibited tumor invasion and metastasis. More importantly, tumor growth inhibitory effects were found to be significantly enhanced in mice combinatorially treated with naringenin and asiatic acid. Lung carcinoma and melanoma developed in an encapsulated growth pattern subcutaneously in mice combinatorially treated with both drugs (25).

Oral administration of naringenin significantly reduced the number of pulmonary metastatic nodules and prolonged the life span of tumor resected mice (26). Moreover, antitumor activity displayed by T cells was superior in naringenin treated mice along with an increased proportion of IFN $\gamma$  and IL-2 expressing T cells (26).

Naringenin suppressed TGF $\beta$ 1-induced migration and invasion of pancreatic PANC-1 and ASPC-1 cancer cells (27). TGF $\beta$ 1/SMAD3 signaling played fundamental role in metastasis. However, naringenin efficiently reduced protein levels and phosphorylated levels of

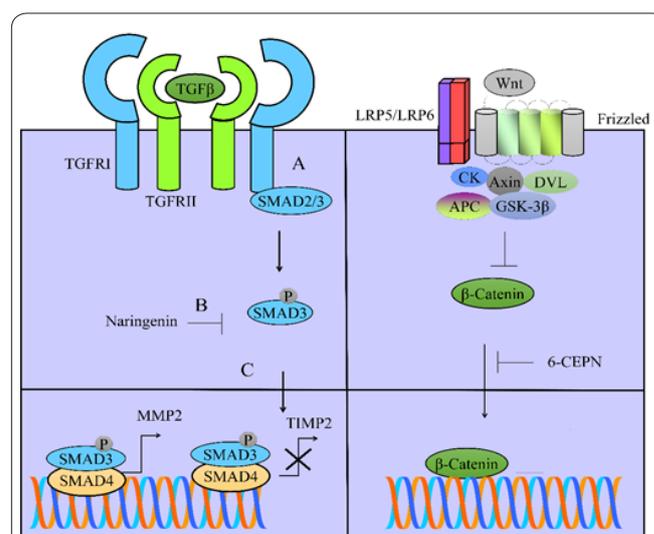
SMAD3. Whereas, SMAD3 overexpression significantly impaired the inhibitory effects of naringenin on TGF $\beta$ 1-induced migration. TGF $\beta$ 1 enhanced EMT and drug resistance by increasing the levels of vimentin, N-cadherin, MMP2 and MMP9. However, naringenin inhibited TGF $\beta$ 1/SMAD3 pathway and markedly suppressed the levels of EMT-markers and matrix metalloproteinases (27).

It has previously been convincingly revealed that tumor growth and metastasis were noted to be considerably enhanced in mice having fibrotic lungs (28). Mice with pulmonary fibrosis are vulnerable to a high risk of lung cancer and metastasis. Therefore, for a better understanding of the key role of pulmonary fibrosis microenvironment as a critical regulator of lung tumor progression, normal lung cells or fibrotic lung cells were co-injected with breast 4T1 cancer cells in BALB/c mice. Pulmonary fibrosis promoted the colonization of cancer cells in the lungs. Naringenin not only inhibited lung metastasis but also increased the survival rate of the mice with pulmonary fibrosis (28).

Semi-natural derivative of naringenin, 6-C-(E-phenylethenyl) naringenin (6-CEPN) was found to be efficient against HCC cells (29). 6-CEPN inhibited the metastatic dissemination of liver cancer cells in xenografted mice. Lung metastasis was found to be considerably inhibited in mice inoculated with Huh7 cells after treatment with 6-CEPN. Intriguingly, 6-CEPN effectively inactivated Wnt/ $\beta$ -catenin signaling by promoting the degradation of  $\beta$ -catenin and inhibition of its nuclear accumulation (Fig. 1). Furthermore, GSK3 $\beta$  upregulation also played critical role in enhancing 6-CEPN-mediated inhibitory effects on Wnt/ $\beta$ -catenin signaling (29).

Naringenin remarkably inhibited the pulmonary invasion of melanoma cells in C57BL6/N mice transplanted with B16-F10 cells (30).

Naringenin and paclitaxel-loaded solid lipid nanoparticles (SLNs) surface modified with cyclic peptides



**Figure 1.** Naringenin-mediated regulation of oncogenic signaling cascades. Hetero-tetramerization of T $\beta$ R1 and T $\beta$ R2 induces signal transduction. SMAD proteins transcriptionally control the expression of target genes. Naringenin inhibited SMAD3 mediated regulation of MMP2 and TIMP2. 6-C-(E-phenylethenyl) naringenin (6-CEPN) inhibited Wnt/ $\beta$ -catenin signaling by promoting  $\beta$ -catenin degradation.

demonstrated noteworthy targeted anti-cancer activity (31). Surface functionalization of SLNs with cyclic peptides significantly improved the release rate and drug absorption performance (31).

Gene expression landscape is a central driver of various steps of cancer and gene expression is fine-tuned by non-coding RNAs. CircFOXM1 is an oncogenic circular RNA reportedly involved in invasion and metastasis (32). CircFOXM1 potentiated the expression of different oncogenic proteins in lung cancer cells. Naringenin dose-dependently reduced circFOXM1 expression in A549 and PC-9 cells. Enforced expression of circFOXM1 reversed the inhibitory effects of naringenin on migration and invasion of A549 and PC-9 cells. SPAG5 (Sperm-associated antigen 5) promoted the migratory and invasive potential of lung cancer cells. CircFOXM1 stimulated the expression of SPAG5 by interfering with miR-3619-5p-mediated targeting of SPAG5 in lung cancer cells (Fig. 2). In xenografted mice studies, circFOXM1 and SPAG5 were found to be downregulated in naringenin group, while miR-3619-5p was induced. However, circFOXM1 overexpression reversed the inhibitory effects of naringenin on tumor growth in xenografted mice (32). Nevertheless, existing evidence is insufficient to support the potential role of naringenin and naringenin in the regulation of non-coding RNAs in cancer. There is a need to identify broader list of circular RNAs as well as long non-coding RNAs which can be activated or inhibited by naringenin for the prevention of metastasis.

Naringenin increased the expression of caspase-3 and reduced the levels of MMP2 and MMP9 in lung cancer cells (33).

### Tangeretin

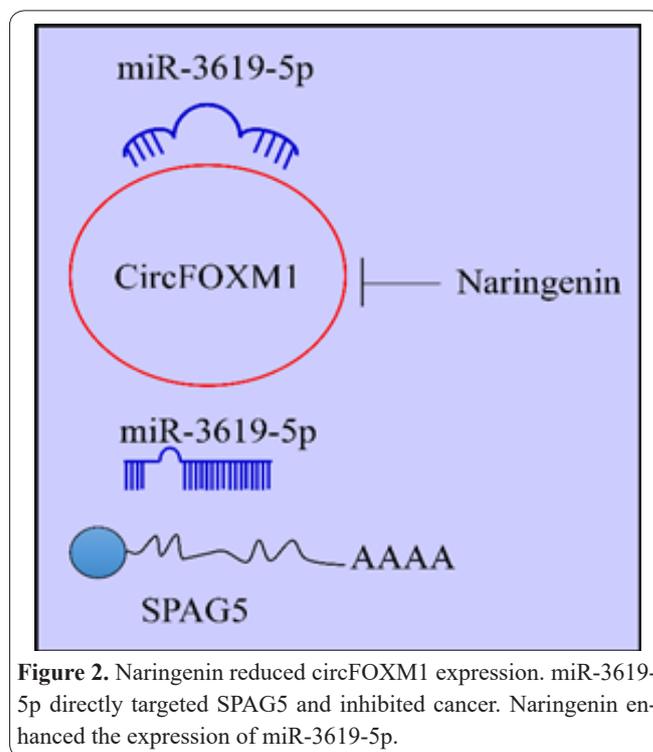
Intraperitoneal injection of tangeretin induced regression of the tumor mass in mice xenografted with MDA-MB-231 cells (34).

It has been shown that radiation induced EMT in gastric cancer cells (35). Radiation caused downregulation of E-cadherin and stimulated the levels of N-cadherin in gastric SGC7901 cancer cells. Importantly, tangeretin caused suppression of radiation-mediated EMT, as evidenced by marked reduction in the levels of N-cadherin and vimentin and notable increase in the levels of E-cadherin. Tangeretin induced downregulation of JAGGED1/2, NOTCH1, HES-1 as well as HEY-1 in irradiated-cancer cells (Fig. 3). Upregulation of miR-410 and simultaneous reduction in the levels of NOTCH1 were observed in the irradiated gastric cancer SGC7901 cells transfected with miRNA-410 mimics. Tangeretin caused alleviation of radiation-induced weight loss in tumor-bearing mice. Tangeretin notably inhibited lung metastasis in irradiated tumor-bearing mice (35).

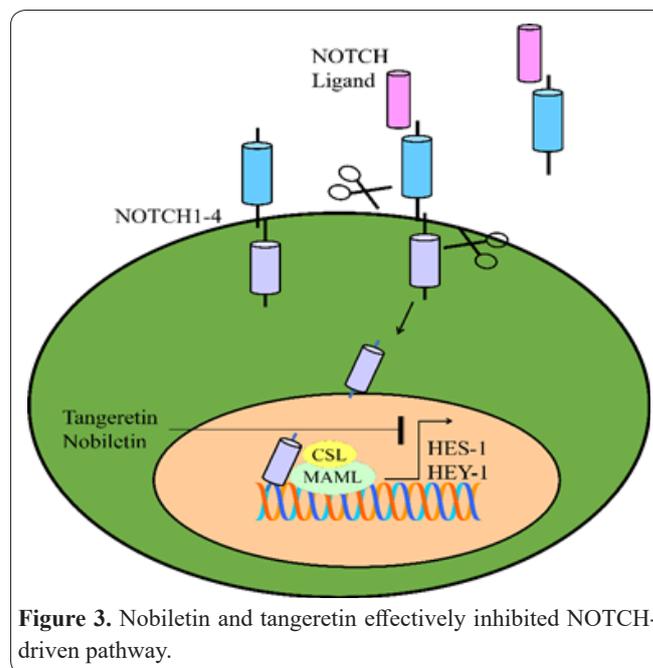
pH-sensitive tangeretin-ZnO quantum dots have been shown to demonstrate high anticancer activity. Tangeretin-ZnO quantum dots efficiently reduced the levels of VEGF, MMP2 and MMP9 in H358 cells (36).

Tangeretin inhibited the growth of the tumors derived from gastric cancer SGC-7901 cells. Tangeretin treatment did not cause reduction in the liver weight and spleen index (37).

Atorvastatin and tangeretin delivered through RGD peptide decorated nanocarriers resulted in significantly



**Figure 2.** Naringenin reduced circFOXM1 expression. miR-3619-5p directly targeted SPAG5 and inhibited cancer. Naringenin enhanced the expression of miR-3619-5p.



**Figure 3.** Nobiletin and tangeretin effectively inhibited NOTCH-driven pathway.

higher distribution of drugs in the tumor (38). More importantly, drugs loaded in nano-systems did not show off-target effects as there was low concentration of drugs in kidney and heart. The blood concentration-time profiles indicated that there was a rapid clearance of free drugs from the circulation as compared to the drugs delivered through nano-systems. Importantly, concentration of the drugs remained high for a longer period of time in the blood when they were delivered through nanoparticles. HT-29 cells were subcutaneously injected into the right fossa axillaris of mice to generate a colon cancer xenograft. Both drugs atorvastatin and tangeretin delivered through RGD peptide decorated nanocarriers efficiently induced regression of the tumor mass (38).

### Nobiletin

Nobiletin is also a critically acclaimed citrus-derived

product having unique properties to inhibit metastasis. Nobiletin has been shown to interfere with TGF $\beta$ /SMAD, NOTCH and NF- $\kappa$ B-mediated regulation of gene networks which fuel metastasis. In this section, we have mapped the protein networks reportedly involved in the progression of metastasis and how nobiletin inactivates these pathways to inhibit metastasis.

PI3K/AKT pathway inhibited GSK3 $\beta$ -triggered  $\beta$ -catenin degradation (39).  $\beta$ -catenin translocated into the nucleus and interacted with lymphoid enhancer factor/T-cell factor (LEF/TCF) transcriptional complexes. TGF $\beta$ -induced nuclear accumulation of  $\beta$ -catenin and stimulated the expression of SLUG. However, nobiletin blocked TGF $\beta$ -induced nuclear accumulation of  $\beta$ -catenin in glioma cells. Nobiletin mediated inhibitory effects were not reported to be significant against glioma U343 cells which did not express SLUG. Nobiletin induced regression of the tumor mass in rodent models bearing U87-Luc implanted xenografts (39).

Nobiletin prevented the transcriptional activity of SMADs induced by TGF $\beta$  in non-small cell lung cancer cells (40). SMAD3 overexpression severely interfered with the ability of nobiletin to inhibit the TGF $\beta$ 1-induced increase in N-cadherin and simultaneous reversal of TGF $\beta$ -stimulated suppression of E-cadherin. Previous studies have shown that SMAD3 transcriptionally controlled the expression of E-cadherin and N-cadherin. More importantly, the number of pulmonary metastatic nodules was reduced to a greater extent in nobiletin-treated A549 xenograft nude mice (40).

Nobiletin remarkably reduced hypoxia-induced EMT, migratory and invasive capacity of H1299 cells, accompanied with reduced expression levels of NOTCH1, JAGGED1/2 and target genes HEY-1 and HES-1 (41). Furthermore, inhibition of NOTCH1 significantly abrogated hypoxia-induced cell migration and marked reduction in levels of SNAIL1, ZEB1/2 and TWIST1 (41).

HGF (Hepatocyte growth factor) and its receptor c-Met transduce the signals intracellularly to play vital role in metastasis (42). Nobiletin considerably inhibited HGF-mediated activation of c-Met as evidenced by reduction in the phosphorylated levels of c-Met. Moreover, nobiletin suppressed HGF-induced activation of AKT and ERK2 in HepG2 cells (42).

CXCR4 has been reported to play instrumental role in migration and metastasis of tumor cells (43). Nobiletin exerted inhibitory effects on metastatic properties of MDA-MB-231 cells by blockade of NF- $\kappa$ B-mediated transcriptional upregulation of CXCR4 (43).

Nobiletin caused suppression of Wnt/ $\beta$ -catenin and NF- $\kappa$ B transduction pathways in hypoxia-stimulated renal cell carcinoma cells (44). Nobiletin stabilized the levels of I $\kappa$ B and inhibited the nuclear accumulation of NF- $\kappa$ B in renal cancer cells (44).

CD36 (cluster of differentiation-36) belongs to the class B scavenger receptor family (45). CD36 has been reported to play contributory role in metastasis. STAT3 transcriptionally upregulated CD36. However, nobiletin inactivated CD36/STAT3/NF- $\kappa$ B signaling axis and efficiently blocked migratory potential of breast cancer cells (45).

Excitingly, nobiletin has been shown to pharmacologically target TGF $\beta$ /SMAD, NOTCH and NF- $\kappa$ B

pathways for the inhibition of metastasis.

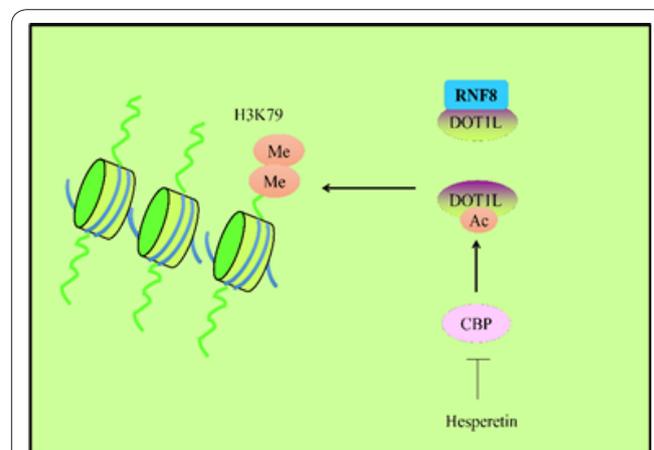
### Hesperetin

Hesperetin inhibited NF- $\kappa$ B-mediated upregulation of P-glycoprotein in A549/DDP cells (46). Hesperetin treatment followed by administration of cisplatin significantly reduced tumor growth in the mice subcutaneously injected with A549/DDP cells (46).

Pioneering research-works have shown that CD8+ T cells play fundamental role in the immunological responses and can efficiently kill tumor cells using CTL functions (47). Additionally, activity of CD4+ T cells is crucial for the cytotoxicity. Importantly, both Th1 cells and Th2 cells are activated and participate in humoral immunological response and cellular immunological response. Maturation of Th1 cells further promotes the maturation of CD8+ T cells. It is worth mentioning that CD8+ T cell maturation is a requisite for effective killing of the tumor cells by secretion of TNF $\alpha$  and IFN $\gamma$ . Inhibition of the functions of Treg cells is yet another advantageous approach to increase immunity. FOXP3 is a central transcriptional factor for regulatory T cells. B16F10 antigen and hesperetin caused significant decrease in the conversion rate of CD4+ T cells into regulatory T cells. Growth rate of B16F10 melanoma is inhibited by immunization with the B16F10 antigen and hesperetin. Size of tumors in the tumor-bearing mice was significantly smaller as compared to B16F10 antigen group. Moreover, survival rate of the mice treated with inactivated B16F10 antigen and hesperetin was significantly higher as compared to B16F10 antigen-treated group (47).

Hesperetin led to an increase in the levels of APAF1, cytochrome C, caspase-9 and caspase-3 (48). Hesperetin caused an increase in Bax and concurrently reduced Bcl-2 levels. Intraperitoneally injected hesperetin caused suppression of tumor growth in mice transplanted with hepatocellular carcinoma cells (48).

Hesperetin dose-dependently reduced histone methylation on H3K79. Hesperetin led to significant reduction in the levels of DOT1L in MKN45 and HGC27 cells (49). CREB-binding protein (CBP) possess intrinsic histone acetyltransferase (HAT) activity. CBP mediated acetylation of DOT1L prevented its binding with E3 ubi-



**Figure 4.** CBP-mediated acetylation of DOT1L protected DOT1L from degradation mainly through impairment of the interaction between DOT1L and RNF8. DOT1L-mediated methylation of H3K79 caused transcriptional upregulation of MMP2, MMP9, N-cadherin and Twist.

quitin ligase and consequent degradation (Fig.4). Hesperetin inhibited CBP-mediated acetylation of DOT1L. Enforced expression of DOT1L (wild-type) increased H3K79me2 in the transcriptional regulatory regions of MMP2, MMP9, N-cadherin and Twist. Knockdown of DOT1L significantly inhibited the metastatic spread of MKN45 cells to the lung in tumor-bearing mice (49).

### Hesperidin

There is an exciting piece of evidence which suggests that gamma-irradiated hesperidin is far more effective as compared to intact hesperidin for the inhibition of tumor growth (50). Gamma-irradiated hesperidin substantially suppressed the number of pulmonary metastatic nodules in C57BL6 mice intravenously injected with B16BL6 cells. Additionally, data also clearly revealed that gamma-irradiated hesperidin achieved noteworthy and significant inhibition of pulmonary metastases of B16BL6 cells (50). Likewise, Hesperidin caused significant reduction in the invasive capacity of MG-63 osteosarcoma cells (51).

### Limonene

Administration of monoterpenes such as perillidic acid and limonene remarkably reduced the metastatic tumor nodule formation (52).

Frequent overexpression of COL8A1 in vascular endothelial cells as well as tumor cells has been extensively reported (53). COL8A1-expressing-Hepal-6 cells clearly exhibited rapid formation of the tumors in xenografted mice. COL8A1 overexpression inhibited D-limonene-mediated tumor regression in xenografted mice (53).

### Concluding remarks

Citrus fruits have wide-ranging health promoting effects. Furthermore, detailed analysis of medicinally important bioactive components present in citrus fruits has shown that many chemicals have considerable cancer chemopreventive activity. In this review we have given an overview of the oncogenic pathways regulated by citrus-fruits derived bioactive constituents.

Recent advancements have been steered by mechanistic studies of key complexes involved in TGF $\beta$ /SMAD signaling. Therefore, the next challenge is to realistically analyze the molecules in action, to precisely identify where on or in the cell they interact and to uncover the dynamics and stoichiometries of multiple signaling complexes. Likewise, interpretation of the context-specific roles of NOTCH will eventually need previously unprecedented understanding of the wiring of regulatory networks in which it operates for carcinogenesis and metastasis.

TGF/SMAD, Wnt/ $\beta$ -catenin, EMT associated markers and non-coding RNAs have been shown to be regulated by different chemicals obtained from citrus fruits. Pharmacological targeting of these interconnected oncogenic pathways will be advantageous because these pathways induce therapeutic resistance and metastasis in xenografted mice. Consequently, drug-resistant metastatic cancers should be screened through a biomarker-driven strategy that validates single-agent or multi-targeted approach depending on the molecular signature

of individual tumors.

### Author contributions

FT, MF and IMY browsed most relevant and high-quality scientific literature. AAF, GB, TCP, NW and RA critically edited the initial draft. AAF, IMY and RA designed the diagrams. TCP and NW cross-checked the authenticity and comprehensive overview of different aspect of the review. All the authors contributed significantly in the literature browsing, preparation of different versions as well as revisions of the draft.

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