

Integrative Bioinformatics Study Reveals Tangeretin Targets and Molecular Mechanisms Against Metastatic Breast Cancer

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Abstract

Background: Agents that target metastasis are important to improve treatment efficacy in patients with breast cancer. Tangeretin, a citrus flavonoid, exhibits antimetastatic effects on breast cancer cells, but its molecular mechanism remains unclear.

Results: Tangeretin targets were retrieved from PubChem, whereas metastatic breast cancer regulatory genes were downloaded from PubMed. In total, 58 genes were identified as potential therapeutic target genes of tangeretin (PTs). Gene ontology analysis with Webgestalt showed that the PTs participate in the biological process of stimulus response, are the cellular components of the nucleus and the membrane, and play molecular roles in enzyme regulation. Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis revealed that the PTs regulate the PI3K/Akt pathway. Genetic alterations for each target gene were MTOR (3%), NOTCH1 (4%), TP53 (42%), MMP9 (4%), NFKB1 (3%), PIK3CA (32%), PTGS2 (15%), and RELA (5%). The Kaplan–Meier plot displayed that patients with low mRNA expression levels of MTOR, TP53, MMP9, NFKB1, PTGS2, and RELA and high expression of PIK3CA had a significantly better prognosis than their counterparts. Further validation of gene expression by using GEPIA revealed that the mRNA expression of MMP9 was significantly lower in breast cancer tissues than in normal tissues, whereas the mRNA expression of PTGS2 showed the opposite. Analysis with ONCOMINE demonstrated that the mRNA expression levels of MMP9 and NFKB1 were significantly higher in metastatic breast cancer cells than in normal tissues. The results of molecular docking analyses revealed the advantage of tangeretin as an inhibitor of PIK3CA, MMP9, PTGS2, and IKK.

Conclusion: Tangeretin inhibits metastasis in breast cancer cells by targeting TP53, PTGS2, MMP9, and PIK3CA and regulating the PI3K/Akt signaling pathway. Further investigation is needed to validate the results of this study.

Background

Breast cancer is a common cause of death among women worldwide (1). Breast cancer was initially considered a local disease, but it can metastasize to lymph nodes and other organs in the body, which is fatal to patients (2). Understanding the molecular mechanisms underlying metastasis is important to improve the clinical management of breast cancer (3). Accordingly, molecular therapeutic agents that target metastasis must be developed to enhance the effectiveness of breast cancer therapy (4).

Tangeretin, a citrus flavonoid (Fig. 1A), may be developed as a specific molecular-targeted anticancer agent because of its antimetastatic effects (5) on cancer cells (6–8). In specific, this compound inhibits metastases of skin, breast, and gastric cancer cells. Tangeretin hampers the invasion of MO4 mouse cells to the embryonic chick heart (9). It also inhibits lung metastasis in melanoma B16F10 cell xenograft (10) and metastasis in 7,12-dimethylbenz(a)anthracene-induced rat breast cancer (11). Moreover, tangeretin alleviates epithelial–mesenchymal transition (EMT), invasion, and migration in gastric cancer cells by downregulating Notch-1, Jagged1/2, Hey-1, and Hes-1 (12). Nonetheless, the molecular target of tangeretin for the metastatic inhibition of breast cancer remains unknown.

In this study, we used a bioinformatics approach to obtain the tangeretin protein target data from PubChem, the metastatic breast cancer regulatory genes from PubMed, and the potential target genes of tangeretin against metastatic breast cancer (PT). We performed gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment, and protein–protein interaction (PPI) network analyses and selected hub genes on the basis of the highest degree score. Selected PTs were further analyzed for their prognostic values by using Kaplan–Meier survival plot and GEPIA. Corroboration of the accuracy of the selected PT in metastatic breast cancer samples was performed using ONCOMINE. Alterations in the selected genes were analyzed using the public database cBioPortal. Molecular docking studies were conducted to identify the interaction between tangeretin and PT. The results of this study emphasized the potential of tangeretin as an antimetastatic agent in breast cancer therapy.

Results

GO and KEGG pathway enrichment analyses

GO analysis was conducted with Webgestalt on the basis of three criteria, namely, biological process, cellular component, and molecular function (Fig. 1C). The PTs participate in the biological processes of stimulus response, metabolic process, and cell proliferation. In addition, the PTs are cellular components of the nucleus and the membrane. The PTs also play a molecular role in protein binding, ion binding, and enzyme regulator activity. Pathway enrichment by KEGG of the PTs (Supplementary Table 3) showed the regulation of ~106 pathways, including PI3K-Akt, breast cancer, and TNF signaling pathway (Supplementary Table 4). Several PTs were involved in PI3K-Akt signaling (e.g., PIK3CA, PRKAA2, RELA, and TP53), breast cancer pathway (e.g., AKT1, MTOR, NOTCH1, PIK3CA, and TP53), and TNF signaling pathway (e.g., MMP9, NFKB1, PIK3CA, PTGS2, and RELA) (Supplementary Table 5).

Analysis of PPI network and selection of hub genes

A PPI network was constructed from 58 proteins (confidence level of 0.4) consisting of 58 nodes, 409 edges, PPI enrichment value of $<1.10 \times 10^{-16}$, and average local clustering coefficient of 0.62 (Fig. 2A). The top 20 highest degree score genes, also known as hub genes, were identified, including TP53, AKT1, STAT3, IL6, and MAPK1 (Fig. 2B, Table 1).

Table 1
Top 20 hub genes ranked by Degree method, analyzed by CytoHubba

Rank	Gene symbol	Gene name	Score
1	TP53	Cellular tumor antigen p53	40
2	AKT1	RAC-alpha serine/threonine-protein kinase	37
3	IL6	Interleukin-6	33
4	STAT3	Signal transducer and activator of transcription 3	31
4	MAPK1	Mitogen-activated protein kinase 1	31
6	MAPK8	Mitogen-activated protein kinase 8	29
7	JUN	Transcription factor AP-1	27
8	CCND1	G1/S-specific cyclin-D1	26
8	CASP3	Caspase-3	26
8	MAPK3	Mitogen-activated protein kinase 3	26
11	VEGFA	Vascular endothelial growth factor A	24
12	INS	Insulin	23
13	MTOR	Serine/threonine-protein kinase mTOR	22
14	MMP9	Matrix metalloproteinase-9	21
15	RELA	Transcription factor p65	20
16	BCL2L1	Bcl-2-like protein 1	19
16	CCL2	C-C motif chemokine 2	19
16	MAPK14	Mitogen-activated protein kinase 14	19
16	PTGS2	Prostaglandin G/H synthase 2	19
16	HMOX1	Heme oxygenase 1	19

Analysis of genetic alterations of potential target genes

Eight PTs were analyzed using cBioportal to explore their genomic alterations across breast cancer studies. MTOR, NOTCH1, PIK3CA, TP53, MMP9, NFKB1, PTGS2, and RELA were selected from KEGG pathway enrichment (Supplementary Table 5), whereas TP53, MTOR, MMP9, RELA, and PTGS2 were selected basing from the highest degree score using CytoHubba. The study BRCA INSERM 2016 (13) was selected for further analysis (Fig 3A). Genetic alterations for each target gene ranged from 3% to 42% of samples, including MTOR (3%), NOTCH1 (4%), TP53 (42%), MMP9 (4%), NFKB1 (3%), PIK3CA (32%), PTGS2 (15%), and RELA (5%) (Fig. 3B). Moreover, most gene alterations belonged to amplification, missense mutation, and truncating mutation (Fig. 3B). Further analysis of mutual exclusivity showed that only one gene pair (NOTCH1-RELA) exhibited significant co-occurrence ($p < 0.05$) in the breast cancer study by the INSERM 2016 project (Table 2), which indicated the pivotal role of NOTCH1 and RELA under tangeretin treatment.

Table 2
Mutual exclusivity analysis of selected genes

A	B	Log2 Odds Ratio	p-Value	Tendency
NOTCH1	RELA	>3	<0.001	Co-occurrence

Kaplan–Meier survival analysis

The Kaplan–Meier plot displayed that patients with low mRNA expression levels of MTOR ($p = 3.95 \times 10^{-5}$), TP53 ($p = 0.00054$), MMP9 ($p = 0.0065$), NFKB1 ($p = 3.3 \times 10^{-16}$), PTGS2 ($p = 0.0019$), and RELA ($p = 0.00088$) had significantly better overall survival rates than the opposite

group (Fig. 4). In addition, patients with a low mRNA level of NOTCH1 had better overall survival rates than those with a high mRNA level of NOTCH1, but the difference was not significant ($p = 0.91$). Moreover, patients with a low mRNA expression of PIK3CA showed significantly worse overall survival than the opposite group ($p = 2 \times 10^{-7}$).

Validation of PTs in breast cancer and metastatic breast cancer samples

Validation of PTs using GEPIA demonstrated that the mRNA expression of MMP9 was significantly higher in breast cancer tissues than in normal tissues (Fig. 5). In addition, the mRNA expression of PTGS2 was significantly lower in breast cancer tissues than in normal tissues. Furthermore, no significant difference in the mRNA expression levels of MTOR, NOTCH1, TP53, NFKB1, PIK3CA, and RELA was observed between breast cancer and normal tissue samples. The validation of target genes by using ONCOMINE showed that in samples from a TCGA study, the mRNA level of MMP9 was significantly higher in metastatic breast cancer cells than in normal breast cells with $p = 2.97 \times 10^{-16}$ (Fig. 6). In addition, samples from a study by Finak et al. (2008) showed that the mRNA level of NFKB1 was significantly higher in metastatic breast cancer cells than in normal breast cells ($p = 3.66 \times 10^{-14}$) (14). Moreover, the mRNA levels of MTOR, NOTCH1, TP53, PIK3CA, PTGS2, and RELA were not different between metastatic breast cancer cells and normal breast cells.

Molecular docking

Simulation of molecular docking and visualization of ligand-protein binding were conducted with MOE software. The protein targets, including PIK3C α , MMP9, PTGS2, COX-2, and IKK, were selected on the basis of the KEGG pathway enrichment analysis, hub gene selection, survival analysis, PT validation, and the uniqueness as drug targets through literature research. Native ligands of each protein consist of PIK3C α , MMP9, PTGS2, COX-2, and IKK complexes comprising ML9 (2-amino-8-[trans-4-(2-hydroxyethoxy)cyclohexyl]-6-(6-methoxypyridin-3-yl)-4-methylpyrido[2,3-d]pyrimidin-7(8H)-one), 7MR ((2R)-2-Amino-3,3,3-trifluoro-n-hydroxy-2-[(4-phenoxyphenyl) sulfonyl] methyl} propanamide), COH (Protoporphyrin IX containing Co), HEM (Protoporphyrin IX containing Fe), and KSA (K-252A). PIK3C α and MMP9 showed a slightly lower docking score than native ligands (ML9 and 7MR) (Table 3). The lower the docking score, the more potent the binding affinity of the ligand, implying that PIK3C α and MMP9 tend to bind and react with tangeretin. Furthermore, tangeretin formed Arene-H between Ile932 and the compound with a bonding distance of 4.07, which was shorter than the Arene-H distance of ML9 with Ile932 (4.22) (Fig. 7). The higher docking score of tangeretin on PTGS2, COX-2, and IKK indicated lower binding affinity compared with native ligands (3X). This phenomenon can be ascribed to the fact that only one amino acid, Gln203, interacted with tangeretin on PTGS2 by Arene-H bond (Table 3). Otherwise, the native ligand of PTGS2 (COH) had four amino acids, which interacted through Arene-H (Gln203, Leu391), arene-cation (His207), and mental contact (His214) (Table 3). A similar phenomenon occurred to 6COX and IKK; the amino acid that interacted with tangeretin was fewer than the native ligands (Table 3).

Table 3
Molecular docking results of tangeretin against the protein targets of PIK3Ca, MMP9, PTGS2, COX-2, and IKK

Protein	Ligand Native		Tangeretin								
	Docking Score	RMSD (Å)	Ligand atom	Amino Acid	Binding Type	Distance	Docking Score	Ligand atom	Amino Acid	Binding Type	Distance
PIK3Ca (4TV3)	-12.3229	1.9820	C	Ile848	Arene-H	4.81	-13.0943	C	Ile932	Arene-H	4.07
			C	Ile932	Arene-H	4.22		C	Trp780	Arene-H	7.51
			N	Val851	Backbone donor-acceptor	4.10		O	Lys802	Sidechain donor	5.20
MMP9 (2OW1)	-11.4732	1.7393	C	Leu188	Arene-H	5.02	-11.5442	C	Arg424	Arene-H	6.41
			C	Tyr423	Arene-H	4.66		O	Tyr423	Backbone donor	5.82
			C	Leu418	Arene-H	4.10		C	Leu418	Arene-H	4.42
			C	7MR502	Arene-Arene			O	Gln402	Sidechain donor	4.16
PTGS2 (5F1A)	-14.8424	1.2559	C	Gln203	Arene-H	3.61	-11.8904	C	Gln203	Arene-H	4.39
			C	Leu391	Arene-H	6.85					
			O-	His207	Arene-cation	4.37					
			O	His214	Metal contact	5.61					
COX-2 (6COX)	-15.6490	1.0546	O	Asn382	Sidechain donor	5.26	-12.0495	C	His386	Arene-H	5.12
			O	Thr212	Backbone donor	3.52		O	Gln203	Arene-H	4.73
			O-	Gln454	Backbone donor	5.73		C	Leu391	Arene-H	6.40
			O	His214	Sidechain donor	5.00					
IKK (4KIK)	-14.0211	0.8232	C	Ile165	Arene-H	3.89	-10.4698	C	Ile165	Arene-H	4.67
			C	Leu21	Arene-H	3.72		O	Leu21	Arene-H	6.94
			O	Cys99	Backbone donor	3.66		O	Cys99	Backbone donor	4.00
			C	Val152	Arene-H	6.07		O	Val152	Arene-H	6.05
			C	Val29	Arene-H	5.80		C	Asp103	Arene-H	4.40
			N	Glu97	Backbone acceptor	6.18					

Discussion

Metastasis is the main cause of death in patients with breast cancer. Utilizing a bioinformatics approach, we identified the PTs and mechanisms of tangeretin in inhibiting metastatic breast cancer. KEGG pathway enrichment analysis resulted in the PI3K-Akt pathway regulated by PT. The top 20 genes with the highest degree score were TP53, AKT1, STAT3, IL6, and MAPK1. Eight PTs that were selected from the KEGG pathway enrichment and with the highest degree scores were analyzed using cBioportal to explore their genomic alterations across breast cancer studies. The highest genetic alterations were found in TP53 (42%), PIK3CA (32%), and PTGS2 (15%). These results indicated the pivotal role of these three genes in the antimetastatic effects of tangeretin on breast cancer cells.

The Kaplan–Meier plot indicated that patients with low mRNA expression levels of MTOR ($p = 3.95 \times 10^{-5}$), TP53 ($p = 0.00054$), MMP9 ($p = 0.0065$), NFKB1 ($p = 3.3 \times 10^{-16}$), PTGS2 ($p = 0.0019$), and RELA ($p = 0.00088$) had better prognosis than the opposite group, whereas patients with low mRNA expression of PIK3CA ($p = 2 \times 10^{-7}$) had a worse prognosis than the opposite group. Validation of gene expression with GEPIA indicated that the mRNA expression of MMP9 was significantly higher in breast cancer tissues than in normal breast tissues, whereas the mRNA

expression of PTGS2 was significantly lower in breast cancer tissues than in normal breast tissues. Validation of PT with ONCOMINE indicated that in samples from a TCGA study, the mRNA level of MMP9 was significantly upregulated in metastatic breast cancer tissues than in normal breast tissues ($p = 2.97 \times 10^{-16}$). Moreover, samples from a study by Finak et al. (2008) showed that the mRNA level of NFKB1 was significantly higher in metastatic breast cancer cells than in normal breast cells ($p = 3.66 \times 10^{-14}$) (14). Collectively, these results indicate the important roles of TP53, PTGS2, MMP9, and NFKB1 in the antimetastatic effects of tangeretin on metastatic breast cancer cells.

This study emphasized the important role of the PI3K/Akt pathway and related genes (TP53, PTGS2, NFKB1, and PIK3CA) in the antimetastatic effects of tangeretin on metastatic breast cancer cells. Here we discussed the important roles of those genes and their potential as tangeretin targets against metastatic breast cancer cells. *TP53* encodes tumor protein p53, a tumor suppressor gene (15). Mutation in TP53 occurs in human epidermal growth factor receptor 2-positive (15), estrogen receptor-positive, and progesterone-positive breast cancer subtypes (16). In addition, the TP53 gene is mutated in 80% of patients with triple-negative breast cancer (17). Loss of p53 or gain of mutant p53 promotes tumor progression and metastasis (18). In addition, loss of p53 induces metastasis via activation of Wnt signaling (19). Moreover, the mutation in TP53 can promote immunogenic activity in breast cancer (20).

Tangeretin regulates p53 expression. Tangeretin increases p53 expression in AGS human gastric cancer cells (21). In addition, tangeretin treatment induces the upregulation of p53 and inhibits metastasis in 7,12-dimethylbenz(a)anthracene-induced rat breast tumor (11). However, the study of TP53 mutation, metastasis, and tangeretin treatment remains elusive.

MMP9 encodes matrix metalloproteinase9 (MMP9), a protease that cleaves the extracellular matrix and is involved in angiogenesis, invasion, and metastasis (22). MMP-9 is dominantly synthesized by tumor cells (23). MMP9 is upregulated in breast cancer cells compared with normal tissue and is correlated with metastasis and recurrence in breast cancer (24). Thus, inhibition of MMP activity is an effective way of preventing metastasis in patients with breast cancer (25). A previous study demonstrated that tangeretin inhibits metastasis in rat mammary carcinoma induced by 7,12-dimethylbenz(a)anthracene by downregulating MMP2, MMP9, and VEGF (11). In addition, tangeretin inhibits the expression and activity of MMP9 in rats with pilocarpine-induced seizures (26). Future study of tangeretin effect against MMP9 activity in metastatic breast cancer is warranted.

PTGS2 encodes prostaglandin-endoperoxide synthase 2, also known as cyclooxygenase-2 (COX-2), which participates in prostaglandin synthesis, regulates inflammation, and promotes cancer progression, invasion, and migration (27, 28). COX-2 is expressed in 40% of human metastatic breast cancers. (29). A previous study showed that tangeretin inhibits COX-2 expression induced by IL-1beta in A549 lung cancer cells by inhibiting NF- κ B, p38 MAPK, JNK, and PI3K signaling (30). Moreover, tangeretin inhibits UVB-induced COX-2 expression by inhibiting MAPK activation and reactive oxygen species elevation (31). Recently, an in silico study has demonstrated that tangeretin can inhibit COX-2 (32). Nevertheless, the effects of tangeretin on COX-2 activity and expression in metastatic breast cancer cells need further exploration.

NFKB1 encodes the nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, a member subunit of NF κ B (33). NFKB1 forms various dimeric complexes with other sub units to activate NF κ B signaling that regulates several biological processes, including inflammation, senescence, apoptosis, cell survival, and cancer progression (33). NFKB1 plays a role in cancer progression and is a potential target for cancer therapy (34). NF κ B signaling is important in the invasiveness of inflammatory breast cancer (35), as well as in chemoresistance mechanism and invasive breast cancer (36). Moreover, NFKB1/RELA induces breast cancer progression by upregulating ETS1 (37). A previous study showed that tangeretin treatment reduces the phosphorylation of I κ B- α and IKK- β , as well as the nuclear translocation of the p65 subunit of NF- κ B in lipopolysaccharide-stimulated microglial cells (38). Hence, the inhibitory effect of tangeretin on invasion and metastasis by targeting NF κ B signaling needs to be explored in future studies.

PIK3CA encodes phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), also known as p110 α , a member of the phosphoinositide 3-kinase (PI3K) family (39). The PI3K signaling pathway is involved in the biological processes of cellular proliferation, apoptosis, survival, motility, and metastasis (40, 41). Mutation in PIK3CA is present in most solid tumors (42) and is found in 12–15% of patients with breast cancers (43). A recent study has shown that mutation in PIK3CA corresponds to a poor prognosis in patients with hormone receptor-positive metastatic breast cancer but a good prognosis in patients with triple-negative breast cancer (44).

Tangeretin inhibits the PI3K signaling pathway. Tangeretin enhances the sensitivity of cisplatin in human ovarian cancer cells by downregulating the PI3K/Akt signaling pathway (45). Tangeretin also inhibits the proliferation and migration of aortic smooth muscle cells through suppressing the PI3K/AKT signaling pathway (46). Another study showed that tangeretin poses a potent neuroprotective activity by triggering the PI3K/Akt signaling pathway in pilocarpine-induced seizures rats (26). Tangeretin also inhibits PI3K and Notch signaling in neonatal asthmatic mice (47). Moreover, tangeretin inhibits EMT in PC-3 prostate cancer cells by downregulating the PI3K/Akt/mTOR pathway (48). However, the effects of tangeretin on PI3K signaling and of PIK3CA mutation on metastatic breast cancer cells need to be clarified in future studies.

KEGG pathway enrichment analysis indicated that the PTs regulate the PI3K/Akt signaling pathway. In this study, we discussed the cross-talk between the PTs in the regulation of the PI3K/Akt pathway. COX2 promotes cell survival by activating the PI3K/Akt pathway in human lung cancer cells (49). Inhibition of COX2 blocks PI3K/AKT kinase activity in ovarian cancer (50) and hepatocellular carcinoma cells (51). In addition,

PI3K/Akt kinase activity induces COX2 expression in lipopolysaccharide-induced murine adrenocortical cells (52). Furthermore, COX2 and PI3K are associated with the progression of colon cancer (53).

PI3K/Akt and mTOR signaling pathways are essential for maintaining the proliferation and survival of cancer cells (54). A recent study has shown that activation of PI3K/AKT/mTOR signaling increases hepatocellular carcinoma resistance to radiotherapy (55). On the one hand, activation of the PI3K/Akt pathway leads to the transcriptional activity of NFκB (56). On the other hand, NFκB activity is important for oncogenic transformation induced by PI3K/Akt signaling (56). Mutation in PI3K signaling regulator, including PIK3CA, leads to cytokine expression upon growth factor deprivation in an NFκB-dependent manner (57). Furthermore, PI3K/Akt/JNK/NFκB signaling plays a pivotal role in the expression of MMP-9 and enlargement in human limbal epithelial cells (58).

Activation of the PI3K/PTEN/AKT/mTOR pathway promotes invasion and metastasis by increasing the expression of MMP9 in hepatocellular carcinoma cells (59) and human breast cancer cells (60). Furthermore, inhibition of Notch1 signaling reduces the proliferation, migration, and invasion of human breast cancer cells by decreasing PI3K/Akt activity (Li et al., 2016). p53 participates in the regulation of cell survival by blocking the PI3K/AKT signaling pathway in cancer cells (61). Moreover, activating mutations in PIK3CA promotes the stimulation of p53 signaling (62). A previous study showed that PI3K/Akt promotes p53 translation in cancer development (63). Inhibition of PI3K/Akt signaling leads to p53 upregulation in leukemic cancer cells (64). A recent study has reported p53 upregulation due to PI3K/Akt signaling inhibition in EMT inhibition in liver cancer cells (65). Collectively, the PI3K/Akt signaling pathway is important for the regulation of metastatic breast cancer and is a potential target of tangeretin in inhibiting metastasis. However, whether or not the inhibitory effect of tangeretin on PI3K/Akt signaling is related to metastatic breast cancer demands further exploration.

In this study, molecular docking analysis emphasized the potential target of tangeretin in inhibiting metastatic breast cancer cells. Tangeretin was shown to inhibit PIK3CA, MMP9, COX2, and IKK. One of the unique targets for cancer drug discovery is PIK3CA because of the high prevalence of its mutations in various human tumors and the progression in the development of personalized cancer medicines (66). The docking results on PIK3CA showed that the docking score of tangeretin was slightly lower than that of the native ligand ML9 ((2-amino-8-[trans-4-(2-hydroxyethoxy) cyclohexyl]-6-(6-methoxypyridin-3-yl)-4-methylpyrido[2,3-d]pyrimidin-7(8H)-one)). A low docking score represents a potent affinity of binding of the ligand, indicating that PIK3CA tends to bend and react with tangeretin instead of the native ligand. The docking results of tangeretin on PIK3CA formed Arene-H between Ile932 and the compound with a bonding distance of 4.07, which was shorter than the Arene-H distance of ML9 with Ile932 (4.22) (Table 3). Furthermore, tangeretin has donor side chains, whereas native ligands have donor-acceptor backbones. Hence, this donor side chain is useful in increasing tangeretin binding to PIK3CA. The docking results on MMP9 showed that docking score of tangeretin was lower than that of the native ligand 7MR ((2R)-2-Amino-3,3,3-trifluoro-n-hydroxy-2-[(4-phenoxyphenyl) sulfonyl] methyl} propanamide)). This result is due to the differences in bond types. In specific, the native ligand has a type of arene-arene bonding, whereas tangeretin has backbone and sidechain donors. This result is in line with the findings of Roshini et al. (2018) that tangeretin, when combined with zinc oxide (Tan-ZnO QDs), can downregulate the expression of metastasis markers, such as MMP2, MMP9, and VEGF (67). Tangeretin showed a higher docking score than native ligands on PTGS2, COX-2, and IKK, suggesting that tangeretin has a lower binding affinity than the native ligands COH (Protoporphyrin IX containing Co), HEM (Protoporphyrin IX containing Fe), and KSA (K-252A) (Table 3).

Molecular docking results on PTGS2 showed that only one amino acid, Gln203, interacted with tangeretin by Arene-H bond (Table 3). Otherwise, the native ligand of PTGS2 (COH) had four amino acids, which interacted by Arene-H (Gln203, Leu391), arene-cation (His207), and metal contact (His214) (Table 3). The results of molecular docking on COX2 showed a lower docking score of tangeretin than native ligands because fewer amino acids on 6COX interacted with tangeretin than native ligands.

The IKK complex plays a pivotal role in NFκB signaling and is an important target for cancer therapy (68, 69). Molecular docking results on IKK with the PDB code of 4KIK showed that the docking scores of tangeretin were lower than those of native ligands because of the lack of one type of bonding, namely, backbone acceptor. However, tangeretin still inhibited COX2 and IKK activities. This results is supported by the previous finding of Chen et al. (2007) that tangeretin inhibits IL-1β-induced COX-2 protein expression by suppressing COX-2 gene expression(30).. Another study also showed that tangeretin significantly inhibits the activation of IKK-β induced by LPS (38). Altogether, although the binding affinity of tangeretin is not much more robust than native ligands, it still has the potency to inhibit PTGS2, COX2, and IKK activities.

Conclusions

Tangeretin inhibits metastasis in breast cancer cells by targeting TP53, PTGS2, MMP9, and PIK3CA. Molecular docking studies revealed the potential of tangeretin as an inhibitor of MMP9 and PTGS2. Furthermore, PI3K/Akt signaling is a potential target of tangeretin in inhibiting breast cancer metastasis. Future in vitro and in vivo investigations are needed to validate the results of this study.

Methods

Data collection and processing

We downloaded 93 tangeretin targets from PubChem (Supplementary Table 1) and 2263 metastatic breast cancer regulatory genes from PubMed (Supplementary Table 2). A Venn diagram was generated using the tangeretin targets from PubChem and the metastatic breast cancer regulatory genes from PubMed, which resulted in 58 genes considered as potential therapeutic target genes of tangeretin (PTs) (Fig. 1B, Supplementary Table 3).

GO and KEGG pathway enrichment analyses

GO and KEGG pathway enrichment analyses were performed using WebGestalt (WEB-based Gene Set Analysis Toolkit) with $p < 0.05$ as the cutoff value (70).

PPI network and selection of hub genes

The PPI network was analyzed using STRING-DB v11.0 (71) with confidence scores of >0.7 and visualized by Cytoscape software (72). Hub genes were selected on the basis of the highest degree score as calculated by CytoHubba plugin (73).

Genetic alteration analysis of PTs

Genetic alterations of the PTs were analyzed using cBioPortal (74, 75). Further connectivity analysis was performed to PTs by using the selected breast cancer study, with a cutoff value of $p < 0.05$.

Kaplan–Meier survival analysis

The prognostic values of the PTs were evaluated with the Kaplan–Meier plot (<http://kmplot.com>) by using the breast cancer database. The cutoff value was $p < 0.05$ (76), and the amount of samples was displayed in each curve.

Validation of PTs in breast cancer and metastatic breast cancer samples

The expression of PTs across breast cancer samples was confirmed using GEPIA (<http://gepia.cancer-pku.cn>), with a cutoff value of $p < 0.05$ (77). The reliability of the PTs in metastatic breast cancer cells was validated using ONCOMINE (<https://www.oncomine.org>) (78).

Molecular docking

Molecular docking was performed to foresee the binding sites of tangeretin with PIK3Ca (PDB ID: 4OVV), MMP9 (PDB ID: 2OW1), PTGS2 (PDB ID: 5F1A), COX-2 (PDB ID: 6COX), and IKK (PDB ID: 4KIK). All computational analyses were conducted using Windows 10, with Intel Core i5-7th Gen processor and 4 GB RAM. The docking simulation, RMSD calculation, and visualization interaction were conducted using MOE 2010 (Licensed from Faculty of Pharmacy UGM). The structure of tangeretin was downloaded from Pubchem (<https://pubchem.ncbi.nlm.nih.gov>) and sought for conformation and minimization by MOE using the energy minimization module. Docking simulations were performed on the binding side of the native ligand based on flexible ligand structures and rigid receptors. The London dG and Triangle matcher were selected for the score function and placement settings, respectively, in the docking simulation. The Forcefield method was used to refine the docking results of 30 settings. Docking simulation was performed using the default settings. The analysis results will conclude which conformations generate the lowest energy when tangeretin binds to the target protein.

Abbreviations

COX2: Cyclooxygenase 2

EMT: Epithelial-to-mesenchymal transition

GO: Gene ontology

KEGG: Kyoto Encyclopedia of Genes and Genomes

MMP9: Matrix metalloproteinase 9

NFKB1: Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1

PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha

PPI: Protein–protein interaction

PT: Potential therapeutic target genes of tangeretin

PTGS2: Prostaglandin-endoperoxide synthase 2

Declarations

- Ethical Approval and Consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors.

- Consent for publication

Not applicable.

- Availability of supporting data

All data produced by the study are disclosed in the manuscript and the additional files

- Competing interests

The authors declare that they have no conflict of interest.

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- Authors' contributions

AH: conception and design of the study, acquisition, analysis and interpretation of data, drafting, and revising the article, HP, NH, and MI: acquisition and analysis of data. All authors had final approval of the submitted manuscript.

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Figures

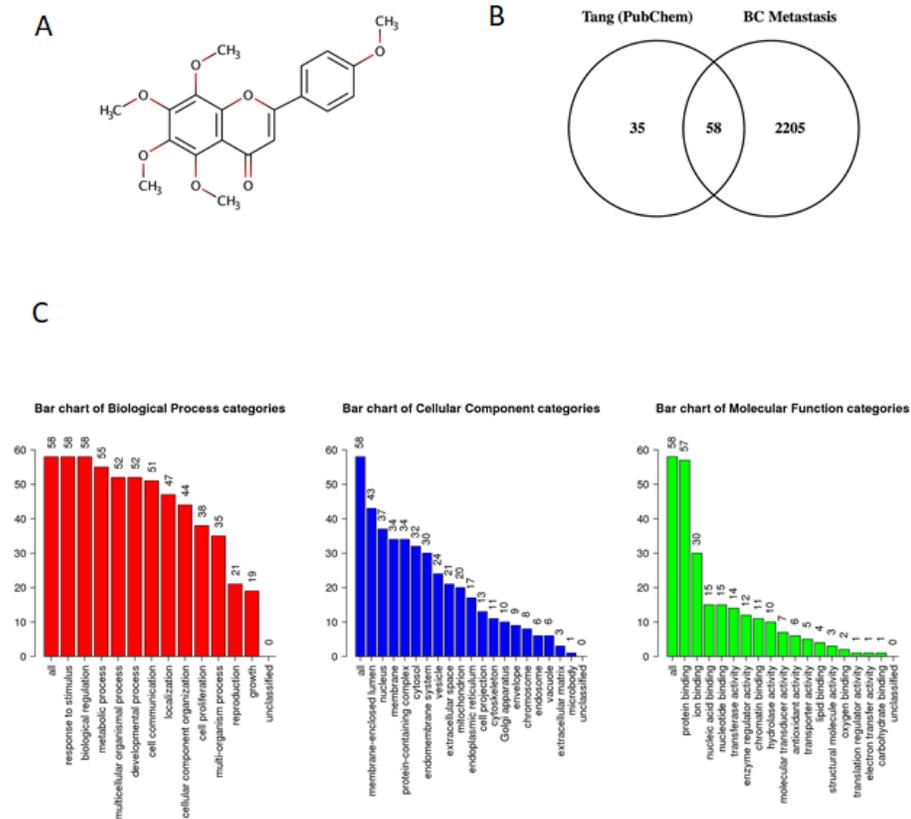


Figure 1

(A). Chemical structure of yangeretin (B). A Venn diagram between tangeretin targets and regulatory genes of breast cancer metastasis. (C). GO enrichment, as analyzed by Webgestalt.

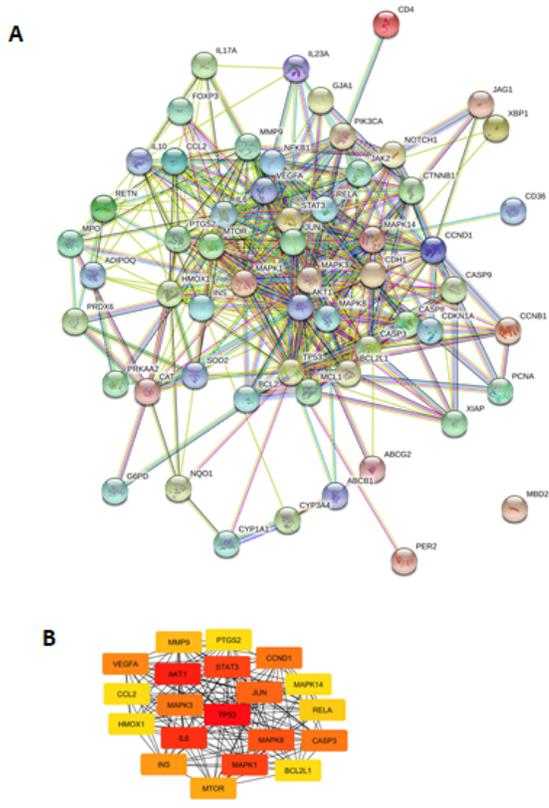


Figure 2

(A). Protein–protein interaction network of potential target genes of tangeretin against metastatic breast cancer, analyzed by STRING. (B). Top 20 hub genes based on highest degree score, analyzed by Cytohubba.

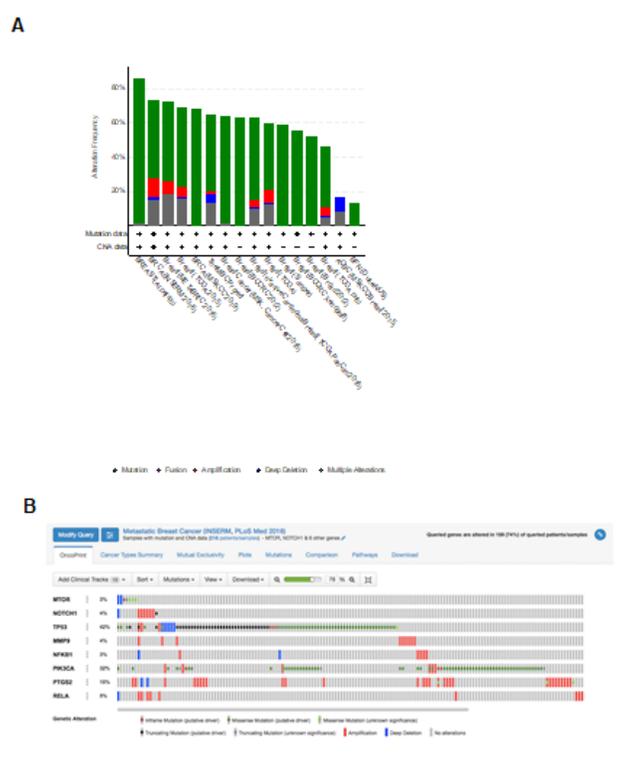


Figure 3

(A). Overview of genetic changes in MTOR, NOTCH1, TP53, MMP9, NFKB1, PIK3CA, PTGS2, and RELA across 16 breast cancer studies, as analyzed by cBioportal. (B). Summary of alterations in MTOR, NOTCH1, TP53, MMP9, NFKB1, PIK3CA, PTGS2, and RELA across breast cancer patients using a study from Lefebvre et al., 2016.

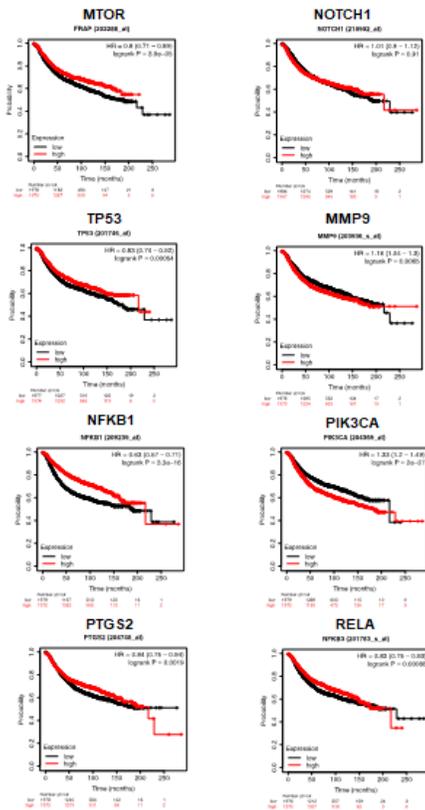


Figure 4

Overall survival of patients with breast cancer related to the mRNA level of MTOR, NOTCH1, TP53, MMP9, NFKB1, PIK3CA, PTGS2, and RELA, as analyzed by GEPIA.

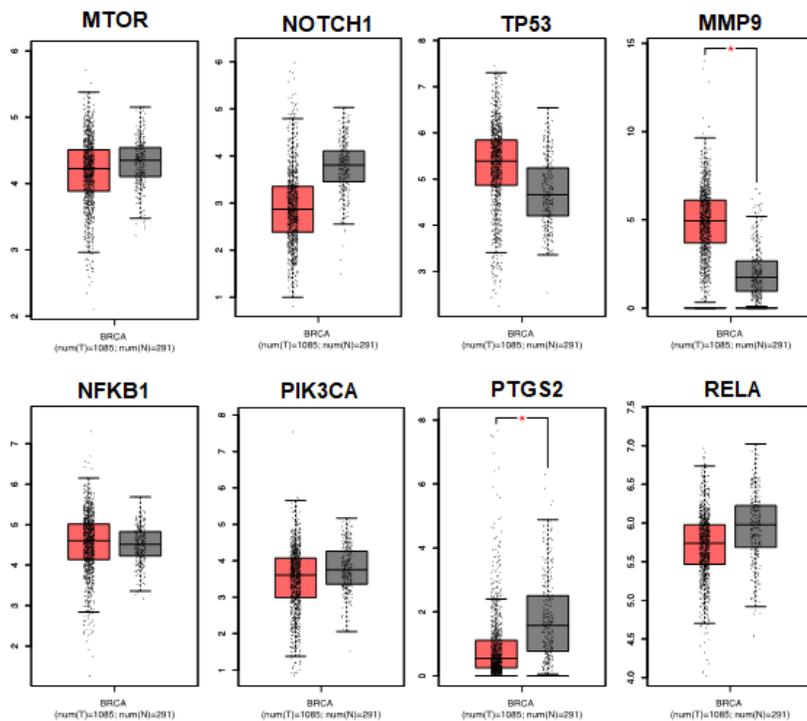


Figure 5

mRNA levels of MTOR, NOTCH1, TP53, MMP9, NFKB1, PIK3CA, PTGS2, and RELA in patients with breast cancer, as analyzed by GEPIA.



Figure 6

mRNA levels of MTOR, NOTCH1, TP53, MMP9, NFKB1, PIK3CA, PTGS2, and RELA in patients with metastatic breast cancer, as analyzed by ONCOMINE.

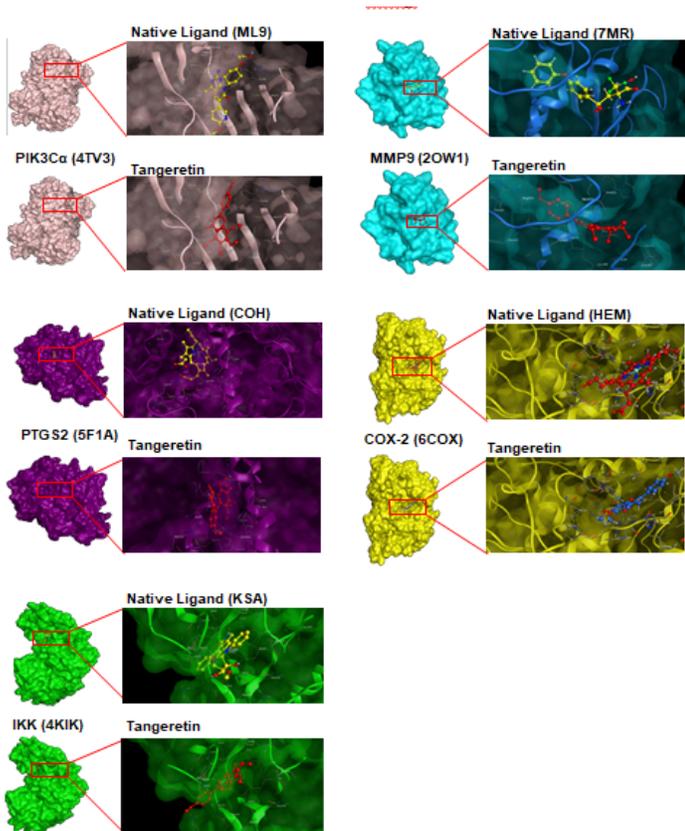


Figure 7

Visualization of ligand interaction to PIK3 Ca, MMP9, PTGS2, and IKK using MOE.

Supplementary Files

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