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Targets and molecular mechanisms of a citrus flavonoid, hesperidin, against luminal breast cancer cells: an integrative bioinformatics analysis

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ABSTRACT

Objective: To identify the potential target and mechanisms of hesperidin in MCF-7 estrogen receptor-positive breast cancer cells using bioinformatics approaches.

Methods: Gene expression profiles were accessed from public database GSE85871. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was carried out with Database for Annotation, Visualization and Integrated Discovery. The protein-protein interaction network was analyzed by STRING-DB and visualized by Cytoscape. Transcription factor regulatory networks were constructed from TRED, TRRUST, RegNetwork and visualized by Cytoscape. Drug association analysis was conducted by WebGestalt.

Results: GO and KEGG pathway enrichment analysis revealed biological processes, cellular components and molecular functions that were related to cancer and estrogen signaling pathways. KEGG pathway enrichment analysis of the genes in transcription factor-differential expression genes regulatory network showed regulation of cancer, estrogen signaling pathways, epidermal growth factor receptor tyrosine kinase inhibitor resistance, and endocrine resistance. Moreover, drug association analysis revealed that hesperidin affected the expression of the same gene as raloxifene.

Conclusions: Hesperidin targets estrogen receptor signaling in estrogen receptor-positive breast cancer cells. Results of this study could trace the molecular mechanism of hesperidin in estrogen receptor-positive breast cancer cells and integrative bioinformatics analysis could accelerate drug discovery and development.

1. Introduction

Breast cancer is still the most commonly diagnosed and leading cause of cancer death among females worldwide[1]. It is classified into five main molecular subtypes, namely luminal A [estrogen receptor (ER)+ and/or progesterone receptor (PR)+, human epidermal growth factor receptor (HER)-2 negative, Ki-67 < 14%], luminal B with HER-2 negative (ER+ and/or PR+, HER-2 negative, Ki-67 ≥ 14%), luminal B with HER-2 positive (ER+ and/or PR+, HER-2+, any Ki-67), HER-2 enriched (ER-, PR-, HER-2+), and basal-like (triple negative) [ER-, PR-, HER-2 negative, CK5/6+ and/or epidermal growth factor receptor (EGFR)+][2]. ER-positive cancer is the most common subtype of breast cancer that responds to endocrine therapy, *i.e.* selective ER modulators (SERMs), such as tamoxifen, selective ER downregulators, such as fulvestran, and aromatase inhibitors, such as letrozole[3]. However, tamoxifen resistance in ER-positive breast cancer intrinsically occurs during

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treatment and causes a major impediment to successful therapy[4]. Accordingly, therapeutic strategies to overcome tamoxifen resistance need to be developed.

Hesperidin (Supplementary Figure 1), a citrus flavonoid, is able to stimulate apoptosis and cell cycle arrest in several types of cancer such as colon[5] and liver[6]. It promotes apoptosis through increased expression of p53 and PPAR-γ and inhibition of NF-κB activation in NALM-6 leukemia series[7]. Combinatorial treatment of hesperidin and chemotherapeutics has been demonstrated in many studies. Hesperidin as adjuvant therapy with doxorubicin improves therapeutic efficacy and reduces tumor resistance to the latter[8]. The hesperidin increases cytarabine cytotoxicity in leukemia cells, so it is potentially developed in acute myeloid leukemia therapy either as a single agent or in combination with cytarabine[9]. Recent research has shown that hesperidin can reduce the rate of liver damage in mice treated with cisplatin in a dose-dependent manner[10]. Hesperidin exerts a cytotoxic effect on doxorubicin-resistant MCF-7 breast cancer cells and has a synergistic effect with doxorubicin through inhibition of P-glycoprotein expression[11]. Hesperidin has low cytotoxic activity in MCF-7 cells and produces a combination that is synergistic with doxorubicin[12]. Nevertheless, the molecular mechanism of hesperidin in ER-positive breast cancer cells, *e.g.* MCF-7 cells, remains unclear.

Integrative bioinformatics approaches are widely used to accelerate drug discovery and development. In this study, we obtained microarray data from public databases, *e.g.* GEO datasets, to obtain differential expression genes (DEGs). Functional annotations were then carried out to predict molecular mechanisms, functions and roles of the DEGs. Furthermore, analysis of the protein-protein interaction (PPI) network was performed. Here we provided information about the possible molecular mechanisms of hesperidin and its molecular targets against ER-positive breast cancer cells. Taken together, we aimed to provide a more complete understanding of the targets and the molecular mechanism of hesperidin against ER-positive breast cancer cells using integrative bioinformatics approaches.

2. Materials and methods

2.1. Data collection and processing

Data of mRNA was obtained from public database GSE85871, entitled, "The gene expression profiles in response to 102 traditional Chinese medicine (TCM) components: a general template for research on TCMs"[13]. Briefly, MCF-7 cells were cultured as previously described and treated with 10 μM hesperidin for 24 h and dimethyl sulfoxide-treated cells were selected as a control. For RNA analysis, the cell viability should be higher than 40% as determined by MTT assay.

The gene expression profiles were assessed using microarray technology with Affymetrix Human Genome U133A 2.0 (Santa Clara, CA, US). Sampling distribution was good (Supplementary Figure 2). Data processing was conducted using GEO2R, an online tool for GEO data analysis based on the R programming language. DEGs between hesperidin and DMSO-treated cells were screened. Adjusted P value < 0.05 and log2 fold change (FC)>1 were used to select significant DEGs. A total of 1 009 genes were extracted from GSE85871, consisting of 389 upregulated and 620 downregulated genes.

2.2. Functional annotation and pathway enrichment analysis

Analyses of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment were conducted by the Database for Annotation, Visualization and Integrated Discovery v6.8[14], with $P < 0.05$ selected as the cutoff value.

2.3. Construction of PPI network and cluster analysis

Analysis of PPI network was constructed with STRING-DB v11.0[15] with confidence scores > 0.9 and visualized by Cytoscape software (version 3.7.1). Genes with a degree greater than 10, analyzed by CytoHubba plugin, were selected as hub genes.

2.4. Transcription factor (TF)–DEGs regulatory network

TRED is a database of cancer-related TFs[16]. TF-related cancer was searched among DEGs. The target genes from TFs were then predicted using the TRRUST[17] and RegNetwork[18] databases and searched among DEGs. TF regulatory network was visualized using Cytoscape software (version 3.7.1).

2.5. Drug association analysis

Predicted drugs with a similar mechanism to hesperidin were analyzed with Overrepresentation Enrichment Analysis (ORA) from WEB-based GEne SeT AnaLysis Toolkit (WebGestalt) with false discovery rate (FDR) < 0.05 as the cutoff value[19]. Briefly, All genes in TF-DEGs regulatory networks were submitted to ORA from WebGestalt, with functional parameter DrugBank.

3. Results

3.1. GO analysis of potential hesperidin target genes

To explore the biological process, cellular components, and molecular function of the DEGs, we performed GO analysis. Among the upregulated genes (Table 1), DEGs take part in the biological process of positive regulation of transcription, DNA-templated, cell adhesion, and positive regulation of cell cycle. The upregulated DEGs are located in the cell surface, extracellular matrix, poteinaceous extracellular matrix, and extracellular space. Moreover, the upregulated DEGs play a molecular function in protein homodimerization activity, calmodulin binding, growth factor activity, transcriptional coactivator activity and calcium ion binding.

The downregulated DEGs (Table 1) are involved in the biological process of immune response, cell-cell signaling, signal transduction, angiogenesis, and aging. The DEGs are located in integral components of the plasma membrane, extracellular space, extracellular region, and cell surface. Also, the downregulated DEGs play a role in protease binding, receptor binding, ion channel binding, cytokine activity, hormone activity, and estrogen response element binding.

Table 1. Top 10 upregulated and downregulated genes in Gene Ontology.

ID	Term	Count	*P* value
Upregulated genes			
Biological process			
GO:0045893	Positive regulation of transcription, DNA-templated	26	3.60E-04
GO:0030198	Extracellular matrix organization	18	3.80E-04
GO:0045787	Positive regulation of cell cycle	17	4.24E-04
GO:0007155	Cell adhesion	46	5.18E-04
GO:0007507	Heart development	9	7.69E-04
GO:0001503	Ossification	12	8.11E-04
GO:0030199	Collagen fibril organization	9	8.13E-04
GO:0001501	Skeletal system development	59	0.001
GO:0070374	Positive regulation of ERK1 and ERK2 cascade	17	0.002
GO:0048008	Platelet-derived growth factor receptor signaling pathway	12	0.002
Cellular component			
GO:0009986	Cell surface	26	1.96E-05
GO:0031012	Extracellular matrix	18	3.03E-05
GO:0005578	Poteinaceous extracellular matrix	17	3.25E-05
GO:0005615	Extracellular space	46	4.85E-05
GO:0030018	Z disc	9	0.001
GO:0045211	Postsynaptic membrane	12	0.002
GO:0005938	Cell cortex	9	0.002
GO:0016020	Membrane	59	0.002
GO:0005925	Focal adhesion	17	0.002
GO:0031410	Cytoplasmic vesicle	12	0.004
Molecular functions			
GO:0042803	Protein homodimerization activity	30	1.53E-04
GO:0005516	Calmodulin binding	13	2.74E-04
GO:0008083	Growth factor activity	12	2.77E-04
GO:0003713	Transcription coactivator activity	14	9.58E-04
GO:0005509	Calcium ion binding	27	0.001
GO:0005201	Extracellular matrix structural constituent	7	0.002
GO:0005515	Protein binding	194	0.002
GO:0003779	Actin binding	14	0.003
GO:0043565	Sequence-specific DNA binding	20	0.005
GO:0001077	Transcriptional activator activity, RNA polymerase Ⅱ core promoter proximal region sequence-specific binding	12	0.006
Downregulated genes			
Biological process			
GO:0006955	Immune response	35	1.50E-07
GO:0007267	Cell-cell signaling	24	2.39E-06
GO:0007165	Signal transduction	62	1.10E-05
GO:0001525	Angiogenesis	21	1.24E-05
GO:0007568	Aging	16	1.29E-04
GO:0007399	Nervous system development	22	1.52E-04
GO:0007584	Response to nutrient	10	3.44E-04
GO:0006954	Inflammatory response	25	4.55E-04
GO:0034220	Ion transmembrane transport	17	5.82E-04
GO:0007204	Positive regulation of cytosolic calcium ion concentration	13	6.80E-04
Cellular component			
GO:0005887	Integral component of plasma membrane	93	6.73E-14
GO:0005886	Plasma membrane	191	3.97E-13
GO:0005615	Extracellular space	83	5.59E-11
GO:0005576	Extracellular region	83	2.05E-07
GO:0009986	Cell surface	38	1.09E-06
GO:0016020	Membrane	96	2.65E-05
GO:0045121	Membrane raft	19	2.89E-05
GO:0016021	Integral component of membrane	187	1.57E-04
GO:0030425	Dendrite	23	2.88E-04
GO:0030054	Cell junction	27	8.14E-04
Molecular functions			
GO:0002020	Protease binding	14	1.04E-05
GO:0005102	Receptor binding	26	7.13E-05
GO:0044325	Ion channel binding	13	1.52E-04
GO:0005125	Cytokine activity	16	2.87E-04
GO:0005179	Hormone activity	11	5.24E-04
GO:0008236	Serine-type peptidase activity	9	5.76E-04
GO:0005088	RAS guanyl-nucleotide exchange factor activity	12	6.98E-04
GO:0008083	Growth factor activity	14	0.001
GO:0046934	Phosphatidylinositol-4,5-bisphosphate 3-kinase activity	8	0.003
GO:0034056	Estrogen response element binding	3	0.005

Table 2. KEGG pathway enrichment of the upregulated and downregulated genes.

Term	Count	*P* value
Upregulated genes		
Chemokine signaling pathway	11	0.006
ECM-receptor interaction	7	0.010
Hematopoietic cell lineage	7	0.010
Protein digestion and absorption	7	0.011
Cytokine-cytokine receptor interaction	12	0.014
Pathways in cancer	16	0.019
Platelet activation	8	0.020
Transcriptional misregulation in cancer	9	0.025
Focal adhesion	10	0.031
Thyroid hormone signaling pathway	7	0.035
Aldosterone-regulated sodium reabsorption	4	0.049
Downregulated genes		
Calcium signaling pathway	21	8.97E-06
Neuroactive ligand-receptor interaction	26	3.00E-05
Type Ⅱ diabetes mellitus	7	0.008
Rap1 signaling pathway	16	0.011
Natural killer cell mediated cytotoxicity	11	0.015
Regulation of actin cytoskeleton	15	0.024
cGMP-PKG signaling pathway	12	0.032
Cytokine-cytokine receptor interaction	16	0.036
Salivary secretion	8	0.040

3.2. KEGG pathway enrichment analysis

Pathway enrichment by KEGG of the upregulated genes (Table 2) showed the regulation of chemokine signaling pathways, ECM-receptor interaction, hematopoietic cell lineage, protein digestion and absorption, cytokine-cytokine receptor interaction, and pathways in cancer. In addition, the downregulated DEGs showed the regulation of signaling pathways such as calcium signaling pathway, neuroactive ligand-receptor interaction, type Ⅱ diabetes mellitus, Rap1 signaling pathway, and natural killer cell mediated cytotoxicity.

3.3. PPI network construction and module selection

To examine the biological role of the DEGs, we constructed a PPI network using the STRING database. A total of 1 009 genes were constructed to the PPI network complex containing 867 nodes and 1 069 edges, with average node degree 2.47 (Supplementary Figure 3). The 50 nodes with degree scores more than 10 were identified, mainly including PIK3R1, GNG11, ADAM10, KRAS, and ITGB3 (Figure 1, Table 3).

3.4. TF–DEGs regulatory network and pathway enrichment

To examine the transcriptional network of the DEGs, we created a TF-DEGs regulatory network. TRED is a database of cancer-related TF. Among DEGs, there are nine transcription factors found in TRED: TFAP2A, EGR4, ESR1, ESR2, HIF3A, MSX2, MYBL1, POU3F2, and RARA. Target genes of eight TFs were then predicted using TRRUST, except for EGR4 target genes, which were predicted using RegNetwork. There are 22 genes among DEGs which are target genes of ESR1, ESR2, TFAP2A and RARA. A TF regulatory network was then constructed and visualized by Cytoscape (Figure 2). KEGG pathway enrichment analysis of the genes in the TF-regulatory network showed regulation of cancer, estrogen signaling pathway, EGFR tyrosine kinase inhibitor resistance, and endocrine resistance (Table 4).

Table 3. Top 50 in protein network interactions ranked by Degree score method.

Rank	Genes	Score	Description
1	*PIK3R1*	53	Phosphatidylinositol 3-kinase regulatory subunit alpha
2	*GNG11*	45	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-11
3	*ADAM10*	24	Disintegrin and metalloproteinase domain-containing protein 10
4	*KRAS*	22	GTPase KRas
5	*ITGB3*	21	Integrin beta-3
6	*GNA14*	21	Guanine nucleotide-binding protein subunit alpha-14
7	*S1PR1*	19	Sphingosine 1-phosphate receptor 1
8	*TBXA2R*	18	Thromboxane A2 receptor
9	*SERPINA1*	18	Alpha-1-antitrypsin
10	*PDGFB*	18	Platelet-derived growth factor subunit B
11	*CCL25*	17	C-C motif chemokine 25
12	*CCR9*	17	C-C chemokine receptor type 9
13	*PPY*	17	Pancreatic prohormone
14	*SST*	17	Somatostatin
15	*NPY1R*	17	Neuropeptide Y receptor type 1
16	*PYY*	17	Peptide YY
17	*CCL27*	17	C-C motif chemokine 27
18	*VWF*	17	Von Willebrand factor
19	*CCR2*	17	C-C chemokine receptor type 2
20	*CX3CL1*	17	Fractalkine
21	*CNR1*	17	Cannabinoid receptor 1
22	*ADORA3*	17	Transmembrane domain-containing protein TMIGD3
23	*PTGDR2*	17	Prostaglandin D2 receptor 2
24	*GPER1*	17	G-protein coupled estrogen receptor 1
25	*GPR183*	17	G-protein coupled receptor 183
26	*HRH3*	17	Histamine H3 receptor
27	*HTR2C*	17	5-hydroxytryptamine receptor 2C
28	*GHSR*	17	Growth hormone secretagogue receptor type 1
29	*F2RL3*	17	Proteinase-activated receptor 4
30	*HCAR3*	17	Hydroxycarboxylic acid receptor 3
31	*ITGAL*	16	Integrin alpha-L
32	*P2RY6*	16	P2Y purinoceptor 6
33	*CCKAR*	16	Cholecystokinin receptor type A
34	*EDNRA*	16	Endothelin-1 receptor
35	*ADRA1A*	16	Alpha-1A adrenergic receptor
36	*EDNRB*	16	Endothelin receptor type B
37	*NPFF*	16	Pro-FMRFamide-related neuropeptide FF
38	*PTGFR*	16	Prostaglandin F2-alpha receptor
39	*NTSR1*	16	Neurotensin receptor type 1
40	*NMBR*	16	Neuromedin-B receptor
41	*TRIO*	16	Triple functional domain protein
42	*AHSG*	15	Alpha-2-HS-glycoprotein
43	*SNAP23*	15	Synaptosomal-associated protein 23
44	*MSLN*	15	Mesothelin
45	*MFI2*	15	Melanotransferrin
46	*ACTN2*	14	Alpha-actinin-2
47	*ARRB1*	14	Beta-arrestin-1
48	*PTPRJ*	14	Receptor-type tyrosine-protein phosphatase eta
49	*ATP8A1*	13	Phospholipid-transporting ATPase IA
50	*TMEM63A*	13	CSC1-like protein 1

3.5. Drug association analysis

The results showed one drug with FDR < 0.05, *i.e.* raloxifene, which has associated genes similar to hesperidin, thus indicating that raloxifene probably affects the expression of the same gene as hesperidin (Figure 3).

Table 4. KEGG enrichment pathway of DEGs involved in TF-regulatory network.

ID	Description	*P* value	FDR	Genes
hsa05200	Pathways in cancer	2.82E-06	9.21E-04	*ERBB2*, *ESR1*, *ESR2*, *HMOX1*, *PRKCA*, *RARA*, *RET*, *TGFA*
hsa04915	Estrogen signaling pathway	1.40E-04	0.022	*ESR1*, *ESR2*, *RARA*, *TGFA*
hsa05206	MicroRNAs in cancer	1.99E-04	0.022	*ERBB2*, *HMOX1*, *PRKCA*, *ZEB1*
hsa05223	Non-small cell lung cancer	3.00E-04	0.023	*ERBB2*, *PRKCA*, *TGFA*
hsa04917	Prolactin signaling pathway	3.58E-04	0.023	*CGA*, *ESR1*, *ESR2*
hsa01521	EGFR tyrosine kinase inhibitor resistance	5.11E-04	0.028	*ERBB2*, *PRKCA*, *TGFA*
hsa04012	ErbB signaling pathway	6.34E-04	0.030	*ERBB2*, *PRKCA*, *TGFA*
hsa05215	Prostate cancer	9.32E-04	0.033	*ERBB2*, *TGFA*, *ZEB1*
hsa01522	Endocrine resistance	9.60E-04	0.033	*ERBB2*, *ESR1*, *ESR2*
hsa04066	HIF-1 signaling pathway	1.02E-03	0.033	*ERBB2*, *HMOX1*, *PRKCA*

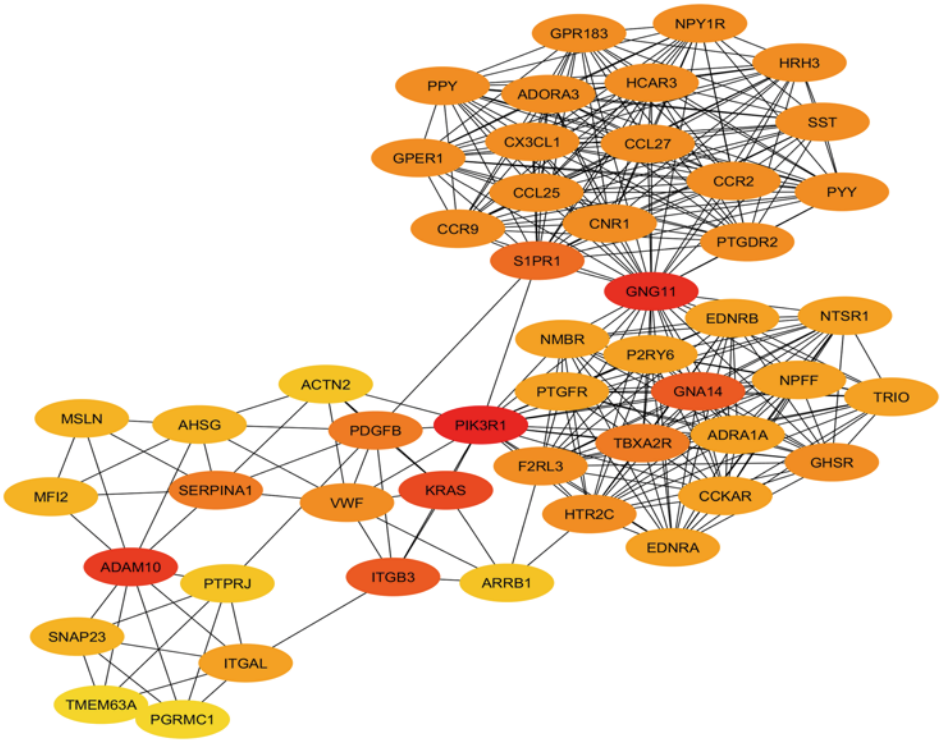

Figure 1. Protein network of top 50 genes with degree score more than 10, analyzed by CytoHubba.

4. Discussion

This study identified the potential target and molecular mechanism of hesperidin in ER-positive breast cancer cells using bioinformatics approaches. GO enrichment analysis showed that upregulated DEGs affect the processes of cell adhesion, positive regulation of transcription, and the cell cycle. Upregulation of RARA promotes epithelial-to-mesenchymal transition, a phenomenon that occurs when cells lose cell-cell adhesion[20]. The upregulated DEGs locate in a cellular component, cell surface, or extracellular matrix. A RARA present in membrane lipid rafts forms complexes with G protein to activate p38MAPK in cancer cells[21]. The upregulated DEGs are involved in transcription coactivator activity. RARA and ERs can cooperate for effective transcriptional activity in breast cancer cells[22].

GO enrichment analysis of downregulated genes affects the biological process of cell-cell signaling, *e.g.* estrogen signaling. The downregulated DEGs are located in several cells, including the extracellular region, membrane raft, and membrane. TGFA encodes transforming growth factor alpha (TGF- α), a ligand which binds to EGFR in the cell membrane and stimulates tyrosine kinase signaling[23]. The downregulated DEGs are involved in estrogen response element binding. The classical mechanism of estrogen signaling starts by binding the hormone to receptors in the nucleus, and continues with receptor dimerization and binding to specific response elements in the promoter of target genes called estrogen response elements[24]. KEGG pathway enrichment analysis of the upregulated and downregulated genes showed regulation of pathways in cancer, and calcium signaling pathway and Rap1 signaling pathway, respectively. Rap1 signaling plays an essential role in cancer migration, invasion and metastasis[25].

PPI network indicated that more than 50 genes possess degree scores of more than ten. *PIK3R1*, *GNG11*, *ADAM10*, *KRAS* and *ITGB3* are the top five genes with the highest degree scores. The expression level of *PIK3R1*, which encodes phosphoinositide-3-kinase regulatory subunit 1 and a constituent of the phosphoinositide 3-kinase, alters tamoxifen anti-proliferative activity in breast cancer cells[26]. Overexpression of *GNG11*, which encodes guanine

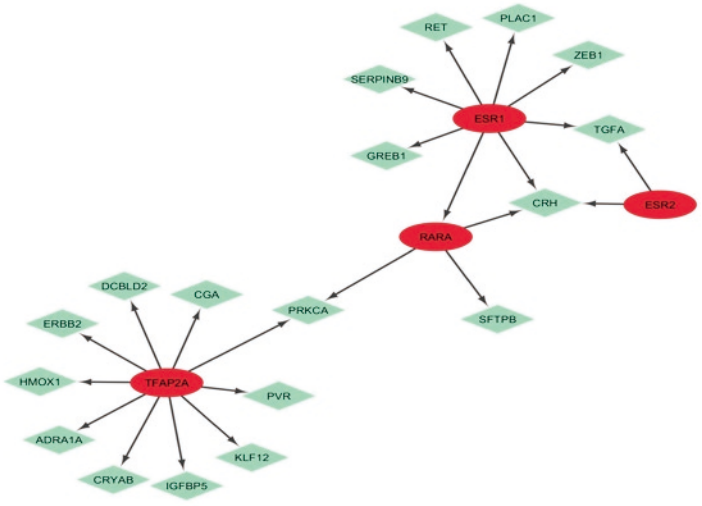

Figure 2. Transcription factors-differential expression genes regulatory network, analyzed by Cytoscape.

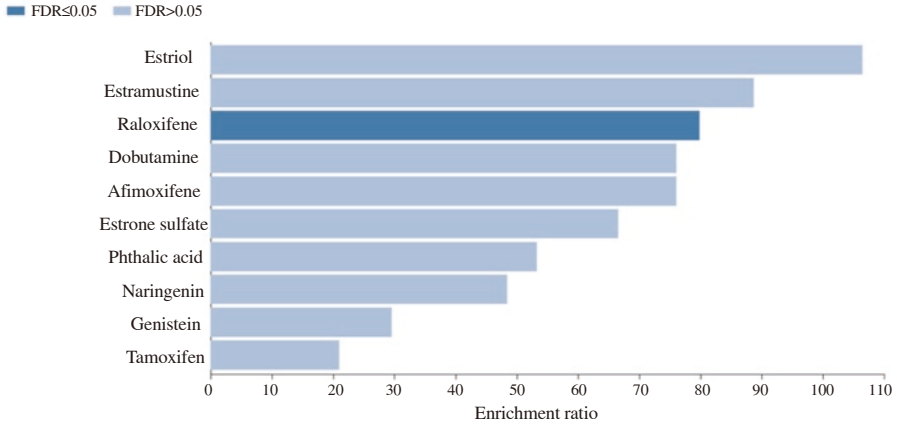

Figure 3. Predicted drugs with similar mechanisms to hesperidin, analyzed by ORA, WebGestalt.

nucleotide-binding protein (G protein), subunit gamma-11, enhances epithelial-to-mesenchymal transition and migration of breast cells toward malignant phenotypes[27]. *ADAM10* encodes a disintegrin and metalloproteinase domain-containing protein 10, and is also known as ADAM 10, a member of the ADAM family, which is involved in breast cancer progression, especially of the basal subtype[28].

Activation of ADAM10, which is mediated by ER signaling, promotes anti-amyloidogenic processing of amyloid precursor protein in Alzheimer's disease in mice[29]. *KRAS* (Kirsten rat sarcoma viral oncogene homolog) is a viral oncogene and a member of the RAS superfamily of proteins which play a role in intracellular signaling associated with carcinogenesis[30]. KRAS-activating mutations activate estrogen signaling in endometrial cancer[31]. *ITGB3* encodes integrin subunit beta 3 which increases proliferation, migration, and invasion of non-small cell lung cancer[32]. Activation of ER signaling promotes estrogen receptor-positive breast cancer invasion by enhancing the expression of integrin β 3[33]. Collectively, those genes are involved in the estrogen signaling pathway. There has been no study to date of those genes in hesperidin-treated ER-positive breast cancer cells yet,.

TF-DEGs regulatory network results showed that there are 4 TFs associated with the effects of hesperidin: ESR1, ESR2, TFAP2A and RARA. TFAP2B encodes transcription factor AP-2 β (TFAP2B) which regulates embryonic organ development and is overexpressed in alveolar rhabdomyosarcoma, a rare childhood malignancy and invasive lobular breast cancer[34]. More importantly, KEGG pathway enrichment analysis of the TF-DEGs regulatory network showed regulation of pathway in cancer, estrogen signaling, EGFR tyrosine kinase inhibitor resistance and endocrine resistance by hesperidin. Binding of estrogen to the ER, a nuclear TF, leads to specific binding to the DNA sequence, called the estrogen response element, and induces expression of estrogen-responsive genes[24]. AKT and MAPK signaling regulate the resistance mechanism of EGFR tyrosine kinase inhibitor. Resistance to gefitinib, an EGFR tyrosine kinase inhibitor in breast cancer, is regulated by MEK/MAPK pathway and AKT signaling pathway[35]. In addition, breast cells can become EGFR-TKI resistant due to the interaction of FAM83A and phosphorylation of c-RAF and PI3K p85, upstream of MAPK and downstream of EGFR[36]. A review conducted by Arpino *et al*. demonstrated that the molecular mechanism of resistance to anti-estrogen therapy, specifically tamoxifen, is associated with increased expression and signaling of EGFR and HER2, as well as a cross-link

between EGFR and ER signaling[37]. Endocrine therapy resistance is associated with TFAP2C since it regulates EGFR and HER2 signaling in luminal breast cancer[38]. Accordingly, those genes are involved in ER signaling. However, analysis of the transcriptional regulatory network of those genes in hesperidin-treated ER-positive breast cancer cells has never been done before.

Drug association analysis showed that raloxifene affects the expression of the same gene as hesperidin. Raloxifene is a selective ER modulator which is antagonistic to ERs in the mammary gland and uterus, and is used to reduce the risk of breast and ovarian cancer[39]. The drug produces estrogen-agonist effects in the skeleton and cardiovascular system and is used to prevent osteoporosis in menopausal women[40]. Raloxifene shows slightly lower efficacy but better safety than tamoxifen in breast cancer[41]. Therefore, hesperidin has the potential as a SERM, although further research is needed.

The present study indicates that hesperidin regulates estrogen signaling and exhibits similar actions as endoxifen as a SERM. A previous study showed that hesperidin had no cytotoxicity on MCF-7 cells at concentrations up to 100 μM over 24 h treatment[12]. Another study has demonstrated that hesperidin at a concentration of 100 μM inhibits the proliferation of MCF-7-GFP-Tubulin cells after 72 h treatment[42]. In addition, a recent study showed hesperidin cytotoxicity on MCF-7 cells with IC_{50} of 9.35 μM at 48 h treatment[43]. Treatment of hesperidin using the same concentration in endometrial cancer cells at 48 h and 72 h showed cytotoxicity and induction of apoptosis[44]. Recently, nanoformulation of hesperidin inhibited cell proliferation and induced p53-dependent apoptosis after 48 h treatment in MCF-7 cells[45]. It exhibits not only cytotoxicity, but also immunomodulatory effect[46] with a good safety profile based on acute and a sub-chronic oral toxicity study in Sprague Dawley rats[47]. To be brief, the present study supports and highlights the development of hesperidin as an anticancer drug that targets ER-positive breast cancer.

Using integrated bioinformatics approaches, this present study found that hesperidin targeted estrogen signaling in luminal breast cancer cells. The results not only contribute to the latest research data but also reveal potential targets for the treatment of ER-positive breast cancer. Genes associated with TFs can be used as biomarkers and for measuring clinical outcomes of hesperidin. More importantly, results of this study can be used as a reference for further work to explore the potential of hesperidin in overcoming the resistance of EGFR tyrosine kinase inhibitors and endocrine, as well as a basis for the development of hesperidin as a SERM. However, this study has several limitations. The data used for PPI network analysis is mRNA, not protein expression data. It is possible to obtain different results when using DEGs from protein expression data because not every mRNA will be translated into protein. The results of this study will also need to be validated *in vitro* and *in vivo* to determine the mechanism of hesperidin in ER-positive breast cancer.

In summary, hesperidin targets ER signaling in ER-positive breast cancer cells. *In vitro* and *in vivo* experiments are needed to validate the results and find out more about the role of these genes in the effectiveness of hesperidin. Future studies are also needed to explore the full therapeutic potential of hesperidin against EGFR tyrosine kinase inhibitor and endocrine resistance.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Authors' contributions

AH contributed in conception and design of the study, acquisition, analysis and interpretation of data, drafting and revising the article and final approval of the version to be published. HP contributed to analysis of data, drafting the article and final approval of the version to be published.

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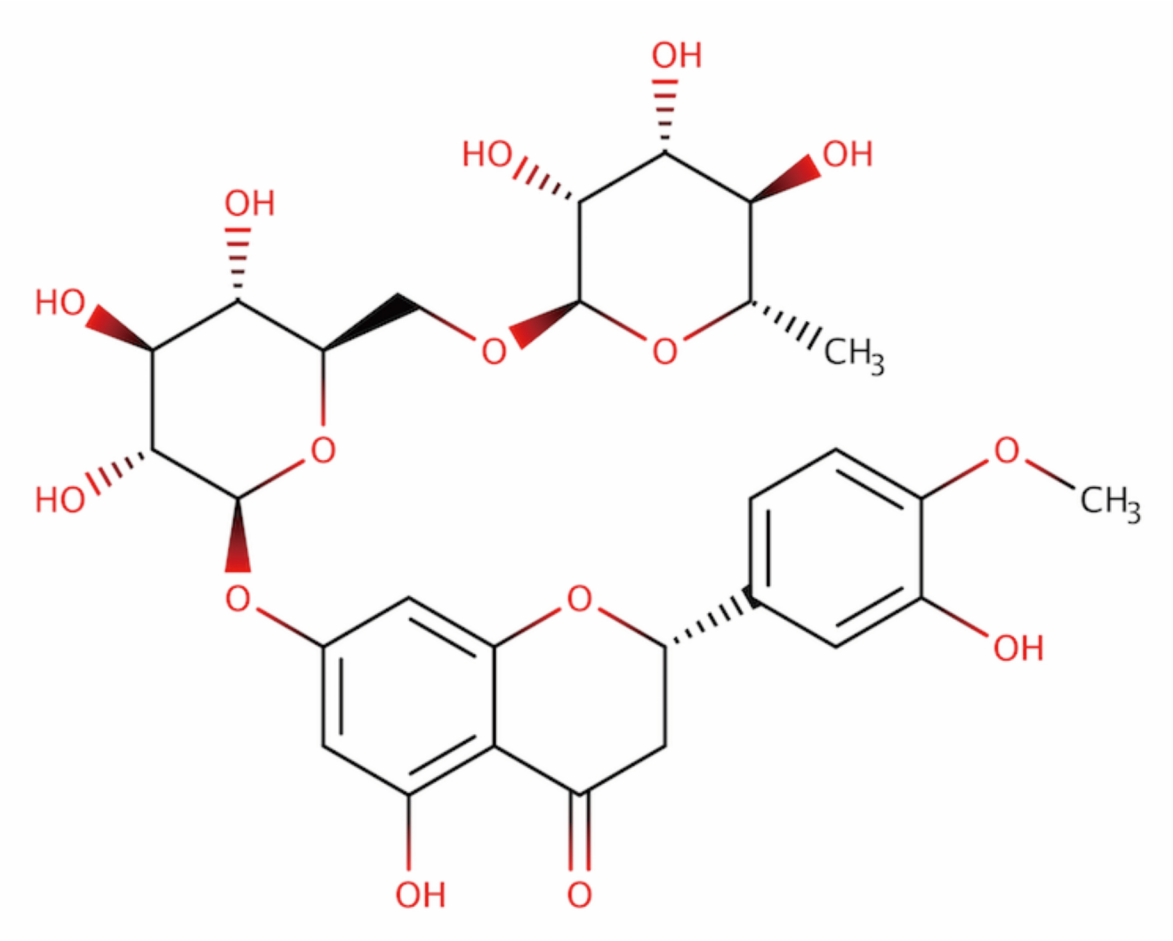
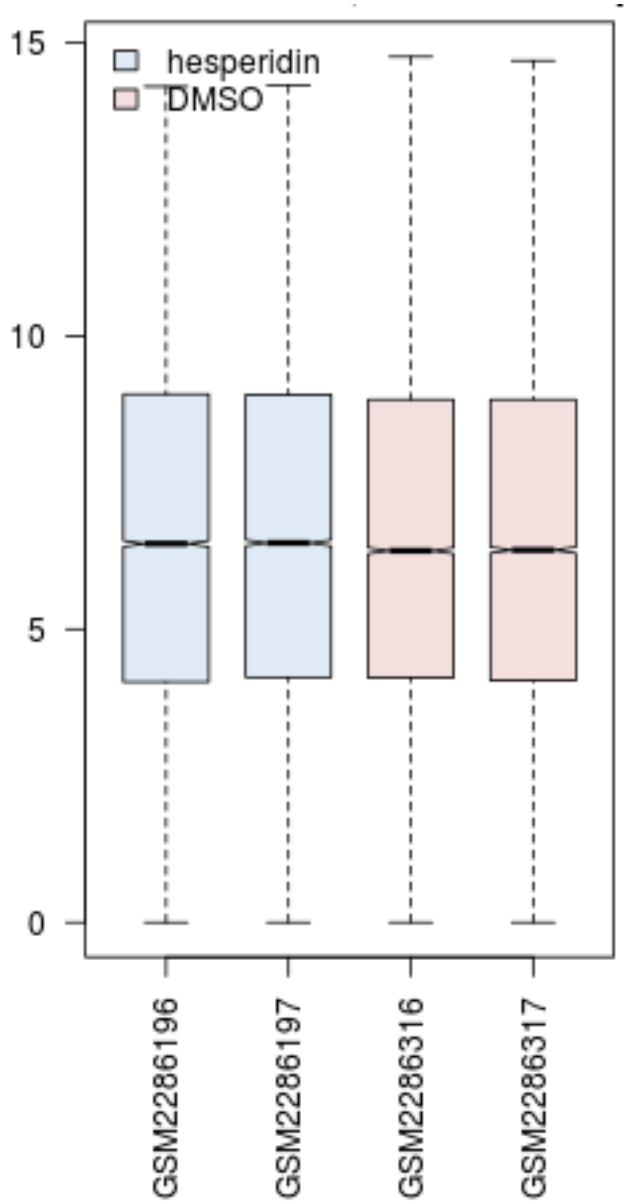
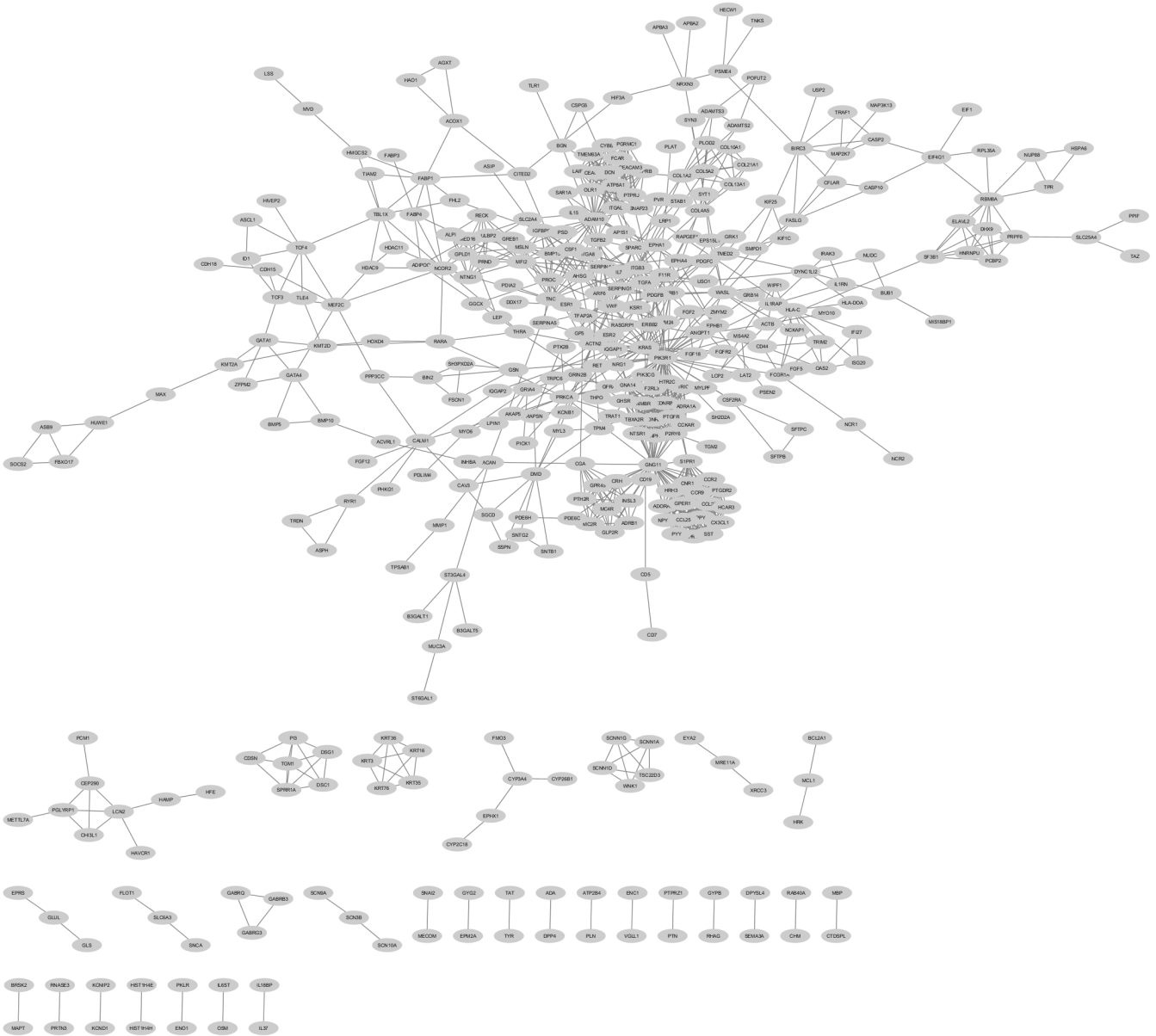


Supplementary Figure 1. Chemical structure of hesperidin.



Supplementary Figure 2. Distribution of samples among GSE85871. Cells were treated with hesperidin and dimethyl sulfoxide as control.



Supplementary Figure 3. Protein-protein interaction network upon treatment of hesperidin.