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Network Pharmacology and Molecular Docking Integrated Strategy to the Screening of Active Components and Mechanisms of Stephaniae Tetrandrae Radix on Breast Cancer

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Abstract: Stephaniae Tetrandrae Radix (STR) is a commonly used herb with a history of thousands of years. Accumulating evidence shows the therapeutic effect on breast cancer (BC) of STR. Here, we aimed to elucidate the active components and mechanisms of STR against BC. The active components and targets were retrieved and screened from the corresponding databases. A target protein–protein interaction (PPI) network was built and Ingenuity Pathway Analysis (IPA) used to analyze and screen key targets and pathways. Subsequently, molecular docking was performed to visualize the patterns of interactions between components and targets. Finally, the main active components of STR in treating BC were confirmed by in vitro experiments, and 34 common targets were obtained. The PPI network and IPA showed that the key targets were TP53, JUN, CASP3, and so on. Additionally, signaling pathways were enriched. Docking verified that the active components have good binding potential with the key targets, especially tetrandrine (Tet) and fangchinoline (Fang). In vitro studies confirmed that they significantly inhibited the viability of MDA-MB-231 cells and increased LDH leakage rate compared to MCF-10A cells. STR participates in many cell processes and regulate multiple targets, thereby playing an anti-breast cancer role. Tet and Fang may be the main active components.

Keywords: stephaniae tetrandrae radix; breast cancer; network pharmacology; molecular docking; molecular mechanism

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1. Introduction

Along with lung and colon cancer, breast cancer (BC) remains one of the three most common cancers in the world [1], and these are still recognized as unsolved problems in the world. At the same time, BC is the most common cancer among Chinese women and the leading cause of mortality among females [2], and it has seriously threatened women's lives and health. Currently, modern medicine mostly relies on surgery, radiotherapy, chemotherapy and hormone therapy to treat BC patients [3]. It is worth noting that for BC patients, the expected treatment goals are to maintain quality of life and prolong life expectancy, but in fact, in most cases, breast surgery is not standard treatment [4,5]. Although chemotherapy is effective in short-term treatment, long-term treatment will mostly lead to adverse reactions and drug resistance [6], which affect the quality of life and physical and mental health of patients. Therefore, researchers are eager to find satisfactory alternative therapies [7]. At this time, traditional Chinese medicine (TCM) therapy with thousands of years of history includes not only traditional acupuncture therapy but also many TCM formulae with better curative effects and less toxicity and side effects, which shows its unique charm in the treatment of BC [8]. There are various types of active

components in TCM that can participate in cell growth and proliferation, cell apoptosis and migration, etc. [9].

Stephaniae Tetrandrae Radix (STR) is derived from the dried root of *Stephania tetrandra* S. Moore, which was first recorded in *Shennong Ben Cao Jing* and is one of the commonly used herbs in TCM [10]. STR, known as the most important medicine for removing rheumatism, has significant diuretic and antirheumatism effects and has been used to treat various diseases in the clinical practice of TCM for thousands of years [11]. Clinically, STR is widely used in the treatment of rheumatism, arthralgia, cardiovascular disease, cancer [12] and other diseases [13]. Modern pharmacological studies have shown that STR has a variety of activities, such as analgesic, anti-inflammatory, antipathogenic, antihypertensive [14], antiarrhythmia, antifibrosis, and antitumor activities [15], which are mostly related to alkaloids, especially bisbenzylisoquinoline alkaloids [16], including tetrandrine (Tet), fangchinoline (Fang), etc. A growing number of evidence shows that STR has good therapeutic effect for BC, but most literature has focused only on Tet or Fang. So far, no systematic study has comprehensively explored the active components and mechanism of STR. Perhaps the original research methods to clarify the synergistic effects of multiple components and multiple targets of TCM is very difficult, which makes it difficult to reveal the specific therapeutic mechanism of TCM. Coincidentally, the advent of network pharmacology has made up for the shortcomings of existing methods. It was first formally proposed by Andrew L. Hopkins, a pharmacologist from the University of Dundee in the United Kingdom in 2007 [17], and is a new discipline with systemic and holistic characteristics developed on the basis of the theory of systems biology. The advent of network pharmacology has broken the existing "single-target, single-drug" research model with a "multitarget, multicomponent" research strategy studying the "complex-protein/gene-disease" pathway by mapping drug targets and molecules related to disease evidence to biomolecular networks, and analyzing the complex relationship between biological systems, drugs and diseases from the perspective of networks [18]. Network pharmacology is based on the combination of pharmacology and bioinformatics. Specific signal nodes are selected to play a predictive role in multitarget drug molecules. TCM network pharmacology has been successfully used in Danshen [19], Poroselle [20], Huanglian Jiedu decoction [21], Fangji Huangqi decoction [22,23], Taohong Siwu decoction [24] and so on. Single-target interventions have been shown to be ineffective and unsuccessful in complex diseases, such as tumors [25]. In this case, network pharmacological strategies are useful for finding multitarget drugs, because they can target multiple targets simultaneously, and if the best one is not pharmacological, other related targets may be helpful. It is worth mentioning that the multichannel regulatory signal can improve the efficiency of molecular mechanism research and new drug discovery, especially for TCM preparations. With the development of virtual screening technology, people increasingly rely on molecular docking technology to screen and predict the active components of TCM. Therefore, the combination of network pharmacology and molecular docking provides a theoretical basis and technical support for the modernization of TCM, and helps to clarify the material basis and underlying mechanism of TCM more quickly [26]. We searched the PubMed database (https://pubmed.ncbi.nlm.nih.gov/, accessed on 19 October 2022) for network pharmacology and molecular docking and finally found 2306 publications, of which 1856 were from the last 3 years. This showed that this research strategy has been widely used in drug development and mechanistic studies, and is mainly used to study the mechanisms of TCM and formulae. In this study, we explored the active components and underlying mechanisms of STR in the treatment of BC with the help of network pharmacology and molecular docking techniques.

2. Materials and Methods

2.1. Screening of Active Components and Targets of STR

All the components of STR were retrieved from the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP, http://old.tcmsp-e.com/

tcmsp.php, accessed on 4 June 2022). In this study, oral bioavailability (OB) and drug likeness (DL) were taken as the main parameters, and the active components were screened based on the criteria of OB $\geq$ 30% and DL $\geq$ 0.15. The main components of STR, Tet, Fang and cyclanoline were found by consulting the literature, and the three were included in this study.

The target corresponding to the component was queried by TCMSP, and the target names were normalized into gene names in the UniProt database (https://www.uniprot.org/, accessed on 10 June 2022). For compounds without target information, Swiss Target Prediction (STP, http://www.swisstargetprediction.ch/, accessed on 10 June 2022) can be used to screen the targets based on the criterion of possibility, and finally the targets were merged and repeat values removed.

2.2. Prediction of Potential Targets of STR against BC

We used "breast cancer" as the search term to collect and merge targets from the Human Gene Database (GeneCards, https://www.genecards.org/, accessed on 12 June 2022), Malacards (https://www.malacards.org/, accessed on 12 June 2022) and Comparative Toxicogenomics Database (CTD, http://ctdbase.org/, accessed on 12 June 2022), and relevant targets were obtained according to the double-median method. Then, the STR data were transferred to Venny 2.1.0 (https://bioinfogp.cnb.csic.es/tools/venny/index.html, accessed on 16 June 2022) to produce a Venn diagram, which indicated the intersection of identified targets of drug and disease, i.e., the potential targets of STR against BC.

2.3. Construction of Common Target PPI Network

The common targets were imported into the STRING (https://www.string-db.org, accessed on 16 June 2022) database to construct a protein–protein interaction (PPI) network, and the obtained data were imported into Cytoscape 3.7.2 (http://cytoscape.org, accessed on 24 June 2022) for topological analysis to screen out the core targets of STR against BC.

2.4. Core Analysis and Construction of Components–Targets–Pathways Network

We uploaded targets of STR against BC to Ingenuity Pathway Analysis (IPA, version 2022.3, biotech (shanghai) Ltd., Shanghai, China, accessed on 2 July 2022) for core analysis, including canonical pathway analysis, upstream regulator analysis, disease and function analysis, and network analysis. The Path Designer module was used to beautify the targets and pathways. Finally, the active components, potential targets, and top 10 pathways were imported into Cytoscape software (version 3.7.2) to build a network.

2.5. Molecular Docking

In order to further analyze the molecular mechanism of STR in the treatment of BC, we selected 10 targets for molecular docking with 6 active components of STR. Firstly, the MOL2 structures of the active components were downloaded from the TCMSP database and energy minimization was performed via Chem 3D software (version 19.0.0.22). Then, high-resolution crystal structures of the targets were obtained through the Protein Data Bank (PDB, https://www.rcsb.org/, accessed on 14 August 2022) platform and imported into PyMOL software (version 1.7.2.1, https://pymol.org/2/, accessed on 20 August 2022) for structural optimization and saved in PDB format. Finally, AutoDock Vina software (version 1_1_2, https://vina.scripps.edu, accessed on 2 September 2022) was used to complete molecular docking between active components and candidate targets. PyMOL software (version 1.7.2.1) was used to generate graphs of the results of molecular docking.

2.6. Validation of Compounds by In Vitro Assays

2.6.1. Cell and Reagents

MDA-MB-231 cells and MCF-10A cells were obtained from the Cell Center of Peking Union Medical College Hospital. MDA-MB-231 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; BI) supplemented with 10% fetal bovine serum (FBS; BI) and

1% antibiotic (100 U/mL penicillin and 100 mg/mL streptomycin; BI) and MCF-10A cells cultured in special medium for MCF-10A cells (Procell, Wuhan, China). They were cultured at 37 °C, 5% CO_2 and relatively saturated humidity. Throughout the entire cell culture process, the principle of sterility was strictly observed. Tet and Fang were purchased from Chengdu Must Bio-Technology Co., Ltd. (Chengdu, China) and dissolved in dimethyl sulfoxide (DMSO) as a 25 mmol/L stock solution.

2.6.2. Cell Viability Assessment by MTT Assay

MCF-10 A and 10AMDA-MB-231 cell viability following treatment with Tet or Fang was measured by the MTT assay. Cells (2.5×10^3 cells/well) were seeded into 96-well plates and cultured for 24 h. Then, we added Tet or Fang solutions with different concentrations (0 μM, 2.5 μM, 5 μM, 7.5 μM, 10 μM, 20 μM) for 24 h. Subsequently, MTT solution (1 mg/mL, 100 μL/well; Solarbio) was added to each well and incubated for another 4 h at 37 °C. The supernatant was then discarded, and 150 mL DMSO was added to each well followed by shaking for 10–15 min. Subsequently, the absorbance at 570 nm in each well was read on a microplate reader (Tecan Austria GmbH, Grödig, Austria). The effects of drugs on cell viability were expressed according to the optical density (OD) values. Cell viability rate formula: treatment group OD value/control group OD value × 100%.

2.6.3. Cytotoxicity Assessment by LDH Assay

Lactate dehydrogenase (LDH) leakage was detected to evaluate the cell toxicity of Tet or Fang on MCF-10A cells and MDA-MB-231 cells according to the instructions of CytoTox 500® nonradioactive cytotoxicity assay kit from Dojindo (Kumamoto, Japan). LDH leakage rate formula: treatment group OD value/control group OD value × 100%.

2.6.4. Statistical Analysis

All experiments were repeated three times. All results are expressed as means ± standard error of mean (SEM). When homogeneity and normality of variance were met, one-way ANOVA was performed between multiple groups using SPSS 26. Otherwise, Dunnett'sT3 and nonparametric tests were conducted between multiple groups. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Selection of Active Components and Targets of STR

Four active components were screened from the TCMSP database. Although Tet, Fang and cyclohexanol were not included, we checked the literature and found that they are the main and unique active components of STR and have good antitumor activity, so they are included in this study. Information on compounds is shown in Table 1.

Table 1. Information of 7 filtered active components.

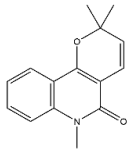
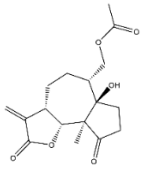
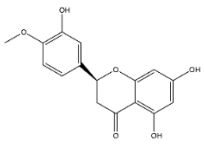
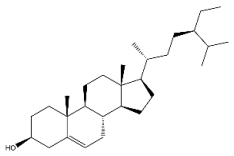
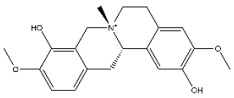
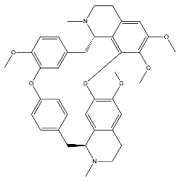
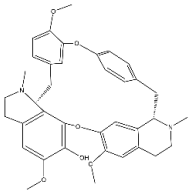
Mol ID	Molecule Name	OB (%)	DL	MW	Structure
MOL002331	N-Methylflindersine	32.36	0.18	241.31	
MOL002333	Tetraneurin A	35.4	0.31	322.39	

Table 1. *Cont.*

Mol ID	Molecule Name	OB (%)	DL	MW	Structure
MOL002341	Hesperetin	70.31	0.27	302.30	
MOL000358	beta-sitosterol	36.91	0.75	414.79	
MOL002344	cyclanoline	2.64	0.57	342.45	
MOL002343	tetrandrine	26.64	0.10	622.82	
MOL002342	fangchinoline	11.73	0.11	608.79	

Collected from the TCMSP database. OB, oral bioavailability; DL, drug likeness; MW, molecular weight.

Sixty targets were obtained from the TCMSP and UniProt databases and 11 targets predicted and screened through the STP database. After the duplication was removed by merging, 66 targets were obtained. Among them, MOL002333 did not screen the target.

3.2. Collection of Potential Targets of STR against BC

In sum, 15,253 BC-related targets were retrieved from GeneCards, 1026 from MalaCards, and 558 from CTD. Then, 2147 BC-related targets were screened. Finally, 34 common targets were obtained by intersecting them with 66 targets of STR (as shown in Figure 1A and Table 2), which were potential targets of STR for BC treatment.

Table 2. 34 potential targets of STR for BC treatment.

No.	UniProtKB	Gene Names	Protein Names
1	P07550	ADRB2	Beta-2 adrenergic receptor
2	Q07812	BAX	Apoptosis regulator BAX
3	P10415	BCL2	Apoptosis regulator Bcl-2
4	P00918	CA2	Carbonic anhydrase 2
5	P42574	CASP3	Caspase-3
6	Q14790	CASP8	Caspase-8
7	P55211	CASP9	Caspase-9
8	P24385	CCND1	G1/S-specific cyclin-D1
9	P11802	CDK4	Cyclin-dependent kinase 4
10	P38936	CDKN1A	Cyclin-dependent kinase inhibitor 1
11	P32297	CHRNA3	Neuronal acetylcholine receptor subunit alpha-3
12	Q01094	E2F1	Transcription factor E2F1
13	P01100	FOS	Proto-oncogene c-Fos

Table 2. *Cont.*

No.	UniProtKB	Gene Names	Protein Names
14	P01579	IFNG	Interferon gamma
15	P60568	IL2	Interleukin-2
16	P01589	IL2RA	Interleukin-2 receptor subunit alpha
17	P24394	IL4	Interleukin-4
18	P05412	JUN	Transcription factor AP-1
19	Q15788	NCOA1	Nuclear receptor coactivator 1
20	Q15596	NCOA2	Nuclear receptor coactivator 2
21	P35228	NOS2	Nitric oxide synthase, inducible
22	P29474	NOS3	Nitric oxide synthase, endothelial
23	P06401	PGR	Progesterone receptor
24	P48736	PIK3CG	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit gamma isoform
25	P27169	PON1	Serum paraoxonase/arylesterase 1
26	P17252	PRKCA	Protein kinase C alpha type
27	P23219	PTGS1	Prostaglandin G/H synthase 1
28	P35354	PTGS2	Prostaglandin G/H synthase 2
29	Q04206	RELA	Transcription factor p65
30	P19793	RXRA	Retinoic acid receptor RXR-alpha
31	P84022	SMAD3	Mothers against decapentaplegic homolog 3
32	O15105	SMAD7	Mothers against decapentaplegic homolog 7
33	P01137	TGFB1	Transforming growth factor beta-1
34	P04637	TP53	Cellular tumor antigen p53

Collected from UniProt database.

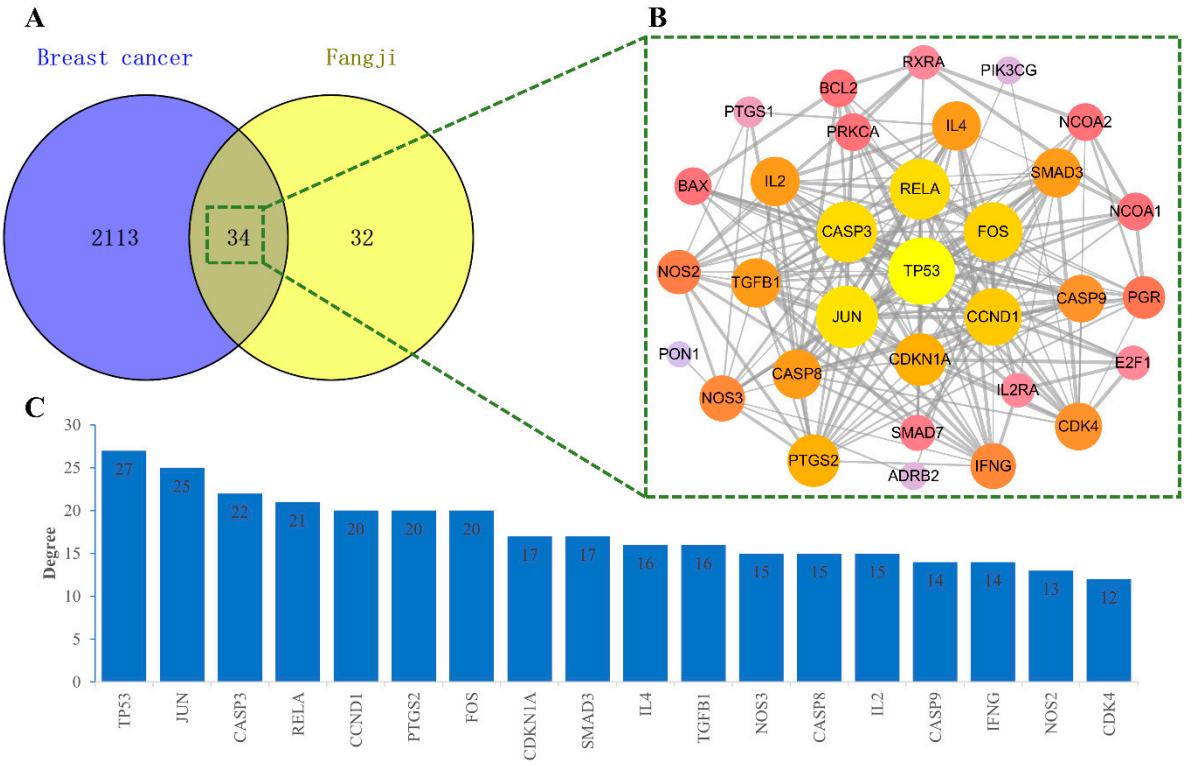


Figure 1. Target acquisition and PPI network diagram of STR for BC. (**A**) Venn diagram of overlapping targets for STR (66 targets) and BC (2147 targets); (**B**) PPI network diagram of common targets; (**C**) bar diagrams of targets that exceed average degree values in the treatment of BC by STR.

3.3. Construction of Common Targets PPI Network

The 34 common targets were uploaded to the STRING database to construct the PPI relationship of the targets for STR against BC, and visualized analysis was performed

by Cytoscape 3.7.2 software, with results shown in Figure 1B. The average node degree value of targets was 11.9, and there were 18 targets that exceeded the average degree value. Ranking from high to low were TP53 (27), JUN (25), CASP3 (22), RELA (21), CCND1 (20), PTGS2 (20), FOS (20), CDKN1A (17), SMAD3 (17), etc. (Figure 1C). It was speculated that these targets may be the main targets and play an important role in the treatment of BC with STR.

3.4. Core Analysis and Construction of Components–Targets–Pathways Network

We uploaded 34 targets to IPA for core analysis to explore the biological process and mechanism of STR against BC. Figure 2A,D shows that targets were involved in multiple disease functions, including organism injury and abnormalities, cell death and survival, and cancer. Meanwhile, 362 canonical pathways were enriched through IPA, and the top 10 pathways were predicted according to $-\log$ (p-value) ≥ 15, including coronavirus pathogenesis pathway, glucocorticoid receptor signaling, molecular mechanisms of cancer, HER-2 signaling in breast cancer, and so on (as shown in Figure 2B). The top 10 pathways and the genes included are shown in Table 3 and Figure 3B. RELA had the highest participation rate, while PIK3CG JUN, TP53, CCND1, TGFB1 and FOS were also involved in eight pathways (Figure 2C).

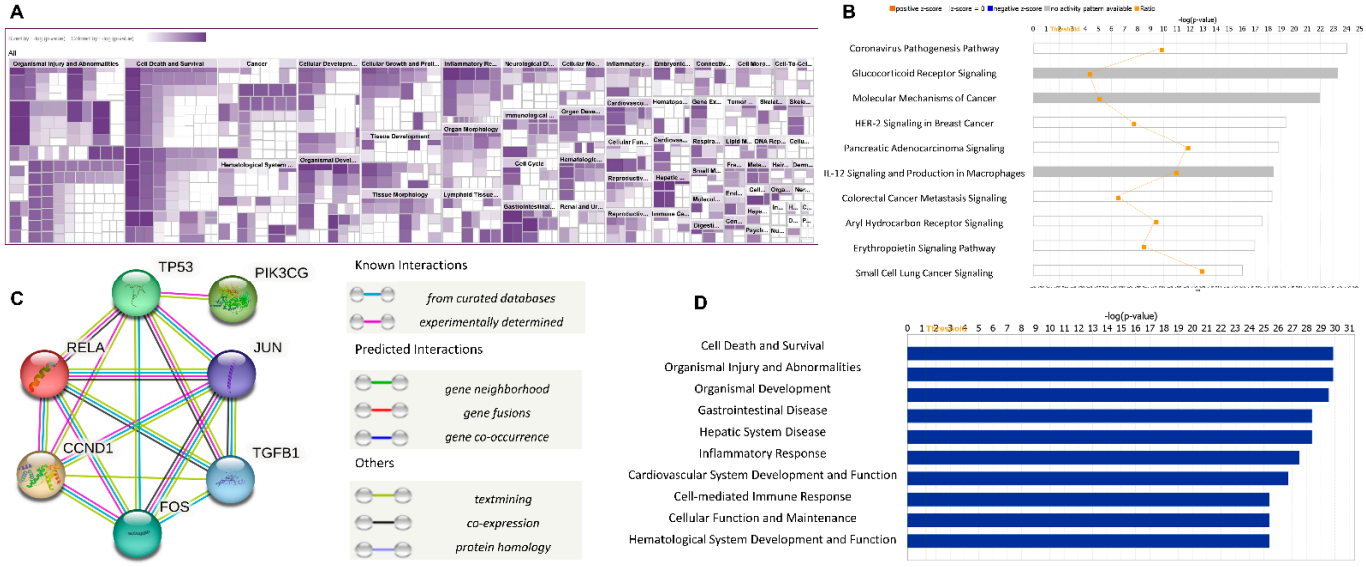


Figure 2. Disease function and upstream regulator analysis of potential targets based on IPA platform. (**A**) Heat map of disease function analysis; (**B**) bar chart of the top 10 canonical pathways predicted; (**C**) PPI network of the most involved targets among the top 10 pathways; (**D**) bar chart of the top 10 disease functions.

Intertarget interaction networks were also predicted by IPA. Based on score, the network ranking first among the 11 networks is shown in Figure 3A. This network is mainly involved in cell death and survival, cell development and other disease functions, among which TP53 is the core target, which is consistent with the previous analysis results. The network diagram of STR-components-targets-pathways-BC drawn by Cytoscape (version 3.7.2) is shown in Figure 3C. Finally, we briefly summarized the comprehensive mechanisms of STR in the treatment of BC (as shown in Figure 3D). We can intuitively see that STR plays a role in the treatment of BC by participating in multiple pathways through the synergistic action of multiple components and multiple targets.

Table 3. Top 10 canonical pathways and targets.

Coronavirus Pathogenesis Pathway	−log(*p*-Value)	Molecules
Glucocorticoid Receptor Signaling	24	BAX, BCL2, CASP3, CASP8, CASP9, CCND1, CDK4, E2F1, FOS, IFNG, JUN, PTGS2, RELA, SMAD3, TGFB1, TP53
Molecular Mechanisms of Cancer	23.3	ADRB2, BCL2, CDKN1A, FOS, IFNG, IL2, IL2RA, IL4, JUN, NCOA1, NCOA2, NOS2, NOS3, PGR, PIK3CG, PTGS2, RELA, RXRA, SMAD3, TGFB1
HER-2 Signaling in BC	22	BAX, BCL2, CASP3, CASP8, CASP9, CCND1, CDK4, CDKN1A, E2F1, FOS, JUN, PIK3CG, PRKCA, RELA, SMAD3, SMAD7, TGFB1, TP53,
Pancreatic Adenocarcinoma Signaling	19.3	CASP3, CASP9, CCND1, CDK4, CDKN1A, FOS, JUN, PGR, PIK3CG, PRKCA, PTGS2, RELA, SMAD3, TP53
IL-12 Signaling and Production in Macrophages	18.8	BCL2, CASP9, CCND1, CDK4, CDKN1A, E2F1, PIK3CG, PTGS2, RELA, SMAD3, TGFB1, TP53
Colorectal Cancer Metastasis Signaling	18.4	FOS, IFNG, IL4, JUN, NCOA1, NOS2, PIK3CG, PON1, PRKCA, RELA, RXRA, TGFB1
Aryl Hydrocarbon Receptor Signaling	18.3	BAX, CASP3, CASP9, CCND1, FOS, IFNG, JUN, NOS2, PIK3CG, PTGS2, RELA, SMAD3, TGFB1, TP53
Erythropoietin Signaling Pathway	17.6	BAX, CCND1, CDK4, CDKN1A, E2F1, FOS, JUN, NCOA2, RELA, RXRA, TGFB1, TP53
Small Cell Lung Cancer Signaling	17	CCND1, FOS, IFNG, IL2, IL4, JUN, NOS3, PIK3CG, PRKCA, RELA, TGFB1, TP53
Coronavirus Pathogenesis Pathway	16	BCL2, CASP9, CCND1, CDK4, E2F1, PIK3CG, PTGS2, RELA, RXRA, TP53

Collected from IPA software (version 2022.3).

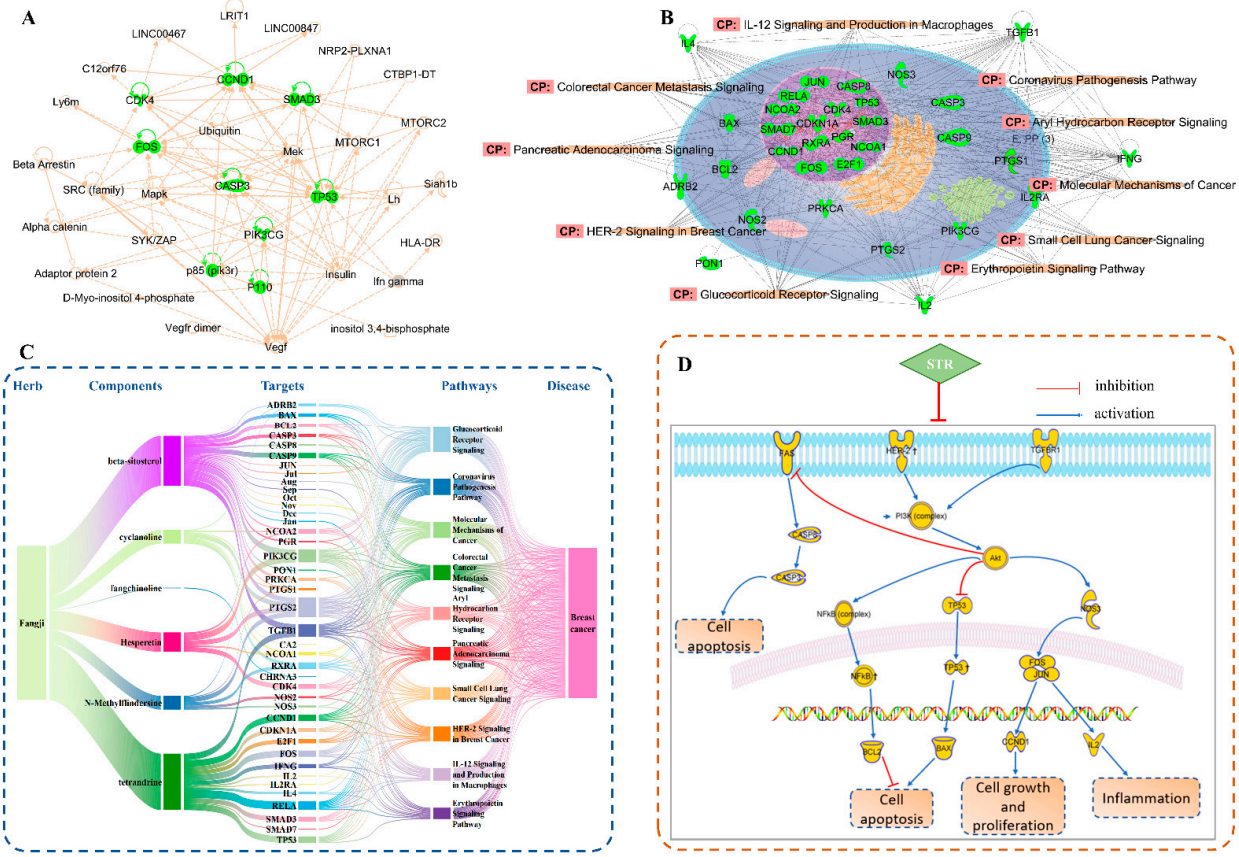


Figure 3. Network diagram of STR–components–targets–pathways–BC. (**A**) Network 1 and its related target diagrams; (**B**) top 10 canonical pathways and their related target diagrams; (**C**) network diagram of STR–components–targets–pathways–BC; (**D**) underlying mechanism of STR in the treatment of BC.

3.5. Molecular Docking

Molecular docking makes it easy to explore the possible interaction mode of components and hub genes. The lower the binding energy, the stronger the binding affinity of the active component to the target, i.e., the greater the possibility of binding. In this study, we evaluated the binding energies of six active components of STR with 10 hub genes: TP53, JUN, CASP3, RELA, PTGS2, NOS3, CASP8, PIK3CG, IL2 and Bcl-2. We chose these targets for our study because they are key nodes in the PPI network and also play an important role in the pathways and upstream regulation. Molecular docking scores are listed in Table 4. We can see that the binding energies of all these 10 targets and 6 active components were less than −6 kcal/mol. Noteworthily, the scores of Tet and Fang were much higher for each target than for the other components. Therefore, it was speculated that these two components may be the most vital components of STR against BC. The partially representative three-dimensional binding modes of Tet and Fang with the targets are shown in Figure 4. Docked components exhibited different binding modes in the active, site including hydrogen bonds, ionic bonds, and H–π and π–π interactions.

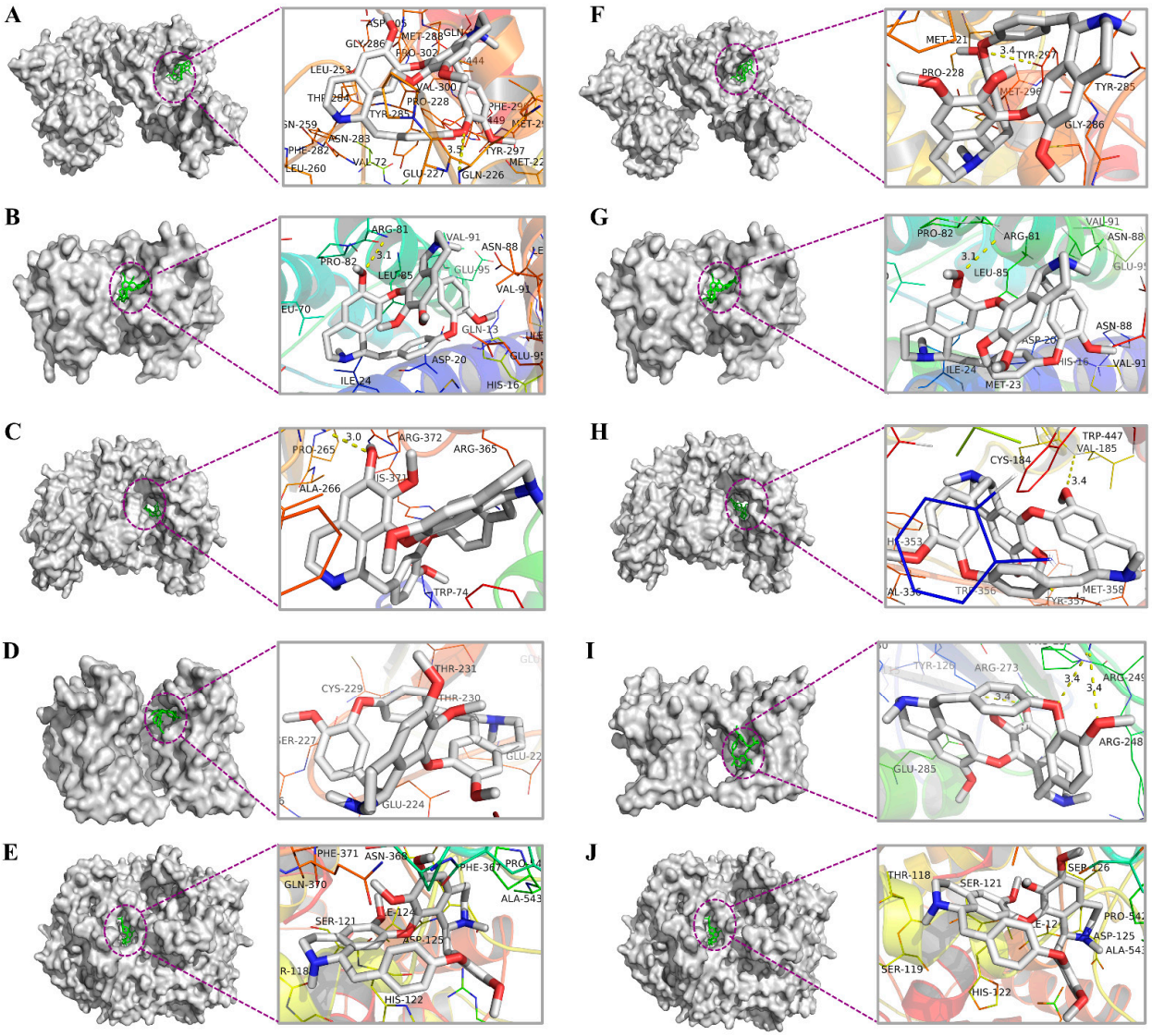


Figure 4. Molecular docking models of Tet and Fang binding to the 10 hub targets. Tet binds to RELA (**A**), IL-2 (**B**), NOS3 (**C**), TP53 (**D**), PTGS2 (**E**), Fang binds to RELA (**F**), IL-2 (**G**), NOS3 (**H**), TP53 (**I**), PTGS2 (**J**).

Table 4. Results of molecular docking studies of 6 components and 10 hub targets.

Gene Name	PDB ID	Tet	Fang	Cyclanoline	Hesperetin	Beta-Sitosterol	N-Methylflindersine
TP53	5O1E	−7.5	−7.8	−7.6	−7.8	−7.2	−8
JUN	5FV8	−7.3	−7.3	−6.4	−6.3	−6.4	−6.2
Casp3	1NMS	−8.5	−8.8	−7.3	−7	−7.2	−7.1
RELA	3QXY	−9.2	−9.5	−8.2	−7.2	−7.1	−8.4
PTGS2	5F19	−10.2	−10.5	−8.2	−8.2	−7.9	−9
NOS3	4D1P	−9.2	−10.3	−8.4	−7.8	−7.6	−8.1
Casp8	6PX9	−7.2	−7.5	−7.1	−7	−7.8	−8.5
PIK3CG	4WWO	−9	−10.6	−7.9	−7.9	−7.8	−7.8
IL2	1M49	−8.6	−9.2	−7.7	−7.4	−8	−6.8
Bcl2	6QGK	−8.7	−9.2	−7.5	−7.2	−7.3	−8.2

3.6. Validation of Compounds by In Vitro Assays

Both Tet and Fang are bisbenzylisoquinoline alkaloids, and their structures are shown in Figure 5A. The results of inverted microscopy showed that the human breast cancer cell line MDA-MB-231 was fusiform and adhered to the wall with fast propagation and compact arrangement. The morphological changes in MDA-MB-231 were obvious after treatment with Tet or Fang at different concentrations for 24 h (Figure 5B). MCF-10A cells and MDA cells were treated with Tet or Fang for 24 h, respectively, to detect cell viability and LDH leakage rate. The results (Figure 5C,D) showed that with the increase in dosage, cell viability decreased in both types of cells treated with Tet or Fang, especially MDA-MB-231 cells. For example, at a dose of 7.5 μM Tet, the viability of MAD-MB-231 cells was inhibited to about 30% and MCF-10A cells to about 75%. Similarly, Fang inhibited the cell viability of MAD-MB-231 to about 30% and MCF-10A to about 90%. We found that cytotoxicity of both was dose-dependent. LDH leaked out of the cell when the cell received enough damage. As shown in Figure 5E,F, with the increased dosage, the LDH leakage rate increased in both types of cells treated with Tet or Fang, especially MDA-MB-231 cells. The LDH leakage rate of MDA-MB-231 cells was significantly increased at doses greater than 7.5 μM. At the same time, the LDH leakage rate of MCF-10A cells was not significantly different at 2.5–10 μM, and only increased significantly at a dose of 20 μM. All results showed that the cytotoxic effect of Tet and Fang on MDA-MB-231 cells was stronger than that on MCF-10A cells, especially at a dose of 5–10 μM.

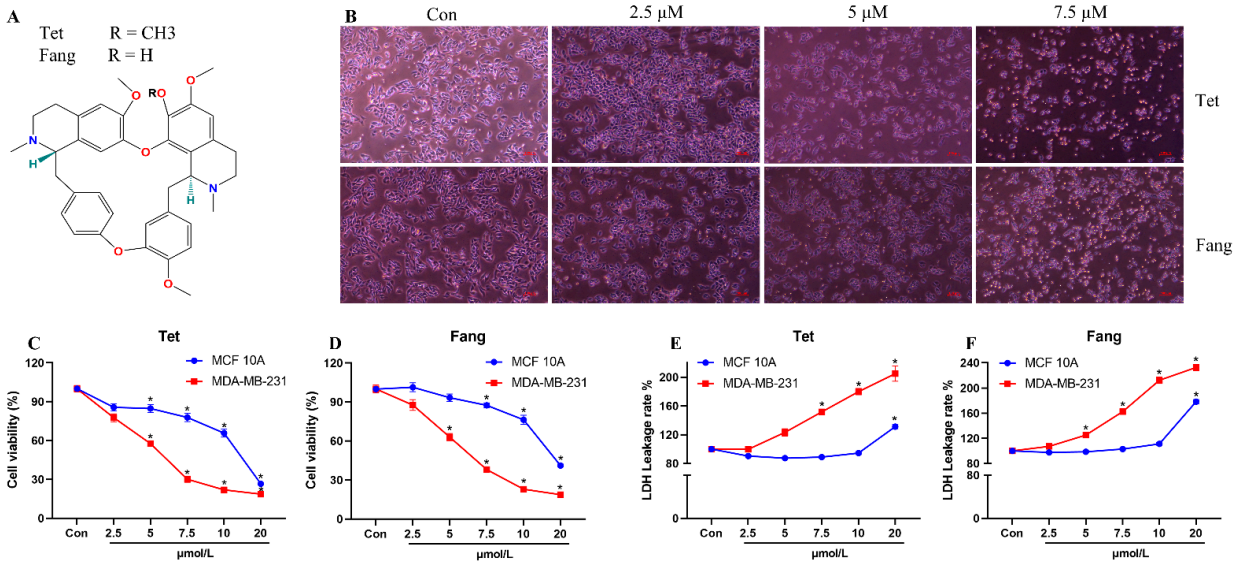


Figure 5. Inhibitory effects of Tet and Fang on human breast cancer cell line MDA-MB-231. (**A**) Chemical structure of Tet and Fang; (**B**) representative images of MDA-MB-231 evaluated by different concentrations of Tet or Fang for 24 h (40×), bar = 100 μm; (**C**,**D**) MTT assays were performed

to measure MCF10a and MDA-MB-231 cell viability following Tet or Fang treatment for 24 h (n = 3); (**E**,**F**) LDH leakage assays were performed to measure MCF10a and MDA-MB-231 cell viability following Tet or Fang treatment for 24 h (n = 3). * $p < 0.05$ compared to Con.

4. Discussion

From the perspective of traditional medicine, BC belongs to the category of "mammary rock." The depletion of bowels and viscera and vacuity of qi and blood are important etiological and pathogenic mechanisms in BC and are also related to genetic and environmental factors [27]. It is obvious to all that in recent years, TCM treatment of BC has made good progress in basic research and clinical application. Therefore, the screening of antitumor drugs from TCM has gradually become the focus of attention of researchers. However, it is worth noting that although TCM has a synergistic multicomponent and multitarget effect, it also brings great difficulties to research on drug mechanism of action and the development of new drugs. Fortunately, network pharmacology was first formally proposed to emphasize the multipathway regulation of signal pathways and help people understand the biological process and mechanism of TCM in treating diseases to a great extent. In contrast to rare single-gene diseases, a very large number of diseases are regulated by signaling networks, and there is more than one component in TCM [28]. In the face of such double difficulties, network pharmacology is undoubtedly the best choice.

As a TCM, STR has a history of more than 2000 years. In recent years, according to clinical experience, it has been found that STR can be combined with other TCMs to treat a variety of tumors, including endometrial cancer, lung cancer, breast cancer, colon cancer, bladder cancer, thyroid cancer, and liver cancer. As we all know, BC remains one of the main diseases leading to the death of women worldwide. A number of studies have reported the anti-breast cancer activity of Tet and Fang. We do agree that these two are the main components of STR, but we do not agree that these two are the only active antitumor ingredients. Unfortunately, to date, there has been no research systematically exploring the active components and potential mechanisms of the anti-breast cancer action of STR. We know that we can find star compounds from TCM to be used as chemical drugs for clinical use, but we must understand how TCM itself functions before this, so as to promote the development of TCM. As an alternative therapy, TCM has a good antitumor effect, not only with multiple targets and multiple ways but also with fewer adverse reactions than chemotherapy drugs [29]. Therefore, taking into account the multicomponent and multitarget nature of STR, our study used network pharmacology and molecular docking strategies, taking active components and targets as the research objects, to comprehensively and systematically reveal the active components, biological processes and signaling pathways of STR for the treatment of BC. Here, we are especially grateful to the developers of various open-source databases. We used these databases to quickly obtain a lot of useful drug components, targets and disease-related information, thus improving our research efficiency and saving research funds.

Table 1 shows that alkaloids are the most active components we screened out, which is consistent with previous reports on STR [30]. To date, compounds isolated and identified from STR include alkaloids, flavonoids and steroids [13]. Studies on the chemical composition of STR revealed that alkaloids were the major components. Qian et al. [31] identified 76 alkaloids using UHPLC-Q-TOF-MS, which can be classified into benzylisoquinoline (BIQ), bisbenzylisoquinoline (BBIQ), aporphine and tetrahydroprotoberberine alkaloids, and so on. Of these classes, the bisbenzylisoquinoline (BBIQ) alkaloids are the most dominant, e.g., Tet, Fang and fenfangjine A-D, G-I and K-S. These components we screened out have been shown to have broad and potent antitumor activity, including BC [32–34], liver cancer [35], lung cancer [36], prostate cancer [37], colon cancer [38,39], cervical cancer [40], bladder cancer [41,42], ovarian cancer [43], stomach cancer [44,45], pancreatic cancer [46], leukemia [47]. The underlying mechanisms are related to a variety of factors, including the induction of autophagy, apoptosis [48] and cell cycle arrest [49], and the inhibition of cell proliferation [50], migration and invasion [51]. The role of autophagy in cancer for good

or bad has been considered a controversial process [52], so we must be extremely careful when we refer to it. Anticancer activity was revealed to be exerted mainly through the regulation of such pathways as MAPK, PI3K/AKT [53], Wnt [54], and Hippo/YAP [55] and caspase cascades and mitochondrial pathways. Here, what we wanted to say was that these pathways have also been shown to be vital roles in the development of BC. MAPK members are divided into seven subgroups, and three classical MAPK pathways involve extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK, which play an important role in cell growth, differentiation and apoptosis [56]. A previous study revealed that Tet can act as a modulator of chemotherapeutic resistance in the treatment of BC. For example, Tet combined with arsenic trioxide amplified the toxic effects on MDA-MB-231 cells, induced activation of MAPK and JNK signaling pathway, and induced S-phase arrest, apoptosis/necrosis, and autophagy [57]. The PI3K/AKT/mTOR (PAM) pathway has been reported to be the most susceptible to abnormal activation in various cancers, promoting excessive cell proliferation and inhibiting apoptosis [58]. Numerous experiments have shown that inhibition of key components of PAM, such as receptor tyrosine kinases (RTKs), including epidermal growth factor receptor (EGFR), insulin-like growth factor-1 (IGF-1) receptor, human epidermal growth factor receptor (HER), PI3K and mTOR, can inhibit cell proliferation, survival and metastasis, and affect tumor microenvironment and angiogenesis [59]. Studies have reported that Tet can exert antitumor effects by inhibiting cell proliferation through the PAM signaling pathway, targeting LC3, p62 and Beclin-1 autophagy genes, and upregulating casp3, Bax and Bid. Downregulation of Bcl-2, survivin, and PARP promoted apoptosis [60]. As the most abundant phytosterol, β-sitosterol produces toxic effects on MCF7 cells, which can upregulate microRNA 10a and inhibit the PI3K/Akt pathway [61], and also activate the Fas signaling pathway to promote cell apoptosis [62]. The Wnt signaling pathway is a highly conserved signaling pathway that plays a key role in cancer progression and is mainly involved in the proliferation and metastasis of BC. Studies have revealed that berberine, an isoquinoline alkaloid, inhibits the proliferation and metastasis of BC cells by regulating Wnt/β-catenin signaling [63]. Hippo/YAP is a cancer suppressor pathway, and its dysregulation will increase the expression of transcription coactivator YAP (Yes-associated protein) and promote tumor formation and growth. In fact, in addition to YAP, a key molecule, many upstream regulators have recently been confirmed to regulate YAP, such as epinephrine, glucocorticoids [64] and estrogen, to participate in tumors. Therefore, inhibitors of these molecules can exert anti-breast cancer effects by inhibiting YAP. By inhibiting MMP-2 and MMP-9 [65], Fang promoted the activation of Casp3,8,9, increased the expression of Bax, and decreased the expression of Bcl2, thereby promoting apoptosis and cell cycle G1 phase arrest. Other studies have revealed the antiproliferation and proapoptotic effects of hesperidin on BC-related cell lines [66].

In this study, we identified 34 common targets between STR and BC, which are considered potential targets for STR for BC treatment. To explore the core targets, we used Cytoscape to construct the PPI network and conduct topological analysis of the targets for the treatment of BC with STR, and sorted them by value from largest to smallest. The top targets were TP53, JUN, CASP3, RELA, CCND1, etc., so we speculated that they played a more important role in the treatment of BC by STR. Then, in order to explore the mechanisms of STR against BC, we conducted core analysis of the target with the help of the IPA platform, which is pathway analysis software that can analyze canonical pathways, diseases and functions, and intermolecular interaction networks, etc. Therefore, we can comprehensively explore the molecular mechanism from multiple levels and perspectives. The disease function results showed that potential targets were involved in multiple disease function processes, such as tissue damage and abnormalities, cell death and survival, and cancer. It is common knowledge that the pathogenesis of BC is extremely complicated. Canonical pathway analysis showed that the target of STR was involved in multiple cancer-related pathways, including glucocorticoid receptor signaling, molecular mechanisms of cancer, and HER-2 signaling in breast cancer, the estrogen receptor signaling

pathway, and the erythropoietin signaling pathway. These mechanisms tend to be highly expressed in tumors. The glucocorticoid receptor signaling pathway has been reported to be dependent on integrin/Src activation and F-actin polymerization to activate YAP in BC, promoting development and metastasis [67]. Some drugs were reported to exert an anti-BC effect through inhibiting the glucocorticoid receptor signaling pathway. However, active components of STR have not been verified in this regard. Human epidermal growth factor receptor-2 (HER2), one of the most thoroughly studied genes in BC [68], is activated in BC and subsequently interacts with the PI3K/AKT pathway to regulate downstream targets. Activated AKT modulates downstream targets to produce different responses, promoting the development of tumors. In recent years, the erythropoietin signaling pathway has been reported to induce the transduction cascade of these signaling pathways, such as PI3K/AKT and MEK/ERK. Erythropoietin silencing inhibited the activation of PI3K/AKT and MAPK pathways, thereby promoting cell apoptosis [69]. We hypothesized that STR exerts antitumor activity by inhibiting these pathways. From our results, we found some targets played core roles in STR treatment of BC. TP53, encoding tumor suppressor protein, is the most commonly mutated gene in human cancers [70], including BC [71]. As a transcription factor, its target genes are mostly related to apoptosis and cell cycle regulation. RELA is present in all types of cells and is involved in the body's inflammatory response and apoptosis [72]. Activated AKT can increase the transcriptional activity of RELA and promote the migration ability of tumor cells. It has been reported that NOS3 can produce NO and promote angiogenesis [73]. NOS3 can promote inflammation and cell proliferation by promoting the binding of FOS and JUN to induce the release of IL-2 and the expression of CCND1 [74]. PTGS2, also known as COX2, is a key enzyme in the biosynthesis of prostaglandin, which is both a dioxygenase and a peroxidase [75]. It is an inflammatory mediator that can lead to the occurrence of inflammation. Among the first 10 pathways, RELA, JUN, TP53, CCND1, TGFB1, FOS, and PIK3CG with high frequency of participation were considered to be important. Combined with the results of PPI and IPA analysis, all 10 hub targets (TP53, JUN, CASP3, RELA, PTGS2, NOS3, CASP8, PIK3CG, IL2 and Bcl-2) were finally selected for molecular docking with the active components of STR to validate our network pharmacological prediction. Molecular docking data showed that the active components of STR had strong binding ability with these key targets, indicating that the active components act to regulate multiple targets simultaneously, rather than a single target agent. Based on the above results, this paper supports the scientific nature of virtual screening. To our knowledge, our cell experiments confirmed for the first time that Tet and Fang have more significant inhibitory effects on MDA-MB-231 cells, but less damage to MCF-10A cells. Although molecular docking results showed that Tet and Fang have better affinity for these targets, several other active components also showed good binding ability, suggesting that if these two major components are not suitable for drug development, other components can also be used as potential drugs.

The mechanisms predicted in this study mainly involved the glucocorticoid receptor signaling pathway, HER-2 signaling pathway, estrogen receptor signaling pathway, p53 signaling pathway, PI3K/AKT/mTOR signaling pathway, etc. These mechanisms have synergistic effects on the occurrence and development of breast cancer. Therefore, the high reliability of results for breast cancer is not in doubt. Compared with chemotherapy drugs, alternative therapy has multicomponent, multitarget, and multipathway therapeutic effects. Of course, this synergistic effect cannot be achieved by combining chemotherapy drugs, which may be a major advantage of all conventional alternative therapies. In complex diseases, especially for tumors, a single way of regulation may play a role, but most can only be effective in the short term, and most of the chemotherapy drugs have a target-based, killing effect on normal cells and adverse reactions occur. The safety of TCM is relatively greater, as shown in in vitro results, so there are fewer adverse reactions. However, this alternative therapy also has multicomponent and multitarget characteristics, which increases the difficulty of research. The mechanism we found is common, and there may still be many unknown black box mechanisms that need to be mined.

Certainly, this study is innovative as the first to systematically explore the mechanisms of STR in the treatment of breast cancer, but there are still some limitations to this study. Firstly, since we used many databases, the accuracy and timeliness of the information needs to be scientifically validated. Secondly, due to financial and time constraints, we only used molecular docking for target validation, which may need to be confirmed by experimental studies in the future. However, there is a large body of research on network pharmacology and molecular docking that has been experimentally validated, so we believe that our results are highly promising.

5. Conclusions

In this study, we systematically revealed six active components of STR (including Tet and Fang) and the underlying molecular mechanisms that inhibit cell proliferation and inflammation and promote apoptosis through the regulation of TP53, JUN, CASP3, RELA, and so on, thereby exerting antitumor effects in the treatment of BC. Our study provided a basis for further study of STR therapy and a reference for the screening of drugs for the treatment of BC.

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