

## Retraction

# Retracted: Identifying and Verifying AR, ERBB2, and VEGFA Are the Targets of Qigesan in the Treatment of Esophageal Adenocarcinoma In Silico and In Vitro

### Scientific Programming

Received 8 August 2023; Accepted 8 August 2023; Published 9 August 2023

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This article has been retracted by Hindawi following an investigation undertaken by the publisher [1]. This investigation has uncovered evidence of one or more of the following indicators of systematic manipulation of the publication process:

- (1) Discrepancies in scope
- (2) Discrepancies in the description of the research reported
- (3) Discrepancies between the availability of data and the research described
- (4) Inappropriate citations
- (5) Incoherent, meaningless and/or irrelevant content included in the article
- (6) Peer-review manipulation

The presence of these indicators undermines our confidence in the integrity of the article's content and we cannot, therefore, vouch for its reliability. Please note that this notice is intended solely to alert readers that the content of this article is unreliable. We have not investigated whether authors were aware of or involved in the systematic manipulation of the publication process.

Wiley and Hindawi regrets that the usual quality checks did not identify these issues before publication and have since put additional measures in place to safeguard research integrity.

We wish to credit our own Research Integrity and Research Publishing teams and anonymous and named external researchers and research integrity experts for contributing to this investigation.

The corresponding author, as the representative of all authors, has been given the opportunity to register their

agreement or disagreement to this retraction. We have kept a record of any response received.

### References

- [1] D. Zhang, L. Guo, X. Wu et al., "Identifying and Verifying AR, ERBB2, and VEGFA are the Targets of Qigesan in the Treatment of Esophageal Adenocarcinoma In Silico and In Vitro," *Scientific Programming*, vol. 2021, Article ID 5463724, 15 pages, 2021.

## Research Article

# Identifying and Verifying AR, ERBB2, and VEGFA Are the Targets of Qigesan in the Treatment of Esophageal Adenocarcinoma In Silico and In Vitro

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Received 14 July 2021; Revised 3 August 2021; Accepted 14 August 2021; Published 25 August 2021

Academic Editor: Gustavo Ramirez

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The Chinese medicine Qigesan can be used to treat esophageal adenocarcinoma in the Chinese mainland widely, but its mechanism is unclear. In order to investigate the mechanism of Qigesan in the treatment of esophageal adenocarcinoma, the concept of network pharmacology was used in this study. The database named TCMSP was used to identify the active therapeutic components as well as targets of Qigesan. The TTD, OMIM, CTD, DrugBank, and GeneCards database were used to identify genes related to esophageal adenocarcinoma. In STRING database, the potential targets were imported to obtain a PPI network, and then Cytoscape software has been used to analyse the results. Subsequently, important components and targets were simulated by molecular docking. Finally, experiments on the cell have been done to verify well docking targets. A total of 124 effective compounds and 646 corresponding targets were filtered. 1478 genes were found to be related to esophageal adenocarcinoma. 68 genes were identified as potential targets for esophageal adenocarcinoma. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of the 68 potential targets indicated that the genes were mainly involved in cell transcription, translation, and apoptosis and mostly expressed in cancer-related pathways. The molecular docking analysis of the hub targets with their corresponding compounds indicated that the well docking targets were AR, ERBB2, and VEGFA. The cell experiments showed that Qigesan can reduce the expression of AR, ERBB2, and VEGFA at transcription and translation level. This network pharmacology study described that the possible targets of Qigesan in treatment of esophageal adenocarcinoma were AR, ERBB2, and VEGFA.

## 1. Introduction

Esophageal cancer is known as the most commonly occurring cancers with incidence rate and mortality among the top ten in all cancers [1]. Esophageal cancer includes esophageal squamous cells (ESSC) and esophageal adenocarcinoma (EAC). There are obvious regional differences between these two types [2, 3]. EAC is mainly distributed in Europe and America, and esophageal squamous cell carcinoma is mainly distributed in Asia, Africa, and South America [4]. Esophageal squamous cell carcinoma accounts

for 90% of all esophageal cancers. However, with the adjustment of dietary structure, the incidence rate of esophageal adenocarcinoma is increasing year by year [5, 6]. There is a significant gender bias in esophageal adenocarcinoma. Men are more likely to suffer from esophageal adenocarcinoma than women [7]. The common risk factors of esophageal adenocarcinoma include obesity, smoking, alcohol abuse, and esophageal reflux [8]. Barrett's esophagus is widely considered as a precancerous lesion of esophageal adenocarcinoma. The patients with esophageal adenocarcinoma have worse than that of most onco patients [9]. Only

1/5 patients have a 5-year survival rate [10, 11]. The main treatment of esophageal adenocarcinoma includes surgery, chemotherapy, radiotherapy, targeted therapy, and immunotherapy [12]. In China, the traditional Chinese medicinal treatment is widely used to treat malignant tumors, which can reduce the symptoms of patients and finally improve the patient's quality of life [13].

Qigesan (QGS) was formulated by Zhongling Cheng, a famous doctor in the Qing Dynasty, and recorded in his book "Yi Xue Xing Wu." Qigesan is composed of seven herbs: *Glehniae Radix* (*Bei sha sheng*), *Radix Salviae* (*Dan shen*), *Wolf* (*Fu ling*), *Fritiliariae Cirrhosae Bulbus* (*Chuan bei mu*), *Curcumae Radix* (*Yu jin*), *Amomum Aurantiacum* (*H. T. Tsai et S. W. Zhao* (*Sha ren*), and *Folium Nelumbinis* (*He ye*). Qigesan is mainly used for the treatment of "Yege," which is a term in Traditional Chinese Medicine equivalent to esophageal cancer in modern medicine. It has been suggested that Qigesan can reduce the invasion of esophageal malignant cells in vitro [14, 15]. Therefore, some Chinese doctors have used Qigesan to treat EAC. In recent years, some clinical observations have suggested that Qigesan may relieve the symptoms and clinical pathology of EAC [16–18]. However, its specific mechanism has not been studied.

In 2007, Hopkins developed the concept of network pharmacology in Nature Biotechnology and, in 2008, suggested that network pharmacology would become the next generation of drug development models [19]. Network pharmacology is used systematically and comprehensively to study the effects of drugs on the body, specifically for "drug-target-diseases" [20]. Network pharmacology is based on diseases considered to be a combination of multifactor and multitarget networks. Traditional Chinese Medicine treats diseases through multiple targets, multiple components, and multiple pathways, in line with the premise of network pharmacology. In order to predict the molecular mechanism of Traditional Chinese Medicine, network pharmacology is an innovative research method.

In the recent studies, the concept of network pharmacology has been applied to predict the molecular mechanism of Qigesan in EAC treatment.

We performed the following steps:

- (1) The TCMSP database has been used to fetch the effective components in Qigesan by employing an ADME approach
- (2) The genes known to be involved in EAC were identified
- (3) Qigesan targets and the EAC-related genes were compared to construct a component-target-pathway network and determine the hub targets of the herb components
- (4) The method of molecular docking simulation was applied to study the binding efficiency of the hub target and their corresponding compounds
- (5) Cell experiments were used to verify the effective targets

Figure 1 shows the workflow we designed.

## 2. Materials and Methods

**2.1. Chemical Components of Each Herb in Qigesan.** An online database named TCMSP was used to determine the chemical components of the 7 herbs in Qigesan. TCMSP is a Chinese herbal pharmacology database and platform that integrates the close relationships between diseases, targets, and drugs [21]. It also contains information about the pharmacokinetic features of herbal compounds such as their oral bioavailability, blood-brain barrier permeability, water solubility, intestinal epithelial permeability, and drug similarities [22].

**2.2. Predicting Active Compounds and Corresponding Targets.** In pharmacokinetics, ADME represents four processes: absorption, distribution, metabolism, and excretion. Oral bioavailability (OB), Caco-2 cell permeability, and drug similarity (DL) are important indexes to evaluate pharmacokinetics [23].

OB (oral bioavailability), one of the key indices in drug research, can be defined as the amount of drug that eventually enters the circulatory system through metabolism. Low oral bioavailability leads to inefficiency and instability, which may lead to unpredictable responses to drugs and candidates that fail to enter the market. According to the information in the TCMSP database, 30% was recommended as an OB filtering standard. Here, we adopted this criterion. In this study, we considered components with an OB value  $\geq 30\%$  to be potentially effective [23].

DL (drug-likeness) is a vague definition widely applied in drug research and development; in general, DL is defined as the molecular similarity of a drug based on their known structures and functions. Drug effectiveness is estimated based on drug molecular structure before it is synthesized and/or tested. We use 0.18 as the threshold standard for DL in this study [23]. Thus, we regarded compounds with a DL value  $\geq 0.18$  as an active compound.

Human colon cancer can develop from Caco-2 cells. The monolayer formed by Caco-2 cells can simulate the absorption of the small intestine and is widely used in the evaluation of true drug uptake. The screening standard using Caco-2 cells is based on an absorbance value of  $-0.4$ , and compounds with a value  $\geq -0.4$  were considered to be adequately absorbed [23].

Therefore, screened candidates must meet the criteria of an OB value  $\geq 30\%$ , a DL value  $\geq 0.18$ , and a Caco-2 absorbance value  $\geq -0.40$  to be included in additional analysis.

Then, we used the TCMSP database to process the screened compounds and identify the corresponding targets. Finally, we used the UniProt database (<http://www.uniprot.org>) so as to convert target description information into the name of genes.

**2.3. Known Therapeutic Targets for Esophageal Adenocarcinoma.** DugBank ([go.drugbank.com](http://go.drugbank.com)), CTD ([ctdbase.org](http://ctdbase.org)), TTD ([db.ibrblab.net/ttd/](http://db.ibrblab.net/ttd/)), OMIM ([omim.org](http://omim.org)), and GeneCards (<http://www.genecards.org>) were used to search for known therapeutic targets of EAC.

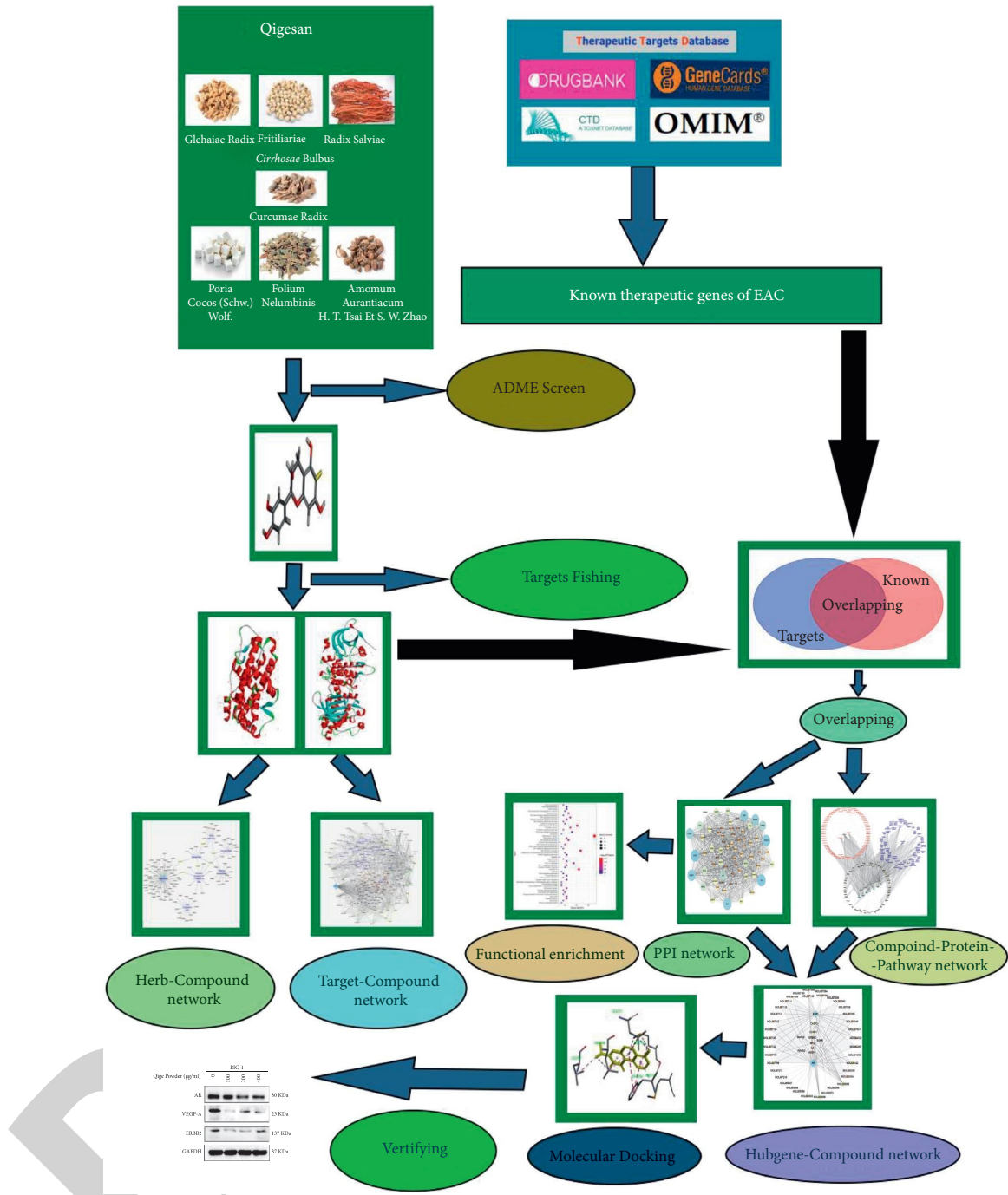


FIGURE 1: Flowchart of data analysis.

DrugBank is an online open source database that includes content about drugs and drug targets [24]. CTD is a database on the relationship between environmental exposure and disease [25]. The Therapeutic Target Database (TTD) presents information about known or currently explored protein and nucleotide targets that can be used for treatment, as well as information about target diseases, target pathways, and drugs/ligands corresponding to these targets [26]. Online Mendelian Inheritance in Man (OMIM) provides continuous updates on human genes and genetically

related diseases with a particular focus on genes and phenotypes [27]. This database aims to raise awareness of the relationship between environmental exposure and disease. It includes information on the interactions between chemicals, genes, and proteins and the relationships among chemical diseases and gene-related diseases [27]. GeneCards includes detailed annotation information of human genes and relevant prediction information [28].

We identified genes in these databases and then removed duplications in the list.

**2.4. Identification of Compound Therapy Genes and Screened Hub Targets.** We took the intersection of EAC-related genes and targets of compound therapy and named the part of intersection genes as compound therapy genes. We introduced compound therapy genes into the STRING online tool (<https://string-db.org>) for constructing the PPI network. We preserved PPI pairs that had a combined score greater than 0.4 and removed duplicates. Subsequently, we imported the results into Cytoscape software (version 3.6.1). We used the software plug-in CytoHubba to calculate the network. Degree, betweenness, and closeness, the three main indicators, were applied for evaluating significance of nodes in the network [29, 30]. Next, we identified the intersection of the top 15 genes in terms of degree, betweenness, and closeness and regarding overlapping parts as hub targets.

**2.5. KEGG and GO Enrichment Analyses of the Compound Therapy Genes.** DAVID, an online bioinformatics analysis tool, was used for KEGG (Kyoto Encyclopedia of Genes and Genomes) and GO (Gene Ontology) pathway enrichment analyses. GO includes MF (molecular function), BP (biological process), and CC (cellular component). In the current study, results with differences of  $P \leq 0.05$  were used as a significant one.

**2.6. Network Visualization.** The network was constructed as follows:

- (1) The herb-candidate compound (H-C) network was built with herb and candidate compounds.
- (2) The compound-target (C-T) network was built with herbs and target candidates.
- (3) The PPI network was constructed by importing compound therapy genes into the STRING database.
- (4) Compounds, targets, and pathways were used to build a compound-candidate target-pathway (C-T-P) network.
- (5) Compounds and hub targets have been used to build a candidate compound-hub target network. Cytoscape version 3.6.1 ([cytoscape.org](http://cytoscape.org)), open source software for data network visualization, was used to build these networks.

**2.7. Molecular Docking.** Discovery studio is widely used in research on protein function and drug action. The process of molecular docking is being used to predict the affinity between a large molecule receptor and the small-molecule based on their structural characteristics and to evaluate the intensity of their effects. This computer simulation method has become one of the important methods for drug design and development. In the current study, we used LibDock, an algorithm used for molecular docking, to evaluate the extent of molecular docking between hub targets and corresponding compounds. We downloaded the hub target 3D crystal structure from RCSB (<http://www.rcsb.org/>) and chose a relatively high-resolution structure (to ensure a high-quality protein structure). PubChem (<http://pubchem.ncbi.nlm.nih.gov>)

was the source of the 3D structures of the corresponding compounds. After docking, we ranked the LibDock scores in descending order for all the docking combinations and calculated the median. Docking combinations (hub targets and corresponding compounds) that had LibDock scores higher than the median were regarded as well docking.

**2.8. Preparation of Qigesan.** Drug composition is as follows: *Salvia miltiorrhiza* 90 g, *Radix Glehniae* 90 g, *Poria Cocos* 90 g, *Fritillaria Fritillariae* 90 g, *Yujin* 15 g, *Amomum Villosum* 15 g, and *Lotus* leaf 15 g. All the herbs were purchased from Xingyuanchun pharmacy in Guangzhou. The above dosage is equivalent to that of 60 kg adult for 3 days. The drug was immersed in 2000 ml ultrapure water for 30 min, subsequently the drug was boiled for 90 min, and then the drug solution was collected. 2000 ml ultra pure water was added to the residue again, and the boiling operation is repeated. The liquid of herbs collected twice was concentrated by rotary evaporation and freeze-drying to obtain 25.6 g Qigesan freeze-dried powder. Before the test, 1 g of Qigesan freeze-dried powder was prepared into 100 mg/ml, filtered and sterilized with 0.22  $\mu\text{m}$ , and stored in the refrigerator at  $-20^{\circ}\text{C}$ .

**2.9. Reagents.** Reagents: DMEM (Gibco, Lot:8120280); trypsin digested solution (Gibco, Lot:2085459); fetal bovine serum (Gibco, Lot: 2168090RP); PBS (Hyclone, Lot: AE29451445); anti-AR antibody (CST,19672S); anti-VEGFA antibody (Abcam, ab46154); anti-ERBB2 antibody (CST, 4290S); anti-GAPDH antibody (Abcam, ab8245); cell counting kit-8 (Beyotime, China); RT reagent kit with gDNA eraser (Takara, Lot: AJ92013 A); and Premix Ex Taq™ II (Takara, Lot: AJ91436A).

**2.10. Cell Lines and Cell Culture.** BIC-1 cell has been brought from Shanghai, the Cell Bank of Chinese Academy of Sciences. Cell culture (10% FBS) was performed in a moist, 5% carbon dioxide,  $37^{\circ}\text{C}$  incubator.

**2.11. CCK8 Assay.** The cells have been well seeded on 96 well plates with  $2 \times 10^3$  cells/well. Subsequently, the cells have been treated with six different concentration gradients (0, 25, 50, 100, 200, and 400  $\mu\text{g}/\text{ml}$ ), and three multiple pores were set for each concentration. CCK8 was detected at 12 h, 24 h, 48 h, and 96 h. When CCK8 was detected, 10  $\mu\text{L}$  CCK8 reaction solution was directly added and incubated in dark at  $37^{\circ}\text{C}$  for 2 h; the absorbance at 450 nm wavelength was measured by enzyme labeled instrument for reading. The cell proliferation rate was the absorbance value of the drug group/the absorbance value of the control group  $\times 100\%$ .

**2.12. Cell Counting.** The cells have been inoculated into 6-well plate according to  $5 \times 10^5$  cells/well. The cells were washed with phosphate buffer (PBS) and digested with trypsin to prepare single-cell suspension. Then, the cells were counted by cell counting plate. Each group is provided with 3 compound holes.

**2.13. qPCR Assay.** The treated cell samples were washed with PBS, and then the total RNA has been extracted with trizol, and the concentration was determined on a microspectrophotometer. The total RNA was translated into cDNA by reverse transcription kit and then amplified by Premix Ex Taq™ II. The system was 10  $\mu$ L with 3 complex pores in each group. The reaction conditions were as follows: pre-denaturation at 95°C for 3 min, 95°C for 5 s, 55°C for 34 s, 72°C for 60 s, and 40 cycles. The expression of gene was analysed by 2<sup>- $\Delta\Delta$</sup>  CT formula.

**2.14. Western Blotting Analysis.** After being treated with Qigesan, BIC-1 was added with protein lysate to collect cell protein samples. After quantitative analysis, the same amount of the multiple protein samples has been separated by the technique named SDS-PAGE electrophoresis and transferred into membrane. 5% skimmed milk powder was sealed for 1 h, and diluted primary antibodies (AR, VEGFA, ERBB2, and GAPDH) were added and incubated at room temperature for 1 hour. After washing, HRP labeled secondary antibody was added and incubated at 25° celsius for 1 hour. After washing, ECL developer was added to take photos, and the software was used for statistical analysis of gray value.

**2.15. Statistical Analysis.** All the data were statistically analysed by using SPSS. The measurement data have been presented as mean  $\pm$  standard error ( $x \pm S.E.M$ ), and the count data were expressed as percentage. The variance analysis has been done to compare the mean of multiple groups.  $P < 0.05$  was considered as statistically significant.

### 3. Results and Discussion

**3.1. Screening of Active Compounds.** After screening with the ADME filter standard that we set, a total of 124 compounds were obtained from 7 herbs contained in Qigesan (Table S1). Among these 124 compounds, *Glehniae Radix* (*Bei sha shen*) had 8 compounds, *Fritillariae Cirrhosae Bulbus* (*Chuan bei mu*) had 13 compounds, *Radix Salviae* (*Dan shen*) had 61 compounds, *Poria Cocos* (*Fu ling*) had 15 compounds, *Folium Nelumbinis* (*He ye*) had 13 compounds, *Amomum villosum Lour* (*Sha ren*) had 10 compounds, and *Curcumae Radix* (*Yu jin*) had 13 compounds (Supplementary Figure 1A). *Bei sha shen*, *Chuan bei mu*, *Sha ren*, and *Yu jin* share the MOL000358 compound. *Chuan bei mu*, *He ye*, and *Yu jin* share the MOL000359 compound. *Bei sha shen* and *Dan shen* share the MOL001942 compound. *Bei sha shen* and *He ye* share the MOL000098 compound. *Bei sha shen* and *Sha ren* share the MOL000449 compound. *Dan shen* and *Sha ren* share the MOL001771 compound. These results suggested that *Dan shen* contained the most compounds of the all.

**3.2. Prediction of Qigesan Therapeutic Targets.** Through the TCMSP database and UniProt database, we identified 646 targets of the identified compounds (Table S2) and removed

duplicates; 116 targets were retained for further analysis (Supplementary Figure 1B).

**3.3. Therapeutic Targets of Esophageal Adenocarcinoma.** The results of searching the DrugBank, TTD, OMIM database, CTD, and GeneCards databases were as follows.

No targets of EAC treatment were found in the DrugBank database or the TTD database; 7 targets were found in the OMIM database; 838 targets were found in the CTD database; and 746 targets were found in the GeneCards database (Table 1). We combined the 7 targets in the OMIM database (Table S3), 838 targets in the CTD database (Table S4), and 746 targets in GeneCards (Table S5), deleted duplications, and obtained 1478 targets finally.

**3.4. Screening Compound Therapy Genes and Hub Targets.**

We ultimately identified 68 compound therapy genes (Figure 2(a)). To further analyse these compound therapy genes, we constructed a PPI network. And the results showed that the complex network had 68 nodes and 668 edges, and the average degree value was calculated to be 19.6 (Figure 2(b)). Indicators of degree, betweenness, and closeness were used to evaluate the importance of gene interactions. The top 15 genes identified on the indicators of degree, betweenness, and closeness were merged and extracted the overlapping genes (Figure 2(c)). Overlapping genes were VEGFA, CCND1, CASP3, EGFR, MAPK8, PPARG, AR, IL6, ESR1, ERBB2, and MYC, and they were regarded as hub targets.

**3.5. Functional Enrichment Analysis of the Compound Therapy Genes.** We found that the compound therapy genes involved 17 cellular components (CCs), 109 biological processes (BPs), 37 molecular functions (MFs), and 48 KEGGs of a significant level (Figure 3).

Top 20 BP terms in gene numbers of the compound therapy genes were mainly enriched involving the positive regulation of transcription from the RNA polymerase II promoter, transcription, negative regulation of the apoptotic process, positive regulation of transcription, response to drugs, positive regulation of gene expression, negative regulation of transcription from the RNA polymerase II promoter, and apoptotic processes (Figure 3(a)).

Top 10 CC terms in gene numbers of compound therapy genes were mainly enriched in the cytoplasm, nucleus, cytosol, nucleoplasm, and plasma membrane (Figure 3(b)).

Top 10 MF terms in gene numbers of compound therapy genes were mainly enriched involved identical protein binding, enzyme binding, protein binding, DNA binding, sequence-specific DNA binding, and transcription factor activity (Figure 3(c)).

Top 10 KEGG pathway terms in gene numbers of compound therapy genes have been enriched in pathways in cancer, microRNAs in cancer, hepatitis B, and proteoglycans in cancer (Figure 3(d)).

TABLE 1: The number of genes in the database that cause EAC.

Database	Gene number
Drugbank	0
TTD	0
OMIM	7
CTD	838
GeneCards	746

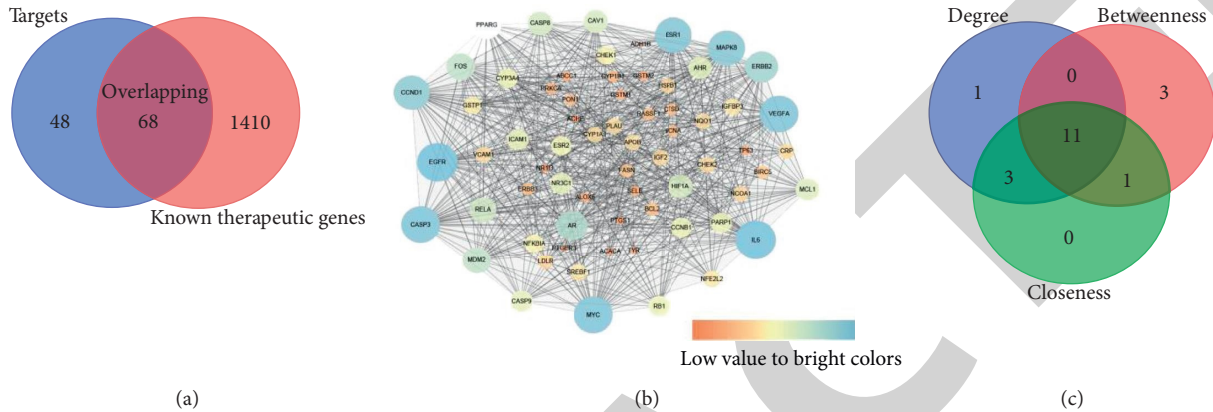


FIGURE 2: (a) Venn diagram of compounds therapy genes for EAC. Overlapping parts: compounds therapy genes for EAC. (b) PPI network of compounds therapy genes for EAC. Node size is proportional to its degree. Node brightness is inversely proportional to its degree. (c) Venn diagram of hub targets in compounds therapy genes for EAC. Overlapping genes were VEGFA, CCND1, CASP3, EGFR, MAPK8, PPARG, AR IL6, ESR1, ERBB2, and MYC, and they were regarded as hub targets.

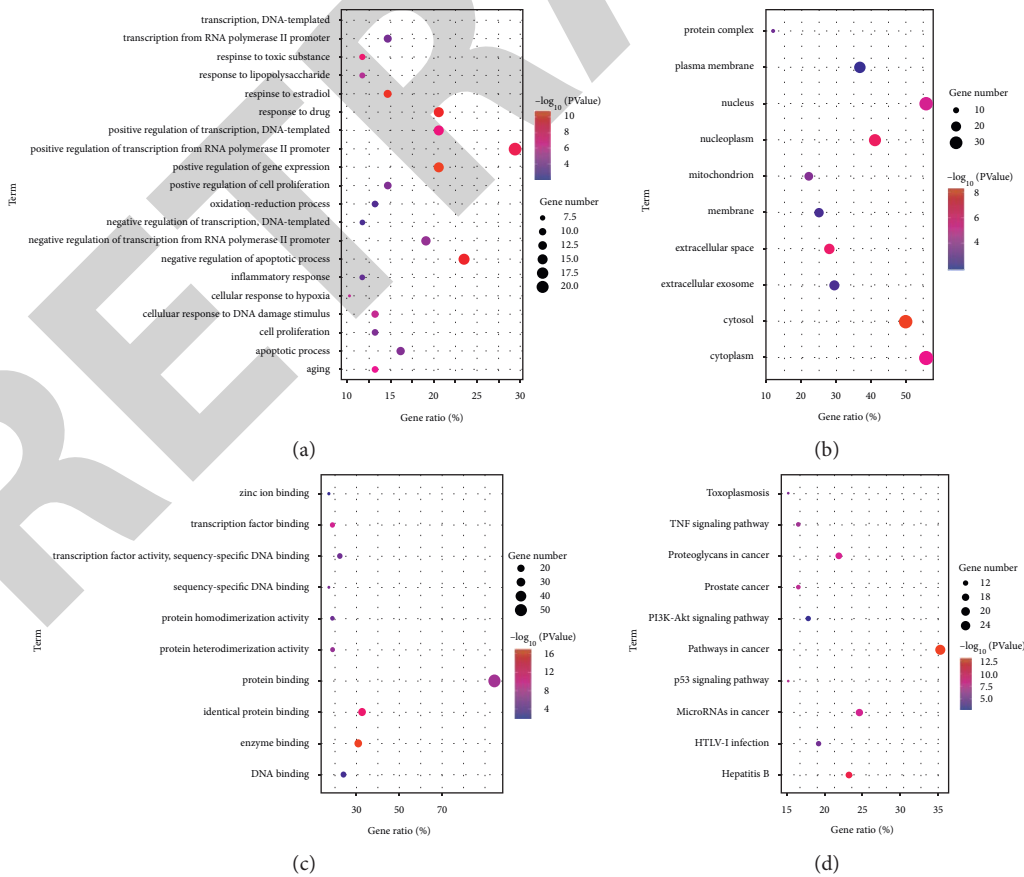


FIGURE 3: (a) Top 20 BP enrichment of compounds therapy genes for EAC. (b) Top 10 CC enrichment of compounds therapy genes for EAC. (c) Top 10 MF enrichment of compounds therapy genes for EAC. (d) Top 10 KEGG pathway enrichment of compounds therapy genes for EAC.

**3.6. Network Construction.** There were 222 nodes and 1292 edges in the compound-target (C-T) network. Out of these 222 nodes, 86 were compounds and 116 were targets. According to the degrees of the nodes, the top four compounds were MOL000098 (quercetin, degree = 73), MOL000422 (kaempferol, degree = 31), MOL000006 (luteolin, degree = 23), and MOL007154 (tanshinone IIa, degree = 21). The top six targets were PTGS1 (degree = 39), CHRM1 (degree = 39), NCOA1 (degree = 34), CHRM3 (degree = 32), ACHE (degree = 31), and ADRA1A (degree = 30) (Supplemental Figure 1B).

The compound-target-pathway (C-T-P) network consisted of 185 nodes and 1388 edges; 185 nodes contained 69 compounds, 68 proteins, and 48 pathways. The top five compounds ranked by degree were MOL000098 (quercetin, degree = 52), MOL000422 (kaempferol, degree = 21), MOL000006 (luteolin, degree = 20), MOL007154 (tanshinone IIa, degree = 12), and MOL004328 (naringenin, degree = 12). The top five targets ranked by degree were PTGS1 (degree = 39), NCOA1 (degree = 35), RELA (degree = 34), ACHE (degree = 31), and AR (degree = 30). The top four pathways ranked by degree were pathways in cancer (degree = 24), microRNAs in cancer (degree = 16), hepatitis B (degree = 15), and proteoglycans in cancer (degree = 14) (Figure 4(a)).

The compounds-hub targets network was composed of 48 nodes and 144 edges. According to the degrees of the compounds, the top four were MOL000098 (quercetin, degree = 8), MOL000006 (luteolin, degree = 6), MOL000422 (kaempferol, degree = 3), and MOL007156 (tanshinone VI, degree = 3). According to the degrees of hub targets, the top four were AR (degree = 28), ESR1 (degree = 23), CASP3 (degree = 7), and CCND1 (degree = 3) (Figure 4(b)).

**3.7. Molecular Docking of the Compounds and Hub Targets.** After removing compounds and hub targets without a 3D structure, 33 compounds and 11 target proteins were assessed (Table S6). LibDock has been used for molecular docking the compounds and hub targets. The LibDock score was used to evaluate the extent of molecular docking. The specific data are presented in Table 2. The median LibDock score was 99.36525. The LibDock scores of the molecular docking that were higher than the median are shown in Table 3 and Figures 5 and 6.

Luteolin, tanshinonVI, quercetin, miltipolone, isotanshinoneII, isorhamnetin, isocryptotanshinone, deoxycryptotanshinone, dehydrotanshinoneIIA, anshexinku d, alloisioimperatorin, and 2-isopropyl-8-methylphenanthrene were active ingredients of Qigesan (Table 3). The potential targets of Qigesan were AR, VEGFA, and ERBB2 (Table 3).

**3.8. Effective Therapeutic Concentration of Qigesan.** We used the CCK8 method to observe the proliferation of BIC-1 cells treated with different concentrations of Qigesan at different time points. At the same time point, the relative proliferation rate was obtained by dividing the absorbance value of each group into blank control group. We selected the relative

proliferation rate of 0.5 as the screening standard of effective concentration of Qigesan. It can be seen from Figure 7(a) that after treating BIC-1 cells with Qigesan for 96 h, the relative proliferation rate of BIC-1 cells at 100  $\mu\text{g}/\text{mL}$ , 200  $\mu\text{g}/\text{mL}$ , and 400  $\mu\text{g}/\text{mL}$  meets the pharmacodynamic standard. In this study, the effect of Qigesan on the proliferation of BIC-1 cells was investigated. The concentration of 100  $\mu\text{g}/\text{mL}$ , 200  $\mu\text{g}/\text{mL}$ , and 400  $\mu\text{g}/\text{mL}$  was selected as concentration of drug, and observation time point was 96 h. The results showed that Qigesan had inhibitory effect on the proliferation of BIC-1 cells, and there was a positive correlation between Qigesan and the dose in a certain range.

**3.9. Effect of Qigesan on Cell Number.** The results (Figure 7(b)) showed that the number of BIC-1 cells treated with Qigesan of different effective concentrations has been detected to be significantly lower than that of the normal group, and there was a certain dose correlation, and the results were statistically significant.

**3.10. Effect of Qigesan on the AR, VEGFA, and ERBB2.** The relative mRNA expression levels of AR, VEGFA, and ERBB2 in BIC-1 cells were detected by qPCR (Figure 7(c)). Compared with the normal group, the mRNA expression levels of AR, VEGFA, and ERBB2 in BIC-1 cells treated with Qigesan were decreased, and there was a certain dose-dependent, and the difference was statistically significant.

The protein expression levels of AR, VEGFA, and ERBB2 in BIC-1 cells treated with Qigesan at different effective concentrations were detected (Figures 7(d) and 8). It was found that the protein levels in the medication group have been detected as significantly lower than those in the usual control group, and their difference has been observed as statistically significant.

## 4. Discussion

EAC is known as a most commonly occurring malignant tumors [31]. Qigesan, a classical prescription for the treatment of esophageal cancer, has been used for nearly 200 years in China. Qigesan is a commonly used adjuvant therapy, which can enhance the effect of other antitumor drugs and improve the patients' quality of life. TCM (Traditional Chinese Medicine) has its own characteristics and advantages for the treatment of EAC, but many of its mechanisms have not yet been elucidated. Network pharmacology, based on the integration and systematization of drug, target, and disease interactions, is based on a complex network model and used to express and analyse the pharmacological properties of a research object. Network pharmacology is especially suitable for explaining the multicomponent and multitarget interaction relationship typical of Traditional Chinese Medicine and is expected to generate breakthroughs in the research of the Traditional Chinese Medicines.

In the current study, we screened databases to obtain 124 target compounds. Among these compounds, *Salvia miltiorrhiza* (*Dan shen*) contained 61 compounds, accounting



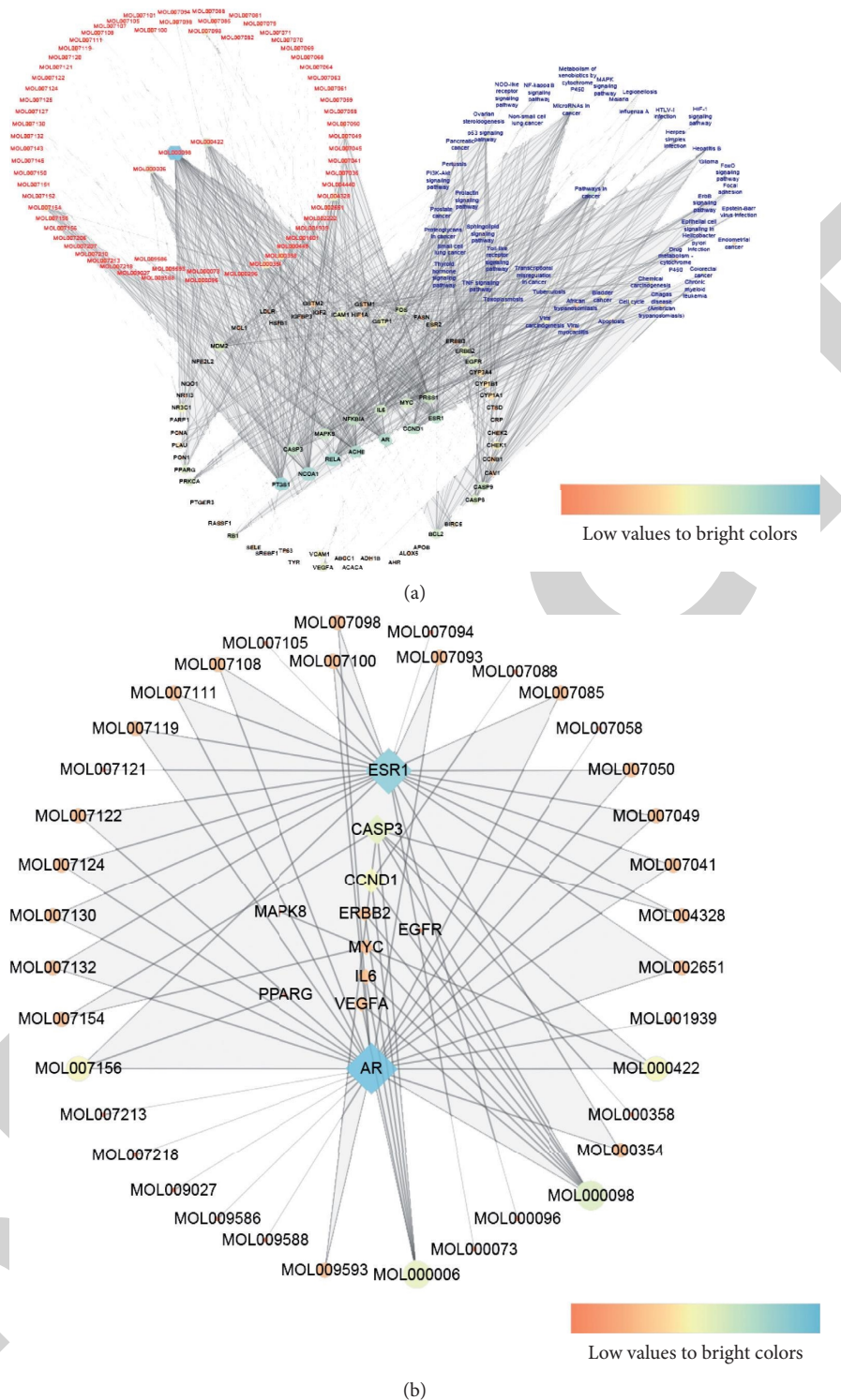


FIGURE 4: (a) Compounds-targets-pathways of compounds therapy genes for EAC. Node size is proportional to its degree. Node brightness is inversely proportional to its degree. (b) Compounds-hub targets network of compounds therapy genes for EAC. Node size is proportional to its degree. Node brightness is inversely proportional to its degree.

for 49.19% of all. According to the theory of *Jun-chen-zuo-shi* in Traditional Chinese Medicines, *Salvia miltiorrhiza* (*Dan shen*) in Qigesan is considered *Jun* medicine. In terms of the number of active compounds contained in the herbs of Qigesan, *Salvia miltiorrhiza* (*Dan shen*) is the most

abundant, reflecting the important role of *Salvia miltiorrhiza* (*Dan shen*) in Qigesan.

According to the corresponding target of the compound, we ultimately identified 116 therapeutic targets. By comparing 116 therapeutic targets of Qigesan with 1478 EAC

TABLE 2: Molecular docking between the hub targets and the corresponding compounds.

Molecule	Gene	LibDock score
Tanshinone VI	AR	103.709
Salvilenone	AR	90.335
Remerin	AR	98.7312
Quercetin	AR	115.901
Nuciferine	AR	71.5803
Miltipolone	AR	108.859
Luteolin	AR	118.843
Isotanshinone II	AR	105.921
Isorhamnetin	AR	112.888
Isocryptotanshinone	AR	109.186
Deoxyneocryptotanshinone	AR	107.894
Dehydrotanshinone II A	AR	99.9993
Dan-shexinkum d	AR	109.177
Alloisimperatorin	AR	106.422
2-Isopropyl-8-methylphenanthrene	AR	101.429
Luteolin	CCND1	95.383
Quercetin	CCND1	97.1556
Cryptotanshinone	CCND1	87.7003
Quercetin	EGFR	82.2235
Quercetin	ERBB2	101.97
Luteolin	ERBB2	117.747
Tanshinone VI	ESR1	44.4754
Salvilenone	ESR1	48.801
Isotanshinone II	ESR1	39.588
Isorhamnetin	ESR1	46.5291
Epidanshenspiroketallactone	ESR1	53.6678
Ent-epicatechin	ESR1	67.6416
Dehydrotanshinone II A	ESR1	35.7066
(-)-Catechin	ESR1	59.1748
Naringenin	ESR1	67.5812
Quercetin	VEGFA	100.195
Luteolin	VEGFA	106.983

TABLE 3: Top 16 molecular docking between the hub targets and the corresponding compounds.

Molecule	Gene	LibDock score
Luteolin	AR	118.843
Luteolin	ERBB2	117.747
Quercetin	AR	115.901
Isorhamnetin	AR	112.888
Isocryptotanshinone	AR	109.186
Dan-shexinkum d	AR	109.177
Miltipolone	AR	108.859
Deoxyneocryptotanshinone	AR	107.894
Luteolin	VEGFA	106.983
Alloisimperatorin	AR	106.422
Isotanshinone II	AR	105.921
Tanshinone VI	AR	103.709
Quercetin	ERBB2	101.97
2-Isopropyl-8-methylphenanthrene	AR	101.429
Quercetin	VEGFA	100.195
Dehydrotanshinone II A	AR	99.9993

disease genes, we obtained 68 genes. These 68 genes were the targets of Qigesan in the treatment of EAC. Sixty-eight targets accounted for 58.62% of all the targets of Qigesan. Quercetin, kaempferol, luteolin, and tanshinone are the most abundant components in Qigesan. For 52 of the therapeutic targets, quercetin was identified as a treatment

for EAC, accounting for 76% of the 68 targeted; 21 genes were identified as targets for kaempferol as a treatment for EAC, accounting for 31% of the 68 genes; 20 genes were identified as targets for luteolin as a treatment for EAC, accounting for 29% of the 68 genes; and 12 genes were identified as targets for tanshinone in the treatment of EAC, accounting for 17% of the 68 genes. Thus, it can be concluded that Qigesan offers some specificity in the treatment of EAC.

Sixty-eight targets were analysed for functional enrichment. According to the results of the GO analysis, the BPs enriched with genes were mainly focused on regulating gene transcription and translation, while the occurrence of cancer was closely related to gene mutations, which may be one of the mechanisms by which Qigesan treats EAC. From the GO analysis results, the MF enriched with genes for which the treatment mainly had an effect was the binding of DNA and protein, which suggest that Qigesan may affect the progress of EAC by regulating the transcription and translation of related genes. Moreover, the results of the KEGG pathway analysis mainly focused on the cancer pathway. We speculate that Qigesan may prevent or delay the progression EAC by affecting cell translation and transcription-related activities, such as proliferation.

In the present research study, we identified hub genes, namely, VEGFA, CCND1, CASP3, EGFR, MAPK8, PPARG, AR, IL6, ESR1, ERBB2, and MYC, by topological structure calculations during the PPI network analysis of the 68 therapeutic targets. The genes related to cancer were VEGFA, CCND1, EGFR, PPARG, ESR1, ERBB2, and MYC [32–38]. These cancer-related genes accounted for more than 60% of the total number of hub genes. Interestingly, when constructing the compound-target-pathway network (Figure 4(a)), it was obvious that most of the hub genes occupied an important position in the network and were closely related to the occurrence of malignant tumors. Qigesan may be able to treat EAC by acting on these targets.

To further elucidate the relationship between important genes in the network and their corresponding targets, we used LibDock to simulate molecular docking. The results of the molecular docking showed that AR, VEGFA, and ERBB2 were well docking. So, we selected AR, VEGFA, and ERBB2 as potential targets for Qigesan in treatment of EAC.

AR, VEGFA, and ERBB2 are closely related to cell proliferation [39–41]. So, we screened the effective concentration of Qigesan by inhibiting the proliferation of BIC-1 cells. The results showed that Qigesan could inhibit the proliferation of BIC-1 cells, and there was a positive dose correlation in a certain range.

Through cell experiment, we found that the level of gene (AR, VEGFA, and ERBB2) transcription and translation was lower than the normal group. AR, VEGFA, and ERBB2 are star molecules in cancer signaling pathway, which are closely related to tumor migration and proliferation.

The incidence rate of some cancers is significantly related to gender [42–45]. The incidence rate of esophageal cancer in men is 3-4 times that of women in global [46]. A research study suggested that hormones may be associated with high incidence rate of EAC in men [47]. Androgen receptor (AR)

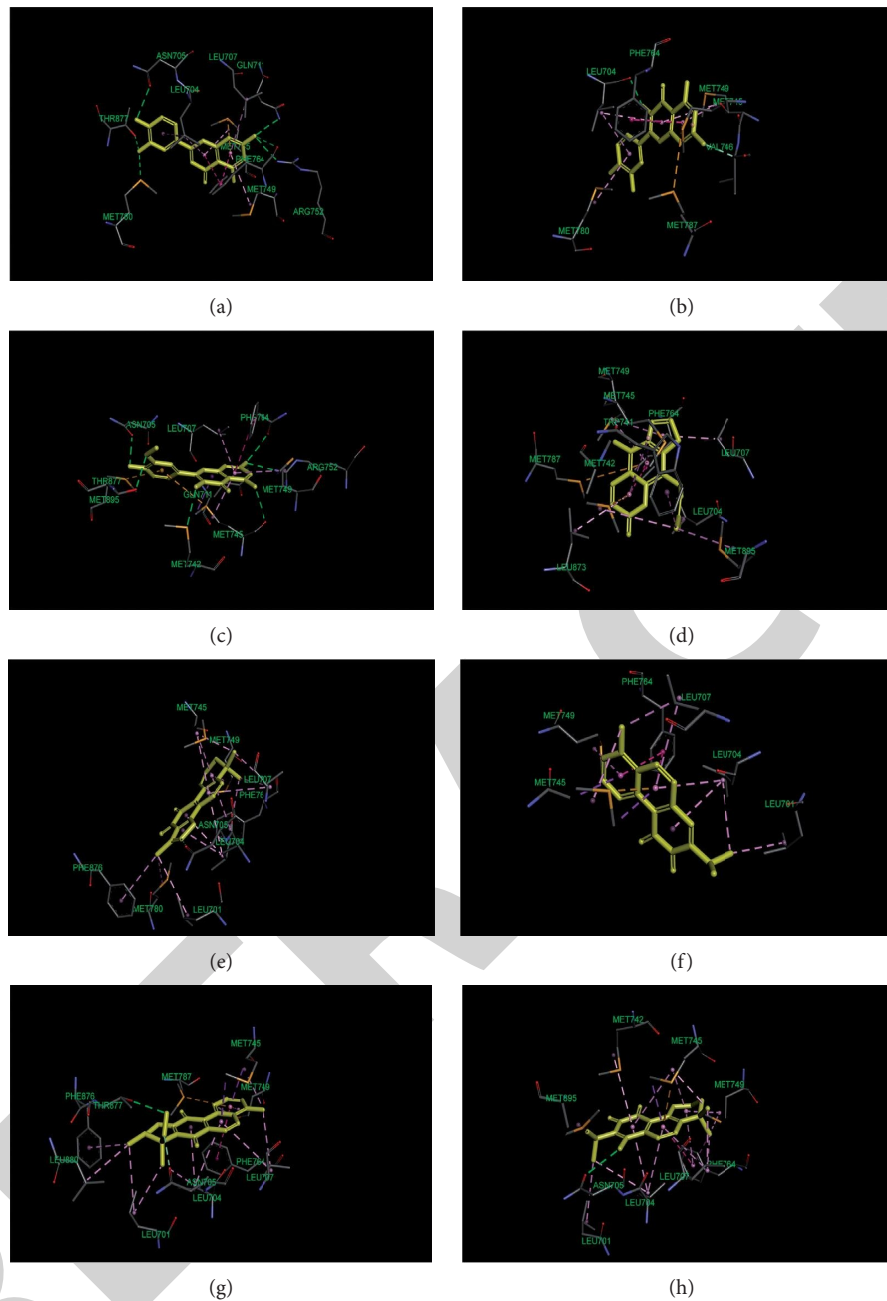


FIGURE 5: Molecular docking between the hub targets and the corresponding compounds. (a) AR-luteolin. (b) AR-quercetin. (c) AR-isorhamnetin. (d) AR-alloisioimperatorin. (e) AR-dehydrotanshinone II A. (f) AR-2-isopropyl-8-methylphenanthrene-3,4-dione. (g) Dan-shexinkun D. (h) AR-deoxyneocryptotanshinone.

is also commonly present in human tissues and is also seen in esophageal cancer [48]. Barrett's esophagus, a precancerous lesion of esophagus, has higher circulating levels of dihydrotestosterone (DHT) and testosterone than normal [49, 50]. A complete analysis of the SEER database showed that patients previously diagnosed with prostate cancer were less likely to develop EAC after androgen deprivation therapy [51]. The presence of AR and the relationship between testosterone and Barrett's esophagus suggest that androgen may involve in the development of EAC, hence proving the male dominance of EAC [52]. The local

transformation of androgen to estrogen may involve in the development of EAC and may explain the interaction of obesity, androgen, and male gender in the development of EAC [53]. Overexpression of AR promotes the proliferation of EAC cells [54]. Awan et al. detected the expression level of AR in 18 EAC patients and 5 ESCC patients by immunohistochemistry and found the expression level of AR in normal esophageal epithelium [55]. Compared with that of the normal tissues, the staining of cytoplasmic AR in 13 specimens from EAC patients and 3 specimens from ESCC patients was obvious. The level of androgen in the EAC

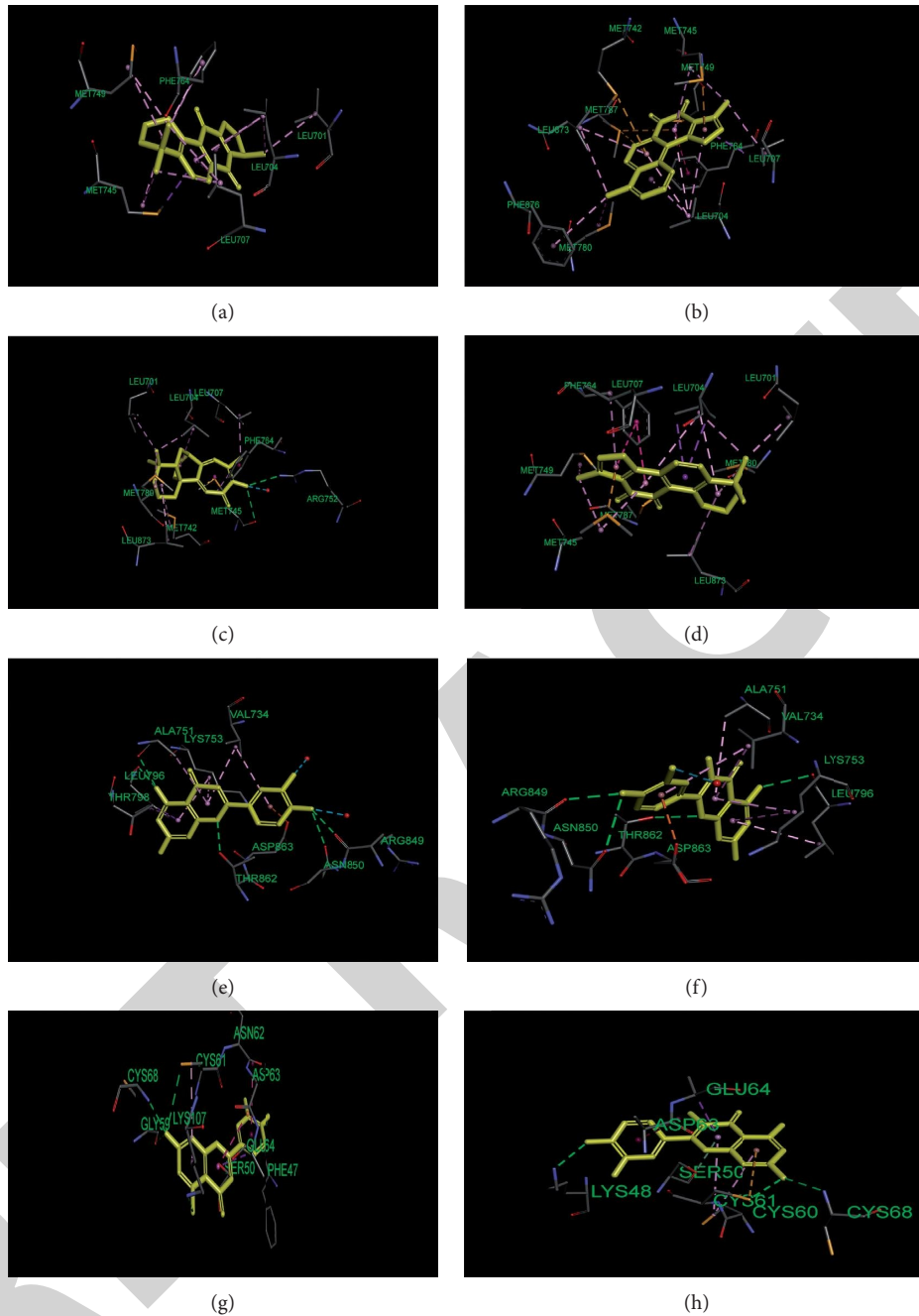


FIGURE 6: Molecular docking between the hub targets and the corresponding compounds. (a) AR-isocryptotanshinone. (b) AR-isotanshinone II. (c) AR-miltipolone. (d) AR-tanshinone. (e) ERBB2-luteolin. (f) ERBB2-quercetin. (g) VEGFA-luteolin. (h) VEGFA-quercetin.

patients was higher than normal before surgery but decreased significantly after the operation, which indicated that the development of EAC might be related to paracrine effects of androgen in EAC tissue [55]. In this experiment, Qigesan reduced the expression of AR and the proliferation of BIC-1 cells to achieve the purpose of anti-cancer.

VEGFA protein is secreted by tumor cells, macrophages, and fibroblasts and is widely distributed in many tissues of the human body. When tumor cells appear, the expression level of the VEGFA protein dramatically increases. VEGFA

plays a crucial role in angiogenesis, tumor growth, and ischemic diseases [56]. Reginald VN Lord et al. quantified the vascularization by microvasculature count and microvascular surface area percentage [57]. The expression of VEGFA was detected by immunohistochemistry. According to the microvasculature count and microvascular surface measurement, the degree of vascularization in superficial cancer was significantly higher than that of other tumors. VEGFA expression was associated with increased angiogenesis. The high abundance of a VEGFA in tissues

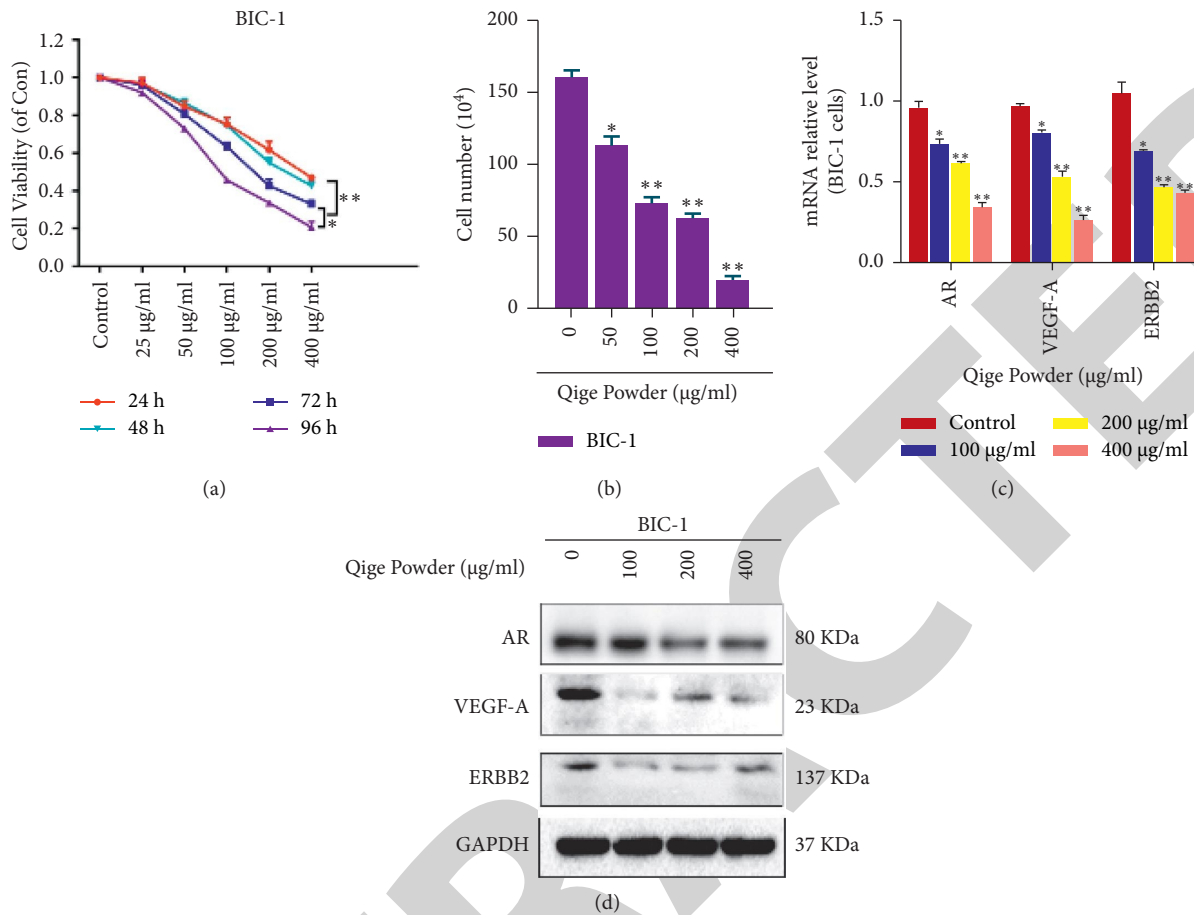


FIGURE 7: Verification experiments of Qigesan in vitro. (a) Different Qigesan concentrations (25 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml, and 400 µg/ml) were used to treat BIC-1. \* indicates 96 h group compared with 72 h group,  $P < 0.05$ ; \*\* indicates 72 h group compared with 48 h group,  $P < 0.01$ . (b) The cell counts of BIC-1 were performed after BIC-1 was treated with different Qigesan concentrations (50 µg/ml, 100 µg/ml, 200 µg/ml, and 400 µg/ml) after 24 hours; \* means compared with blank control group,  $P < 0.05$ ; \*\* means compared with blank control group,  $P < 0.01$ . (c) AR, VEGFA, and ERBB2 were detected by qPCR after BIC-1 was treated with different Qigesan concentrations (100 µg/ml, 200 µg/ml, and 400 µg/ml) for 24 h; \* means compared with blank control group,  $P < 0.05$ ; \*\* means compared with blank control group,  $P < 0.01$ . (d) After different Qigesan concentrations (100 µg/ml, 200 µg/ml, and 400 µg/ml) treated BIC-1 for 24 hours, AR, VEGFA, and ERBB2 were detected by Western Blot.

suggested the risk of distant metastasis. Barrett's esophageal precancerous lesions and early cancers have high angiogenic characteristics. Barrett esophagus is an important precancerous lesion of EAC. Couvelard et al. detected the expression of VEGFA, COX-2, and Ki-67 in Barrett's esophagus samples by immunohistochemistry and evaluated the results using a qualitative scoring method. The results showed that VEGFA was expressed in endothelial cells of all the samples [58]. VEGFA, COX-2, and Ki-67 were highly expressed in most of Barrett's esophagus specimens. The malignant degree of the metaplasia was negatively correlated with VEGFA expression and positively correlated with Ki-67 expression.

The results of a meta-analysis showed that the positive expression of VEGF suggested poor prognosis of EAC and ESCC [59]. Furthermore, VEGFA is involved in tumor angiogenesis [60]. Qigesan can reduce the expression of VEGFA, which may be the purpose of inhibiting tumor angiogenesis, growth, and metastasis.

ERBB2 is one of the most frequently amplified proto oncogenes in esophageal adenocarcinoma. Trastuzumab can inhibit the proliferation of esophageal adenocarcinoma cells [61]. ERBB2 is overexpressed in 10–70% of EAC [62]. The high expression of ERBB2 may be one of the riskers of increased dysplasia and extensive adenocarcinoma [63]. ERBB2 is overexpressed in Barrett's esophageal adenocarcinoma and is closely related to survival and prognosis [64]. In the current research study, Qigesan can reduce the expression of ERBB2 and inhibit the proliferation of tumor cells.

In recent studies, the concept of network pharmacology was used to predict the mechanism of Qigesan, and AR, VEGFA, and ERBB2 were found to be potential targets for Qigesan in treatment of EAC (Figure 9).

However, as a new subject in pharmacology, network pharmacology has many limitations: ① the accuracy and integrity of the databases are not reliable, and the research hotspots in database can easily lead to selectivity bias; ② the

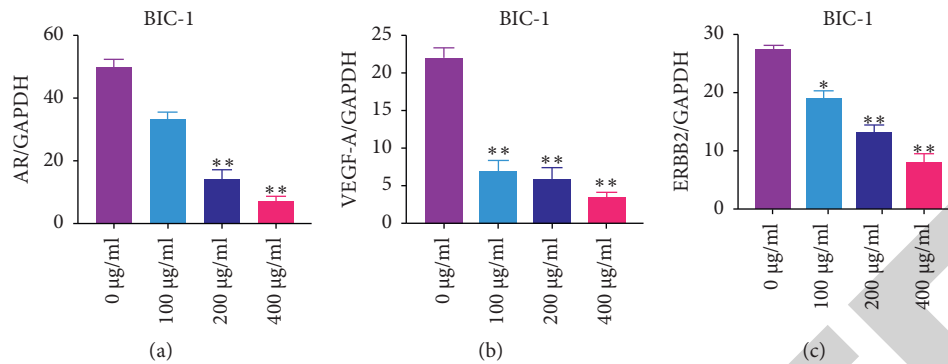


FIGURE 8: Qigesan decreased the protein expression of AR, VEGFA, and ERBB2. The bar graph showed significant dose-dependent inhibition. The relative protein density (normalized to GAPDH) of AR, VEGFA, and ERBB2 was compared with control group. The results came from three separate experiments. \* means compared with blank control group,  $P < 0.05$ ; \*\* means compared with blank control group,  $P < 0.01$ .

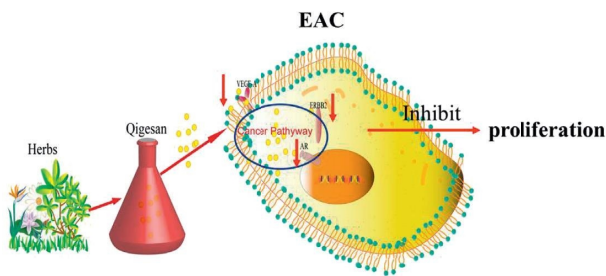


FIGURE 9: Mechanism chart. Qigesan can treat EAC may through the cancer-related pathways mediated by AR, VEGFA, and ERBB2.

number of small-molecule compounds and their targets are limited, such that the complete pharmacological effect of compound Chinese medicine is not discernable; ③ currently, there are difficulties in the experimental validation of network pharmacological prediction results, making it challenging to check the consistency of the results in different experimental platforms; and ④ there is the lack of experimental verification in vivo.

## 5. Conclusions

Through the analysis of network pharmacology, it is speculated that the active components of Qigesan in the treatment of EAC may be luteolin, tanshinonVI, quercetin, miltipolone, isotanshinoneII, isorhamnetin, isocryptotanshinone, deoxyneocryptotanshinone, dehydrotanshinoneIIA, danshexinkun D, alloisimperatorin, and 2-isopropyl-8-methylphenanthrene, and the effective targets may be AR, VEGFA, and ERBB2. In vitro experiments have confirmed that Qigesan can inhibit the proliferation of BIC-1 cells by reducing the expression of AR, VEGFA, and ERBB2, which may be one of the mechanisms of Qigesan against EAC.

## Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

## Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

## Authors' Contributions

In this study, Dong Zhang was responsible for writing the manuscript; Lang Guo, Cheng Jiang, Tao Wang, and Xiaoting Wu were responsible for in silico analysis; Xinyi Liang and Meng Luo completed the experimental verification; Qiuping Zou and Yong Gao guided this study.

## Acknowledgments

This work was supported by National Natural Science Foundation of China (Grant no. 8207033830).

## Supplementary Materials

Supplementary Figure 1: Herb-Compound network and Compound-Target network. Table S1: Compounds contained in Qigesan. Table S2: 646 targets of the identified compounds. Table S3: 7 targets in the OMIM database. Table S4: 838 targets in the CTD database. Table S5: 746 targets in Gene Cards. Table S6: 33 compounds and 11 target proteins. (*Supplementary Materials*)

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