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Prognostic model construction and target identification of Si-Wu-Tang against breast cancer

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ABSTRACT

The targets and mechanisms of Si-Wu-Tang (SWT) against (Breast cancer) BRCA were identified and a survival model and nomogram was construted by network pharmacology, bioinformatic analysis and *in vitro* experiments. A total of 72 anti-breast cancer SWT targets were selected, among which eleven genes (*MAOA*、*SQLE*、*CACNA2D1*、*GLI1*、*RORB*、*ITGB3*、*TACR1*、*NR3C2*、*CA3*、*RBP4 and PTK6*) were used to construct a novel prognostic model of breast cancer. The anti-breast cancer activity of SWT was related to the modulation of the receptor tyrosine kinases signaling pathways. Moreover, two compounds, mairin and senkyunone were found to bind directly to ITGB3 and RORB proteins. Finally, mRNA and protein expression of ITGB3 and RORB was observed to be significantly down-regulated after incubation of MCF-7 cells with SWT. Overall, our results indicated that mairin and senkyunone were the key ingredients present in SWT, and ITGB3 as well as RORB proteins were the major targets affected by SWT. The prognostic model can be used to predict the outcome of BRCA patients.

1. Introduction

Breast cancer is a malignant tumor which originates from the mammary epithelial tissue with breast mass, nipple discharge, and axillary lymph node enlargement as the common clinical manifestations. Based on molecular typing, breast cancer can be divided into four different types: Luminal A, Luminal B, human epidermal growth factor receptor 2 (HER2) positive, and triple negative [1]. In 2020, the International Agency for Research on Cancer (IARC) reported that the incidence and mortality of breast cancer was 47.8/100,000 and 13.6/100,000 respectively [2], and it has thus emerged as the cancer with the highest incidence in the world which can adversely affect both the physical and mental health of affected women. The various risk factors for breast cancer include high endogenous estrogen level, obesity and family history of breast cancer [3], whereas the exact pathogenesis has not been completely elucidated. Currently, the treatment for breast cancer includes surgery, endocrine therapy, chemotherapy, targeted therapy and immune therapy [4–6]. Especially, the application of Antibody-drug conjugate (ADC) in BRCA, having both targeted and chemotherapy effects, prolong the overall survival time of patients, which has promising prospects [7]. Despite advances in breast cancer treatment,

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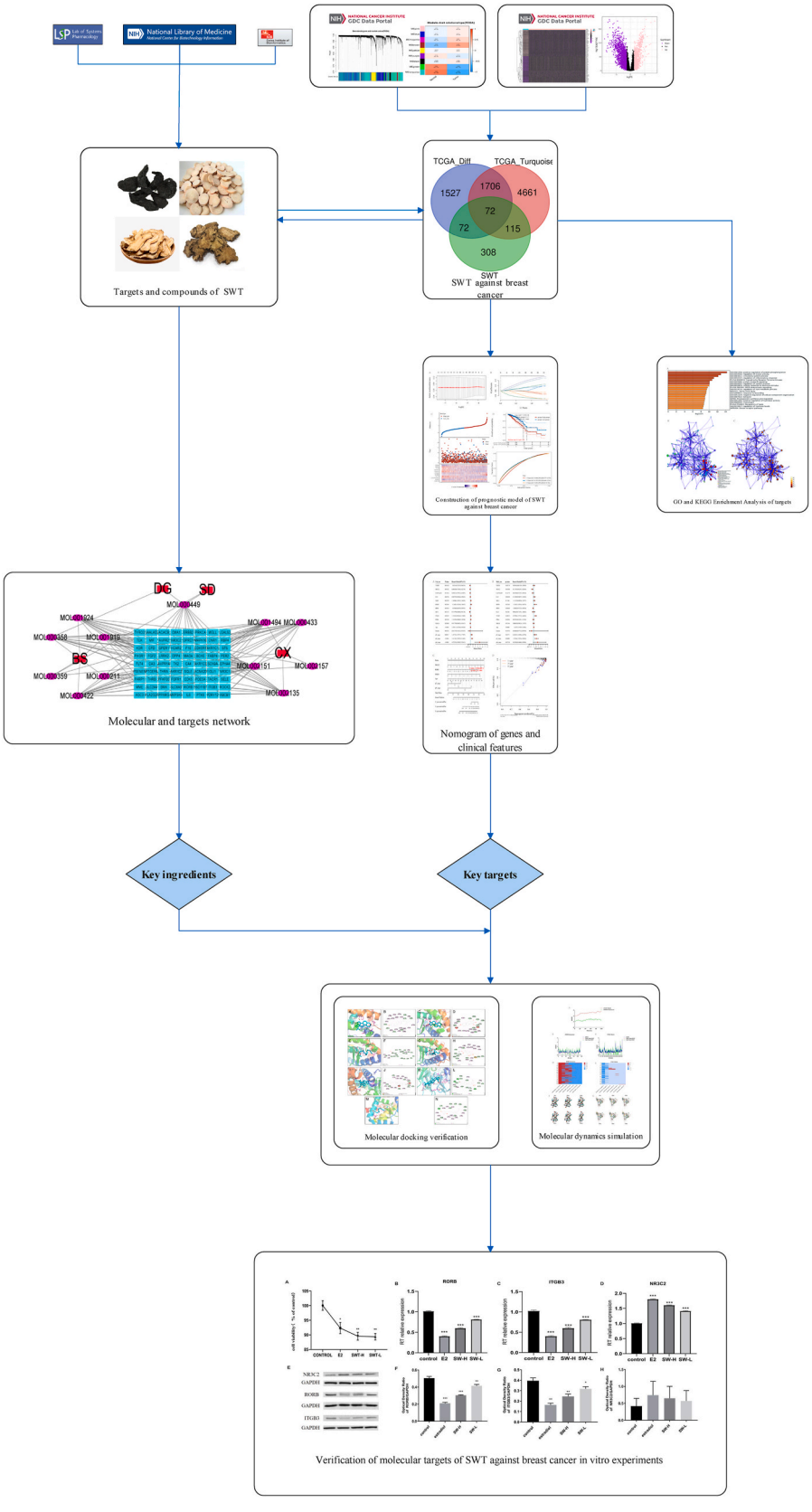


(caption on next page)

Fig. 1. Workflow of the study design.

the survival rate for patients in stage IV is merely 26 % over a five-year period [8]. Therefore, development of novel therapeutic strategies for the treatment of BRCA is urgently required.

Si-Wu-Tang (SWT) is a traditional Chinese medicine (TCM) formula, which is composed of the following four herbs: Shudihuang (*Rehmannia glutinosa*), Danggui (*Angelica sinensis*), Baishao (Paeonia lactiflora Pall), ChuanXiong (*Conioselinum anthriscoides*). Plant names have been verified at www.theplantlist.org. SWT has been extensively used to treat various gynecological diseases in East Asia since ancient times. It has also been employed to treat hematopoietic disorders, orthopedic diseases etc. [9,10]. Of note, SWT has an anti-cancer effect (Shan et al., 2021) [11]. A number of previous studies have indicated that the molecular mechanism of SWT against breast cancer is closely related to its potential effects on modulation of estrogen activity [12,13]. By regulating ER pathway, the expression level of estrogen receptor β subtype (ER β) protein is increased, and the ratio of ER α/ER β is reduced, thus leading to the attenuation of the growth of estrogen-dependent tumor cells [14,15]. In addition, the molecular mechanism of SWT on non-triple-negative breast cancer was found to be related to its effect on regulating the energy metabolism and apoptosis [16]. However, the potential targets and mechanisms of SWT against BRCA remains unclear.

Network pharmacology is a discipline that uses omics high-throughput screening network visualization as well as network analysis techniques to reveal the specific drug disease network based on the theories of pharmacology, system biology, and computational biology [17]. The determination of the pharmacological actions, mechanisms and safety of TCM can lead to the development of novel therapies and can be of great clinical significance [18]. Therefore, network pharmacology, bioinformatic analysis and the various *in vitro* experiments were combined to unravel the molecular targets and mechanisms of SWT against BRCA in this study.

2. Materials and methods

2.1. Identification of the ingredients and targets of SWT

The detail steps of this study has been shown in Fig. 1. The criteria required an Oral Bio-availability (OB) of at least 30 % and a Drug Likeness (DL) value greater than or equal to 0.18, and the ingredients of SWT were acquired from the TCM systems pharmacology (TCMSP) database (http://lsp.nwu.edu.cn/tcmsp.php). It has been established that TCMSP database is a unique pharmacology platform that captures the potential relationships between Chinese herbal medicines, targets, and diseases. The database comprises various chemicals, targets and drug–target networks, along with essential pharmacokinetic properties, such as oral bioavailability, drug likeness, intestinal epithelial permeability, blood–brain barrier penetration, and aqueous solubility. Moreover, in order to comprehensively collect SWT targets, PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and Swiss Target Prediction database (http://www.swisstargetprediction.ch/) were used to obtain additional targets. Specifically, the Canonical SMILES of ingredients were obtained from PubChem database and then were imported in Swiss Target Prediction database to carry out the target prediction. Finally, the targets of all the compounds were obtained and Perl language was used to map the candidate ingredients and targets.

2.2. Identification of anti-breast cancer targets of SWT

Breast cancer-associated genes were retrieved from two different sources. First of all, the expression data for BRCA and metadata file were downloaded from The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/). Thereafter, we conducted the differentially expressed analysis to screen the differentially expressed genes (DEGs) by using the "limma" package in the R language with the criteria of $\log|FC| > 1$ and an adjusted P value < 0.05. Subsequently, the "WGCNA" package in the R software was also employed to identify the genes which were modulated with significant differences between the normal and the tumor groups. This was achieved this by constructing a dynamic tree cut with a minimum number of modular genes to 50 and a shear height of 0.30. Finally, the therapeutic targets of SWT against breast cancer were identified by integrating the targets of SWT and the DEGs.

2.3. Construction of a prognostic model of SWT against BRCA

The anti-breast cancer targets of SWT were used to carry out Univariate Cox regression analysis to screen for the various survival related genes of BRCA. Thereafter, the different redundant factors were removed and a prognostic model was constructed by Least absolute shrinkage and selection operator (LASSO) regression analysis. The LASSO-Cox model has gained extensive usage in predicting cancer outcomes and identifying associated genes due to its ability to pinpoint small sets of genes with exceptional prognostic performance. The survival difference between the high risk and low risk group of the model was detected by Kaplan–Meier (KM) survival analysis, and the P value of <0.05 was considered to be significant. Additionally, the performance of the prognostic model was also assessed using Receiver Operating Characteristic (ROC) curves to gauge its predictive capabilities.

2.4. Nomogram construction of SWT against BRCA

In order to construct a nomogram of SWT against BRCA, the various genes of the prognostic model were combined with age, gender and TNM stages by univariate cox analysis and multivariate cox analysis. The different factors that exhibited significant differences in the univariate cox analysis were considered as risk factors associated with BRCA prognosis, while other factors that had significant

differences in multivariate cox analysis were considered as independent prognostic risk factors for BRCA. We included the factors that showed significant differences in the multivariate cox analysis in the construction of the nomogram. In the nomogram, each factor was given a score. By adding the scores of all the different factors the total score was obtained and the patient's survival was predicted. Moreover, the c-index was used for the evaluation of the nomogram reliability, and the correction curves were used to assess the fit of the model.

2.5. Mechanisms analyses of SWT against BRCA

Based on the criteria: P value < 0.01 and minimum enrichment >1.5, the mechanisms of action of SWT against BRCA were detected by the Metascape database (http://metascape.org/gp/index.html#/main/step1). Furthermore, in order to analyze the potential relationship between these biological functions and pathways, an interaction network was constructed.

2.6. Prediction of the candidate anti-breast cancer ingredients

In order to identify the pivotal candidate ingredients of SWT against BRCA, the Cytoscape version 3.7.2 software was used to construct a potential molecule target network. The various ingredients that can target the hub genes related to the survival status of BRCA were used for the further analysis.

2.7. Molecular docking simulation

In order to verify the performance of the potential binding abilities between ingredients and proteins, molecular docking simulations were performed. The structural formula of the active ingredients were downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/), and thereafter the corresponding 3D structure were made by the Chem3D software and output it in mol*2 format. PDB format of the hub proteins were downloaded from the PDB database (http://www.nlm.nih.gov/).://www.rcsb.org/) and then were dehydrated and dephosphorylated by Pymol software. Subsequently, AutoDockTools 1.5.6 software was used to convert the PDB format of the active ingredients of the drugs and core protein gene files into PDBQT format to facilitate identification of active pockets. Finally, Vina script was run to calculate the molecular binding energy and analyze the results of the molecular docking. In addition, Discovery Studio 2019 was used to search for the various docking sites and LibDockScore of flexible binding was calculated, and then the obtained molecular docking results were imported into PyMOL software for the conformation display. If the binding energy was found to be less than 0, the ligand could spontaneously bind to the receptor. When the Vina binding energy was less than -5.0 kcal mol^{-1} and LibDockScore >100, the two could form a stable docking. The results of the molecular docking of the ligand-receptor complex were displayed in 3D and 2D to evaluate the reliability of the bioinformatics analysis prediction.

2.8. Molecular dynamics analysis

In order to further analyze the binding process between the core proteins and active ingredients, molecular dynamics simulation of the ligand-receptor complex was carried out. Position parameter was ten acquired from the modules of Simulation and Standard Dynamics Cascade in Discovery Studio 2019 software. Charmm36 Force Field was employed for the determination of the molecular parameters of the ligands in the process of simulation, while Charmm Force Field was used for the analysis of the molecular parameters of the receptor proteins. The ligand-receptor complex was solvated in the computational process in Solvation module. Thereafter, the molecular dynamics simulation was performed, including 5 stages: I Minimization, I Minimization2, Heating, Equilibration, and Production. Afterwards, animation of the change of conformation was shown to display the changes associated with the process of the molecular dynamics simulation. After the molecular dynamics calculation was completed, the module of Analyze Trajectory was finally used to analyze the structural characteristics of the molecular dynamics trajectory, such as geometric properties (distance, Angle, and dihedral Angle), the number of non-key interactions per simulated frame, Root-Mean-Square Deviation (RMSD) and Root-Mean-Square Fluctuation (RMSF) between the different conformations.

2.9. Vitro experiments

2.9.1. Pharmacological serum preparation

SWT was provided by Beijing Dongzhimen Hospital, which composed of the following four herbs: 15 g Shudihuang (*Rehmannia glutinosa*), 10 g Danggui (*Angelica sinensis*), 10 g Baishao (Paeonia lactiflora Pall), 6 g ChuanXiong (*Conioselinum anthriscoides*). All herbs were identified by Professor Piwen Zhao (Beijing University of Chinese Medicine, Beijing, China). The extract of SWT was prepared according to the method of Chinese Pharmacopoeia. SWT was soaked in 2 L cold water for 30 min and then decocted together for 60 min to get the first decoction. Add 1.5 L water to SWT and boil it for 30 min to get the second soup. Finally, the first and second decoctions are mixed and filtered with gauze to concentrate the final extract. The quality of herbal medicine has been identified by the research group and meets the requirements [16]. Positive drug was estradiol valerate (E2, trade name: progynova), Bayer pharmaceutical company production (batch number: 195A5).

22- day-old female Sprague-Dawley (SD) mice (50 ± 2 g) were purchased from SBF Experimental Technology Co., Ltd. (Peking, China). The animal's license number is No. SCXK [Jing] 2017-0005. Animals are subject to European Community guidelines (EEC Directive 1986; 86/609/EEC) for the use and care of laboratory animals. The immature mice were randomly assigned into four

different groups (n = 5/group) and were oral administrated with either normal saline, estradiol (E2, 0.16 mg/kg/d), high dose group of SWT (2.08 mg/kg/d), low dose groups of SWT (1.04 mg/kg/d) for 4 days.

One hour after the last gavage of immature mice, blood was collected from the abdominal aorta, and the complement was inactivated in a water bath at 56 °C for 30 min. Thereafter, the pharmacological serum was stored at −20 °C until use.

2.9.2. Culture conditions

MCF-7 cells were procured from the Cell Center of Peking Union Medical College Hospital and cultivated in DMEM (Hyclone) medium supplemented with 10 % fetal bovine serum. The cells were incubated at 37 °C with 5 % CO2 in an environment of adequately saturated humidity.

2.9.3. Western blot analysis

The previously prepared drug-containing serum was incubated with MCF-7 cells treated with different drugs at a ratio of 5 %. The protein was extracted from the cells with lysis buffer and the protein concentration was measured using the BCA assay kit. The protein samples were separated using SDS-polyacrylamide gel (SDS-PAGE) (10 %) and proteins were transferred to PVDF membranes. After the membrane was blocked with 10 % BSA for 1.5 h, it was incubated with the different target antibodies: anti-NR3C2 (Proteintech), anti-ITGB3 (Abcam), anti-RORB (Abcam), and anti-GAPDH at 4 °C for 24 h. After washing with TBST, the membrane was incubated with goat anti-rabbit IgG or goat anti-mouse IgG secondary antibody for 2 h at the room temperature. After washing the membrane with TBST again, it was imaged with ECL photoluminescence solution. The test results were analyzed by using Image J software.

2.9.4. Quantitative RT-qPCR analysis

For the quantitative RT-PCR total RNA was extracted from MCF-7 cells using TRIzol (Invitrogen) according to the manufacturer's instructions. The RNA was reverse transcribed into cDNA and the conditions used for RT-qPCR were as following: pre-denaturation at 95 °C for 5min, denaturation at 95 °C for 10s, annealing at 58 °C for 20s, extension at 72 °C for 20s, and 40 cycles. The protein sequences have been shown in Table 1.

2.9.5. Statistical analysis

Graphpad Prism 8.0 software was used for the statistical analysis of the data. Student's *t*-test was used to compare the two different groups of data. In addition, one-way ANOVA was employed to analyze the data of more than two groups. $P < 0.05$ indicated that the difference was statistically significant, Moreover, RT-qPCR data was analyzed using the 2-△△Ct algorithm.

3. Results

3.1. Identification of anti-breast cancer targets of SWT and identification of its ingredients

The candidate SWT ingredients that were obtained from the TCMSP included 13 different compounds from BS, 7 from CX, 2 from DG and 2 from SD. Among them, there were 6 compounds in each of BS and CX, and 2 compounds in each of DG and SD against BRCA. Table 2. A total of 567 targets of SWT were acquired by intersecting the various candidate compounds and potential targets.

A total of 3377 and 6554 DEGs were identified in the differentially expressed analysis and WCGNA analysis in TCGA dataset, respectively. Fig. 2A–D. Finally, 72 SWT against breast cancer targets were identified by mapping the SWT targets with the DEGs. Fig. 2E.

3.2. Construction of a prognostic model of SWT against BRCA

Univariate Cox regression analysis identified eleven different genes (*MAOA, SQLE, CACNA2D1, GLI1, RORB, ITGB3, TACR1, NR3C2, CA3, RBP4 and PTK6*) that significantly affected patient survival based on the 72 identified anti-breast cancer targets of SWT. Fig. 3A and B. The LASSO regression method was employed to remove the redundant factors and eleven genes were used to build a prognostic model with the risk formula:

(−0.0404)*MAOA+(0.0777)*SQLE+(−0.1421)*CACNA2D1+(−0.1464)*GLI1+(0.2422)*RORB+(0.2133)*ITGB3+(−0.0552)*TACR1+(−0.0492)*NR3C2+(0.0534)*CA3+(−0.0221)*RBP4+(0.101)*PTK6. With the increasing risk scores, the number of BRCA patients that died increased significantly. Fig. 3C. KM survival analysis also revealed a significant difference between the high- and

Table 1
The sequence of proteins.

Target Name		Primer(5′-3′)
NR3C2	F	AGCTGGAATGAATTTAGGAGGAT
	R	ATAGTTCATACATGGCAGACTGAT
RORB	F	CCGAGGAGGAGATCGCTTTG
	R	CAAACTGCCGTGATGGTTGG
ITGB3	F	TTGGAGACACGGTGAGCTTC
	R	TTAGGTTCAGCTTGGGCCTG

Table 2
Identification of SWT candidate compounds and therapeutic targets against breast cancer.

Molecular ID	Molecular Name	OB (%)	DL	Origin
MOL000359	sitosterol	36.91	0.75	BS
MOL000358	beta-sitosterol	36.91	0.75	BS
MOL000422	kaempferol	41.88	0.24	BS
MOL001919	(3S,5R,8R,9R,10S,14S)-3,17-dihydroxy-4,4,8,10,14-pentamethyl-2,3,5,6,7,9-hexahydro-1H-cyclopenta[*a*] phenanthrene-15,16-dione	43.56	0.53	BS
MOL001924	paeoniflorin	53.87	0.79	BS
MOL000211	Mairin	55.38	0.78	BS
MOL001494	Mandenol	42	0.19	CX
MOL002135	Myricanone	40.6	0.51	CX
MOL002151	senkyunone	47.66	0.24	CX
MOL002157	wallichilide	42.31	0.71	CX
MOL000359	sitosterol	36.91	0.75	CX
MOL000433	FA	68.96	0.71	CX
MOL000359	sitosterol	36.91	0.75	SD
MOL000449	Stigmasterol	43.83	0.76	SD
MOL000358	beta-sitosterol	36.91	0.75	DG
MOL000449	Stigmasterol	43.83	0.76	DG

low-risk groups (P = 8.39e-06). Fig. 3D. Furthermore, the AUC values were 0.668, 0.705 and 0.693 for 1-, 3-, and 5-year survival, respectively, suggesting that the prognostic model exhibited a good efficiency to predict the potential outcomes of patients with BRCA. Fig. 3E.

3.3. Nomogram construction of SWT against BRCA

In order to construct a nomogram of SWT against BRCA, univariate cox analysis and multivariate cox analysis were performed on 11 genes related to the prognostic model, age, sex and TNM stages. The results showed that TACR1, SQLE, RORB, RBP4, GLI1, age and TNM stages can serve as prognostic factors of BRCA. Fig. 4A. It was specifically found that RORB, ITGB3, NR3C2, age and TN stages acted as independent risk factors for BRCA. Fig. 4B. By incorporating the different factors with significance level among the multivariate cox analysis into the nomogram, we constructed the nomogram using NR3C2, RORB, ITGB3, age and TN stages. Fig. 4C. The results indicated that the C-index of the nomogram was 0.736 and P value < 0.001, thereby indicating that the nomogram displayed a good performance. Fig. 4C. In addition, the fitting curve of the nomogram fluctuated slightly around the diagonal and was close to overlap, thus suggesting that the fitting degree of the nomogram was good. (Fig. 4D).

3.4. Mechanisms analyses of SWT against BRCA

The 72 therapeutic targets of SWT against BRCA were used to carry out GO and KEGG analysis. The results suggested that these genes were primarily involved in the regulation of kinase activity. In addition, the key mechanisms of SWT action against BRCA were associated with the modulation of the receptor tyrosine kinases signaling pathway. Fig. 5.

3.5. Prediction of candidate anti-breast cancer ingredients of SWT

The pivotal candidate ingredients of SWT anti-breast cancer were predicted by using the 12 different compounds and 72 therapeutic targets of SWT to construct a molecule target network. Within the network, six distinct compounds from BS, six from CX, two from DG, and two from SD were identified to potentially have anti-breast cancer activity. Among them, there were 5 specific compounds targeting NR3C2, 1 targeting ITGB3 and 1 targeting RORB. Therefore, subsequently validation using the molecular docking and molecular dynamics was performed. Fig. 6.

3.6. Molecular docking and molecular dynamics of target proteins and candidate ingredients

The results of the molecular docking showed that mairin and senkyunone had better binding affinities to the ITGB3 and RORB proteins than those of other complexes. It was found that senkyunone displayed the docking score of −9.3 with the RORB protein ranking top one. Fig. 7.M – N, Table 3. Therefore, mairin and senkyunone were selected to explore their potential binding modes to the ITGB3 and RORB proteins.

The solvated water model with a definite periodic boundary was then used to solve problem. 5659 water molecules, 15 sodiums and 19 chlorides were added into RORB-Senkyunone ligand-receptor complex, whereas 22698 water molecules, 73 sodiums and 60 chlorides were added into ITGB3-Mairin ligand-receptor complex to analyze the physiological environment. In order to investigate the structural stability of the protein-ligand complex during MD simulation, the RMSD value was also calculated in the process of complex

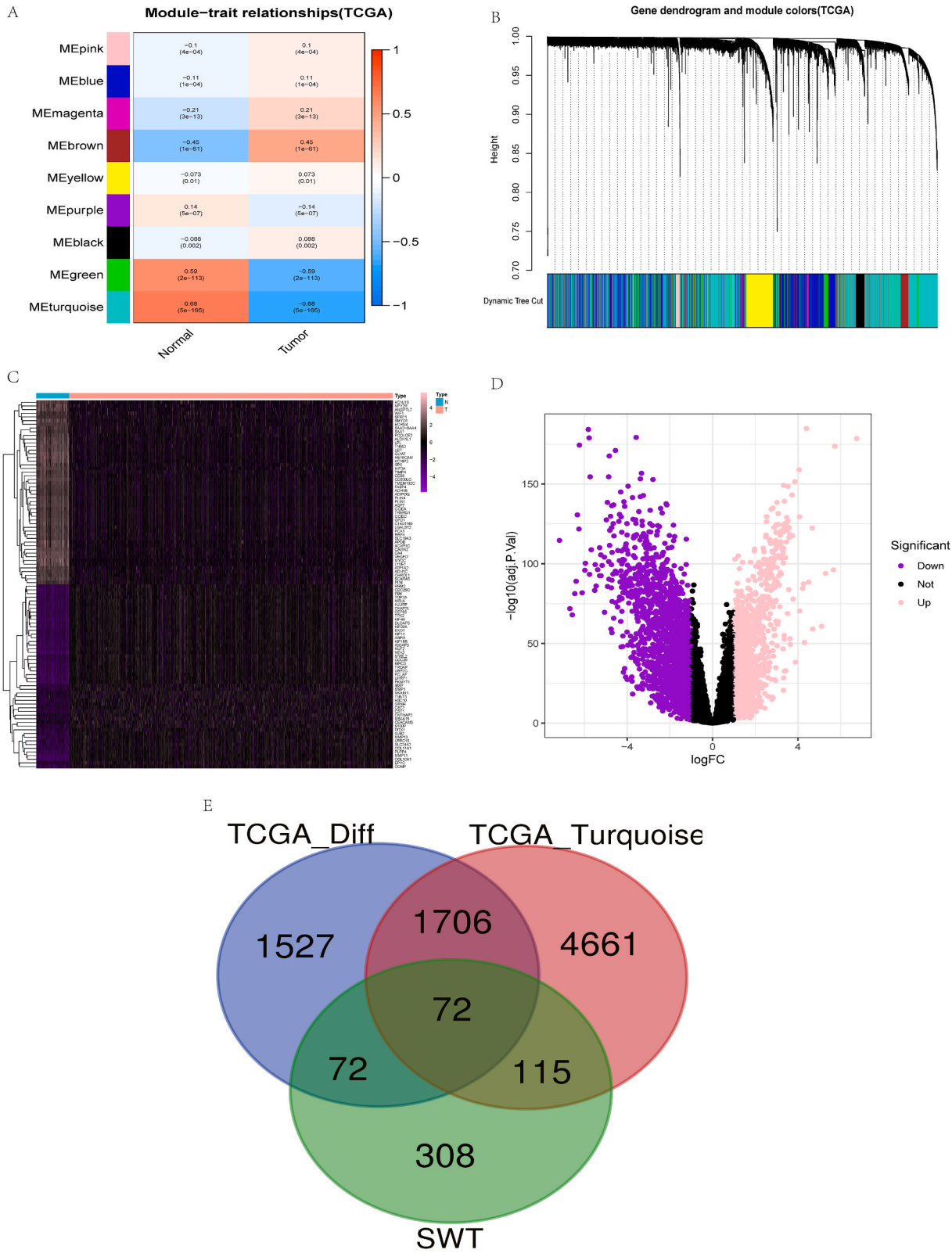


Fig. 2. Identification of therapeutic targets of SWT against breast cancer. (A–B) WGCNA analysis of TCGA LUAD; (C–D) Differentially expressed analysis of TCGA LUAD; (E) Integrating the targets of SWT and DEGs.

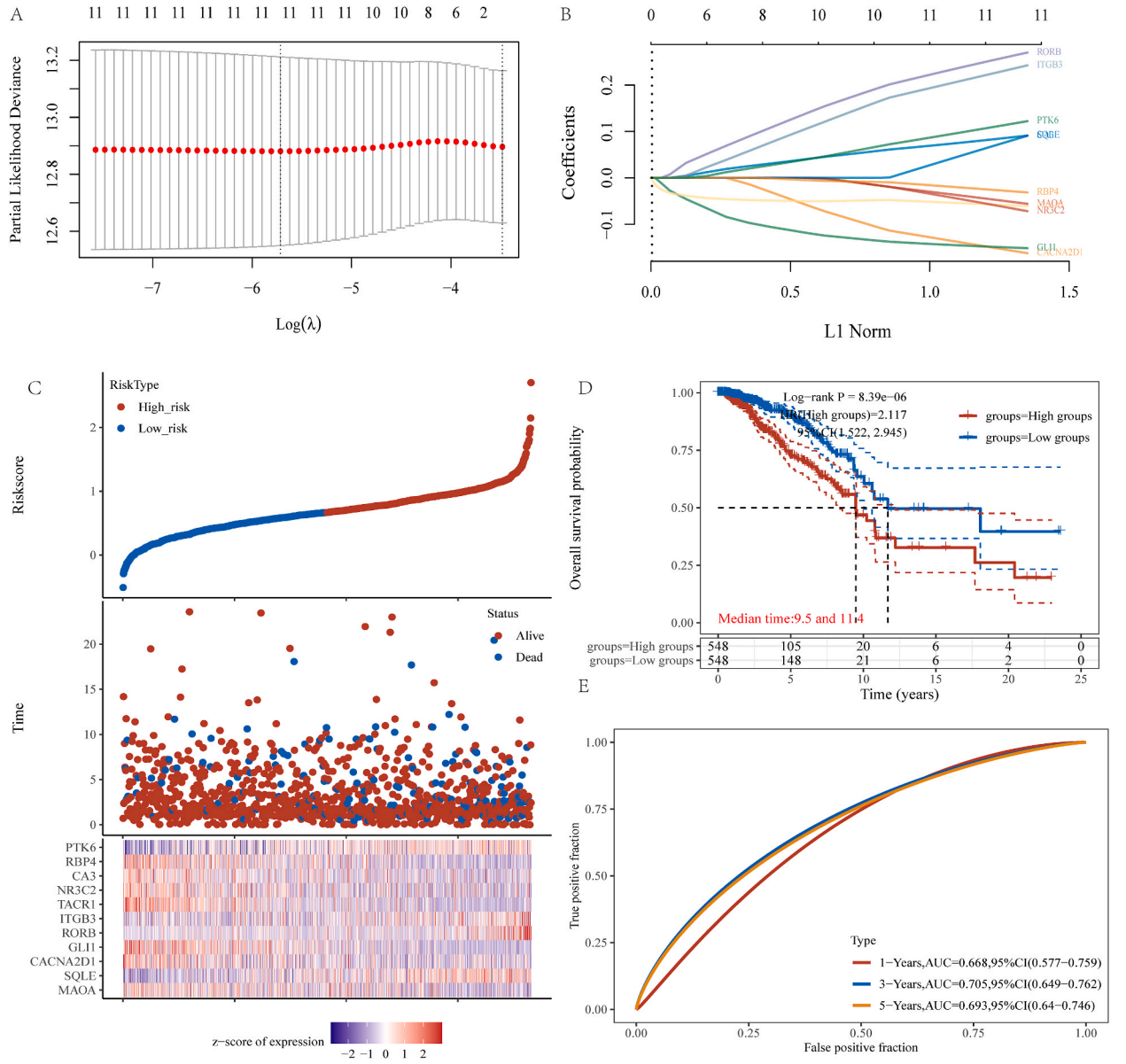


Fig. 3. Construction of prognostic model of SWT against breast cancer. (A–B) LASSO regression removed redundant factors; (C) Risk curve of scores; (D) KM survival analysis divided patients in two groups based on median of survival time; (E) ROC curve of prognostic model.

in 100ns. As shown in Fig. 8A, the complexes in both the systems stabilized after 100 ns of MD simulation. In addition, RMSD of RORB-Senkyunone complex mainly fluctuated between 1.0032 and 1.53518, with an average RMSD of 1.30024, while that of the RMSD of ITGB3-Mairin complex primarily fluctuated between 2.00237 and 2.81534, with an average RMSD of 2.48494, thus indicating that the structures of the complex were in equilibrium after the simulation.

Subsequently, in order to analyze the fluctuation of the various amino acids in the complex during MD simulation, RMSF values of all amino acids used in the simulation were calculated. The results indicated that amino acid Ile492, Val493 and Val494 fluctuated significantly around RORB-Senkyunone complex, while amino acid Ser121, Tyr122, Ser123, Ile380, Pro381, Gly382, Lys689, Cys687 and Pro688 fluctuated markedly around ITGB3-Mairin. Fig. 8B-C. The RMSF value of amino acids in the complex RORB-Senkyunone was substantially lower than in the complex ITGB3-Mairin thus suggesting that slight oscillation of amino acids might stabilize the complex RORB-Senkyunone. Moreover, the heatmap of hydrogen bond showed that the mutual effect was existed in both the conformation especially displayed in red columns thus indicating durable and stable functions. Fig. 8D and E. The dynamic change of the complex exhibited that the various proteins and ingredients remained stable after binding to each other. Fig. 8F and G.

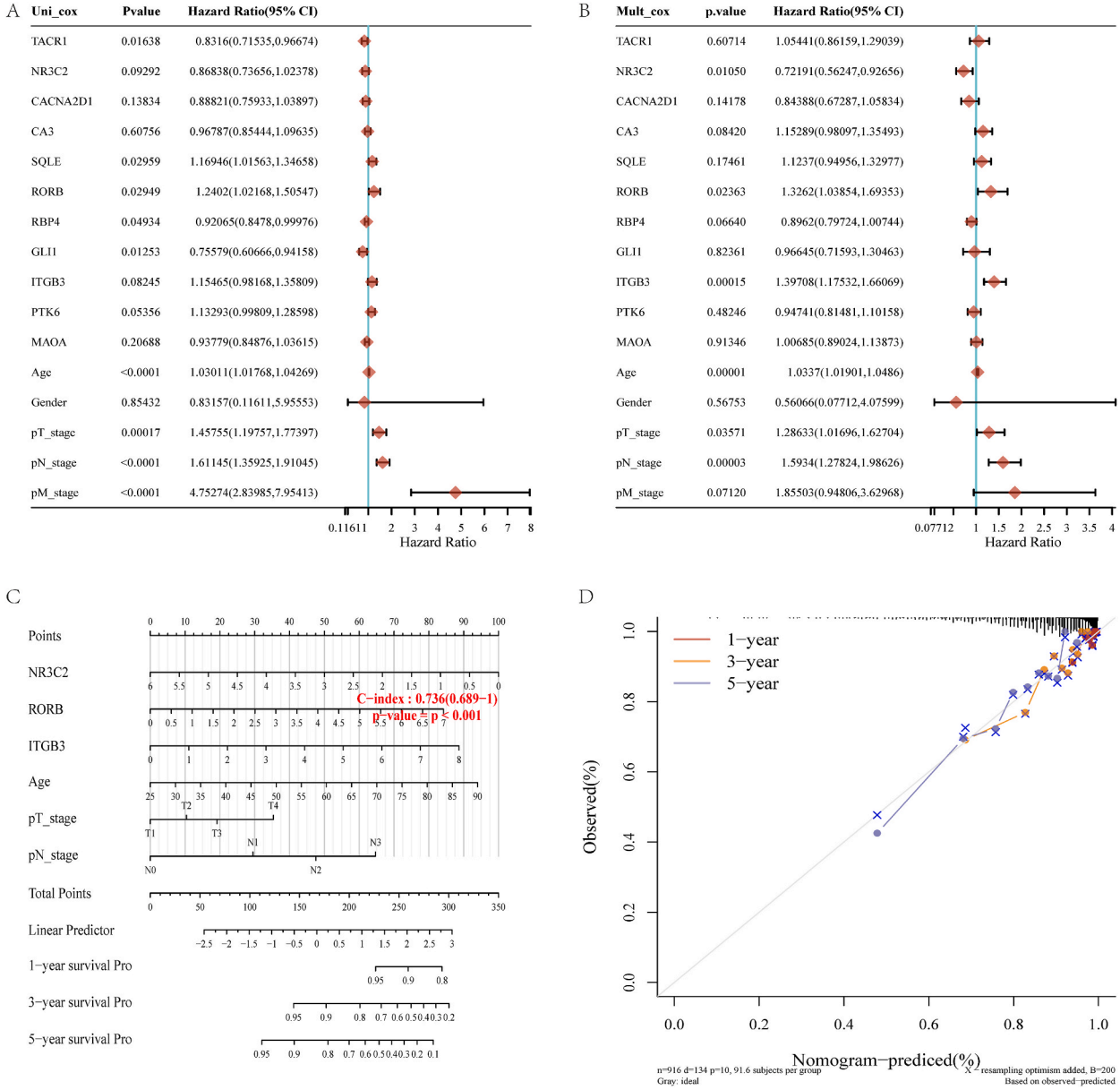


Fig. 4. Construction of nomogram of SWT against breast cancer. (A) Univariate cox analysis; (B) Multivariate cox analysis; (C) Nomogram of SWT against breast cancer; (D) Fit curve of nomogram.

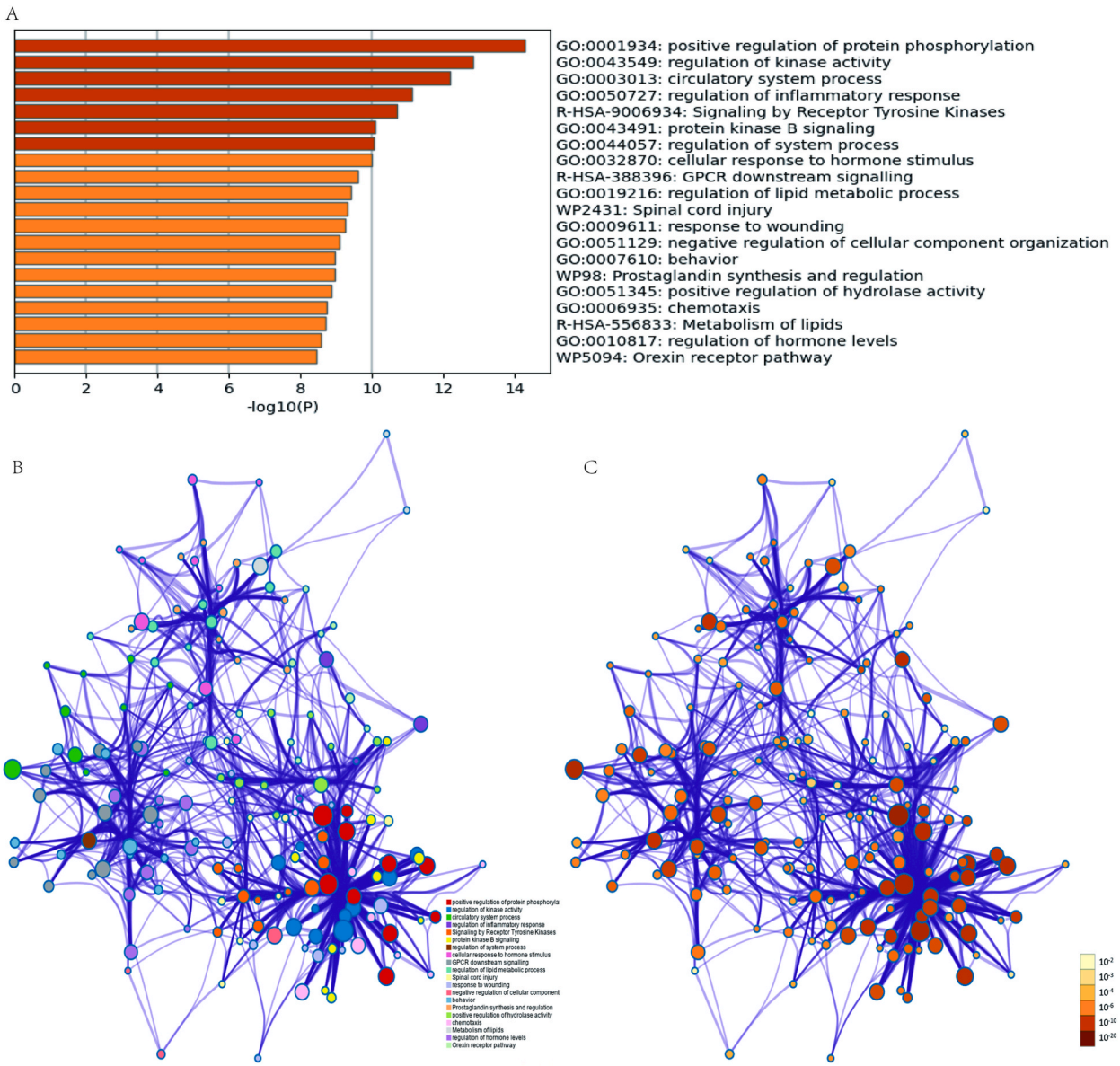


Fig. 5. GO and KEGG functional enrichment analyses. (A) Molecular functions and pathways; (B–C) Protein interaction network of GO and KEGG.

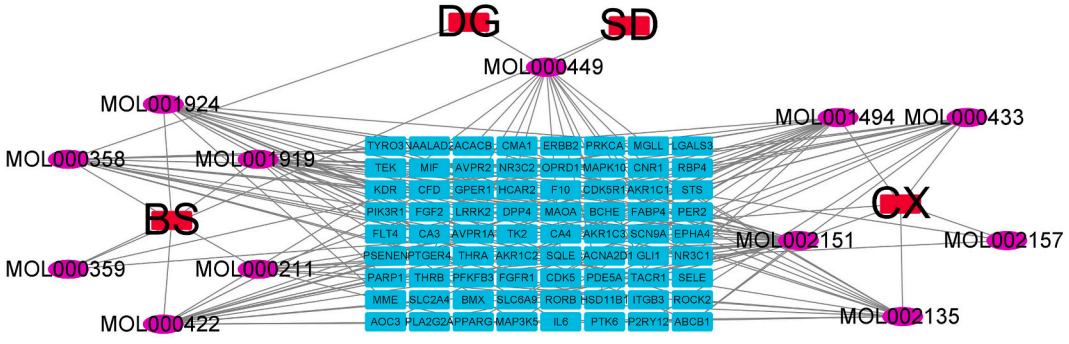


Fig. 6. Prediction of candidate compounds against breast cancer based on molecular and targets network.

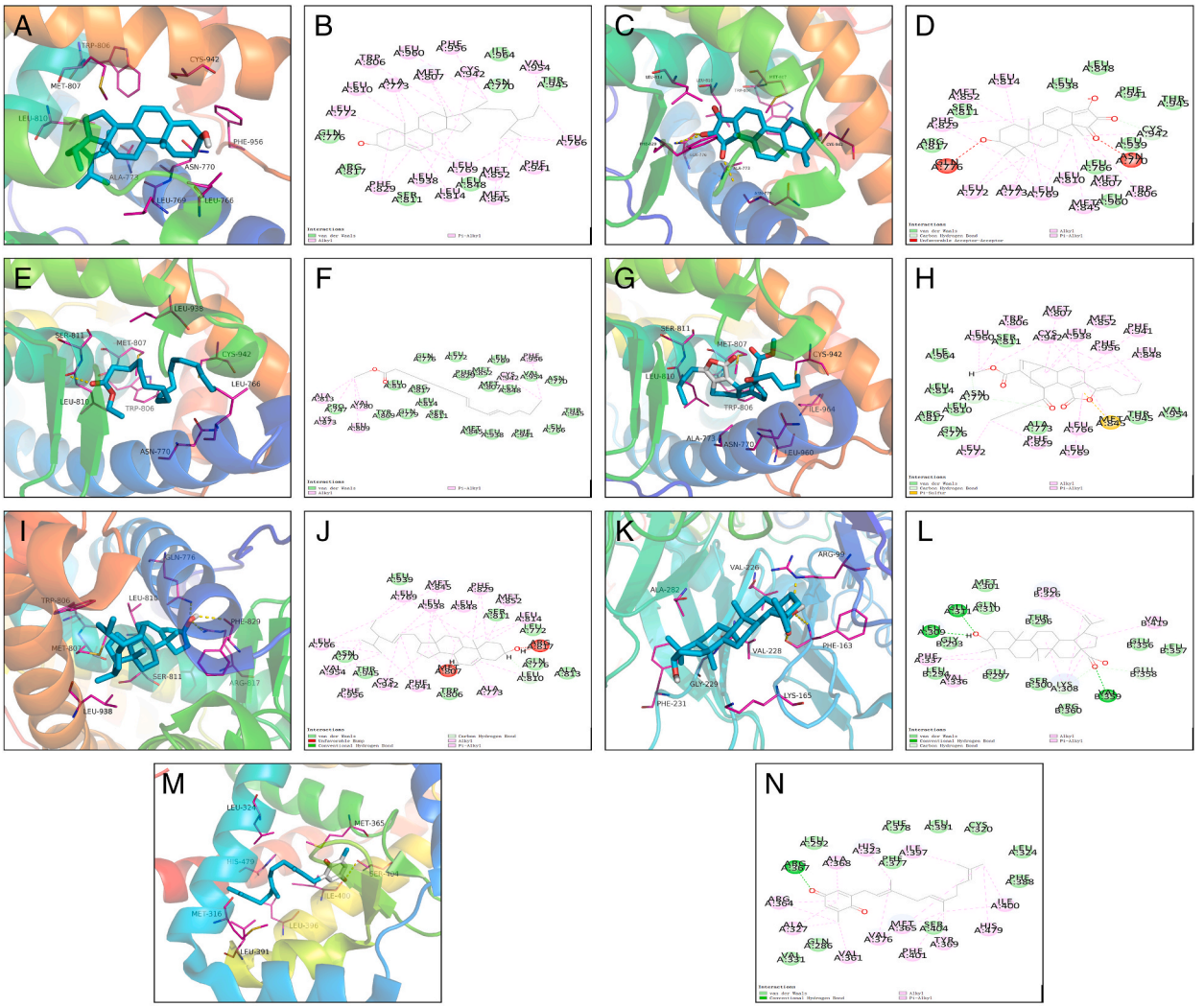
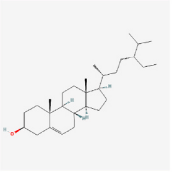
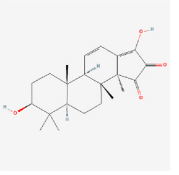
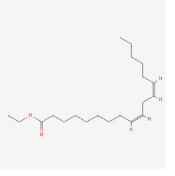
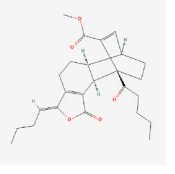


Fig. 7. Molecular docking. (A–B) Position and energy decomposition of NR3C2 docked with sitosterol; (C–D) Position and energy decomposition of NR3C2 docked with (3S,5R,8R,9R,10S,14S)-3,17-dihydroxy-4,4,8,10,14-pentamethyl-2,3,5,6,7,9-hexahydro-1H-cyclopenta[*a*]phenanthrene-15,16-dione; (E–F) Position and energy decomposition of NR3C2 docked with Mandenol; (G–H) Position and energy decomposition of NR3C2 docked with wallichilide; (I–J) Position and energy decomposition of NR3C2 docked with Stigmasterol; (K–L) Position and energy decomposition of ITGB3 docked with mairin; (M – N) Position and energy decomposition of RORB docked with senkyunone.

3.7. Validation of compounds by in vitro assays

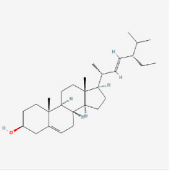
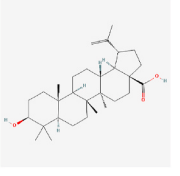
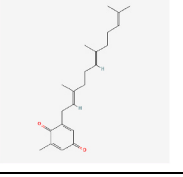
In order to further verify the results of the molecular docking and molecular dynamic, we prepared pharmacology serum of SWT that was incubated with MCF-7 cell. The results showed that the group of low concentration of SWT exhibited the most pronounced effect against MCF-7 cell in comparison to the control group with a p value < 0.01. Fig. 9A. Subsequently, the mRNA expressions of *NR3C2*, *ITGB3* and *RORB* were detected by qRT-PCR after incubating with the different concentration of SWT and estradiol. Moreover, compared with the control group, the mRNA expressions of *NR3C2* in all SWT group markedly showed an over-expression ($P < 0.001$), while compared with the blank group, the mRNA expressions of *NR3C2* and *ITGB3* in the SWT group were found to be significantly decreased ($P < 0.001$). Fig. 9B–D. In addition, it was observed that the protein levels of ITGB3 and RORB were up-regulated in all SWT groups in comparison to the control group ($P < 0.05$). It was also noted that NR3C2 was up-regulated more significantly than ITGB3 compared to the control group, which further confirmed the above results of the molecular docking and molecular dynamic. Fig. 9E–G. Nevertheless, although the protein level of NR3C2 was found to be up-regulated, the protein expression in the SWT groups were observed to be not statistically significant compared with the control group, which might be possibly related to the sample difference. Fig. 9H. Overall, these results further proved that SWT exhibited anti-cancer effects against MCF-7 cells by primarily targeting RORB and ITGB3 proteins.

Table 3
Molecular docking of hub ingredients and proteins.

Gene	Compound	Structure	Vina (kcal·mol^{-1})	RMSD	DS (LibDockScore)	Hydrogen bond interaction	Hydrophobic interaction
NR3C2 (1Y9R)	**sitosterol**		−3.1	2.693	115.2	–	VAL:954,LEU:766,PHE:941,MET:852,MET:845, LEU:769,LEU:814,LEU:938,PHE:829,LEU:772, LEU:810,ALA:773,TRP:806,MET:807,LEU:960, PHE:956,CYS:942
NR3C2 (1Y9R)	**(3S,5R,8R,9R,10S,14S)-3,17-dihydroxy-4,4,8,10,14-pentamethyl-2,3,5,6,7,9-hexahydro-1H-cyclopenta [*a*] phenanthrene-15,16-dione**		−6.7	1.294	113.2	CYS:942,	LEU:814,MET:852,PHE:829,GLN:776,LEU:772, ALA:773,LEU:769,LEU:810,MET:845,MET:807, TRP:806,ASN:770
NR3C2 (1Y9R)	**Mandenol**		−5.9	1.157	85.48	–	ALA:813,LYS:873,LEU:809,VAL:780,CYS:942, PHE:956
NR3C2 (1Y9R)	**wallichilide**		−5.9	1.368	98.5	–	LEU:960,TRP:806,CYS:942,MET:807,LEU:938, MET:852,PHE:956,PHE:941,LEU:848,MET:845, LEU:766,LEU:769,PHE:829,LEU:772

(*continued on next page*)

Table 3 (*continued*)

Gene	Compound	Structure	Vina (kcal·mol^{-1})	RMSD	DS (LibDockScore)	Hydrogen bond interaction	Hydrophobic interaction
NR3C2 (1Y9R)	**Stigmasterol**		−2.3	1.237	103	LEU:810	LEU:769,LEU:938,MET:845,LEU:848,PHE:829, MET:852,LEU:814,ARG:817,ALA:773,MET:807, PHE:941,CYS:942,PHE:956,VAL:954,LEU:766
ITGB3 (1JV2)	**Mairin**		−7.9	1.859	104.7	GLU:311, LEU:309, LYS:308, VAL:359,	PRO:326,VAL:419,VAL:336,PHE:337
RORB (6cn6)	**senkyunone**		−9.3	1.063	136.4	ARG:367	ALA:368,HIS:323,ILE:397,ILE:400,HIS:479, TYR:369,PHE:401,VAL:376,MET:365,VAL:361, ALA:327,ARG:364

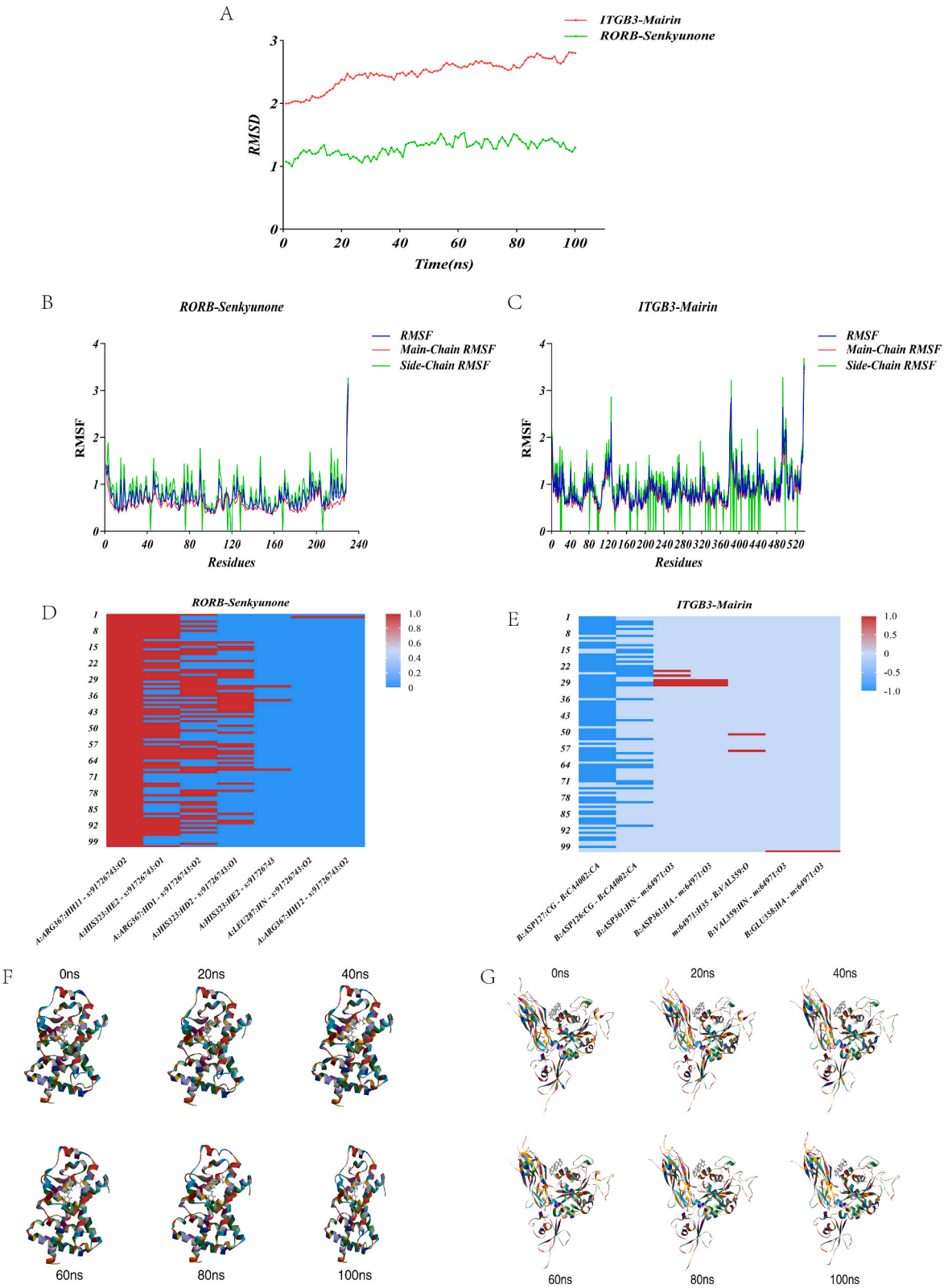


Fig. 8. Molecular dynamic. (A) RMSD of ITGB3 docked with mairin and RORB docked with senkyunone; (B) RMSF of RORB docked with senkyunone; (C) RMSF of ITGB3 docked with mairin; (D) Heatmap of amino acid of ITGB3 docked with mairin; (E) Heatmap of amino acid of RORB docked with senkyunone; (F) Motion trail of RORB in senkyunone in different times; (G) Motion trail of ITGB3 in mairin in different times.

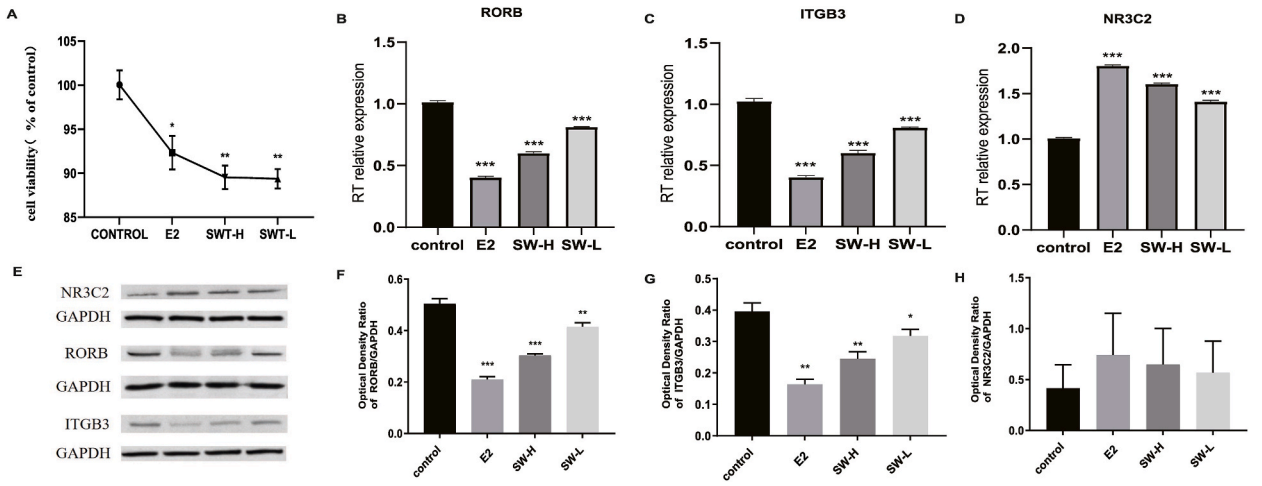


Fig. 9. Verification of molecular targets of SWT against breast cancer *in vitro* experiments. (A) CCK8 assay of SWT pharmacological serum against MCF cell; (B–D) Expressed level of mRNA of RORB, ITGB3 and NR3C2 was verified by RT-PCR; (E–H) Expressed level of protein of RORB, ITGB3 and NR3C2 was verified by WB.

4. Discussion

BRCA has overtaken lung cancer as the world's most common cancer and is the leading cause of cancer deaths among women worldwide in 2020. Presently, surgical resection is the major treatment choice for BRCA, in combination with chemotherapy and radiotherapy [19]. Although these methods can prolong the survival of patients, postoperative complications still remain inevitable [20]. SWT is a common TCM used to treat the various gynecological diseases and is reportedly often employed as a basic prescription drug owing to its significant antitumor effects. A number of studies have has demonstrated that SWT can exhibit an estrogen-like effect and inhibit the proliferation of MCF-7 cells by regulating the ratio of estrogen receptor α as well as β and by affecting the phosphorylation of amino acids, so as to play significant anti-breast cancer role [12,13].

Even if the methods such as network pharmacology has been widely used for the study of Chinese medicine, there still lacking a systematic integrating method in this filed. Besides, although some studies digged out some potiential targets and compounds, their validation methods are relatively simple [21,22]. An increasing number of scientific study instruments or methods will benefit the study of Chinese medicine in the future. In this study, we integrated network pharmacology, molecular docking and dynamics simulation, bioinformatic analysis, clinical prognostic model and experiments, which was able to provide a more reliable result.

In this study, 72 targets of SWT against breast cancer were acquired from TCGA database, and GO and KEGG analyses indicated that the therapeutic effect was primarily related to the various biological processes such as the regulation of the kinase activity, as well as the modulation of the receptor tyrosine kinases signaling pathways. The tyrosine kinase receptor family is a key regulator of different important cellular processes, which are involved in the regulation of the cell proliferation, apoptosis, cell migration and other physiological processes [23]. The RTK family contains multiple subfamily receptors, such as epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGFR), fibroblast growth factor receptor (FGFR) etc. and over-activation of RTK pathways has been associated with increased aggressiveness and poor prognosis of BRCA [24]. NF-κB activation can effectively cause down-regulation of ER expression and EGFR over-activation in inflammatory breast cancer IBC, while EGFR/COX-2 axis can induce EMT to promote BRCA stem cell-like cell (CSC) phenotype and increase IBC invasiveness. VEGF signaling activation can induce the tumor growth and angiogenesis, and VEGFR1 activation can also promote EMT of the cancer cells to further augment the ability of breast cancer metastasis. In addition, RTK signaling pathway can activate PI3K/Akt signaling cascade and induce expression of the multi-drug resistance proteins as well as the membrane transporters in the cancer cells, which have been closely linked to the drug resistance in BRCA cells [25].

In order to explore the targets and mechanisms of SWT against BRCA, and to construct a prognostic model, 72 anti-BRCA targets of SWT were screened by a combination of network pharmacology and bioinformatics analysis. Univariate cox analysis and LASSO regression analysis were thereafter performed on 72 targets. Redundant factors were then removed and 11 prognostic genes were selected to construct the prognostic models. Survival analysis indicated that the survival time of the high-risk group was significantly shorter than that of the low-risk group. In addition, the AUC area was also calculated by ROC curve of the prognostic model, and the AUC at 1, 3 and 5 years was 0.668, 0.705 and 0.693, respectively, thereby suggesting that the prognostic model had good predictive efficacy for BRCA. Thereafter, univariate cox and multivariate cox analysis was performed to construct a nomogram based on 11 genes and clinical characteristics, including age, sex, and TNM stages. The results showed that RORB, NR3C2, ITGB3, age and TN stages acted as independent prognostic factors and could effectively evaluate the prognosis of patients, with a C index of 0.736.

RORB belongs to the thyroid hormone receptor subfamily, also known as RARP-associated orphan receptor -β, and is a circadian rhythm gene [26]. It has been established that evasion of the circadian rhythms is considered an emerging marker of cancer. A pan-cancer study confirmed that RORB upregulation in cancer can significantly disrupt the circadian clock. The mechanism of RORB

affecting the biological CLOCK of BRCA was related to the mechanism of the interlocking feedback loop formed by ARNTL (BMAL1) -clock/NPAS2 complex and PER and CRY, which were primarily activated by ARNTL heterodimer transcription [27]. Member 2 (NR3C2) of nuclear receptor subfamily 3C is the *MR* gene, a transcription factor that plays a pivotal role in the regulation of electrolyte balance. A number of the prior studies have shown that when the diagnostic efficacy of NR3C2 in BRCA was verified by ROC curve, the AUC value was as high as 0.908, thus suggesting that NR3C2 might play an important role in BRCA progression [28]. Moreover, *in vitro* experiments have demonstrated that Mir-301B-3p targeted inhibition of the binding site sequence on NR3C22 3′UTR, markedly reduced *NR3C2* mRNA and protein expression, and then induced migration and invasion of BRCA [29]. ITGB3 is an important member of the integrin β family and a key participant in mediating the interaction between the cells and their environment. ITGB3-mediated endocytosis of extracellular vesicles (EV) can affect the sensitivity of BRCA cells against the tumor microenvironment [30]. As a transmembrane cell-surface receptor, IGTB3 has been reported to participate in EMT through srC-mediated receptor phosphorylation pathway in the mammary epithelial cells, thereby inducing the migration and invasion of these cancer cells [31].

In order to further clarify the components targeting RORB, NR3C2 and ITGB3, a molecular target network was established to screen out 7 components targeting the three different genes. As observed from the molecular target network, 6 compounds from BS, 6 from CX, and 2 from SD and DG were identified. Among them, there were 5 compounds targeting NR3C2, 1 compound targeting ITGB3 and 1 compound targeting RORB.

To verify the reliability of these results, molecular docking and molecular dynamics simulations were performed. Molecular docking results indicated that the binding energies of Mairin and ITGB3, and Senkyunone and RORB were −7.9 and −9.3, respectively. Therefore, anti-breast cancer activity of SWT might be achieved by targeting ITGB3 and RORB by the above two compounds. To further validate this result, molecular dynamics simulations were used to evaluate the possible combinations of the compound and the target. Interestingly, it was found that the combination of Mairin and ITGB3, senkyunone and RORB was stable overall and senkyunone and RORB appeared to be substantially better than Mairin and ITGB3.

Finally, in order to verify whether SWT can significantly regulate the three key core genes, *RORB*, *NR3C*2 and *ITGB3*, RT-PCR and WB experiments were performed. The results indicated that SWT could significantly down-regulate the expression of RORB and ITGB3 at mRNA and protein levels. It was noted that the group of RORB compared with the control group showed a higher level of significance than ITGB3 compared with the control group, which was consistent with the results of our molecular docking and molecular dynamics simulations. In addition, although SWT was observed to significantly up-regulate *NR3C2* expression at mRNA level, there appeared to be no significant difference at the protein level. Therefore, we concluded that SWT effect against BRCA might be through regulating the expression of *RORB* and *ITGB3*.

5. Conclusions

To be summary, the pivotal targets and mechanisms of SWT against BRCA were explored and a prognostic model was constructed by using an integration methods in this study. The hub targets and ingredient of SWT were further validated based on various *in vitro* experiments. Overall, our results suggested that anti-neoplastic effects of SWT against BRCA might be achieved by targeted inhibition of *RORB* and *ITGB3* expression by senkyunone and mairin. Of course, this study also has some limitations. The BRCA patients we included have heterogeneity, which may have some bias in the prognostic model when applying in the patients with different molecular subtypes.

Consent for publication

Not applicable.

Availability of data and materials

The data of this study were acquired from TCGA database, TCMSP database, Pub Chem database and Swiss Target Prediction database.

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Ethics approval and consent to participate

Not applicable.

CRediT authorship contribution statement

Zeye Zhang: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Visualization, Writing – original draft, Writing – review & editing. **Zexin Zhang:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft. **Jinqin Song:** Data curation. **Wenfeng Wu:**

Data curation. **Yiqi Chen:** Writing – original draft. **Jing Li:** Writing – original draft. **Yongchen Wang:** Writing – original draft. **Piwen Zhao:** Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Lists of abbreviations

SWT: Si-Wu-Tang
AUC: Area under the curve
ROC: Receiver operating characteristic
DEGs: Differentially expressed genes
SD: Shudi
DG: Danggui
CX: Chuanxiong
BS: Baishao
GO: Gene Ontology
KM: Kaplan–Meier
KEGG: Kyoto Encyclopedia of Genes and Genomes
LASSO: Least absolute shrinkage and selection operator
RMSD: Root-mean-square deviation
RMSF: Root-mean-square fluctuation
RT-PCR: Real-time polymerase chain reaction
WB: Western bolt
TCGA: The Cancer Genome Atlas
TCM: Traditional Chinese medicine
TCMSP: Traditional Chinese Medicine Systems Pharmacology