

Identification of Key Therapeutic Targets of Triptolide Against Breast Cancer by Integrated Transcriptomic Analysis and Co-expression Network Approach

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Abstract

Background: Triptolide, a natural product extracted from *Tripterygium wilfordii*, has shown anti-tumor activities against breast cancer (BC). This study aimed to identify potential therapeutic targets of triptolide for treating breast cancer using bioinformatics approaches.

Methods: The transcriptomic data of breast cancer tissues and triptolide-treated breast cancer cells were obtained from public databases. Differentially expressed genes (DEGs) were identified using limma package. Weighted gene co-expression network analysis (WGCNA) was performed to detect modules related to breast cancer progression and triptolide treatment. Hub genes in relevant modules were identified as candidate targets based on module membership and gene significance. The common hub genes were determined as potential therapeutic targets. Functional enrichment was conducted to investigate the biological functions and pathways involved in these therapeutic targets. Then, PPI networks were constructed to explore interactions between them and identify key therapeutic targets. Finally, independent datasets were utilized to validate the expression and diagnostic value of key targets.

Results: A total of 5206 and 2113 targets were identified associated with breast cancer progression and triptolide treatment, respectively. By integrating these two groups of targets, 122 common targets were determined as candidate therapeutic targets of triptolide, which were significantly enriched in tumor-related metabolic pathways, such as pyruvate metabolism, glycolysis/gluconeogenesis, citrate cycle. PPI network analysis identified 10 hub targets, including VIM, DLD, ACAT1, RABIF, ALDH2, RPS20, BIN1, TUBB6, CALM1 and PINK1. Their aberrant expression in cancer was validated, and triptolide could reverse this aberrance. ROC analysis showed their potential as diagnostic markers and therapeutic targets.

Conclusions: Integrated transcriptomic and network analysis identified potential therapeutic targets of triptolide against breast cancer, including key therapeutic targets VIM, DLD, ACAT1, RABIF, ALDH2, RPS20, BIN1, TUBB6, CALM1 and PINK1. Our findings provide novel insights into the mechanisms of triptolide against breast cancer.

1 Introduction

Breast cancer (BC) is one of the most common malignancies and the leading cause of cancer-related death in women globally (Wilkinson and Gathani 2022). According to GLOBOCAN 2020 estimates, there were about 2.26 million new breast cancer cases and 0.68 million deaths worldwide in 2020 (Sung et al. 2021). The development of breast cancer involves complex interactions between genetic, reproductive, and environmental risk factors (Coughlin 2019). Currently, the major treatments for breast cancer include surgery, chemotherapy, radiotherapy, endocrine therapy, etc (Trayes and Cokenakes 2021). In recent years, with the improvement in early diagnosis and therapeutic methods, the prognosis of breast cancer patients has been improved to some extent. However, the overall cure rate for breast cancer remains relatively low (Liang et al. 2020). Due to the lack of effective targeted therapies, distant metastasis is still the major obstacle for the survival of breast cancer patients (Liang et al. 2020, Hosonaga, Saya and

Arima 2020). Therefore, identifying novel therapeutic targets and drugs for breast cancer is the key focus of current research.

Triptolide, a natural product extracted from *Tripterygium wilfordii*, has shown anti-tumor activities against breast cancer and other cancers (Tong et al. 2021). Mechanistic studies have demonstrated that triptolide could inhibit tumor cell proliferation, induce apoptosis, suppress angiogenesis, reverse drug resistance through multiple pathways (Luo et al. 2019, AbdulHussein et al. 2023). Moreover, triptolide can also sensitize tumor cells to chemo- and radiotherapies without increasing the toxicity to normal cells (Jiang et al. 2016, Zhang et al. 2016, Lin et al. 2013). Thus, triptolide holds great potential for cancer treatment. However, the molecular mechanisms mediating the anti-tumor effects of triptolide remain largely unclear. Identifying the key targets and signaling pathways of triptolide action will facilitate its clinical application as a novel therapeutic agent for cancers.

With the development of high-throughput omics technologies and bioinformatics analysis, researchers are able to explore molecular signatures associated with cancer progression and drug response on a genome-wide scale (Li et al. 2020). Weighted gene co-expression network analysis (WGCNA) is a systems biology method used for describing the correlation patterns among genes across transcriptomic datasets. It can be utilized to identify modules of highly correlated genes and hub genes, which are considered as candidate biomarkers or therapeutic targets (Kakati et al. 2019). Integration of WGCNA with other bioinformatics approaches provides an effective strategy to uncover molecular mechanisms and screen targets related to cancer and drug treatments.

In this study, we performed integrated transcriptomic and bioinformatics analysis to elucidate the mechanisms of triptolide against breast cancer and screen potential therapeutic targets. We compared the transcriptomic profiles of breast cancer tissues and triptolide-treated breast cancer cells to identify aberrantly expressed genes. Weighted gene co-expression network analysis (WGCNA) was utilized to detect gene modules and hub genes associated with breast cancer progression or triptolide treatment. Through comparing hub genes from both analyses, we identified common hub genes as candidate targets of triptolide. Functional enrichment analysis was conducted to investigate their potential involvement in biological processes and pathways. Furthermore, protein-protein interaction (PPI) analysis was performed to explore the interactions between candidate targets and determine key targets. The expression and diagnostic value of key targets were further validated in independent datasets. Our study provides an effective strategy to elucidate the molecular mechanisms of triptolide against breast cancer and identify key mediators of its anti-tumor activities. These findings will facilitate the development of triptolide and its derivatives as novel treatment options for breast cancer.

2 Methods

2.1 Data Collection and Preprocessing

The transcriptomic data of 1091 breast cancer samples and 113 normal breast tissue samples were downloaded from The Cancer Genome Atlas (TCGA) database (<https://portal.gdc.cancer.gov/>). Gene expression data of another cohort containing 104 breast cancer samples and 17 normal breast tissue samples (GSE42568) were obtained from the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>). The transcriptomic data of breast cancer cells treated with triptolide (GSE16760) were also obtained from GEO database. This dataset included 36 samples of breast cancer cell line A549 treated with different concentrations of triptolide (0.05, 0.15, 0.45 μM) or control medium for 1, 2 and 4 hours (3 replicates per group). Raw data were preprocessed using limma package in R, including background correction, normalization and log2 transformation.

2.2 Identification of DEGs

The differentially expressed genes (DEGs) between breast cancer and normal breast samples were identified using the limma package in R separately for TCGA-BC dataset. Genes with $P < 0.05$ and $|\text{fold change}| > 1$ were considered as DEGs significantly associated with breast cancer. DEGs between triptolide-treated and control A549 cells were also identified by limma package. Genes with $P < 0.05$ and $|\text{fold change}| > 1$ were considered as DEGs significantly associated with triptolide treatment.

2.3 Weighted Gene Co-expression Network Analysis

The “WGCNA” package in R was utilized to construct two separate co-expression networks using DEGs identified from TCGA-BC dataset and GSE16760 dataset, respectively.

For TCGA-BC dataset, the DEGs between breast cancer and normal samples were input into WGCNA. A signed Pearson correlation matrix was computed for all DEG pairs. Then the adjacency matrix was calculated by raising the correlation matrix to a power β to fulfill a scale-free topology. The adjacency matrix was further transformed into a topological overlap matrix (TOM). Genes were hierarchically clustered based on TOM dissimilarity. Modules were identified from the clustering dendrogram and similar modules were merged. Then, module eigengenes (MEs) were calculated for each module. The association between MEs and breast cancer status was evaluated to identify modules significantly related to breast cancer ($P < 0.05$). Within these modules, hub genes were defined as genes with high module membership ($\text{MM} > 0.5$) and high gene significance ($\text{GS} > 0.5$) for breast cancer status. These hub genes were considered as breast cancer-related targets.

For GSE16760 dataset, the DEGs between triptolide-treated and control cells were utilized to construct a weighted co-expression network following the same procedure. Module-trait correlation analysis was performed between module MEs and triptolide dosage/time to identify triptolide-related modules. Specifically, we calculated the correlation between MEs and dosage under each time point, and the correlation between MEs and time under each dosage. Modules significantly correlated with both variables were selected. Hub genes were identified with thresholds of $\text{GS} > 0.5$ and $\text{MM} > 0.5$. These hub genes with dose-dependent and time-dependent expression changes were considered as triptolide-related targets.

The overlapping hub genes between breast cancer-related modules from TCGA-BC dataset and triptolide-related modules from GSE16760 dataset were identified as candidate therapeutic targets of triptolide against breast cancer.

2.4 Functional Enrichment Analysis

Gene ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed for the identified candidate therapeutic targets using R package clusterProfiler to investigate their associated biological functions and signaling pathways. For GO analysis, the candidate targets were mapped to GO terms in GO database (<http://geneontology.org/>) to identify enriched terms, including biological processes (BP), cellular components (CC) and molecular functions (MF). For KEGG pathway analysis, the candidate targets were mapped to KEGG database (<https://www.kegg.jp/kegg/pathway.html>) to find enriched pathways. Fisher's exact test was applied to evaluate the statistical significance of enrichment for each GO term and KEGG pathway. GO terms and KEGG pathways with $P < 0.05$ were considered significantly enriched.

2.5 PPI Network Construction and Analysis

Protein-protein interaction (PPI) network analysis was performed to investigate the interactions among the candidate therapeutic targets and further identify key targets. The PPI network was constructed by mapping the targets to STRING database (<https://string-db.org/>). Cytoscape software (version 3.7.2) was utilized to visualize the network. CytoHubba plugin in Cytoscape was used to calculate hub scores and rank nodes based on Maximal Clique Centrality (MCC) algorithm. Top 10 ranked nodes with the highest MCC scores were identified as hub proteins in the PPI network, which may play critical roles in regulating other targets. Therefore, these hub proteins were considered as key therapeutic targets, which likely play important roles in mediating the anti-tumor mechanisms of triptolide. In addition, KEGG pathway enrichment analysis for these key targets was performed using clusterProfiler package in R to further explore the signaling pathways involved. Terms with $P < 0.05$ were considered as significantly enriched pathways.

2.6 Validation of Key Targets in Breast Cancer and Triptolide Treatment

To validate the expression and diagnostic value of the identified key targets in breast cancer, we first compared their expression profiles between breast tumor and normal tissue samples from TCGA-BC cohort and GEO dataset GSE42568 using Wilcoxon rank-sum test. $P < 0.05$ was considered statistically significant. Then, receiver operating characteristic (ROC) curve analysis was performed to evaluate the capability of key targets for discriminating breast cancer from normal breast tissues using R package "pROC". ROC curve is a widely used statistical method to assess the performance of a classifier in distinguishing two groups. The diagnostic power is reflected by the area under ROC curve (AUC), and $AUC > 0.6$ indicates a good discrimination.

Furthermore, we analyzed the expression changes of key therapeutic targets in response to triptolide treatment based on dataset GSE16760. Student's t-test was used to compare the statistical differences in target expression between triptolide-treated groups (0.05, 0.15, 0.45 μM) and control group (0 μM) at different time points. Differences were considered statistically significant when $P < 0.05$. ROC curve analysis was also conducted to validate the power of key targets for distinguishing triptolide-treated breast cancer cells from untreated controls.

3 Results

3.1 Identification of breast cancer-related targets

Differential expression analysis between 1091 breast cancer samples and 113 normal controls from TCGA-BC database identified 15,991 DEGs, including 6052 down-regulated and 9939 up-regulated DEGs in cancer samples compared to normal tissues (Fig. 1A). WGCNA analysis was performed on these DEGs to identify gene modules and hub genes associated with breast cancer. With a soft thresholding power β of 4 (Fig. 1B), a total of 22 gene modules were identified using average linkage hierarchical clustering and dynamic tree cut (Fig. 1C). Among them, 20 modules were significantly correlated with breast cancer status ($P < 0.05$) (Fig. 1D). Based on $\text{MM} > 0.5$ and $\text{GS} > 0.5$, a total of 5206 hub genes were identified from these 20 relevant modules as breast cancer-related targets, including 4084 up-regulated and 1122 down-regulated hub genes in breast cancer tissues (Fig. 1E).

3.2 Identification of triptolide-related targets

Differential expression analysis between triptolide-treated and control breast cancer cell samples in GSE16760 dataset identified 6337 DEGs, including 4542 up-regulated and 1795 down-regulated genes in triptolide-treated cells compared to controls (Fig. 2A). WGCNA was performed on these DEGs and identified 14 gene modules with a soft thresholding power β of 22 (Fig. 2B, C). Among them, 4 modules were significantly correlated with both triptolide dosage and treatment time ($P < 0.05$) (Fig. 2D). Based on $\text{MM} > 0.5$ and $\text{GS} > 0.5$, a total of 2113 hub genes were identified from these 4 modules as triptolide-related targets, including 1883 up-regulated and 230 down-regulated hub genes in triptolide-treated cells (Fig. 2E).

3.3 Identification of Candidate Therapeutic Targets

By comparing the 5206 breast cancer-related and 2113 triptolide-related hub genes, 122 overlapping targets were identified as candidate therapeutic targets of triptolide against breast cancer. These included 90 up-regulated targets in breast cancer tissues but down-regulated after triptolide treatment, and 32 down-regulated targets in breast cancer but up-regulated after triptolide treatment (Fig. 3).

3.4 Functional Enrichment Analysis of Candidate Targets

To explore the potential mechanisms of triptolide against breast cancer, GO and KEGG functional enrichment analysis of the 122 candidate targets was performed. The top 10 most significantly enriched GO terms were displayed in Fig. 4. GO analysis suggested that these targets were involved in several biological processes such as positive regulation of cell proliferation, carbohydrate metabolic process, ubiquitin-dependent protein catabolic process, positive regulation of protein kinase B signaling, gluconeogenesis, electron transport chain (Fig. 4A). The candidate targets were mainly enriched in many cellular components including cytosol, cytoplasm, nucleus, membrane, nucleoplasm, extracellular exosome (Fig. 4B). And they exerted multiple molecular functions such as protein binding, identical protein binding, RNA binding, GTPase activity, protein heterodimerization activity, electron carrier activity (Fig. 4C). KEGG enrichment analysis revealed that the candidate targets were primarily enriched in pathways including metabolic pathways, pyruvate metabolism, pathways in cancer, valine, leucine and isoleucine degradation, tryptophan metabolism, lysine degradation, glycolysis / gluconeogenesis, glyoxylate and dicarboxylate metabolism, citrate cycle (TCA cycle), propanoate metabolism, fatty acid degradation (Fig. 4D). Notably, the candidate targets were significantly enriched in tumor-related metabolic functions and pathways, suggesting metabolism may play an important role in mediating the anti-tumor effects of triptolide against breast cancer.

3.5 PPI Network Analysis and Identification of Key Targets

To investigate the interactions between the 122 candidate targets, we constructed a protein-protein interaction (PPI) network using the STRING database. The PPI network contained 119 nodes and 582 edges, indicating extensive interactions between the candidate targets (Fig. 5A). Topological analysis of the network revealed that nodes including VIM (degree = 31), DLD (degree = 28), ACAT1 (degree = 25), RABIF (degree = 25) and ALDH2 (degree = 23) had the highest degree values, suggesting their hub roles in the network. Furthermore, to identify key therapeutic targets among the candidates, we utilized the CytoHubba plugin to calculate the MCC scores and rank the nodes. The top 10 ranked hub nodes with the highest MCC scores were identified as key therapeutic targets and used to construct an interaction network, including VIM, DLD, ACAT1, RABIF, ALDH2, RPS20, BIN1, TUBB6, CALM1 and PINK1 (Fig. 5B). Functional enrichment analysis revealed that these key targets were significantly enriched in cancer-related metabolic pathways, including tryptophan metabolism, pyruvate metabolism, valine, leucine and isoleucine degradation, lysine degradation, glyoxylate and dicarboxylate metabolism, fatty acid degradation, and glycolysis/gluconeogenesis (Fig. 5C).

3.6 Validation of Key Therapeutic Targets in Breast Cancer and Triptolide Treatment

We first validated the aberrant expression of identified key targets in breast cancer tissues compared to normal controls using TCGA-BC and GSE42568 datasets. The results showed VIM, DLD, ACAT1, ALDH2, RPS20, BIN1, TUBB6, CALM1 and PINK1 were significantly down-regulated in breast tumor tissues ($P < 0.05$), while RABIF was up-regulated ($P < 0.05$) (Fig. 6A, B). ROC curve analysis further indicated these key

targets could effectively distinguish breast tumor from normal tissues, with AUC values all greater than 0.6. Among them, RABIF achieved the highest AUC of 0.972 in TCGA-BC cohort, and RPS20 had the best diagnostic power with AUC of 0.929 in GSE42568 dataset (Fig. 7A, B).

We then analyzed the expression changes of key targets in response to triptolide treatment using dataset GSE16760. The results showed noticeable alterations of key target expression in response to triptolide treatment. The expression of VIM, DLD, ACAT1, ALDH2, RPS20, BIN1, TUBB6, CALM1 and PINK1 exhibited positive correlation with triptolide dosage and treatment time. In contrast, RABIF expression was negatively correlated with dosage and time. At 4 h treatment, compared to control cells treated with 0 μ M triptolide, these key targets showed significant expression changes in cells treated with 0.15 μ M and 0.45 μ M triptolide ($P < 0.05$) (Fig. 8). ROC curve analysis further demonstrated that the key targets could effectively distinguish triptolide-treated cells from untreated controls, with AUC values all greater than 0.6 (Fig. 9).

4 Discussion

Breast cancer is the most prevalent malignancy among women worldwide, posing a great threat to women's health (Wilkinson and Gathani 2022). For many years, surgery, chemotherapy, and adjuvant endocrine therapy have been the mainstay treatment for breast cancer (Trayes and Cokenakes 2021). Although survival rates for early-stage breast cancer have improved, therapies for metastatic and recurrent disease remain limited (Trayes and Cokenakes 2021, Liang et al. 2020, Hosonaga et al. 2020). Therefore, developing novel targeted therapies is imperative. Triptolide, a natural product derived from traditional Chinese medicine, has displayed anti-tumor activities against breast cancer and other cancers (Luo et al. 2019). However, its specific mechanisms of action are still not well elucidated. In this study, we utilized integrated bioinformatics approaches to systematically identify the potential targets of triptolide against breast cancer, providing insights into developing triptolide as an anti-cancer drug.

Firstly, based on TCGA-BC dataset, we identified 5206 targets associated with breast cancer progression through WGCNA analysis. We then analyzed the transcriptomic changes of breast cancer cells in response to triptolide treatment, and identified 2113 triptolide-responsive targets. These genes showed expression changes correlated with triptolide dosage and treatment time, suggesting their potential involvement in the anti-tumor effects of triptolide. By comparing breast cancer-related and triptolide-related targets, we identified 122 common targets as candidate therapeutic targets of triptolide. These common targets were aberrantly expressed in breast cancer and could be regulated by triptolide, thus likely mediating the anti-tumor action of triptolide.

Functional enrichment analysis results showed that these therapeutic targets were mainly enriched in tumor-related metabolic pathways, including pyruvate metabolism, glycolysis/gluconeogenesis, citrate cycle (TCA cycle), valine, leucine and isoleucine degradation, tryptophan metabolism, lysine degradation, glyoxylate and dicarboxylate metabolism, propanoate metabolism, fatty acid degradation. It is known that tumor cells exhibit significant metabolic reprogramming, which enables them to survive and

proliferate rapidly in the nutritionally deficient tumor microenvironment (Wang, Jiang and Dong 2020). Pyruvate metabolism, glycolysis/gluconeogenesis and citrate cycle (TCA cycle) are pathways closely related to tumor cell energy metabolism (Gray, Tompkins and Taylor 2014, Lu, Tan and Cai 2015). Pyruvate is a key intermediate metabolite of glucose metabolism in tumor cells (Gray et al. 2014), while glycolysis/gluconeogenesis is one of the major pathways for tumor cells to generate energy from glucose (Lu et al. 2015). Valine, leucine and isoleucine degradation, tryptophan metabolism and lysine degradation pathways are mainly involved in amino acid metabolism. Valine, leucine, and isoleucine are branched-chain amino acids (BCAAs) that can promote tumor cell proliferation through multiple pathways, including mTOR signaling, contributing to cellular metabolism, and providing substrates for protein synthesis (Nie et al. 2018). Tryptophan is an essential amino acid, and increased tryptophan metabolism is beneficial to the aggregation of immunosuppressive T cells and inhibits tumor cell apoptosis (Peyraud et al. 2022). Increased lysine metabolism can produce more acetylated histones to activate oncogenes, inhibit tumor suppressors, and promote tumorigenesis (Moreno-Yruela et al. 2022, Zhang et al. 2019, Guo et al. 2018). Glyoxylate/dicarboxylate metabolism, propanoate metabolism, and fatty acid degradation involve carbohydrate and fat metabolism. As a TCA cycle replenishment pathway, glyoxylate/dicarboxylate metabolism provides sugar skeletons for anabolism and gluconeogenesis in tumor cells (Pan et al. 2021). Propanoate metabolism produces methylmalonic acid to facilitate invasion and metastasis in breast cancer (Gomes et al. 2022). Park et al. (2021) found that sodium propionate can inhibit the proliferation of breast cancer cells and induce apoptosis by inhibiting STAT3, increasing ROS levels and activating p38. Additionally, fatty acid degradation generates acetyl-CoA through β -oxidation to fuel the TCA cycle and provide energy to tumor cells (Currie et al. 2013). Notably, some studies found triptolide affects these same metabolic pathways. For instance, triptolide inhibited head and neck cancer cell growth and metastasis by suppressing glycolysis/gluconeogenesis (Cai et al. 2021). Metabolomics analysis revealed triptolide treatment altered TCA cycle, amino acid, and fatty acid metabolism in mice (Zhao et al. 2018). However, whether triptolide exerts its anti-breast cancer effects through similar metabolic mechanisms warrants further investigation.

To elucidate connections between the 122 candidate targets and determine key therapeutic targets, we constructed a PPI network and performed topological analysis. Based on the MCC algorithm, we identified 10 key targets, including VIM, DLD, ACAT1, RABIF, ALDH2, RPS20, BIN1, TUBB6, CALM1 and PINK1. Furthermore, we analyzed their expression patterns in breast cancer versus normal breast tissues, as well as in triptolide-treated versus control breast cancer cells. The results showed that compared with normal breast tissues, RABIF was down-regulated in breast cancer tissues, while the other key targets were up-regulated in breast cancer tissues. Triptolide reversed the aberrant expression of these key targets in a time- and dose-dependent manner. Moreover, ROC analysis validated the potential of these key targets as diagnostic biomarkers and therapeutic targets for breast cancer. Notably, many studies have demonstrated the importance of these key targets in breast cancer and other cancers.

For example, VIM expression is downregulated in breast cancer cells compared to normal breast cells (Sharp et al. 2008) and associated with bone metastasis (Senga et al. 1986). The key target DLD encodes dihydrolipoamide dehydrogenase (DLD), which is a component of several multienzyme complexes,

including the pyruvate dehydrogenase complex, α -ketoglutarate dehydrogenase complex and branched-chain α -keto acid dehydrogenase complex (Yan and Wang 2023). DLD is downregulated in breast cancer, correlating with poor prognosis (Jiang et al. 2022, Zhang et al. 2022). It has been shown that decreased expression of DLD will lead to metabolic reprogramming of immune cells (Palmieri et al. 2023). It may have the same effect in tumor cells, but still unclear. ACAT1, a lipid metabolism gene downregulated in breast cancer predicts poor prognosis when expressed at low levels (Kuldeep et al. 2023, Maldonado et al. 2021). Zhang et al. (2023) found that ACAT1 encoded Acetyl-CoA acetyltransferase 1 could inhibit breast cancer cell migration and invasion by binding to METTL3. ALDH2 (aldehyde dehydrogenase 2) is an enzyme related to Glyoxylate and dicarboxylate metabolism. Its low expression can enhance the glycolytic pathway in cells (Ma et al. 2023). ALDH2 deficiency promoted hepatocellular carcinoma through activating oncogenic signaling including JNK, STAT3, BCL-2, and TAZ (Seo et al. 2019). ALDH2 was also found to facilitate colon cancer growth and metastasis via β -catenin signaling (Wei et al. 2023). While direct evidence for ALDH2 in breast cancer is lacking, it represents a breast cancer biomarker (Han et al. 2023). Rab (Ras-related in brain) interacting factor (RABIF) is a GTPase activating protein that can activate the GTPase activity of the Rab family and participate in the regulation of intracellular membrane trafficking (Gulbranson et al. 2017). RABIF is up-regulated in breast cancer, and it can promote invasion, metastasis of breast cancer cells and induce drug resistance (Huang et al. 2021). PTEN-induced kinase 1 (PINK1) is an important kinase mainly located in mitochondria and participates in maintaining mitochondrial function. PINK1 protects against neurotoxin-induced mitochondrial damage, and its mutation or loss of function leads to ROS-mediated mitochondrial damage (Gautier, Kitada and Shen 2008). PINK1 is downregulated in most tumors, holding important prognostic value (Zhu et al. 2020). Miyahara et al. (2021) showed that knockdown of PINK1 promoted breast cancer cell growth, demonstrating the tumor suppressive role of PINK1. BIN1 (Myc box-dependent-interacting protein 1), a tumor suppressor, is down-regulated in various tumor diseases (Wang et al. 2017). Studies have shown that BIN1 inhibits MYC-mediated malignant transformation (Sakamuro et al. 1996) and induces apoptosis in cancer cells (Ge et al. 2000). Additionally, studies have found CALM1 is downregulated in hepatocellular carcinoma (Han et al. 2021) and TUBB6 is downregulated in breast cancer (Nami and Wang 2018), though their anti-breast cancer effects and mechanisms remain unclear. In summary, these key targets play significant roles in breast cancer progression and may serve as diagnostic biomarkers and therapeutic targets. Our study suggests triptolide may exert its anti-breast cancer activities by modulating these targets, but further experimental validation is needed.

5 Conclusions

In summary, in this study, we identified potential targets of triptolide for treating breast cancer using integrated bioinformatics approaches. A total of 5206 targets related to the progression of breast cancer were obtained based on TCGA-BC cohort and 2113 triptolide-associated targets were identified based on transcriptomic data of triptolide-treated breast cancer cells. Functional enrichment analysis indicated that the identified targets were significantly enriched in tumor-related metabolic pathways, including pyruvate metabolism, glycolysis/gluconeogenesis, citrate cycle, amino acid metabolism, and fatty acid

metabolism. PPI network analysis identified 10 key therapeutic targets, including VIM, DLD, ACAT1, RABIF, ALDH2, RPS20, BIN1, TUBB6, CALM1 and PINK1. The aberrant expression of these key targets in breast cancer samples was validated using independent datasets. ROC analysis also confirmed their sensitivity and specificity as diagnostic markers and therapeutic targets. Our study provides an effective strategy to elucidate the mechanisms of triptolide against breast cancer and identify key mediators of its anti-tumor activities. These findings will facilitate the development of triptolide and its derivatives as novel treatment options for breast cancer. However, further experimental studies are required to validate the functional roles of identified targets in mediating the anti-cancer effects of triptolide.

Declarations

Author Contribution

LY and LH performed the experiments, collected the data, and wrote the initial draft of the manuscript. JX and WC conducted the statistical analyses. WY and ZL assisted with data acquisition, analysis, and interpretation, and provided critical revisions to the manuscript. All authors reviewed the manuscript.

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Figures

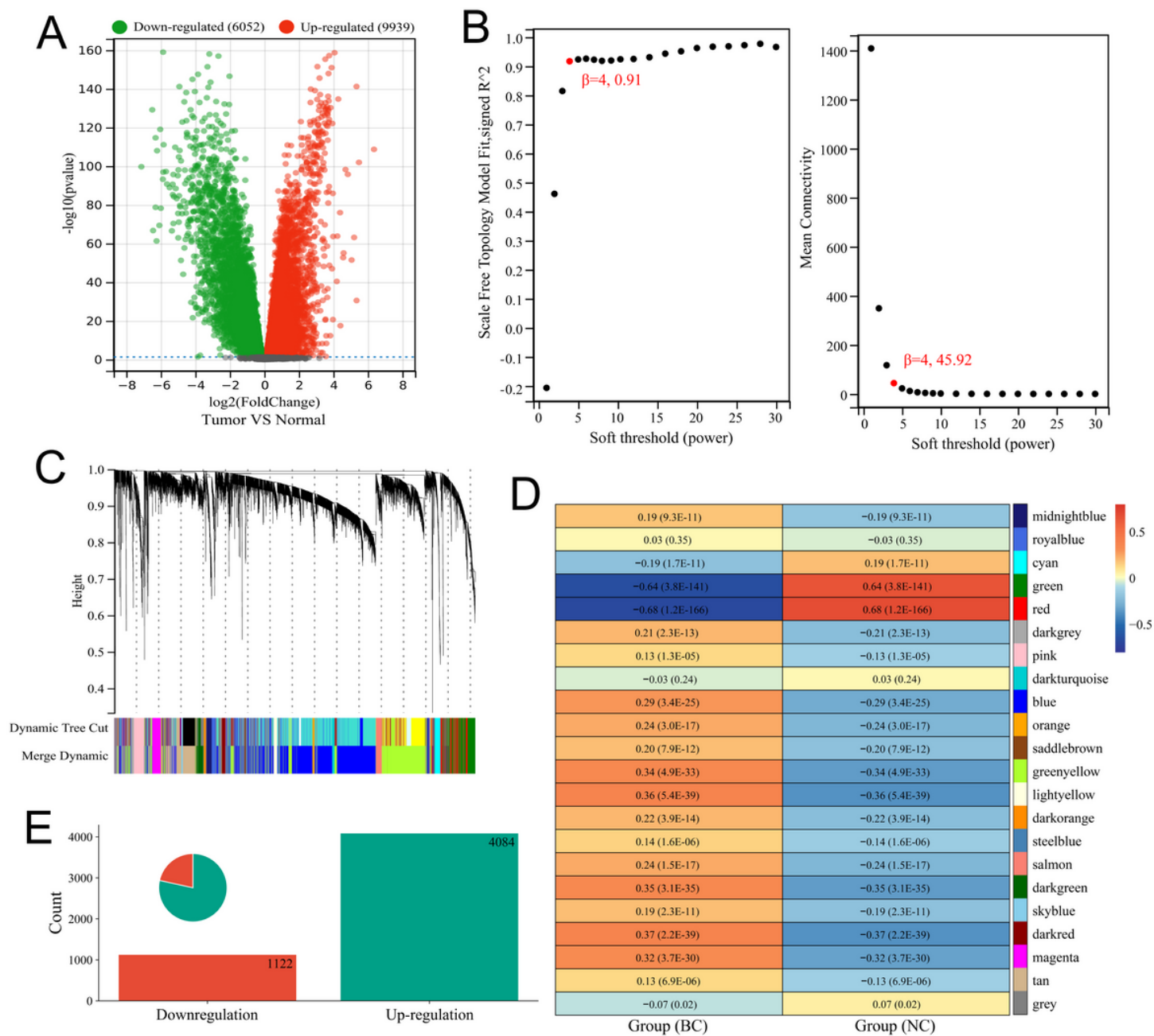


Figure 1

Identification of targets associated with breast cancer progression in TCGA-BC cohort. **(A)**Volcanic map of differential expression analysis. **(B)** Analysis of the scale-free index and mean connectivity for various soft-thresholding powers. **(C)**Dendrogram of DEGs clustered based on based on TOM dissimilarity. Different colors represent different modules. **(D)** Heatmap of module-trait relationships. Each row corresponds to a module eigengene, column to a trait. Each cell contains the corresponding correlation

and p-value (in brackets). **(E)** Bar plot showing number of breast cancer-related hub genes identified from modules.

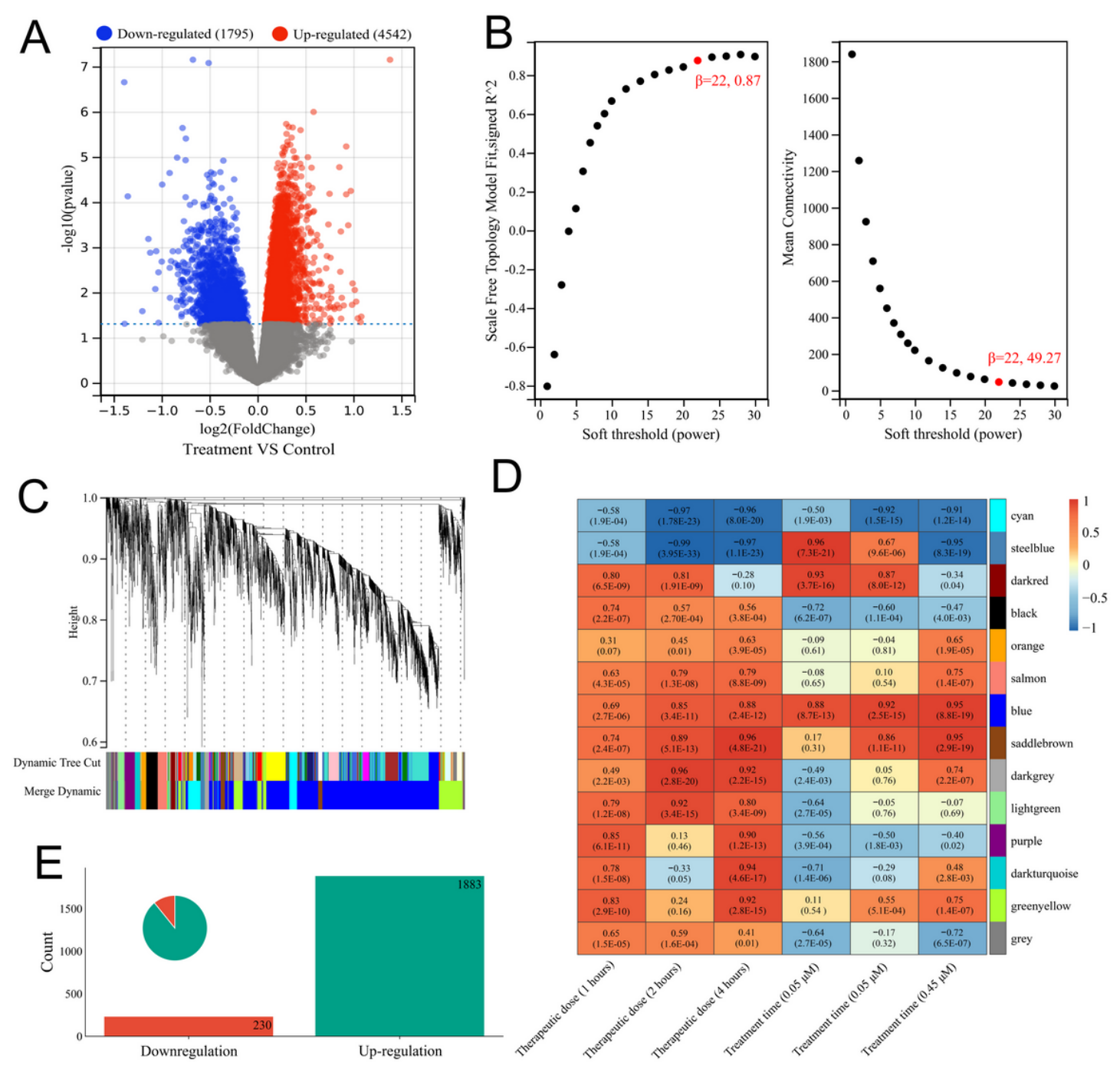


Figure 2

Identification of triptolide-related targets in GSE16760 dataset. **(A)** Volcanic map of differential expression analysis. **(B)** Analysis of the scale-free index and mean connectivity for various soft-thresholding powers. **(C)** Dendrogram of DEGs clustered based on TOM dissimilarity. Different colors represent different modules.. **(D)** Heatmap of module-trait relationships. Each row corresponds to a

module eigengene, column to a trait. Each cell contains the corresponding correlation and p-value (in brackets). **(E)**Bar plot showing number of triptolide-related hub genes identified from modules.

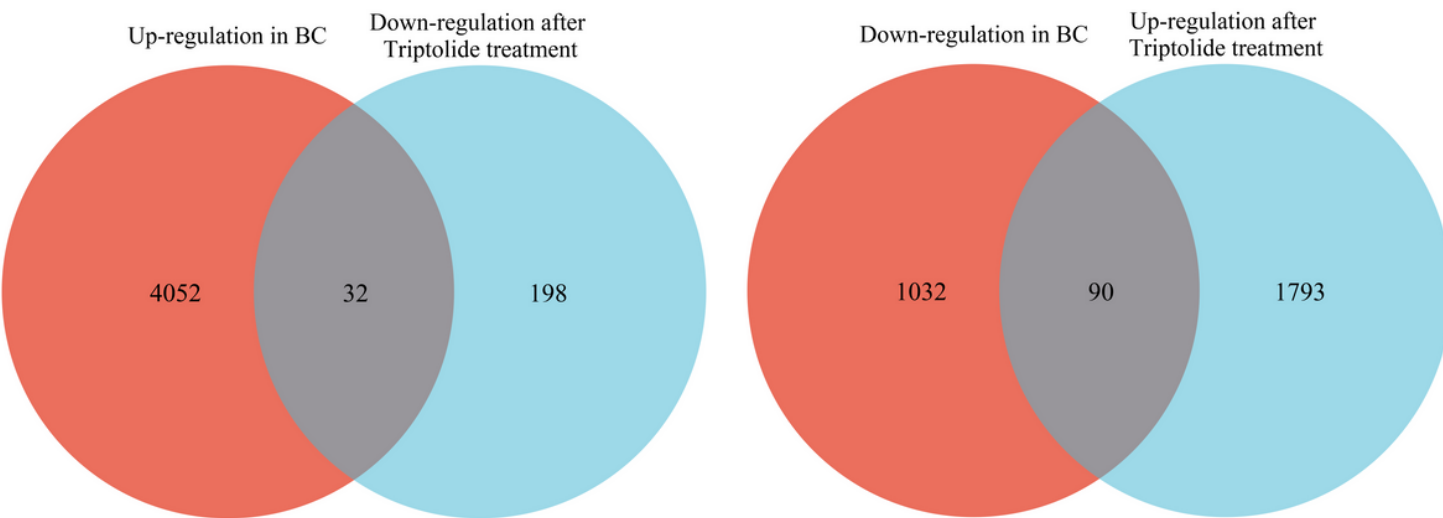


Figure 3

Venn diagram showing the overlap between breast cancer-related and triptolide-related hub genes. 122 common hub genes were identified as candidate therapeutic targets of triptolide against breast cancer.

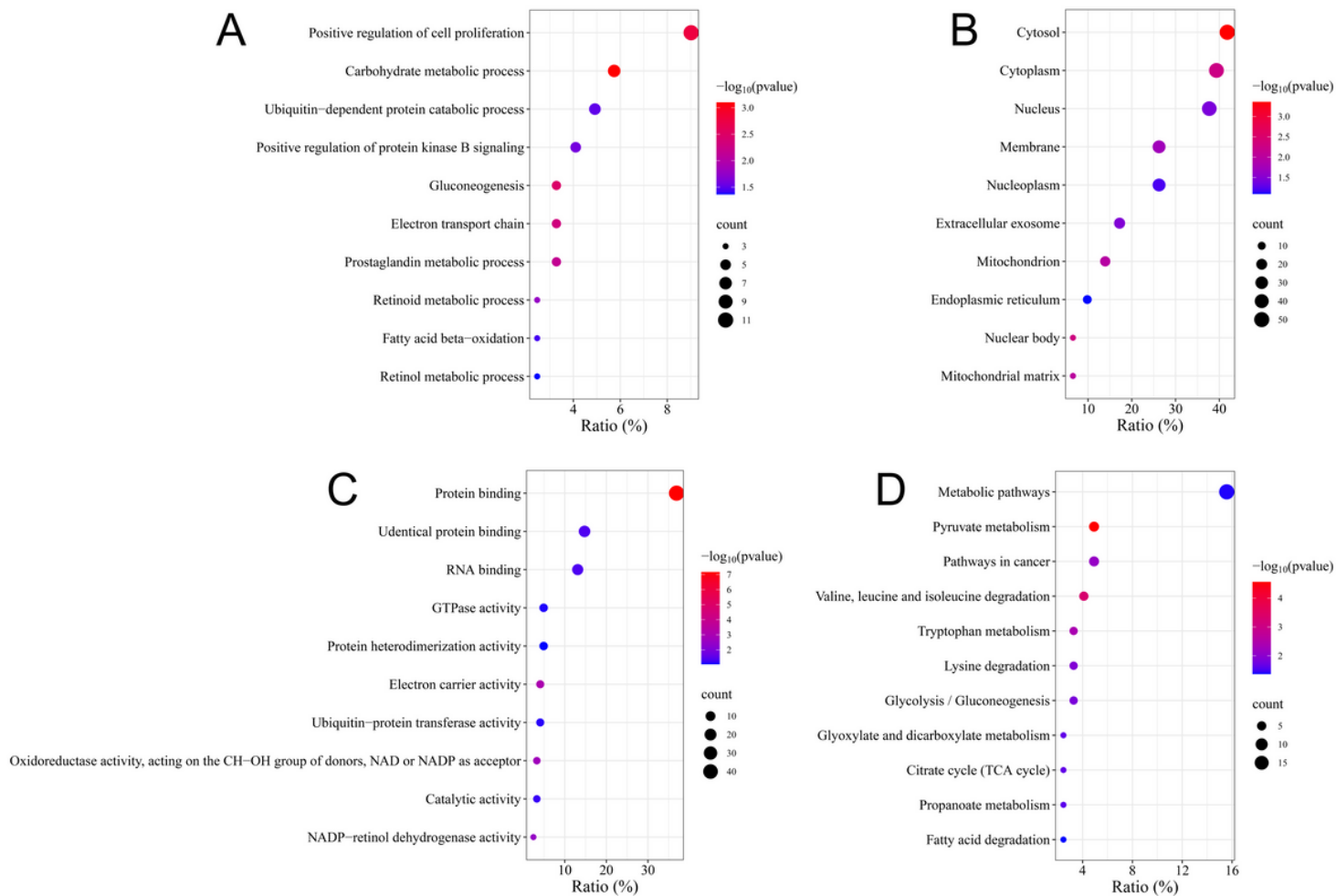


Figure 4

Functional enrichment analysis of the 122 candidate targets. Top 10 enriched terms/pathways of **(A)** Biological Process, **(B)** Cellular Component, **(C)** Molecular Function, and **(D)** KEGG pathway.

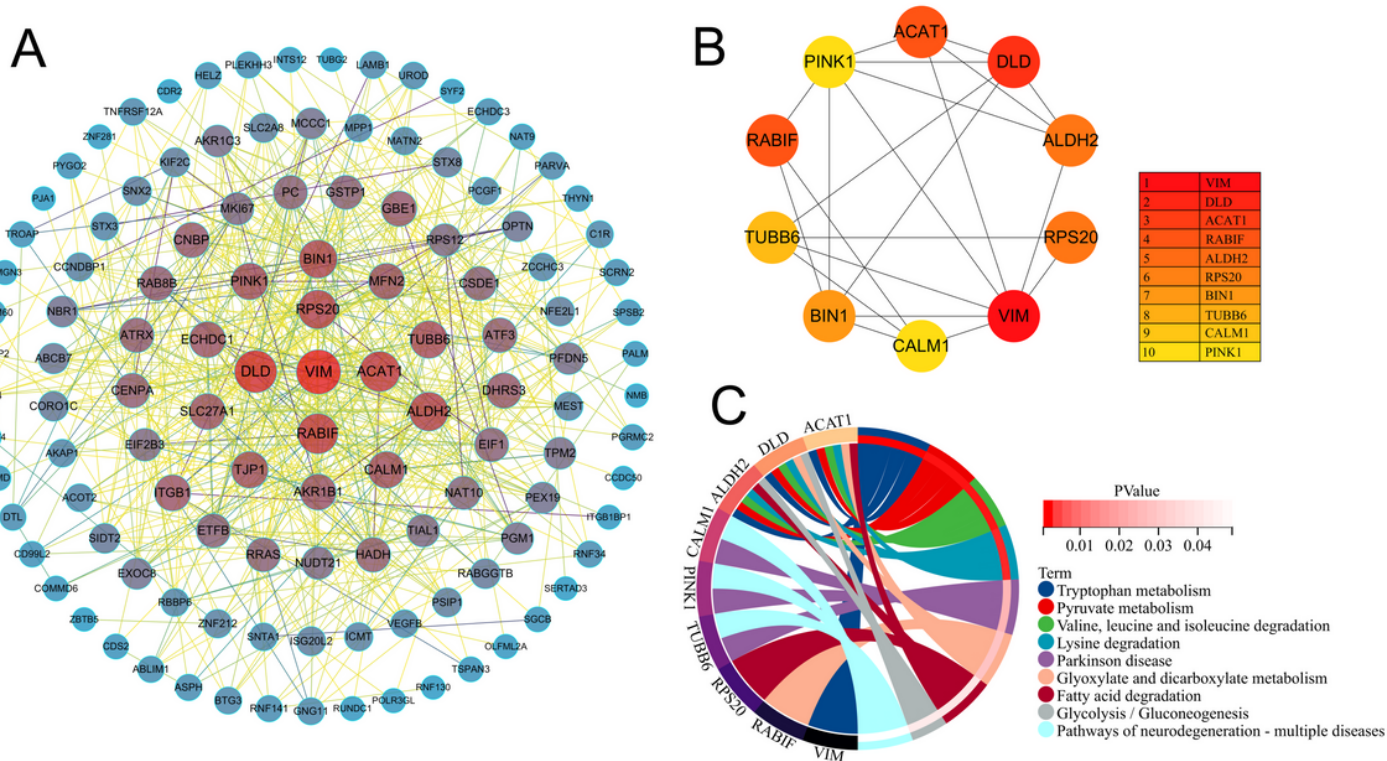


Figure 5

PPI network analysis and identification of key therapeutic targets. **(A)** PPI network of the 122 candidate targets constructed using STRING. Nodes with darker color indicate higher degree values. **(B)** Subnetwork of the 10 key targets. Nodes with darker color represent higher MCC scores. **(C)** KEGG pathways enriched in the 10 key targets.

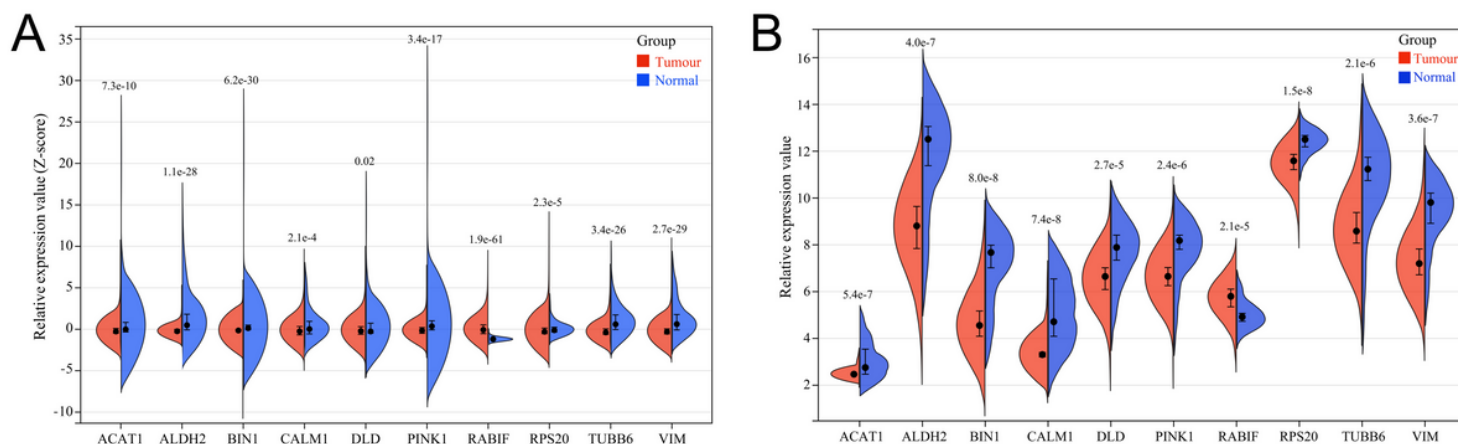


Figure 6

Validation of key target expression in breast cancer tissues compared to normal controls. Violin plots of key target expression in **(A)** TCGA-BC cohort and **(B)** GSE42568 dataset. Targets are arranged in the order of ACAT1, ALDH2, BIN1, CALM1, DLD, PINK1, RABIF, RPS20, TUBB6, and VIM.

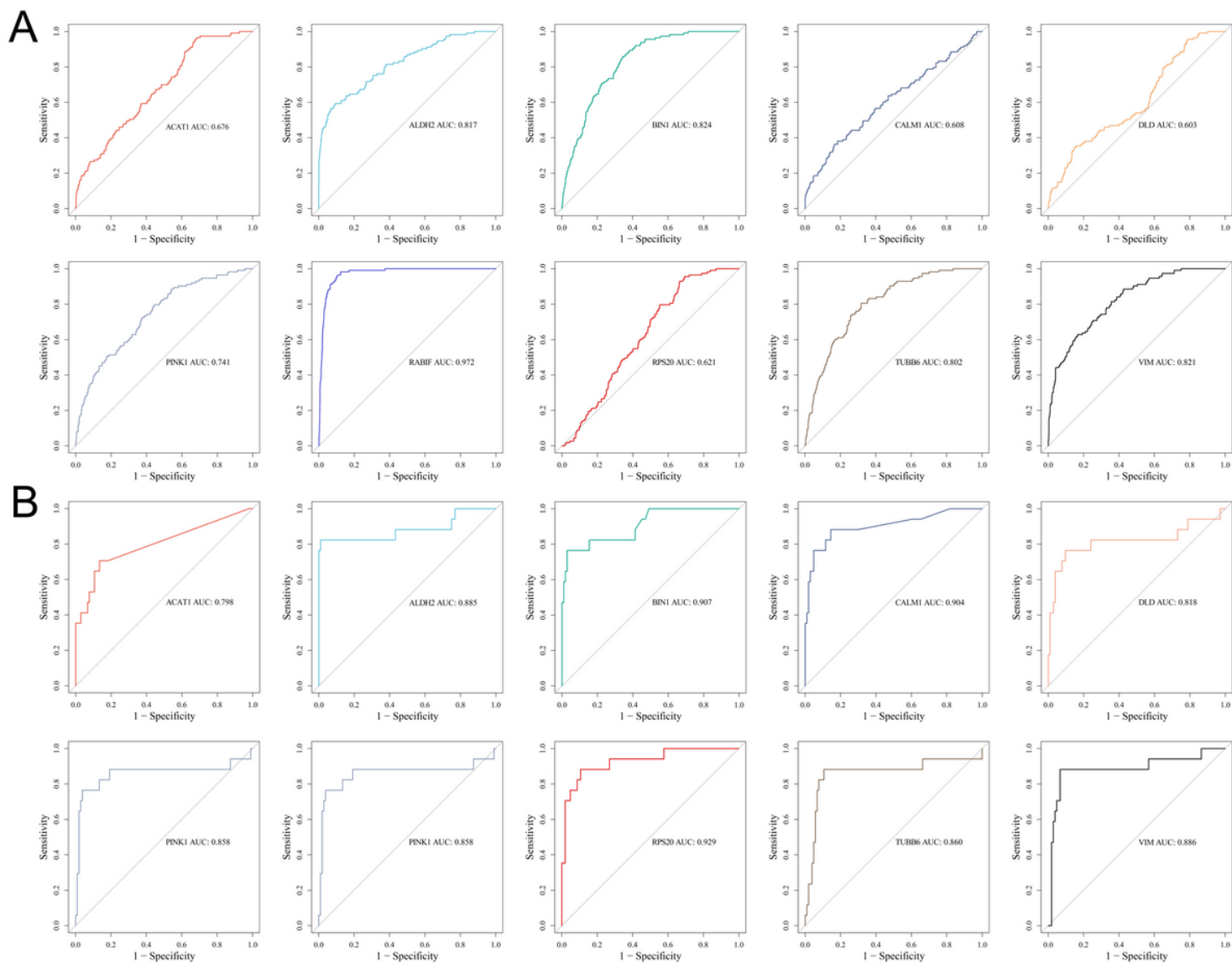


Figure 7

ROC curve analysis of key targets for discriminating breast cancer from normal tissues. ROC curves of the 10 key targets were plotted in the order of ACAT1, ALDH2, BIN1, CALM1, DLD, PINK1, RABIF, RPS20, TUBB6, and VIM based on their expression profiles in **(A)** TCGA-BC cohort and **(B)** GSE42568 dataset.

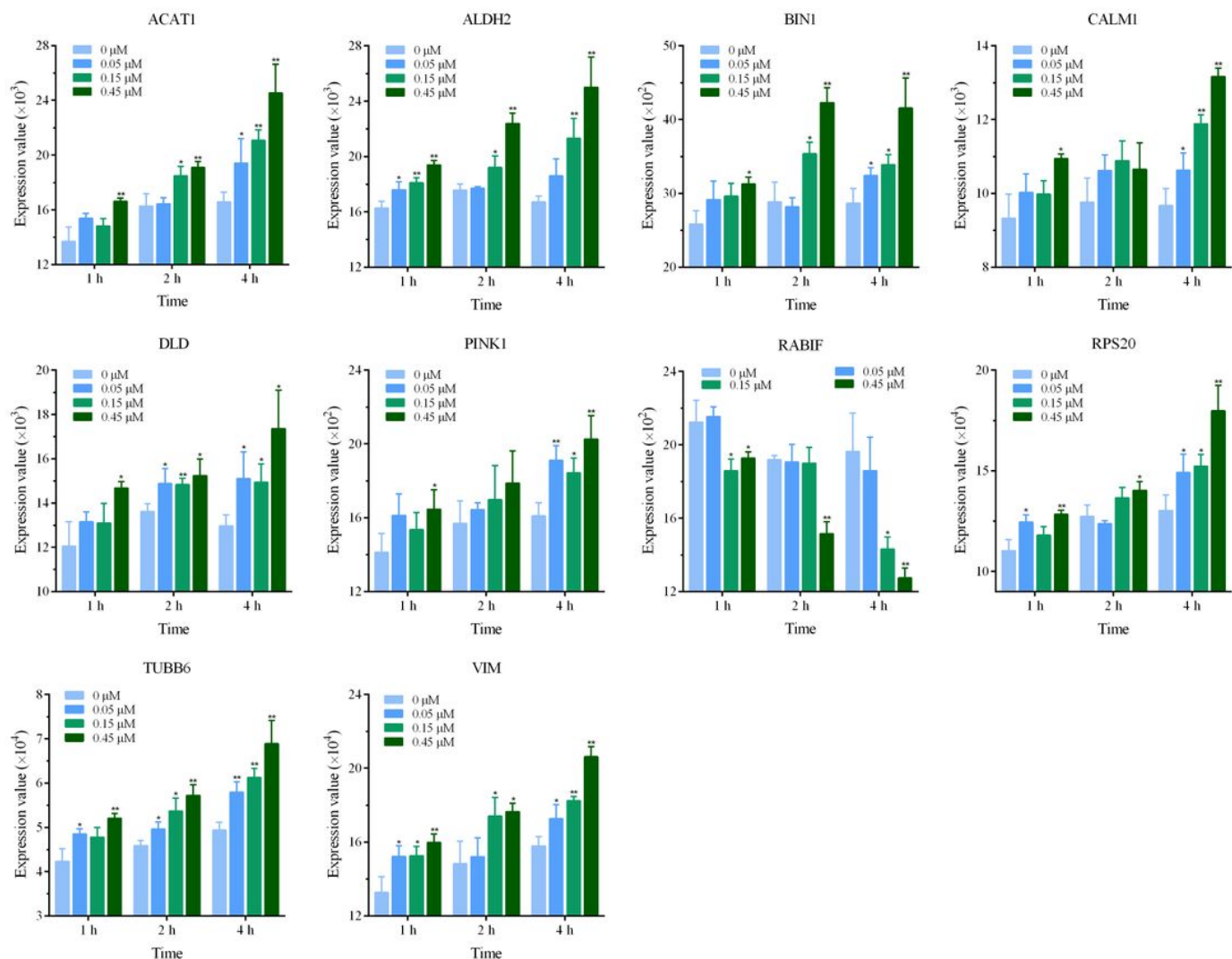


Figure 8

Expression changes of key targets in breast cancer cells treated with triptolide. Bar plots showing expression changes of the 10 key targets after 1, 2, 4 h treatment with different concentrations of triptolide compared to control group (0 μM). The targets are arranged in the order of ACAT1, ALDH2, BIN1, CALM1, DLD, PINK1, RABIF, RPS20, TUBB6, and VIM based on GSE16760 dataset. * indicates significant difference compared to control group (0 μM) at corresponding time points ($P<0.05$).

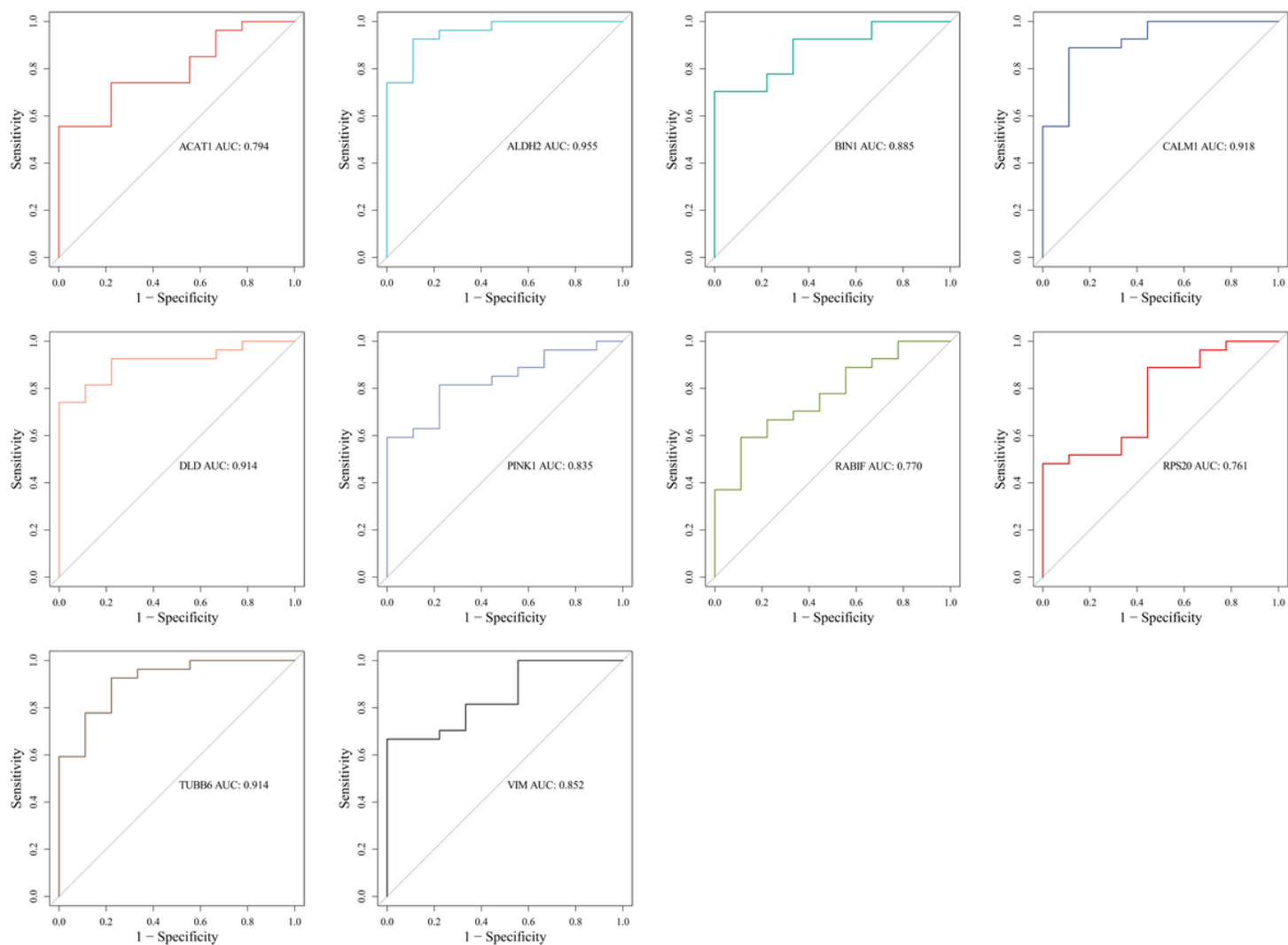


Figure 9

ROC curve analysis of key targets for discriminating triptolide-treated cells from untreated controls. Targets are arranged in the order of ACAT1, ALDH2, BIN1, CALM1, DLD, PINK1, RABIF, RPS20, TUBB6, and VIM.