

Identification of lymph node metastasis-related microRNAs in breast cancer using bioinformatics analysis

Guangyu Gao, BSc, Xinya Shi, BSc, Zhen Yao, BSc, Jiaofeng Shen, BSc, Liqin Shen, PhD*

Abstract

Background: Lymph node metastasis is a significant problem in breast cancer, and its underlying molecular mechanism is still unclear. The purpose of this study is to research the molecular mechanism and to explore the key RNAs and pathways that mediate lymph node metastasis in breast cancer.

Methods: GSE100453 and GSE38167 were downloaded from the Gene Expression Omnibus (GEO) database and 569 breast cancer statistics were also downloaded from the TCGA database. Differentially expressed miRNAs were calculated by using R software and GEO2R. Gene ontology and Enriched pathway analysis of target mRNAs were analyzed by using the Database for Database of Annotation Visualization and Integrated Discovery (DAVID) and R software. The protein–protein interaction (PPI) network was performed according to Metascape, String, and Cytoscape software.

Results: In total, 6 differentially expressed miRNAs were selected, and 499 mRNAs were identified after filtering. The research of the Kyoto Encyclopedia of Genes and Genomes (KEGG) demonstrated that mRNAs enriched in certain tumor pathways. Also, certain hub mRNAs were highlighted after constructed and analyzed the PPI network. A total of 3 out of 6 miRNAs had a significant relationship with the overall survival ($P < .05$) and showed a good ability of risk prediction model of over survival.

Conclusions: By utilizing bioinformatics analyses, differently expressed miRNAs were identified and constructed a complete gene network. Several potential mechanisms and therapeutic and prognostic targets of lymph node metastasis were also demonstrated in breast cancer.

Abbreviations: CCR4 = carbon catabolite repression 4, DAVID = Database of Annotation Visualization and Integrated Discovery, F2RL2 = coagulation factor II thrombin receptor like 2, FBXO17 = F-box protein 17, FOXJ2 = forkhead box J2, GEO = Gene Expression Omnibus, GPI = glucose-6-phosphate isomerase, HK1 = hexokinase 1, INTS7 = integrator complex subunit 7, KEGG = Kyoto Encyclopedia of Genes and Genomes, miRNAs = microRNAs, OBSL1 = obscurin like cytoskeletal adaptor 1, PPI = protein-protein interaction, RBAK = RB-associated KRAB zinc finger, RPS29 = ribosomal protein S29, TUBA1C = α -tubulin.

Keywords: bioinformatics, breast cancer, lymph node metastasis, miRNA-mRNA network

1. Introduction

By 2018, 1,762,450 new tumor patients are been made definite diagnosed and 606,880 among them died in the United States. Of

them, breast carcinoma makes up 30% (268,600) of all new female tumor cases and 15% (41,760) of all tumor-related deaths.^[1] Though we have made great progress in pre-diagnosis and treatment, medicine resistance and molecular heterogeneity have influenced the active treatment of drugs. Every year, the increasing prevalence of breast carcinoma in the United States and the relevant treatment of malignant breast carcinoma bring huge pressure to the national economy and health care industry.^[2] According to recent treatment views, like radiotherapy and adjuvant chemotherapy, have promoted the overall survival of patients, particularly in early stage breast tumor of women who were diagnosed timely.^[3] However, among these treatments, side effects like psychological effects, gastrointestinal discomfort, adverse physical, and psychological effects on sexuality often exist which influences the quality of life of patients. Besides, they can also take the possibility of mortality like bone loss, increased risk for secondary cancers and cardiac toxicity.^[4,5]

MicroRNAs (miRNAs) are small, noncoding RNAs, having the function of regulating after gene transcription.^[6] According to previous studies, miRNAs can regulate many target genes or one type of miRNAs can be regulated by many genes.^[7] In particular, Thomas et al found that some miRNAs can improve the therapeutic effect by improving the drug sensitivity of cancer cells.^[8] Xuan et al found that miR-374a, miR-92a, and miR-106a increase drug resistance and promote growth and metastasis of

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Department of Oncology, The Second Affiliated Hospital of Soochow University, Suzhou, Jiangsu, PR China.

* Correspondence: Liqin Shen, The Second Affiliated Hospital of Soochow University, 1055 Sanxiang Road, Suzhou 215004, Jiangsu, PR China (e-mail: 737443291@qq.com).

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lung cancer.^[9] Kania et al also reported that miR-9-3p and miR-9-5p decrease DNA topoisomerase II α expression levels in acquired resistance to etoposide and may act as biomarkers of responsiveness to TOP2-targeted therapy.^[10] However, the mechanisms of miRNAs in the occurrence and development of lymph node metastasis breast cancer remain unknown.

In the present study, microarray data for GSE100453 and breast cancer sample data in the TCGA (The Cancer Genome Atlas) database facilitated the investigation of differently expressed miRNAs in lymph node non-metastasis cancer tissues and lymph node metastatic cancer tissues. The functions of the target mRNAs were assessed by using GO annotation, KEGG, PPI network and the relationship between identified miRNAs and the overall survival of patients with cancer. In conclusion, we conducted this research to discover the key miRNAs and mRNAs of lymph node metastasis and to find potential new cancer therapy targets.

2. Methods

2.1. Microarray data

The GEO (<https://www.ncbi.nlm.nih.gov/gds>) database is a public functional genomics dataset that helps users download statistics and achieve gene expression introduction. In our research, Gene expression profile data (GSE100453 and GSE38167) were obtained from GEO. Forty-three lymph node non-metastasis breast cancer tissues and 25 lymph node metastasis breast cancer tissues were included. The array data were acquired from the Agilent-070156 Human miRNA [GPL20712; miRNA version] and Agilent-029297 Human miRNA [GPL14943; miRNA version]. Besides, we downloaded breast cancer statistics from the TCGA database which included 44 normal tissues and 525 tumor tissues. The flow of this study is shown in Figure 1.

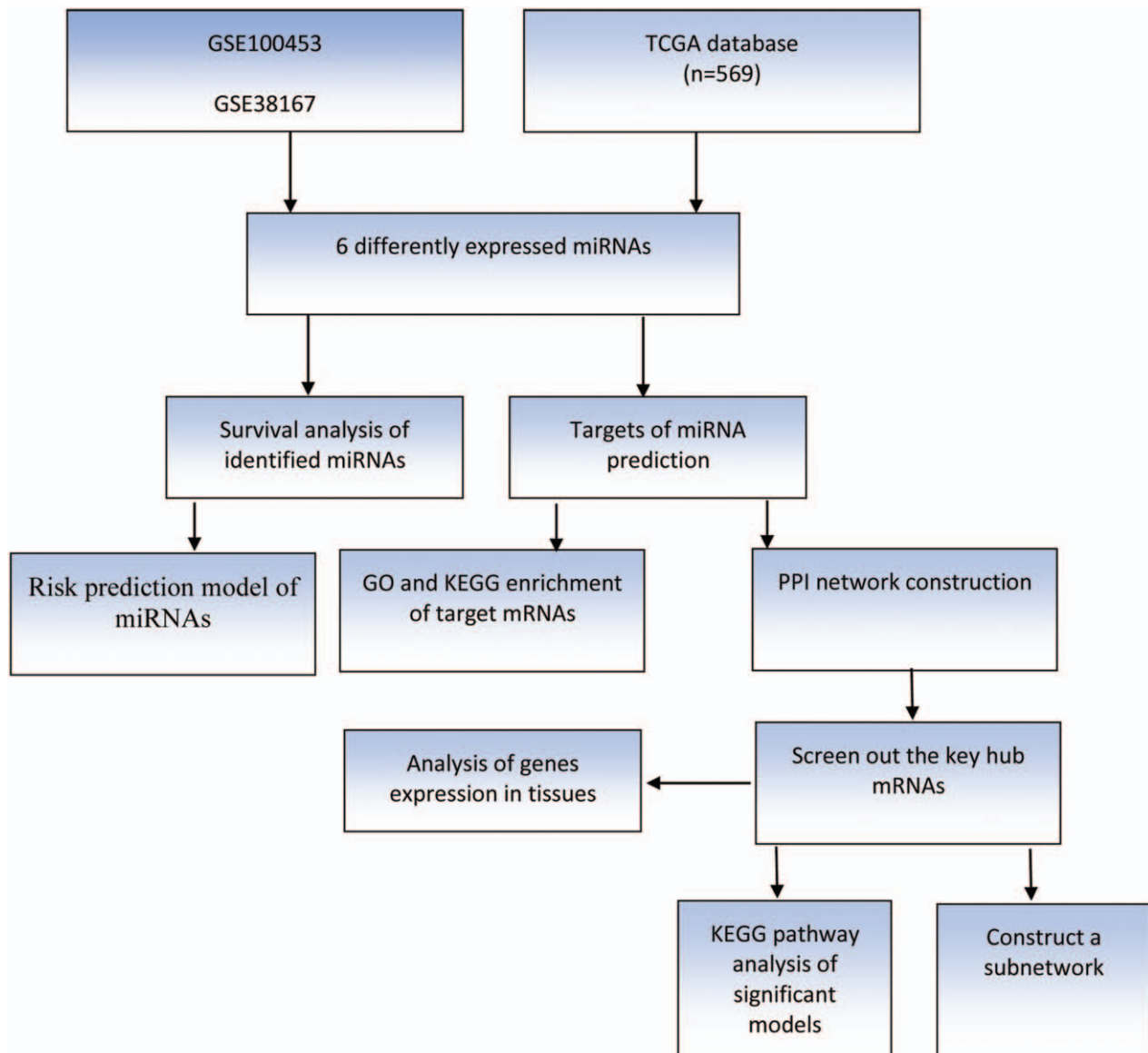


Figure 1. Flow chart of this study.

2.2. Differently expressed miRNAs analysis

R software was utilized to compare two groups of tissues to identify different genes under identical experimental factors. Besides, we used $|\log_2FC| \geq 2$ and $P < .05$ as a cut-off criterion and an obvious statistical difference would be considered if the statistics met our standards.^[11]

2.3. Targets of miRNA prediction

To predict the miRNA and mRNA interaction, we used the database of miRDB (<http://mirdb.org/>) and TargetScan (http://www.targetscan.org/vert_72/). All these websites are used for predicting miRNA binding sites, which are very effective for the prediction of miRNA binding sites in mammals and the interaction between miRNA and various types of RNA. We used cumulative weighted context score ≤ -0.4 and target score ≥ 65 as a cut-off criterion, and the intersection of two screening results was identified as our target mRNAs. We also constructed the network between the identified miRNAs and their target genes by using Cytoscape.

2.4. Functional and pathway enrichment analysis

KEGG pathway analysis and GO functional analysis of the differently expressed mRNAs we identified were conducted by taken advantage of the DAVID (<https://david.ncifcrf.gov/>) and R software. DAVID is a biological information database, which integrates biological data and analysis tools, provides a comprehensive system of biological function annotation information for the large-scale gene or protein lists (hundreds of gene ID or protein ID lists), and helps users extract biological information from them. GO analysis was divided into the cellular component (CC), biological process (BP) and molecular function (MF), and a P -value of $< .05$ was considered to indicate a statistical difference. In terms of the KEGG pathway analysis, they were identified according to the P -value of $< .05$. DAVID includes many functional annotation programs to analyze plenty of biological information of genes, and data that existed in DAVID was vital for the achievement of high-throughput gene functional analysis. We also used R software to analyze datasets.

2.5. PPI network analysis

PPI network analysis was conducted by utilizing the Search Tool for the Retrieval of Interacting Genes (STRING). STRING supplies messages related to proven and predicted interactions among huge numbers of proteins. Differently expressed mRNAs that were selected would then input into STRING. We would consider mRNAs to be meaningful which had a score of > 0.9 .^[12] The PPI network was built using Cytoscape and Metascape. Besides, degrees > 10 was set as the cutoff criterion. Molecular Complex Detection (MCODE) was then used to analyze the sub-modules of the PPI network, and the criteria we set was the number of nodes > 6 and MCODE score > 5 .

2.6. Analysis of mRNA expression in breast cancer

Hub genes expression in breast cancer tissues and normal tissues were extracted from the human protein atlas (www.proteinatlas.org). mRNAs expression we identified were determined through analysis of TCGA databases, which are available through

TCGAportal (<http://tumorsurvival.org/>). High and low groups were defined as above average and below average respectively.

2.7. Analysis of the miRNAs and their relationship with breast cancer prognosis

The Kaplan–Meier Plotter (www.kmplot.com/) is a non-parametric statistic that is utilized to evaluate the overall survival statistics from recorded data. It was constructed from gene expression and survival data which were downloaded from GEO and TCGA database.^[13] Every miRNA which was selected would be put into the online tool to evaluate the survival of patients with breast cancer for the Kaplan–Meier curve.

2.8. Independent prognostic ability of the miRNA signature

To better understand the relationship between the selected miRNAs and the prognosis of breast cancer patients, we constructed a risk prediction model. By using “survivalROC” package, we calculated area under curve (AUC) of 3 years and 5 years dependent receiver operating characteristic (ROC) curve to assess the predictive power of identified miRNAs. A block diagram was also drawn to show the risk score of the model.

3. Results

3.1. Identification of the miRNAs between lymph node non-metastasis tissues and lymph node metastasis tissues

R software was used to research the gene expression profiles from the GSE100453 and GSE38167. According to the cut-off criteria ($P < .05$ and $|\log_2FC| \geq 1$), 30 differentially expressed miRNAs were identified. After analyzing 569 samples of the TCGA database, 80 differentially expressed miRNAs were identified and 6 of them exist in both filter results which were consisted of 3 downregulated and 3 upregulated miRNAs. The result is shown in Table 1.

3.2. Target prediction and GO analysis

The target mRNAs of those 6 differently expressed miRNAs were downloaded from two miRNA target prediction websites (targetscan and miRDB). Four hundred ninety-nine mRNAs were identified after filtering. The Network between miRNAs (2 upregulated miRNAs and 2 downregulated miRNAs) and target mRNAs was shown in Figure 2. To learn more about the function of these mRNAs, we uploaded them into DAVID to perform GO and KEGG analysis. In the CC ontology, upregulated mRNAs were enriched in “adherens junction” (29 mRNAs), “focal

Table 1
Key differently expressed miRNAs of joint screening from GEO and TCGA database.

ID	P	logFC
hsa-miR-4274	.00535	2.674835
hsa-miR-6880-3p	.02268	2.435096
hsa-miR-4689	.0287	2.349269
hsa-miR-30b-3p	.01508	-2.142329
hsa-miR-670-5p	.04815	-2.171476
hsa-miR-1-3p	.01365	-2.5698

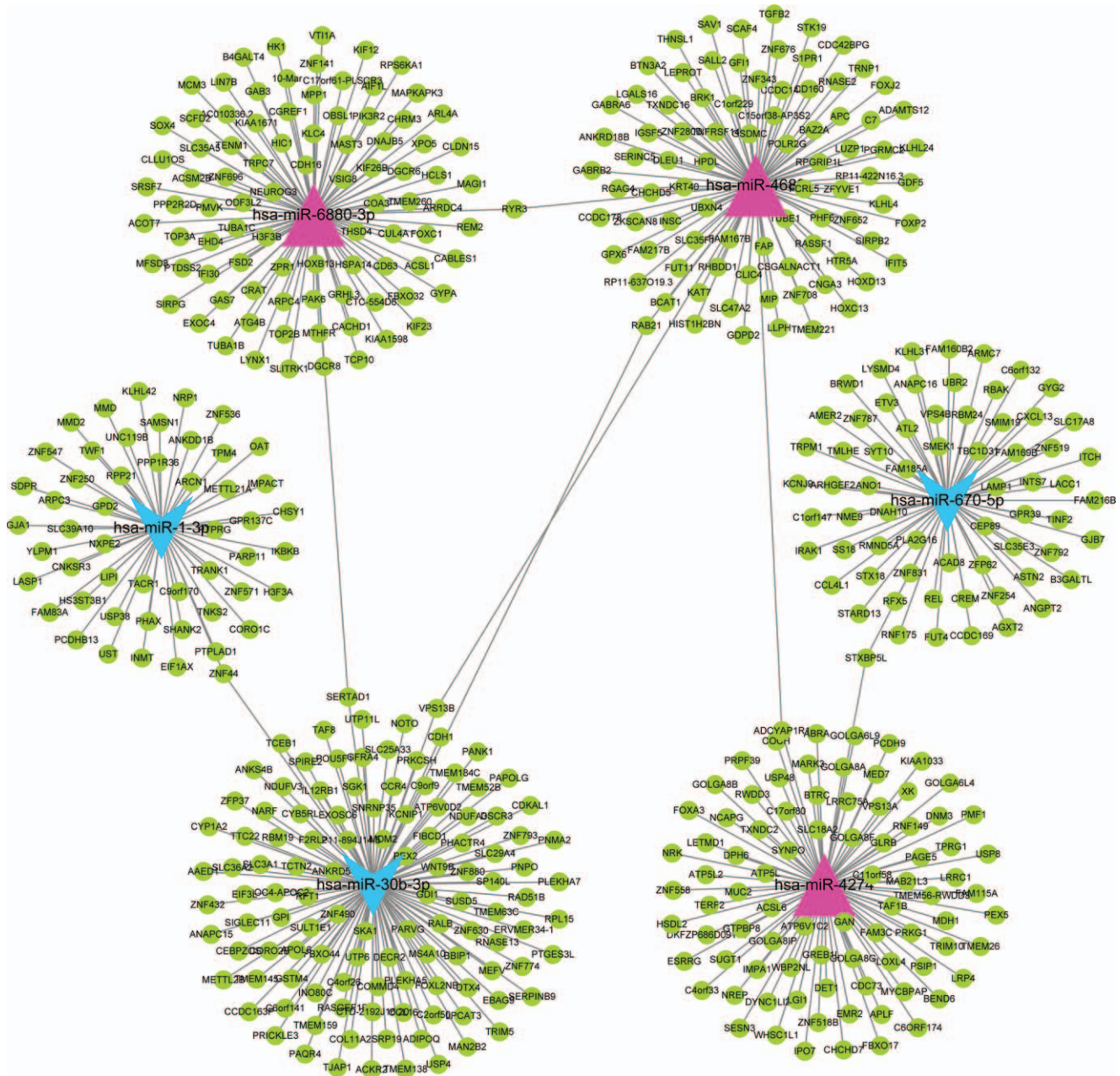


Figure 2. The interactions between differentially expressed miRNAs and target mRNAs.

adhesion” (24 mRNAs) and “cell-substrate adherens junction” (24 mRNAs), downregulated mRNAs were enriched in “nuclear envelope” (27 mRNAs), “focal adhesion” (27 mRNAs) and “nuclear speck” (26 mRNAs). In the BP ontology, upregulated mRNAs were enriched in “regulation of cytoskeleton organization” (27 mRNAs), “myeloid cell differentiation” (23 mRNAs), and “regulation of microtubule-based process” (16 mRNAs), downregulated mRNAs were enriched in “regulation of gene silencing by miRNA” (18 mRNAs), “regulation of gene silencing” (17 mRNAs), and “regulation of posttranscriptional gene silencing” (17 mRNAs). In the MF ontology, upregulated mRNAs were enriched in “proximal promoter sequence-specific DNA binding” (22 mRNAs), “protein serine/threonine kinase

activity” (20 mRNAs) and “enzyme activator activity” (20 mRNAs), downregulated mRNAs were enriched in “protein heterodimerization activity” (32 mRNAs), “transcription coregulator activity” (31 mRNAs) and “protein serine/threonine kinase activity” (31 mRNAs) (Fig. 3).

3.3. KEGG pathways of differentially expressed mRNAs

The KEGG pathway analysis identified many significant enriched pathways. Upregulated mRNAs were enriched in “Hepatitis B,” “Epstein-Barr virus infection,” “Kaposi sarcoma-associated herpesvirus infection,” “Thyroid cancer,” and “Human T-cell leukemia virus 1 infection,” downregulated mRNAs revealed

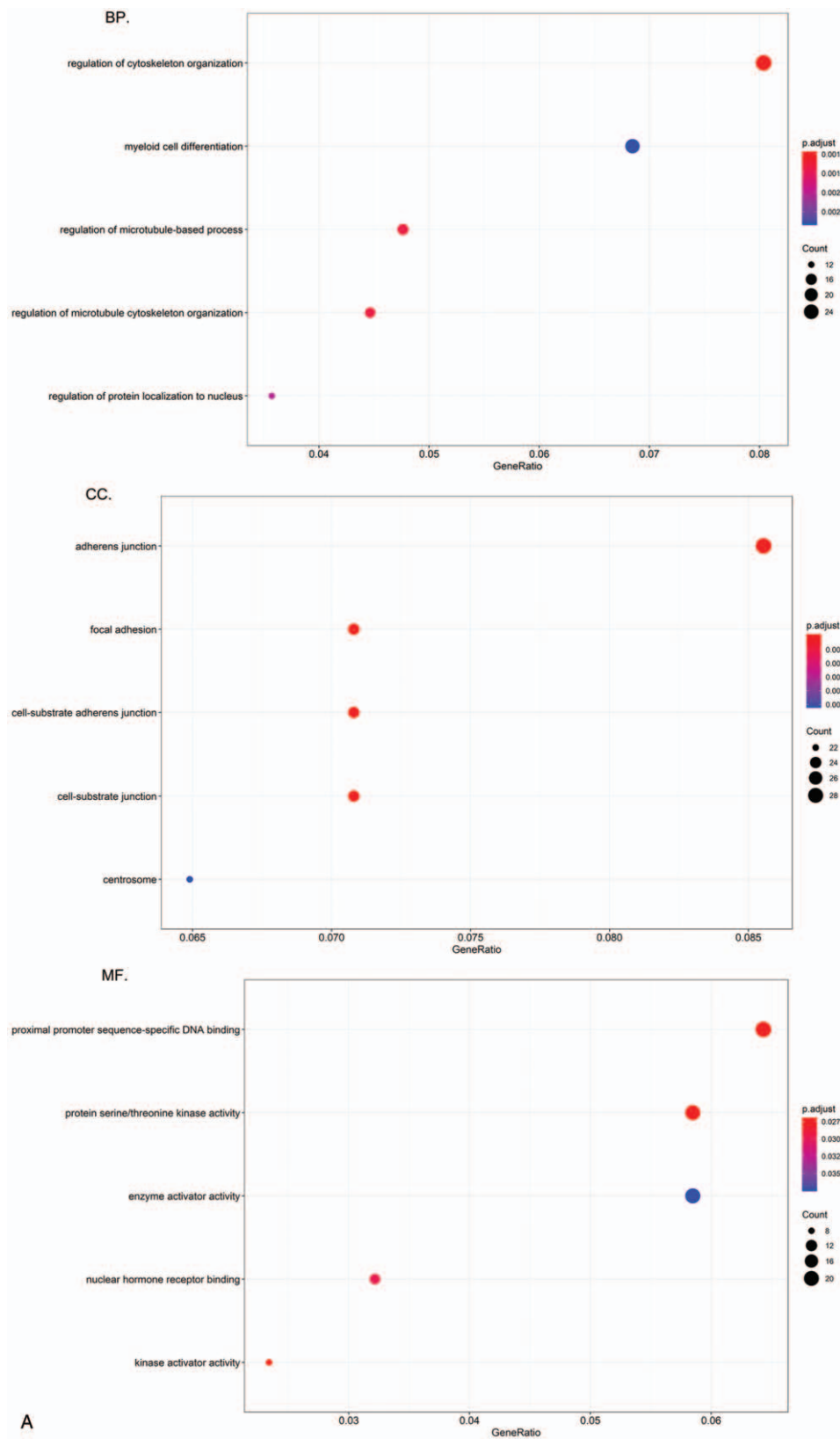


Figure 3. Differentially expressed mRNAs analyzed by GO enrichment. (A) Up-regulated mRNAs analyzed by GO enrichment. (B) Down-regulated mRNAs analyzed by GO enrichment. BP=biological process, CC=cellular component, MF=molecular function.

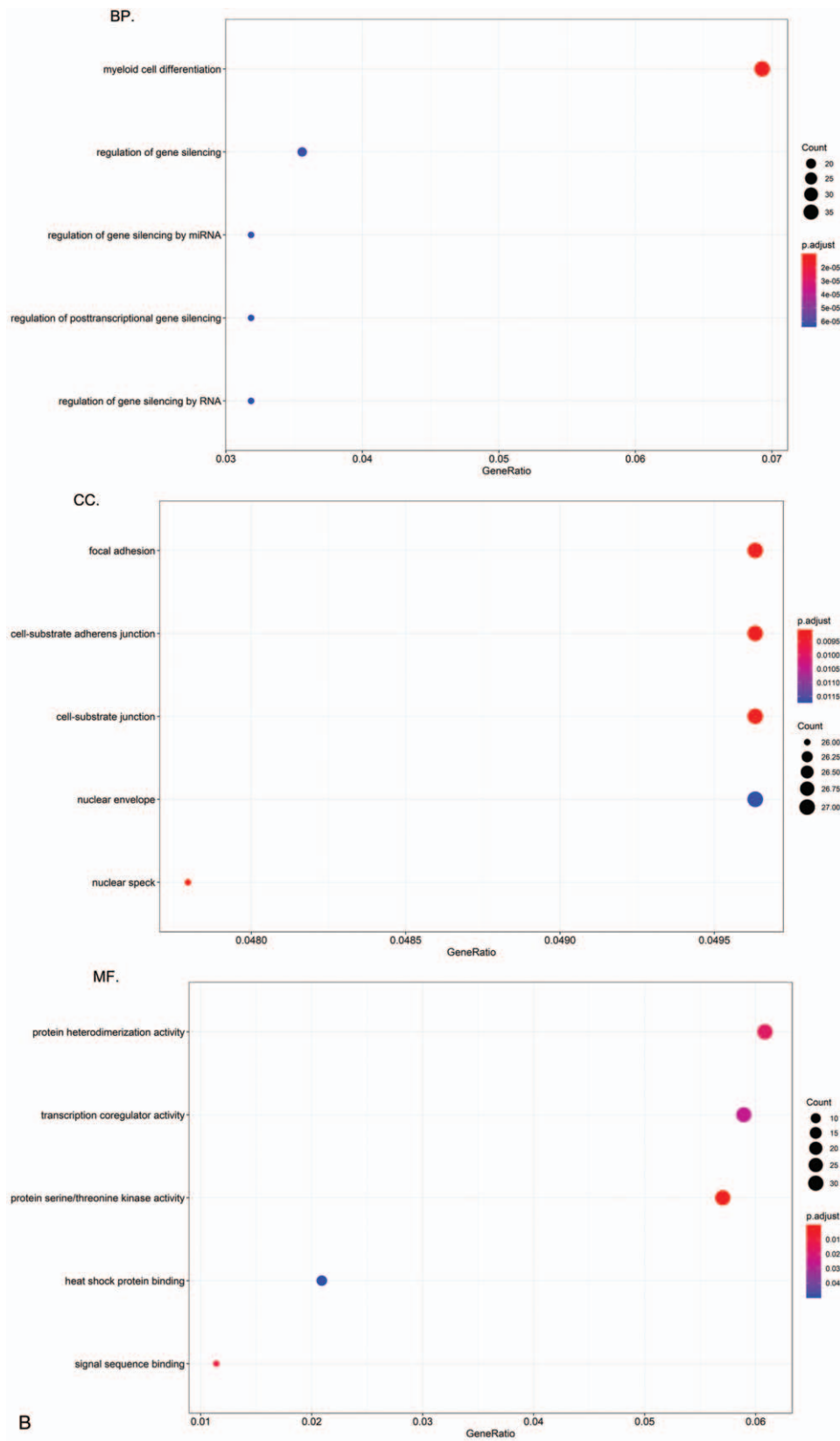


Figure 3. (Continued).

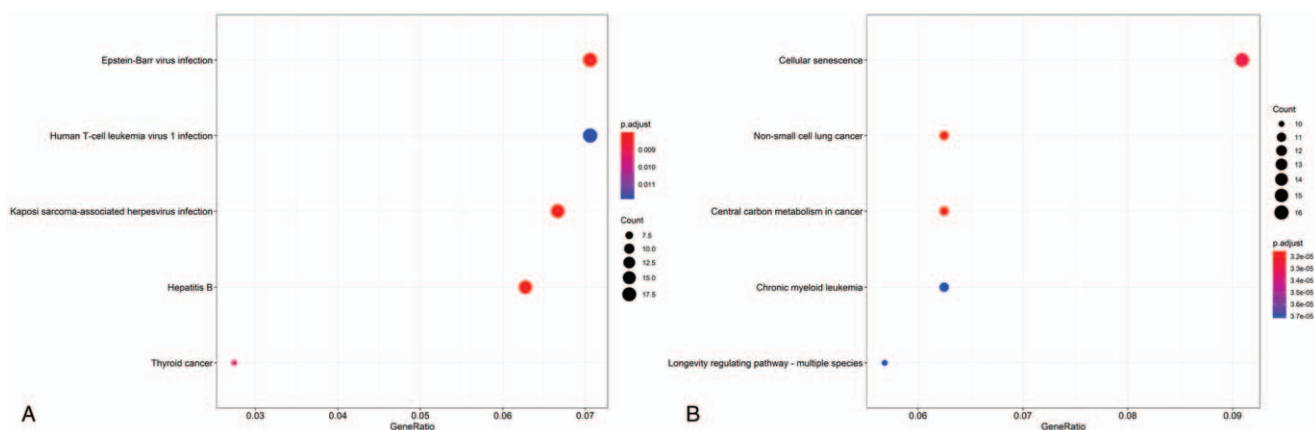


Figure 4. KEGG pathway enriched by up- and down-regulated mRNAs. (A) Up-regulated mRNAs analyzed by KEGG enrichment. (B) Down-regulated mRNAs analyzed by KEGG enrichment.

involvement in “Non-small cell lung cancer,” “Central carbon metabolism in cancer,” “Cellular senescence,” “Chronic myeloid leukemia,” and “Longevity regulating pathway” (Fig. 4).

3.4. Construction of a PPI network

Four hundred ninety-nine mRNAs were entered into the Metascape website to get interactive data. Then, if the combined score was ≥ 0.9 , we would choose the selected mRNAs to build a PPI network (Fig. 5). In the PPI network, 11 modules, including RBAK, TUBA1C, CCR4, HK1, FBXO17, INTS7, F2RL2, RPS29, FOXJ2, OBSL1, and GPI were identified. The outcomes of the KEGG pathway between modules were related to “Oxidative Stress Induced Senescence,” “Class A/1,” “Antigen processing: Ubiquitination and Proteasome degradation,” “RNA polymerase II transcribes snRNA genes,” “G alpha (q) signaling events,” and “Formation of the ternary complex, and subsequently, the 43S complex” (Table 2). Based on the key modules and related miRNAs which were identified by using miRDB and TargetScan, we constructed a miRNA–mRNA network (Fig. 6), and it may become potential therapeutic targets and new biomarkers for lymph node metastasis breast cancer.

3.5. Analysis of the key mRNAs expression in normal tissues and cancer tissues

The human protein atlas was utilized to research the expression of human proteins in different tissues. RBAK, TUBA1C, and HK1 were selected from 11 key mRNAs. After entering them into the database, we found that three mRNAs have a positive strong expression in breast cancer tissues and negative weak expression in normal tissues (Fig. 7A). These mRNAs expression was detected by the polyclonal antibody using immuno-histochemistry and sections were counterstained lightly with hematoxylin. The scale bar was 20 μm . To verify our conclusion, TCGAportal was then used. It is a website that downloads statistics from the TCGA database and contains 1102 breast cancer sample tissues. After inputting relevant mRNAs, we also found that the three mRNAs level was higher in cancer tissues than that in normal tissues (Fig. 7B).

3.6. Analysis of the miRNAs and their relationship with breast cancer prognosis

To research the prognosis of patients with breast cancer, we used the Kaplan–Meier Plotter. After uploaded 6 miRNAs, we got 6 survival graphs. The results indicated that overexpression of hsa-miR-4274, hsa-miR-6880-3p, and hsa-miR-670-5p (Fig. 8) were related to worse overall survival in patients with breast cancer ($P < .05$). However, the expression level of has-miR-149, has-miR-1-3p, and has-miR-30b-3p may have no significant relationship with the overall survival ($P > .05$). This suggested that the selected miRNAs may be potential targets.

3.7. Evaluation of the 6-miRNA signature for overall survival

A multivariate analysis of miRNAs with significant differences in prognostic analysis was performed to establish an optimal model using kaikie Information Criterion (AIC). The risk model established in this paper consists of 6 miRNA expression values. According to this risk model, patients were divided into high and low risk groups. The results show that this model can well predict the clinical outcomes of patients. The AUC of 3 years survival for the 6-miRNA signature achieved 0.809 and the AUC of 5 years survival achieved 0.981, which proved that the model has good performance in predicting the survival risk of breast cancer patients. Besides, the box diagram also proved our conclusion (Fig. 9).

4. Discussion

Lymph node metastasis is a common problem in breast cancer, which seriously affects the survival and prognosis of patients. Therefore, it is more and more urgent to understand mechanisms and find a specific and sensitive treatment method for patients. At present, many important advances have been made in breast cancer research using bioinformatics. For example, genes with prognostic value in the Breast cancer microenvironment^[14] and breast microcalcification diagnosis^[15] have been researched through the combination of a variety of database analysis. In this present research, gene expression data of GSE100453 and GSE38167 were searched from the GEO. Five hundred sixty-nine

Table 2
KEGG enrichment analysis of hub target mRNAs.

MCODE	GO	Description	Log10(P)
MCODE_2	R-HSA-2559580	Oxidative Stress Induced Senescence	-5
MCODE_4	R-HSA-373076	Class A/1 (Rhodopsin-like receptors)	-11.3
MCODE_5	R-HSA-983168	Antigen processing: Ubiquitination and Proteasome degradation	-9.5
MCODE_6	R-HSA-6807505	RNA polymerase II transcribes <i>snRNA</i> genes	-10.1
MCODE_7	R-HSA-416476	G alpha (q) signalling events	-8.2
MCODE_8	R-HSA-72695	Formation of the ternary complex, and subsequently, the 43S complex	-10.8

breast cancer statistics were searched from the TCGA database. Six differently expressed miRNAs (hsa-miR-4274, hsa-miR-6880-3p, hsa-miR-4689, hsa-miR-1-3p, hsa-miR-670-5p, hsa-miR-30b-3p) were identified by combining two screening results. Among them, some miRNAs have been shown to affect tumor proliferation, migration, and prognosis. For example, a previous study reported miR-4274 promotes breast cancer cell proliferation, migration, and invasion through suppressing lncRNA SNHG15,^[16] it also serves as a prognostic biomarker, or as a potential therapeutic target, in cutaneous melanoma patients.^[17] As for hsa-miR-146a, it may play an important role in enhancing the proliferative activity related to liver cancer by regulating the expression of PROX1 at the post-transcriptional level.^[18] Another study also reported hsa-miR-1-3p was significantly down-regulated in gastric cancer and overexpression of miR-1-3p inhibited proliferation and invasion in gastric cancer by inhibiting stanniocalcin 2 expressions.^[19] Besides, it may play a tumor suppressor role by directly regulating ADAM9 and MMP7 in breast cancer.^[20] However, the regulation mechanism of some miRNAs in the tumor has not been reported yet, so more

researches need to be conducted in the future, especially in breast cancer.

To learn more about the regulatory mechanism of the 6 miRNAs in breast cancer, we chose the intersection of two screening results from targets can and miRDB, 499 mRNAs were identified after filtering. Function annotation indicated that these miRNAs were primarily related to adherens junction, regulation of cytoskeleton organization, and protein heterodimerization activity. KEGG pathway analysis of DEGs revealed involvement in “Influenza A,” “Hepatitis C,” and “NOD-like receptor signaling pathway.” “Hepatitis B,” “Epstein-Barr virus infection,” “Kaposi sarcoma-associated herpesvirus infection,” “Non-small cell lung cancer,” and “Central carbon metabolism in cancer.” According to previous studies, the NOD-like receptor signaling pathway participates in a diverse array of important cellular processes, including the survival, proliferation, differentiation, and activation of different cell types.^[21] Another study reported the treatment for influenza A has been long used as a treatment for Parkinson’s disease.^[22] Also, recent researches showed that hepatitis C involved in invasion, metastasis, and

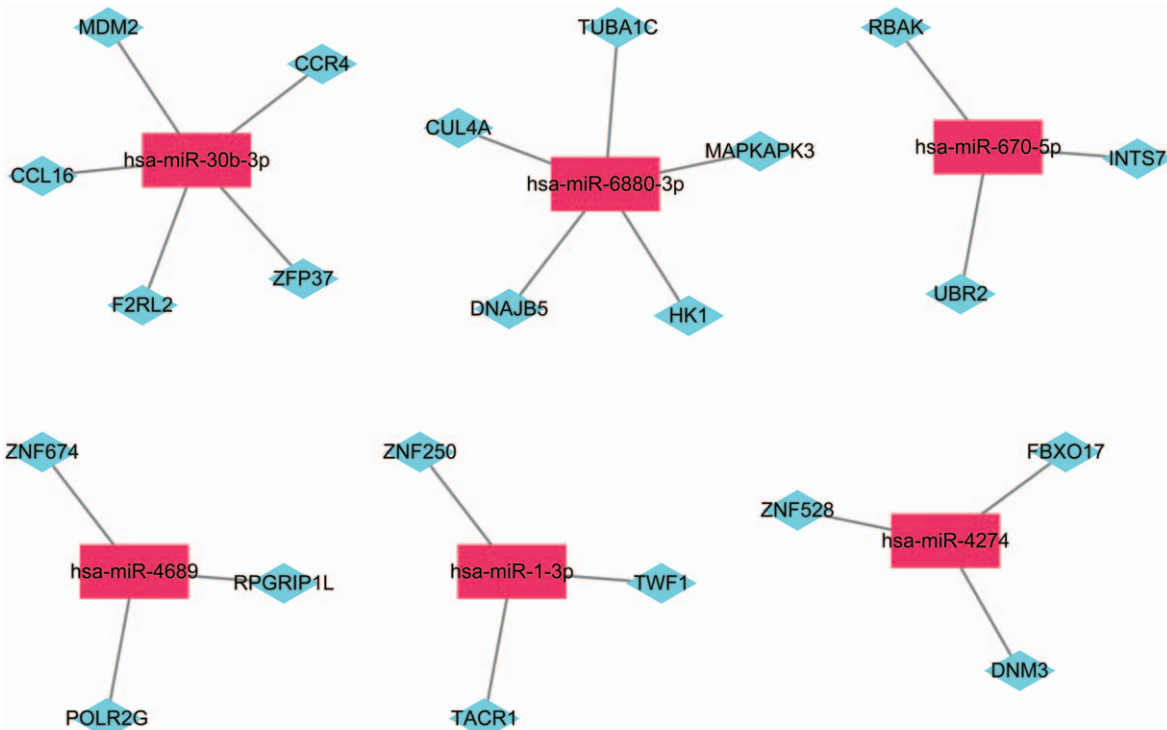


Figure 6. miRNA-mRNA regulatory pairs from the 6 hub miRNA targets and their regulated miRNAs.

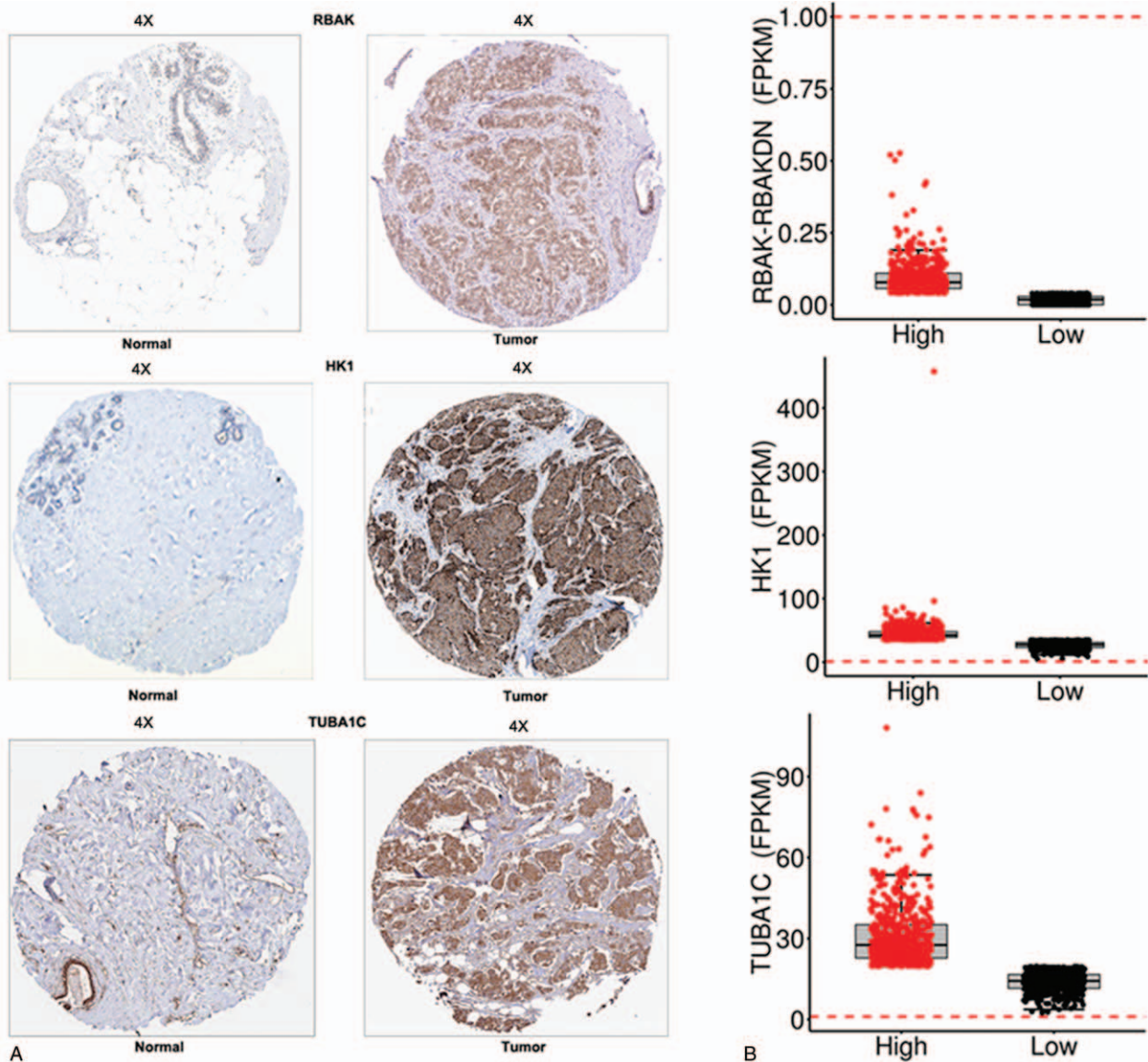


Figure 7. Genes expression in human breast carcinoma specimens. (A) RBAK, TUBA1C, and HK1 expression in normal breast tissue and breast carcinoma specimens. (B) TCGA data showing genes expression in normal vs tumor.

prognosis of metastatic melanoma and may be potential therapeutic targets.^[23]

Besides, PPI network analysis indicated that 11 hub genes including RBAK, TUBA1C, CCR4, HK1, FBXO17, INTS7, F2RL2, RPS29, FOXJ2, OBSL1, and GPI may be used as new targets in breast cancer with lymph node metastasis which had higher degrees of interaction. RBAK, RB-associated KRAB zinc finger, was found upregulated in non-small cell lung cancer^[24] and taking part in the regulation of the cell cycle.^[25] A previous study reported that the high expression of RBAK is related to the short survival time of prostate cancer patients after radical prostatectomy,^[26] but its prognostic value and molecular function in breast cancer are not clear. As for TUBA1C, a component of tubulin is highly expressed in tumor tissues than the normal tissues according to previous studies.^[27] Ji Wang et al reported that TUBA1C may promote the migration and proliferation of hepatoma cells possibly through the cell cycle

signal pathway.^[28] As for FBXO17, previous results showed that FBXO17 expression in tumor tissues of hepatocellular carcinoma (HCC) patients was markedly higher than that in adjacent tissues.^[29] It might promote the malignant progression of HCC by inhibiting the wnt/ β -catenin pathway.^[30] In lung adenocarcinoma cells, FBXO17 was observed to promote cell proliferation through activation of Akt.^[31] Furthermore, the relationship between the 6 miRNAs and the overall survival curves for patients with breast cancer revealed that overexpression of hsa-miR-4274, hsa-miR-6880-3p, and hsa-miR-670-5p was related to worse overall survival. However, the expression level of has-miR-149, has-miR-1-3p, and has-miR-30b-3p may have no significant relationship with the overall survival of patients. Meanwhile, evaluation of the 6-miRNA signature for overall survival by the ROC curve displayed better predictive ability.

Nowadays, with the development of precision medicine, more and more attention has been paid to individual differences in

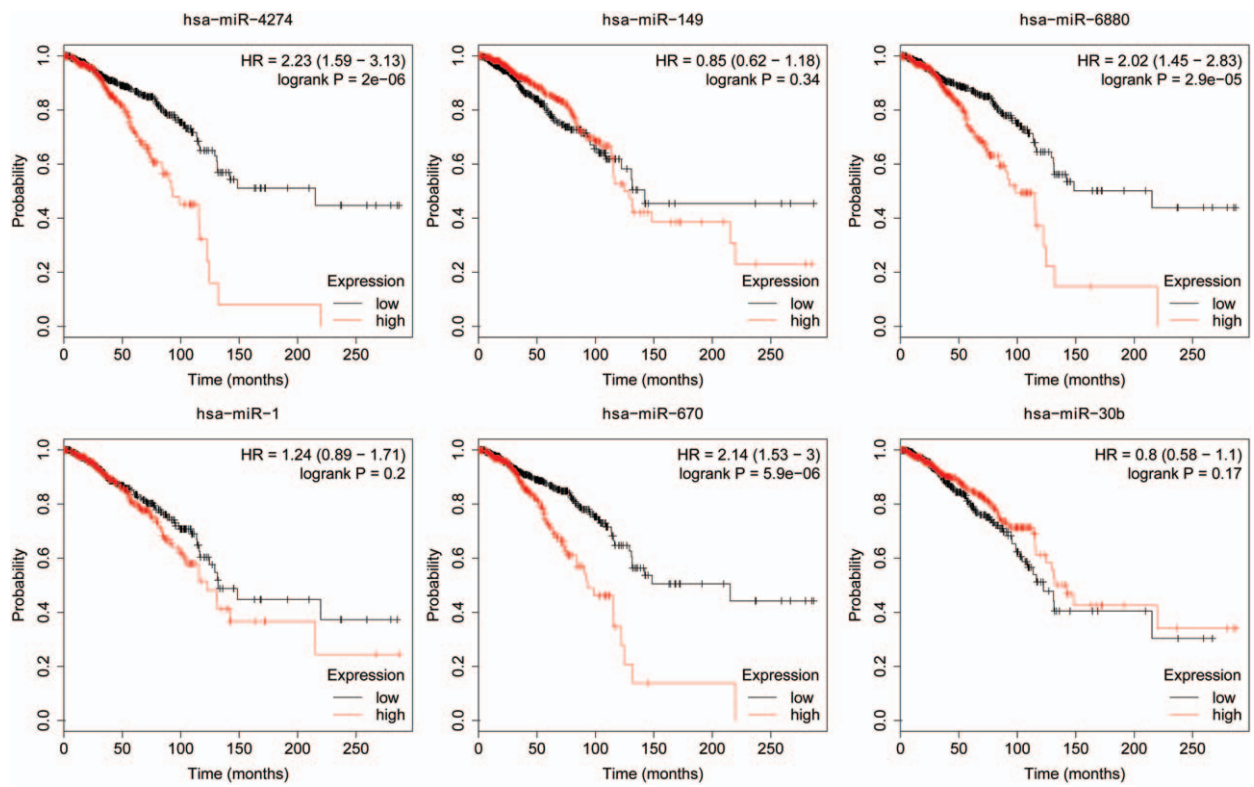


Figure 8. The association between miRNAs and breast cancer prognosis.

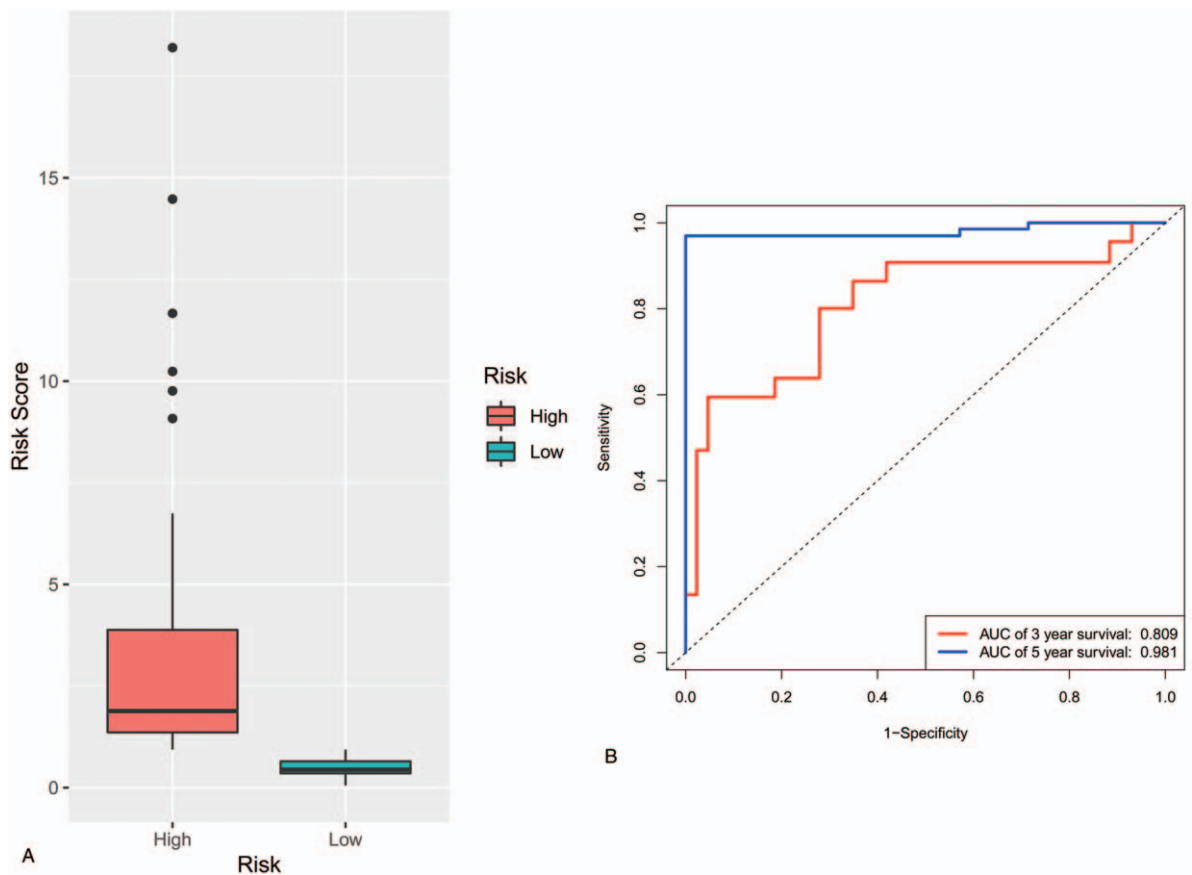


Figure 9. Risk prediction model of 6 identified miRNAs. (A) The AUC of 3 years and 5 years curve. (B) Box diagram of the risk score. AUC=area under curve.

treatment methods. Therefore, it is necessary to find new biomarkers and treatment methods for patients. Our findings approved that many differentially expressed mRNAs and miRNAs involved in the lymph node metastasis of breast cancer by certain signaling pathways and had prognostic value. Since all our data were achieved from the GEO database and TCGA by bioinformatics tools, as well as the limited number of relevant samples, more data analysis, and clinical experiments should be further performed for verification.

5. Conclusion

Our study not only concluded certain mechanisms of lymph node metastasis in breast cancer but also constructed a significant 6-miRNA risk prediction model for overall survival. Bioinformatics methods were utilized to identify the differently expressed miRNAs. The results of the enrichment analysis of GO and KEGG pathways showed that these miRNAs were significantly correlated with tumor progression. Also, 11 selected genes were identified according to the PPI network. Based on selected miRNAs and mRNAs, we constructed a miRNA-mRNA network. Compared with previous biomarkers, this network has an improved prediction of targeted therapy for patients. Meanwhile, we found that overexpression of hsa-miR-4274, hsa-miR-6880-3p, and hsa-miR-670-5p were related to the worse overall survival. These conclusions from the present research may offer a significant understanding of the mechanisms of lymph node metastasis in breast cancer. However, these findings need to be considered for future investigation.

6. Supplementary material

The Supplementary Material for this article can be found online at: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE100453> and <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE38167>, <http://links.lww.com/MD/E910>.

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Author contributions

Conceptualization: Guangyu Gao.

Investigation: Xinya Shi, Guangyu Gao.

Methodology: Guangyu Gao.

Project administration: Guangyu Gao.

Resources: Guangyu Gao, Zhen Yao.

Software: Jiaofeng Shen, Zhen Yao.

Supervision: Guangyu Gao, Xinya Shi.

Validation: Guangyu Gao, Zhen Yao.

Visualization: Guangyu Gao, Zhen Yao.

Writing – original draft: Guangyu Gao.

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