Original Article

Identification of three miRNAs signature as a prognostic biomarker in breast cancer using bioinformatics analysis

Meijie Sang[1,2], Aiying Li[3], Xu Wang[1], Can Chen[4], Kun Liu[5], Lin Bai[6], Ming Wu[7], Fei Liu[2], Meixiang Sang[2]

[1]Department of Surgical Nursing, Hebei University of Chinese Medicine, Shijiazhuang 050017, China; [2]Research Center, the Fourth Hospital of Hebei Medical University, Shijiazhuang 050017, China; [3]Department of Biological Chemistry, [4]Department of Basal Nursing, [5]Department of Laboratory Medical Science, [6]Department of Gynecological Pediatrics Nursing, Hebei University of Chinese Medicine, Shijiazhuang 050017, China; [7]Department of Histology and Embryology, Hebei Medical University, Shijiazhuang 050017, China

Contributions: (I) Conception and design: M Sang, F Liu, M Sang; (II) Administrative support: M Sang, F Liu; (III) Provision of study materials or patients: M Sang, A Li, X Wang; (IV) Collection and assembly of data: M Sang, X Wang, C Chen; (V) Data analysis and interpretation: F Liu, L Bai, M Wu; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Meixiang Sang; Fei Liu. Research Center, the Fourth Hospital of Hebei Medical University, No. 12 Jiankang Road, Shijiazhuang 050017, China. Email: mxsang@hotmail.com; kyzxlf@hotmail.com.

Background: Accumulating evidences indicated that some miRNAs are dysregulated in breast cancer and involved in cell growth, migration and invasion, differentiation, cell cycle arrest, apoptosis, and autophagy. Our study aims to identify a novel set of biomarkers for predicting the prognosis of breast cancer patients.

Methods: We downloaded clinical information and raw sequencing data from The Cancer Genome Atlas (TCGA) database. We selected samples with miRNA sequencing data and relevant clinical prognostic data for subsequent analysis. The association between miRNA and prognosis function was analyzed by Cox regression analysis. The potential biofunctions of target miRNAs were investigated through bioinformatic analysis.

Results: We identified 84 differentially expressed miRNAs (DEmiRNAs), among them, 17 were downregulated and 67 were upregulated. We used Kaplan-Meier survival analysis to evaluate the prognostic value of three miRNAs (mir-105-1, mir-301b and mir-1258). We also found that the three-miRNA signature is independent prognostic factors for breast cancer by using Cox regression analysis. It might be participated in different signaling pathways associated with cancer by using functional enrichment analysis, including adherens junction, autophagy, and TGF-beta signaling pathway, ErbB signaling pathway, FoxO signaling pathway.

Conclusions: Taken together, three-miRNA signature might be used as a potential predicting prognostic biomarker in breast cancer.

Keywords: Breast neoplasms; microRNAs (miRNAs); The Cancer Genome Atlas (TCGA); prognosis

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Introduction

Breast cancer is the most common neoplasm in females and the second leading reason of cancer death in females. It is estimated that approximately 62,930 cases of cancer *in situ* of the female mammary gland were newly diagnosed in 2019 (1). Although progress has been made in neoadjuvant chemotherapy and surgical techniques, some breast cancer patients still have poor prognosis, especially for human epidermal growth factor receptor-2 (HER-2) positive breast cancer or triple negative breast cancer (TNBC) patients (2). Therefore, uncovering the pathogenesis and

identifying new biomarkers are urgently needed for early diagnosis, judge prognosis, and direct treatment for breast cancer.

MicroRNAs (miRNAs) are small noncoding RNAs that inhibit the expression of target genes by targeting the 3'UTR of mRNA (3). Accumulating evidences showed that miRNAs are unusually expressed in many different kinds of neoplasms and play important roles in tumorigenesis and development (4). It has been demonstrated that miRNAs modulated a variety of oncogenic processes, including cell viability (5) apoptosis (6), autophagy (7), migration, invasion (8), and cell maturation (9). Therefore, identification of tumor-specific miRNAs will have important implications for predicting prognosis in breast cancer patients.

In the current study, we identified differentially expression miRNAs (DEmiRNAs) between normal breast tissues and breast cancer tissues by analyzing the sequencing data of miRNAs downloaded from The Cancer Genome Atlas (TCGA) database. Moreover, we found that three miRNA signatures may validly predict the prognosis of patients. Additionally, we evaluated the function and signaling pathway of predicting gene with Enrichr database, which might provide further understand the molecular mechanism of breast cancer.

Methods

Data acquisition and characteristics

We downloaded clinical information and raw sequencing data from the official TCGA website (https://cancergenome.nih.gov/). We selected samples with miRNA sequencing data and relevant clinical prognostic data for subsequent analysis. Finally, our study included 1,166 samples, including 104 normal samples and 1,062 breast cancer samples. Clinical information including age at diagnosis, gender, clinical stage, lymph-node status, T stage, metastasis, progesterone receptor (PR) status, estrogen receptor (ER) status, and Her2 status, and is shown in *Table 1*.

Raw-count miRNA sequencing data were conducted with Edge-R to screen DEmiRNAs between normal tissues and breast cancer tissues. $Log_2|$fold changes (FCs)$|$ >2.0 and

Table 1 Clinical characteristics of breast cancer patients

Variables	Case, n (%)
Age at diagnosis (years)	
≥60	494 (46.5)
<60	568 (53.5)
Gender	
Male	12 (1.1)
Female	1,050 (98.9)
Metastasis	
M0	879 (82.8)
M1	21 (2.0)
MX	162 (15.3)
Lymph-node status	
N0	498 (46.9)
N1–3	544 (51.2)
NX	20 (1.9)
Clinical stage	
I+II	779 (73.4)
III+IV	261 (24.6)
NA	22 (2.1)
T stage	
T1+T2	892 (84.0)
T3+T4	167 (15.7)
TX	3 (0.3)
ER status	
Positive	782 (73.6)
Negative	232 (21.8)
NA	48 (4.5)
PR status	
Positive	680 (64.0)
Negative	331 (31.2)
NA	51 (4.8)
Her2 status	
Positive	173 (16.3)
Negative	748 (70.4)
NA	141 (13.3)

NA, non-available.

FDR values <0.05 were set as cut-off criteria for identifying DEmiRNAs.

Correlation analysis between DEmiRNAs and prognosis of breast cancer

The prognostic value of each DEmiRNA were estimated by log-rank test and Kaplan-Meier method. We first identified miRNAs correlated with overall survival (OS) and then performed binary logistic regression analysis. Then, we constructed prognostic miRNA signature, and it can count risk score for each patient. Based on the median risk score of miRNA, patients with breast cancer were divided into high- and low-risk groups. Next, we assessed the difference in survival between the two groups by using Kaplan-Meier method.

Target gene prediction and functional enrichment analysis

Three miRNAs-targeted mRNAs were predicted by using three online analytical softwares: miRDB, TargetScan, and miRDIP. In order to further improve the credibility of bioinformatics analysis, we then used Venn diagram to screen the overlapping target genes. Finally, we evaluated the function and signaling pathway of predicting gene with Enrichr bioinformatics tool (http://amp.pharm.mssm.edu/Enrichr/).

Statistical analysis

The relationship between patient clinicopathological parameters and miRNA expression level was assessed by using *t*-test and Chi-square test, as appropriate. The correlation between three-miRNA signature and patients' survival was assessed by Kaplan-Meier method. The prognostic value of three miRNAs were analyzed by Cox analysis. All statistical analyses were performed using R (v.3.6.0) software and SPSS v.24.0 software (IBM Corp.).

Results

Identification of DEmiRNAs in breast cancer

Using FDR <0.05 and $|\log_2 FC| \geq 2$ as the cut-off criteria. We identified 84 differentially expressed miRNAs (DEmiRNAs), among them, 17 were downregulated and 67 were upregulated (*Table 2*). The relationships between $\log_2 FC$ and FDR of each DEmiRNA were shown by the volcano plot (*Figure 1*).

Screening for miRNAs correlated with OS

To further understand the correlation between differently expressed miRNAs and OS, we performed Kaplan-Meier analysis. The results indicated that miR-105-1, miR-301b and miR-1258 were significantly related with breast cancer patients' OS (*Figure 2*). As shown in *Table 3*, the correlation between the expression of three kinds of miRNA and clinicopathological parameters was assessed in breast cancer patients. Our research revealed that miR-105-1 was obviously correlated with ER and PR; miR-301b was correlated with Age, Metastasis, PR, ER, and HER-2. miR-1258 was correlated with age, clinical stage, PR, and ER.

Prognostic value of three-miRNA signature

We used Kaplan-Meier method to assess the survival performance of three-miRNA signature, and estimated the risk score for every breast cancer patient. According to the median risk score, we divided breast cancer patients into low and high risk groups. As shown in *Figure 3*, the OS rate of breast cancer patients with high-risk scores was lower than that of patients with low-risk scores (P=0.0078).

Then, we performed univariate Cox regression analysis for each clinicopathological factor. As shown in *Table 4*, age (HR =1.031, P<0.001), Metastasis (HR =6.417, P<0.001), lymph node metastasis (HR =2.314, P<0.001), Clinical stage (HR =2.702, P<0.001), ER (HR =0.683, P=0.043), and three-miRNA signature (HR =1.777, P=0.001) were significant related with survival in breast cancer patients. Subsequently, we carried out the multivariate Cox regression analysis of the factors, and observed that three-miRNA signature (HR =1.574, P=0.014) was an independent prognostic factor for poor prognosis of breast malignant tumor patient (*Table 4*).

Pathway enrichment analysis of predicted target genes

We used miRDB, TargetScan and miRDIP online analysis softwares to predict target genes for three miRNAs. The overlapping genes of miR-150-1, miR-301b and miR-1258 were identified as 30, 261 and 51, respectively (*Figure 4*). To further elucidate the biological roles of differently expressed miRNAs, we conducted Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology (GO) analysis to indicate the molecular function of predicted

Table 2 DEmiRNAs between breast cancer samples and normal samples (top 30)

miRNA	logFC	P value	FDR
Upregulated			
hsa-miR-122	7.024277	8.59E-15	2.39E-14
hsa-miR-1269a	6.219576	2.25E-21	9.05E-21
hsa-miR-1269b	6.175296	1.14E-16	3.58E-16
hsa-miR-767	6.047708	1.42E-21	5.80E-21
hsa-miR-105-2	5.999491	2.13E-20	8.38E-20
hsa-miR-105-1	5.985519	1.53E-21	6.23E-21
hsa-miR-449a	5.736712	3.68E-27	1.98E-26
hsa-miR-3156-1	4.752563	2.21E-18	7.79E-18
hsa-miR-592	4.52489	9.63E-81	2.39E-79
hsa-miR-449c	4.442171	7.59E-19	2.70E-18
hsa-miR-3156-3	4.419179	1.92E-17	6.37E-17
hsa-miR-184	4.393167	5.11E-24	2.35E-23
hsa-miR-449b	4.357878	3.22E-19	1.17E-18
hsa-miR-4724	4.221601	9.71E-36	7.91E-35
hsa-miR-3156-2	4.198612	7.71E-15	2.17E-14
hsa-miR-522	3.581905	4.43E-12	1.06E-11
hsa-miR-4501	3.540763	1.41E-23	6.30E-23
hsa-miR-190b	3.524598	2.84E-47	3.45E-46
hsa-miR-196a-1	3.428353	1.03E-42	1.13E-41
hsa-miR-96	3.397427	9.58E-109	3.64E-107
hsa-miR-1251	3.344711	3.58E-13	9.18E-13
hsa-miR-196a-2	3.284369	5.45E-41	5.54E-40
hsa-miR-4652	3.264641	9.98E-15	2.75E-14
hsa-miR-187	3.260218	2.57E-24	1.22E-23
hsa-miR-519a-1	3.230734	1.78E-12	4.43E-12
hsa-miR-210	3.191708	3.98E-52	5.40E-51
hsa-miR-183	3.073046	4.16E-102	1.32E-100
hsa-miR-7-3	3.013492	2.96E-25	1.47E-24
hsa-miR-301b	2.993863	7.22E-39	6.53E-38
hsa-miR-7705	2.975935	1.72E-48	2.18E-47
Downregulated			
hsa-miR-133b	−6.64828	1.77E-187	1.12E-185
hsa-miR-1-2	−5.66393	3.31E-261	3.78E-259
hsa-miR-1-1	−5.64985	2.02E-246	1.65E-244
hsa-miR-486-1	−4.56451	2.99E-307	5.67E-305
hsa-miR-486-2	−4.56384	1.12E-306	1.59E-304
hsa-miR-206	−3.78198	4.67E-24	2.16E-23
hsa-miR-4732	−3.7358	3.35E-110	1.47E-108
hsa-miR-451a	−3.29369	5.93E-144	3.38E-142
hsa-miR-139	−2.90964	1.04E-255	9.84E-254
hsa-miR-144	−2.86216	8.29E-107	2.78E-105
hsa-miR-204	−2.5508	1.09E-61	1.88E-60
hsa-miR-6715a	−2.5086	3.15E-38	2.76E-37
hsa-miR-145	−2.24857	9.89E-198	7.05E-196
hsa-miR-1258	−2.21095	2.29E-54	3.43E-53
hsa-miR-378a	−2.17398	3.13E-133	1.62E-131
hsa-miR-5683	−2.16241	8.46E-41	8.31E-40
hsa-miR-551b	−2.14579	5.10E-66	9.69E-65

DEmiRNAs, expressed miRNAs; miRNA, MicroRNAs.

genes. Biological process was obviously enriched in signal transduction, regulation of transcription, activation of protein kinase activity. Additionally, KEGG signaling pathways were mainly concentrated in adherens junction, autophagy, and TGF-beta signaling pathway.

Discussion

Breast cancer is the second leading reason of cancer death in females (10). Survival rate of breast cancer patients will be significantly improved if cancer behavior can be reliably predicted in the initial diagnosis of the disease. Accordingly, it is extremely significant to explore the molecular mechanisms of breast cancer development and to identify the specific and sensitive biomarkers.

In this study, we identified 84 differentially expressed miRNAs (DEmiRNAs). Among them, 17 were downregulated and 67 were upregulated, and three of them were related with the OS in breast cancer patients. It has been indicated that combinations of differently miRNAs might be more specific and sensitive than single miRNA biomarkers (11). In our study, we constructed a three miRNAs signature (miR-105-1, miR-301b and miR-1258)

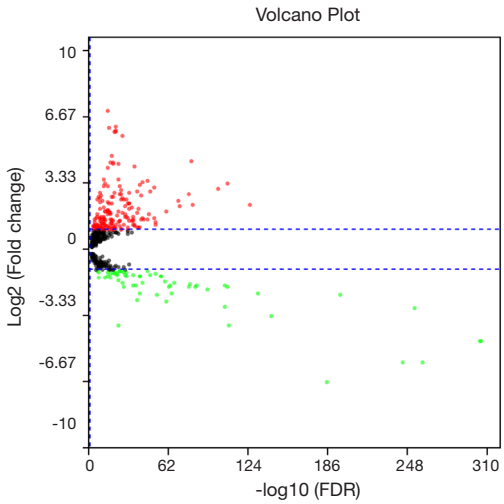

Figure 1 Volcano plot of the DEmiRNAs, red and green dots represent up- and down-regulated miRNAs, respectively.

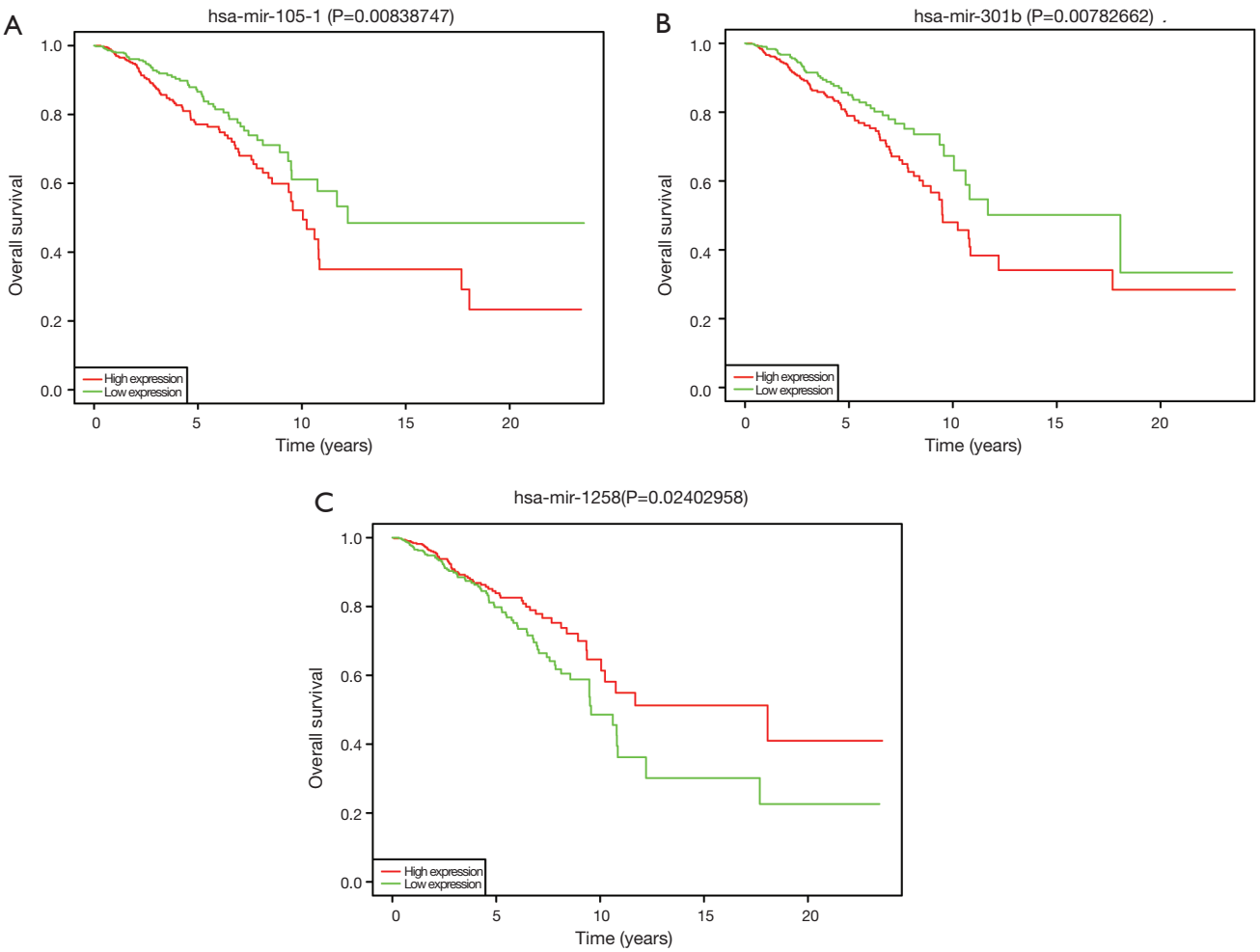

Figure 2 Kaplan-Meier survival analysis for three miRNAs of breast cancer patients from TCGA. (A) miR-105-1; (B) miR-301b; (C) miR-1258. TCGA, The Cancer Genome Atlas.

Table 3 Association of three miRNAs and clinical features

Variables	Case	miR-105-1			miR-301b			miR-1258		
		High	Low	P	High	Low	P	High	Low	P
Age at diagnosis				0.977			0.014			0.026
≥60	494	250	244		223	271		234	260	
<60	568	281	287		308	260		297	271	
Gender				0.082			0.082			0.082
Male	12	9	3		9	3		3	9	
Female	1050	522	528		522	528		528	522	
Metastasis				0.125			0.023			0.135
M0	879	437	442		449	430		441	438	
M1	21	14	7		16	5		14	7	
Lymph-node status				0.457			0.590			0.860
N0	498	255	243		250	248		227	271	
N1-3	544	266	278		264	280		245	299	
Clinical stage				0.397			0.153			0.002
I+II	779	380	399		395	384		369	410	
III+IV	261	140	121		119	142		152	109	
T stage				0.546			0.361			0.564
T1+T2	892	442	450		451	441		443	449	
T3+T4	167	87	80		78	89		87	80	
ER status				<0.001			<0.001			<0.001
Positive	782	335	447		323	459		419	363	
Negative	232	169	63		180	52		89	143	
PR status				<0.001			<0.001			<0.001
Positive	680	276	404		277	403		373	307	
Negative	331	226	105		225	106		133	198	
HER-2 status				0.074			0.003			0.444
Positive	173	97	76		103	70		83	90	
Negative	748	363	385		352	396		383	365	

and found that this signature was an independent prognostic indicator for breast cancer patients. Furthermore, we used three online bioinformatics tools to predict target genes for these three miRNAs, and evaluated cellular functions and signal pathway of those three miRNAs by GO and KEGG pathway analysis. In the last decade, some evidences have indicated that several miRNAs play key roles in various pathological activities, such as cell growth, migration and invasion, differentiation, cell cycle arrest, apoptosis, and autophagy (12-16). It has been suggested that miRNAs could act as novel prognostic biomarkers and therapeutic targets (17,18). Nevertheless, previous researches were based on various detection platforms, and the number of miRNAs was relatively limited. In the current study, we analyzed the sequencing data of miRNA and found that upregulation of miR-105-1 and miR-301b expression

and downregulation of miR-1258 expression were clearly correlated with the prognosis of the patients with breast cancer. Previous studies indicated that exosomal miR-105 can promote cancer cell migration and distant metastasis by targeting zona occludens protein 1 (ZO-1) (19). Jin *et al.* showed that miR-105 promotes epithelial mesenchymal transition (EMT) via upregulation of myeloid cell leukemia-1 (MCL-1) in non-small cell lung cancer (NSCLC) (20). miR-105 was also showed to activate Wnt/β-catenin signaling by targeting secreted frizzed related protein (SFRP1), and promote chemoresistance, stemness, and metastasis in TNBC. In addition, it has been shown that the expression of miR-301 was up-regulated in some cancers, including gastric, pancreatic, hepatocellular, lung, and breast cancers (21-25). Overexpression of miR-301b can increases autophagy, viability and radioresistance in prostate cancer (26,27). Song *et al.* demonstrated that miR-301b can play a carcinogenic role in TNBC by targeting cylindromatosis (CYLD) (28). miR-1258 was reported to suppress breast cancer brain metastasis by inhibiting heparanase (29). Other studies also indicated miR-1258 may function in oral squamous cell carcinoma, colorectal cancer, NSCLC, and osteosarcoma (30-33). Our results showed that miR-105-1 was obviously correlated with ER and PR; miR-301b was associated with age, metastasis, ER, PR, and HER-2; miR-1258 was associated with age, clinical stage, ER, and PR, indicating that these three-miRNAs were participated in the progression of breast cancer.

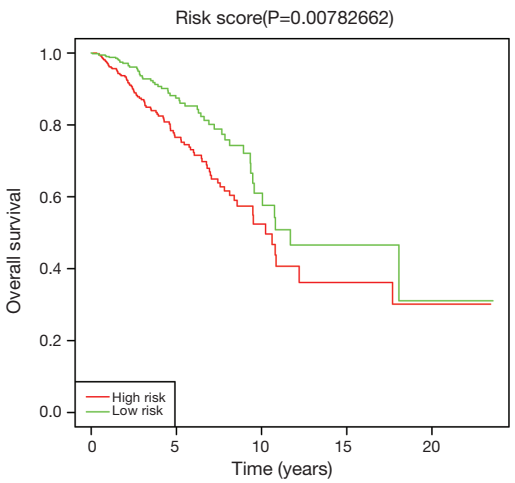

Figure 3 Kaplan-Meier survival curves for the three-miRNA signature in patients with breast cancer.

In our present study, we observed that miR-105-1, miR-301b and miR-1258 were significantly correlated with OS in patients with breast cancer. We then established a three-miRNA signature, and found that this signature was a potential independent prognostic factor for breast cancer patients.

In order to further explore the biology functions of these three miRNAs, we conducted KEGG pathway and Gene Ontology analysis to indicate the molecular function of predicted genes. We found that these three miRNAs are involved in many crucial signaling pathways, including adherens junction, autophagy, and TGF-beta, FoxO,

Table 4 Univariate and multivariate cox regression analysis in breast cancer patients

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P	HR (95% CI)	P
Age (≥60 *vs.* <60)	1.031 (1.018–1.044)	<0.001	1.031 (1.018–1.044)	<0.001
Gender (male *vs.* female)	0.833 (0.116–5.965)	0.855		
Metastasis (M1 *vs.* M0)	6.417 (3.795–10.851)	<0.001	3.540 (1.962–6.385)	<0.001
Lymph-node status (N1-3 *vs.* N0)	2.314 (1.608–3.329)	<0.001	1.709 (1.099–2.658)	0.017
Clinical stage (III+IV *vs.* I+II)	2.702 (1.930–3.781)	<0.001	1.917 (1.233–2.979)	0.004
T stage (T3+T4 *vs.* T1+T2)	1.219 (0.807–1.842)	0.346		
ER status (positive *vs.* negative)	0.683 (0.472–0.988)	0.043	0.579 (0.390–0.861)	0.007
PR status (positive *vs.* negative)	0.727 (0.516–1.023)	0.067		
HER-2 status (positive *vs.* negative)	1.150 (0.697–1.896)	0.585		
Three-miRNA signature (high risk *vs.* low risk)	1.777 (1.271–2.484)	0.001	1.574 (1.098–2.257)	0.014

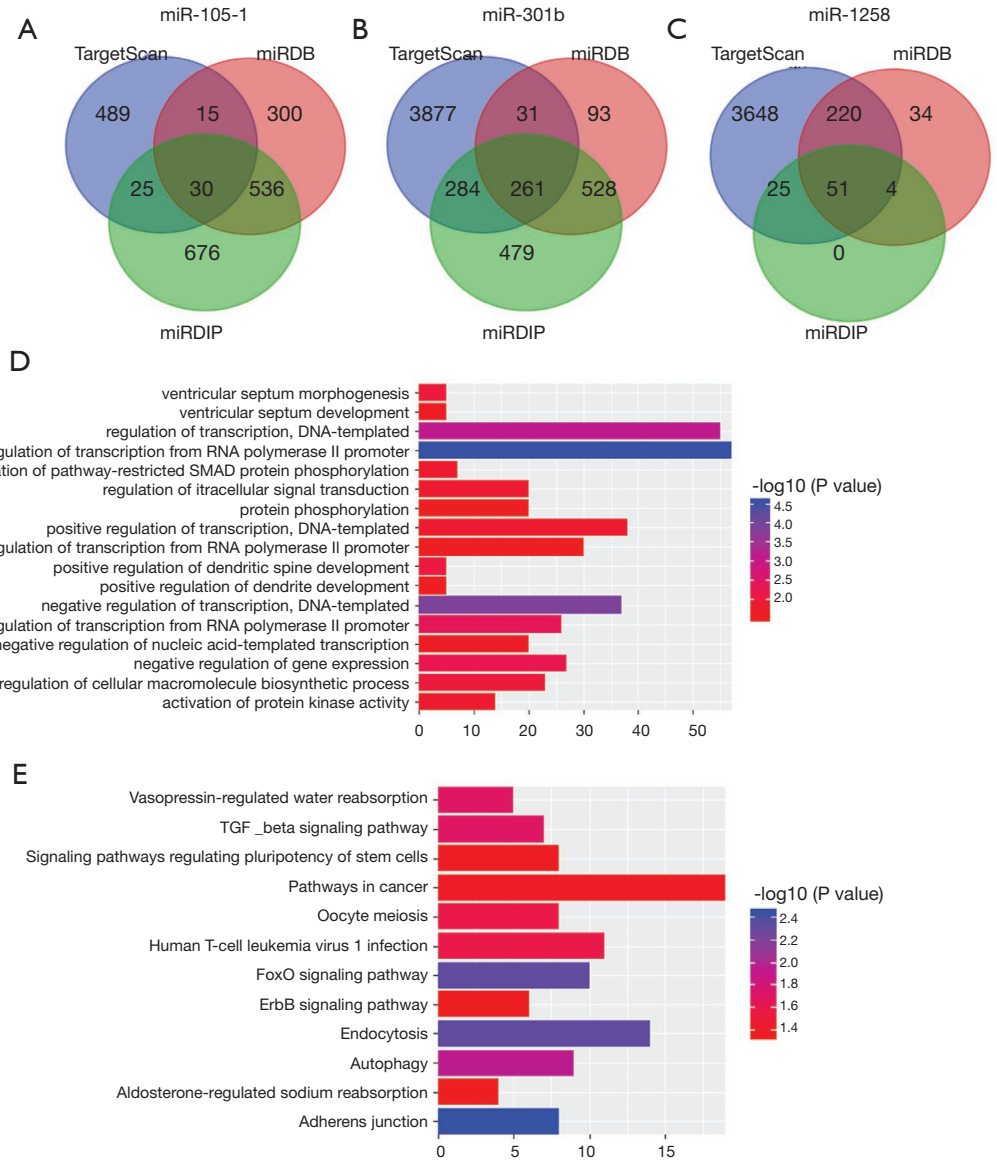

Figure 4 Pathway enrichment analysis of predicted target genes. The overlapping target genes were identified by using miRDB, TargetScan, and miRDIP software programs. (A) miR-105-1; (B) miR-301b; (C) miR-1258; (D) GO biological processes analysis; (E) KEGG pathway analysis. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

and ErbB signaling pathway. Moreover, accumulating evidences showed that autophagy was correlated with the progression of breast cancer, including proliferation, invasion and metastasis (34). TGF-β was involved in various biological functions including cell differentiation, metastasis, proliferation, angiogenesis, and cellular microenvironment (35). Inhibition of TGF-β pathway is a potential new therapeutic strategy for breast cancer (36). In addition, Bullock *et al.* reported that the PI3K/AKT/FOXO signaling axis plays a crucial role in the hormone-independent growth of many breast cancers (37). However, further experiments are needed to verify these predictions.

In conclusion, we found that a three-miRNA signature could validly predict the prognosis of the patients with bre

ast cancer. Nevertheless, further functional experiments are required to verify the biological functions of this signature in breast cancer progression.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2020.02.21). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Institutional ethical approval and informed consent were waived.

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