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ORIGINAL ARTICLE

Identification of genes associated with survival of breast cancer patients

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Abstract

Background We aimed to investigate the potential of microRNA expression profiles to predict survival in breast cancer.

Methods MicroRNA and mRNA expression data of breast cancer were downloaded from The Cancer Genome Atlas. LASSO regression was used to identify microRNAs signature predicting survival of breast cancer patients. Transfection experiment was conducted to explore the influence of microRNAs on their potential targets.

Results We identified 56 differentially expressed microRNAs in breast cancer tissues compared to adjacent normal tissues. 10 microRNAs with non-zero coefficient were selected from the 56 microRNAs using LASSO Cox regression. After predicting the targets for the 10 microRNAs, we further obtained 155 targets that were associated with overall survival of breast cancer patients. Spearman's correlation analysis found that the expression of SCUBE2, SCRN3, YTHDF3, ITFG1, ITPRIPL2, and JAK1 was an inversely correlated with their microRNAs. Transfection experiment showed that YTHDF3 was down-regulated in cells transfected with miR-106b-5p mimics compared with those transfected with negative control of mimics (fold change 4.21; $P < 0.01$).

Conclusions In conclusion, we identified a 10-miRNA signature associated with prognosis of breast cancer patients. The expression of YTHDF3 was down-regulated by miR-106b-5p.

Keywords MicroRNA · Breast cancer · Prognosis · Survival

Min Liu and Siying Zhou are equal contributors.

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Introduction

It is estimated that about 252,710 cases of breast cancer are diagnosed, and 71,280 women are die from the disease in 2017, making breast cancer the second leading cause of cancer deaths in United States women [1]. Many factors could influence the prognosis of breast cancer, such as age of the patient, menopause status, tumor size, lymph node status and tumor grade [2]. However, these factors alone are inadequate for determining prognosis of breast cancer patients. A number of research has been devoted to develop and validate molecular biomarkers that can provide not only prognostic information but also can predict response to therapy [3].

MicroRNAs (miRNAs, miRs) are a class of small non-coding RNAs (ncRNAs) with 18–25 nucleotides that negatively control gene expression at the messenger RNA (mRNA) and protein level [4]. It has been demonstrated that miRNAs play important roles in tumorigenic processes of breast cancer, such as sustaining proliferation, evading growth suppressors, resisting cell death, metastasis, angiogenesis, replicative immortality, deregulating metabolism,

avoiding immune destruction, and genome instability [5]. Moreover, accumulating evidence has suggested aberrant expression of miRNAs is associated with prognosis for breast cancer, especially for invasive breast cancer [6]. For example, higher expression level of miR-21 and miR-210 was associated with poorer outcome of patients with breast cancer [7].

The Cancer Genome Atlas (TCGA) is a project that gathered comprehensive genomic data and robust clinical data from over 30 types of cancer [8]. More than 1000 breast cancer samples enables us to explore the association between miRNA expression level and survival of breast cancer patients. Here, we used data from the TCGA to identify miRNAs signature predicting survival of breast cancer patients using the least absolute shrinkage and selection operator method (LASSO) [9].

Materials and methods

Data set of TCGA

Level 3 miRNA-seq isoform quantification (The calculated expression for each individual miRNA sequence isoform observed, per sample), RNA-Seq data (HTSeq-Counts) and clinical data for 1066 female patients were downloaded from the data portal for TCGA (accessed may 2018) [10].

Statistical analysis of differentially expressed miRNAs

To obtain differentially expressed miRNAs between 1066 breast cancer tissues and 104 adjacent normal tissues, raw counts of miRNAs were normalized and differential miRNA expression was calculated using DESeq2 [11] in the R software (version 3.3.2). Raw read counts were filtered to remove the genes with a zero read count in 10% samples or genes with a median raw read counts less than 100. Benjamini and Hochberg method [12] was used to correct for multiple testing. Differentially expressed genes between tumor tissues and normal tissues were identified, using fold change >2 and adjusted $P<0.05$ as selection criteria.

LASSO penalized regression analysis

LASSO [9] penalized regression analysis was used to identify miRNAs signature predicting survival of breast cancer patients. In this section, we only included 1018 breast cancer patients with a survival time longer than 30 days. The patients were randomly allocated to two sets, training set and test set. We compared two data sets using χ^2 test for categorical variables and the t test for continuous variables. The training set was subjected to LASSO Cox regression model to select most useful prognostic markers from differentially expressed miRNAs. Depending on the regulation weight λ, LASSO shrinks all regression coefficients towards zero and sets the coefficients of many irrelevant features exactly to zero. The λ yielded minimum cross validation error in 10-fold cross validation was chosen as optimal λ. A risk score was calculated using the sum of normalized read counts weighted by the coefficients from the LASSO regression [13]. We calculated the risk score for all the samples and then separated the samples into low- and high-risk groups using the median as a cutoff. The association between the risk and overall survival was assessed using the log-rank test and survival curve was generated using the Kaplan–Meyer method. We used Cox Proportional Hazards to adjust for potential confounders. The hazard ratios (HRs) were given with their 95% confidence intervals (CIs). The "glmnet" and "survival" package in R software (version 3.3.2) were used to do these analyses and a P value <0.05 was considered statistical significance. Using the LASSO method, 10 miRNAs were identified with non-zero regression coefficients.

Prediction of target genes

TargetScan (Release 7.1) [14] and miRDB [15] were used to predict the target genes of the 10 miRNAs with non-zero coefficients. Given that the prediction programs often suffer from high false positive rates, only the target genes predicted by both two independent tools were taken into account. Cytoscape software 3.4.0 was utilized to construct the possible functional network of the miRNAs and their targets [16].

Identification of predicted miRNA targets associated with survival

Raw read counts were filtered to remove the genes with a zero read count in 10% samples, and then normalized using DESeq2 [11]. The expression data of the predicted targets of the 10 miRNAs was stratified into 'high' and 'low' expression based on median cutoff. The genes with a zero normalized read count in 50% samples were removed. The association between expression level of a gene and overall survival was assessed using the log-rank test. The correlation of targets associated with survival and miRNAs was assessed using Spearman's correlation.

Transfection experiment

Human breast cancer cell line MDA-MB-231 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). MiR-9-5p mimics, miR-106b-5p mimics and negative control of mimics were synthesized

by RiBoBio (Guangzhou, China). The MDA-MB-231 cells were transfected with miRNA mimics or negative control of mimic using a Nepa21 pulse generator (Nepa Gene, Chiba, Japan) as previously described [17].

Real-time quantitative PCR (RT-qPCR)

Total RNA was reverse transcribed using PrimeScript RT reagent kit (TaKaRa Bio Inc., Shiga, Japan) according to the manufacturer's protocol. Expression of genes was analyzed using Light Cycler® 480 SYBR Green I Master (Roche, USA) on Roche LightCycler 480 II. Table S1 presents the primers used in this study. The expression of genes was normalized to β-actin. Each sample was analyzed in duplicates for each specific genes.

Results

Differentially expressed miRNAs

Compared to adjacent normal tissues, there were 56 differentially expressed miRNAs in breast cancer tissues including 34 up-regulated miRNAs and 22 down-regulated miRNAs (Fig. 1a). The fold change of the 56 miRNAs is presented in Table S2.

Identification of a 10-miRNA signature associated with prognosis

To identify miRNAs associated with overall survival of breast cancer patients, LASSO regression was performed using expression data of the 56 differentially expressed miRNAs. The 1018 patients were randomly allocated to two sets, training set and test set. The characteristics of the two set patients were similar (Table 1). Then LASSO Cox regression model was used to build a prognostic classifier with the training set, and 10 miRNAs with non-zero coefficient were selected from the 56 miRNAs (Fig. 1b, c). The 10 miRNAs and coefficients are presented in Fig. 2a. Risk score of each patient was calculated using the normalized read counts and the coefficients from the LASSO regression. The patients were separated into high- and low- risk groups using the median risk score as cutoff, which was 0.337. Figure 2b shows that high-risk patients had shorter survival time and more deaths, comparing to low-risk patients. The median survival time of the low-risk group was 4267 days and the median of the high-risk group was 2520 days (Fig. 2c, HR 2.55, 95% CI 1.62–4.00; $P < 0.001$). After adjusting age, race and stage of cancer, the difference was still significant (HR 3.32, 95% CI 1.97–5.60; $P < 0.001$).

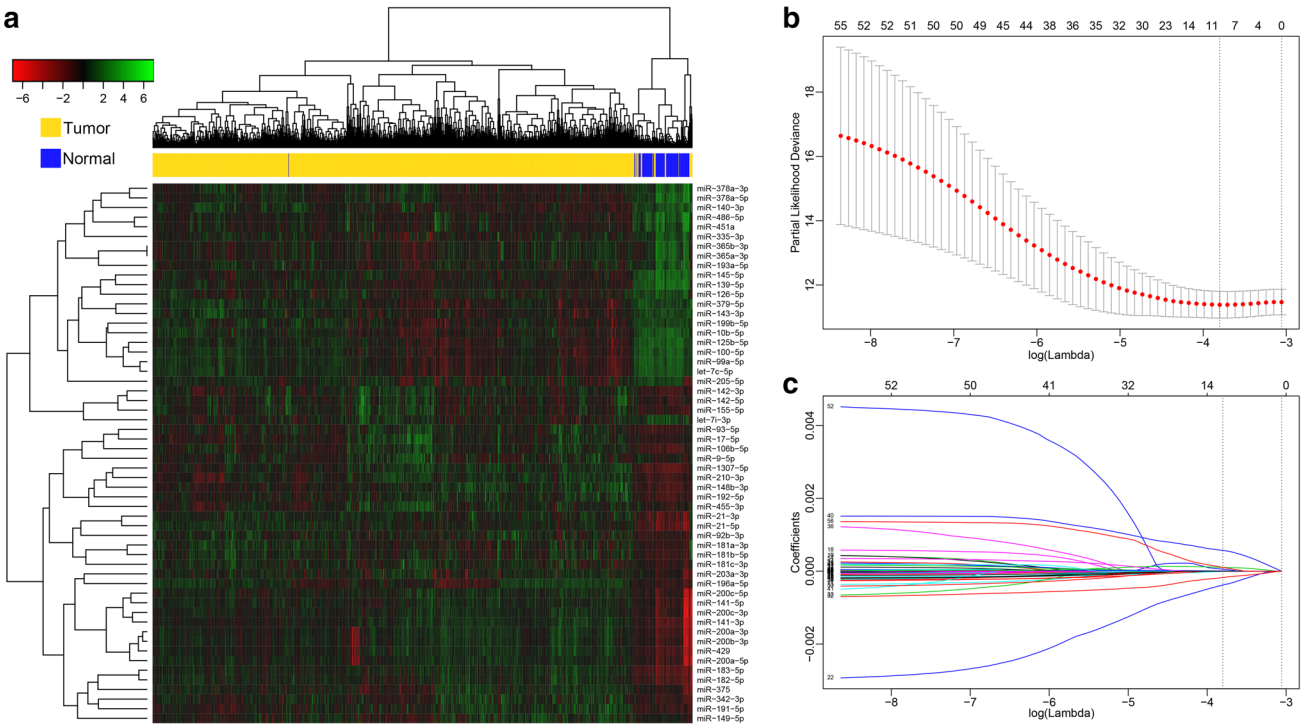


Fig. 1 Heatmap and LASSO Cox regression of the 56 differentially expressed miRNAs. **a** Heatmap of the 56 differentially expressed miRNAs in breast cancer tissues compared to adjacent normal tissues. **b** Ten miRNAs were selected by LASSO Cox regression analysis. **c** LASSO coefficient profiles of the 56 differentially expressed miRNAs

Table 1 Characteristics of breast cancer patients

Characteristic	All ($n=1018$)	Training set ($n=509$)	Test set ($n=509$)	*P* value
Age, years, mean ± SD	58.29 ± 13.23	59.10 ± 13.35	57.56 ± 13.11	0.063
Age, *n* (%)				
≤ 65 years	726 (71.32)	351 (34.48)	375 (36.84)	0.096
> 65 years	292 (28.68)	158 (15.52)	134 (13.16)	
Race, *n* (%)				
White	710 (69.74)	356 (69.94)	354 (69.55)	0.892
Others	308 (30.26)	153 (30.06)	155 (30.45)	
Stage, *n* (%)				
I	177 (17.81)	97 (19.48)	80 (16.13)	0.394
II	572 (57.55)	280 (56.22)	292 (58.87)	
III	227 (22.84)	110 (22.09)	117 (23.59)	
IV	18 (1.81)	11 (2.21)	7 (1.41)	
Tumor size, *n* (%)				
T1	272 (26.80)	144 (28.29)	128 (25.30)	0.455
T2	580 (57.14)	290 (56.97)	290 (57.31)	
T3	128 (12.61)	61 (11.98)	67 (13.24)	
T4	35 (3.45)	14 (2.75)	21 (4.15)	
Node status, *n* (%)				
N0	472 (47.25)	236 (47.39)	236 (47.11)	0.973
N1	345 (34.53)	169 (33.94)	176 (35.13)	
N2	109 (10.91)	56 (11.25)	53 (10.58)	
N3	73 (7.31)	37 (7.43)	36 (7.19)	
Metastases, *n* (%)				
M0	834 (97.66)	416 (97.20)	418 (98.12)	0.371
M1	20 (2.34)	12 (2.80)	8 (1.88)	
Status, *n* (%)				
Alive	887 (85.54)	427 (83.89)	445 (87.43)	0.107
Death	150 (14.46)	82 (16.11)	64 (12.57)	
Survival time (days), mean ± SD	1301.48 ± 1192.16	1258.00 ± 1085.62	1330.86 ± 1268.24	0.325

SD standard deviation

Risk score validation in the test set and all the patients

Patients were separated into low- and high-risk groups using the cutoff established above. The survival time and status of each patient and expression patterns of the 10 miRNAs are shown in Fig. 3 according to risk score (Fig. 3a for test set; and Fig. 3b for all patients).

Regarding to test set, median survival time of high-risk patients in the test set was 3873 days, and more than a half low-risk patients were still alive (Fig. 3c, HR 1.74, 95% CI 1.06–2.87; $P=0.030$). After adjusting age, race and stage of cancer, the HR of high-risk patients was 1.75 (95% CI 1.03–2.96; $P=0.038$).

We further validate the risk score in all the samples. Median survival time of low- and high-risk patients was 4367 and 3409 days, respectively (Fig. 3d, HR 1.99, 95% CI 1.43–2.78; $P<0.001$). After adjusting age, race and stage of cancer, high-risk patients had a HR of 2.08 (95% CI 1.46–2.96; $P<0.001$).

The potential targets of the 10 miRNAs

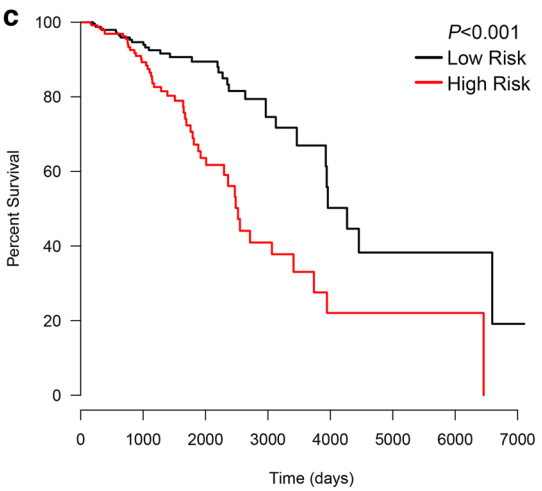
We predicted the target genes of the 10 miRNAs with non-zero coefficients using TargetScan and miRDB, and obtained 2345 miRNA/mRNA interactions (Table S3).

Predicted miRNA targets associated with survival

We used the log-rank test to assess the association between expression level of a target gene and overall survival. 155 target genes were associated with overall survival of breast cancer patients (Fig. 4). The correlation of the 155 target genes and miRNAs was assessed using Spearman's correlation (Table S4). Six significant miRNA/mRNA interactions with an inverse correlation of at least 0.25 were identified

a

Correlation	miRNAs	Coefficients
Positive correlation	hsa-miR-148b-3p	5.69E-04
	hsa-miR-365b-3p	1.04E-04
	hsa-miR-193a-5p	1.03E-04
	hsa-miR-365a-3p	5.40E-05
	hsa-miR-21-3p	6.21E-06
	hsa-miR-196a-5p	2.43E-06
	hsa-miR-9-5p	5.07E-07
Inverse correlation	hsa-miR-99a-5p	-4.82E-06
	hsa-miR-106b-5p	-1.77E-04
	hsa-miR-142-5p	-3.79E-04



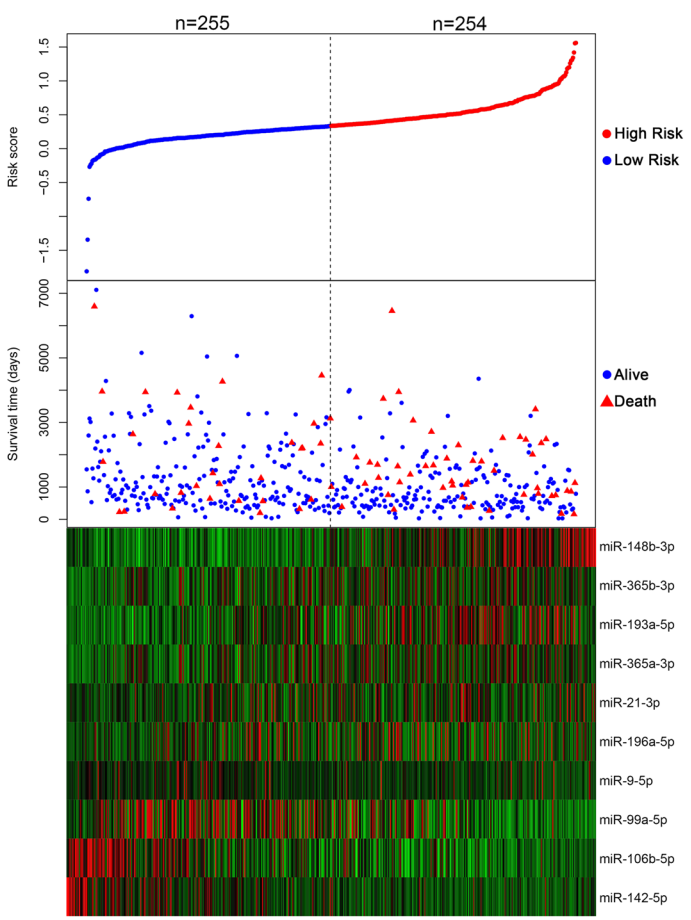


Fig. 2 Ten-miRNA-based classifier in training set. **a** Ten miRNAs and their coefficients. **b** Characteristics of the patients order by their risk score. Dotted line: the median of the risk score (0.337). Top panel: the distribution of the risk score for the patients. Middle panel: survival time and status of the patients in the high- and low-risk groups, as defined by the risk score. Bottom panel: expression patterns of the ten miRNAs. **c** Low-risk group has significantly longer survival times than those in the high-risk group. The median survival time of the low- and high-risk patients is 4267 days and 2520 days, respectively (HR 2.55, 95% CI 1.62–4.00; $P < 0.001$)

(Table 2). Although median survival time of patients with low-expression SCUBE2 or JAK1 was slightly longer than those with high-expression levels, we found that patients with low-expression SCUBE2 or JAK1 had a much shorter 10-year survival (Figure S1). Figure S2 shows miR-9-5p and miR-106b-5p binding sites in the six candidate target genes.

Influence of miR-9-5p and miR-106b-5p on their targets

We transfected miR-9-5p mimics, miR-106b-5p mimics, and negative control of mimics into MDA-MB-231 cells, respectively. After 24 h, we detected the expression of the targets in Table 2. We found that only YTHDF3 was down-regulated in cells transfected with miR-106b-5p mimics compared with those transfected with negative control of mimics (fold change 4.21; $P < 0.01$; Fig. 5).

Discussion

LASSO approach is a popular method for regression with high dimensional predictors and has been used to identify relevant miRNA and gene expression signatures in large datasets [18, 19]. In present study, we identified a 10-miRNA signature associated with prognosis of breast cancer patients using LASSO regression. Our results indicated that the breast cancer patients can be successfully categorized into high-risk and low-risk groups according to their risk score. We further analyzed the targets of the ten miRNAs and identified six potential targets that may be associated with the survival of breast cancer patients.

Several miRNAs used in our classifier have been investigated in previous studies. MiR-21, a well-known oncomiR, is the most significantly up-regulated miRNA in various cancers, including breast cancer [20]. A recent meta-analysis showed that higher expression levels of miR-21 significantly

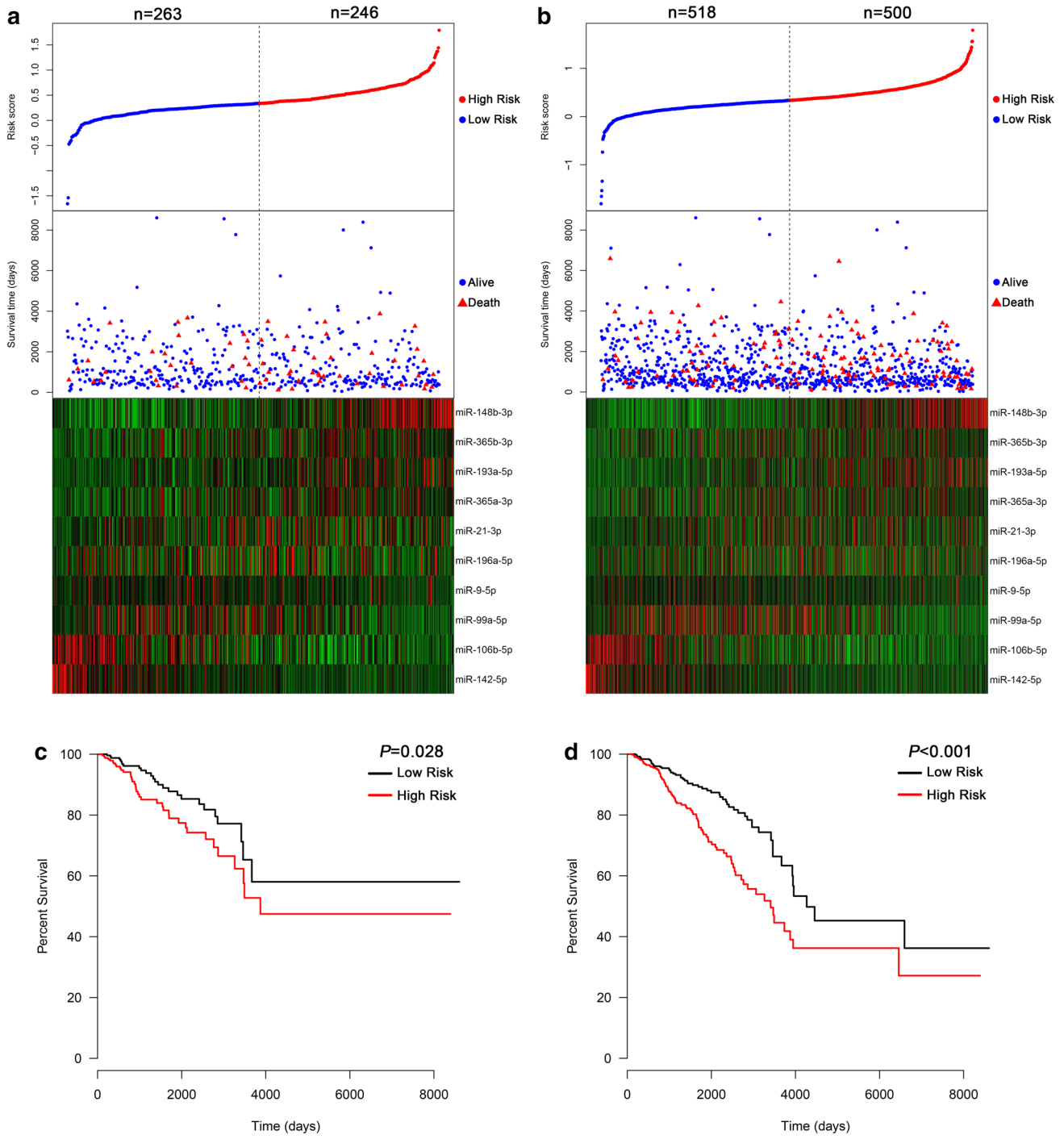


Fig. 3 Validation of the ten-miRNA-based classifier in test set and all the patients. **a** Characteristics of the patients in the test set order by their risk score. Dotted line: the median risk score of the training set (0.337). Top panel: the distribution of the risk score. Middle panel: Survival time and status of the patients. Bottom panel: expression patterns of the ten miRNAs. **b** Characteristics of the patients in the all the patients order by their risk score. Dotted line: the median risk score of the training set (0.337). Top panel: the distribution of the risk score. Middle panel: Survival time and status of the patients. Bottom panel: expression patterns of the ten miRNAs. **c** Low-risk patients have significantly longer survival times than high-risk patients in test set. Median survival time of high-risk patients is 3873 days, and more than a half low-risk patients are still alive (HR 1.74, 95% CI 1.06–2.87; $P=0.030$). **d** Low-risk patients have significantly longer survival times than high-risk patients in all the patients. Median survival time of low- and high-risk patients was 4367 and 3409 days, respectively (HR 1.99, 95% CI 1.43–2.78; $P<0.001$)

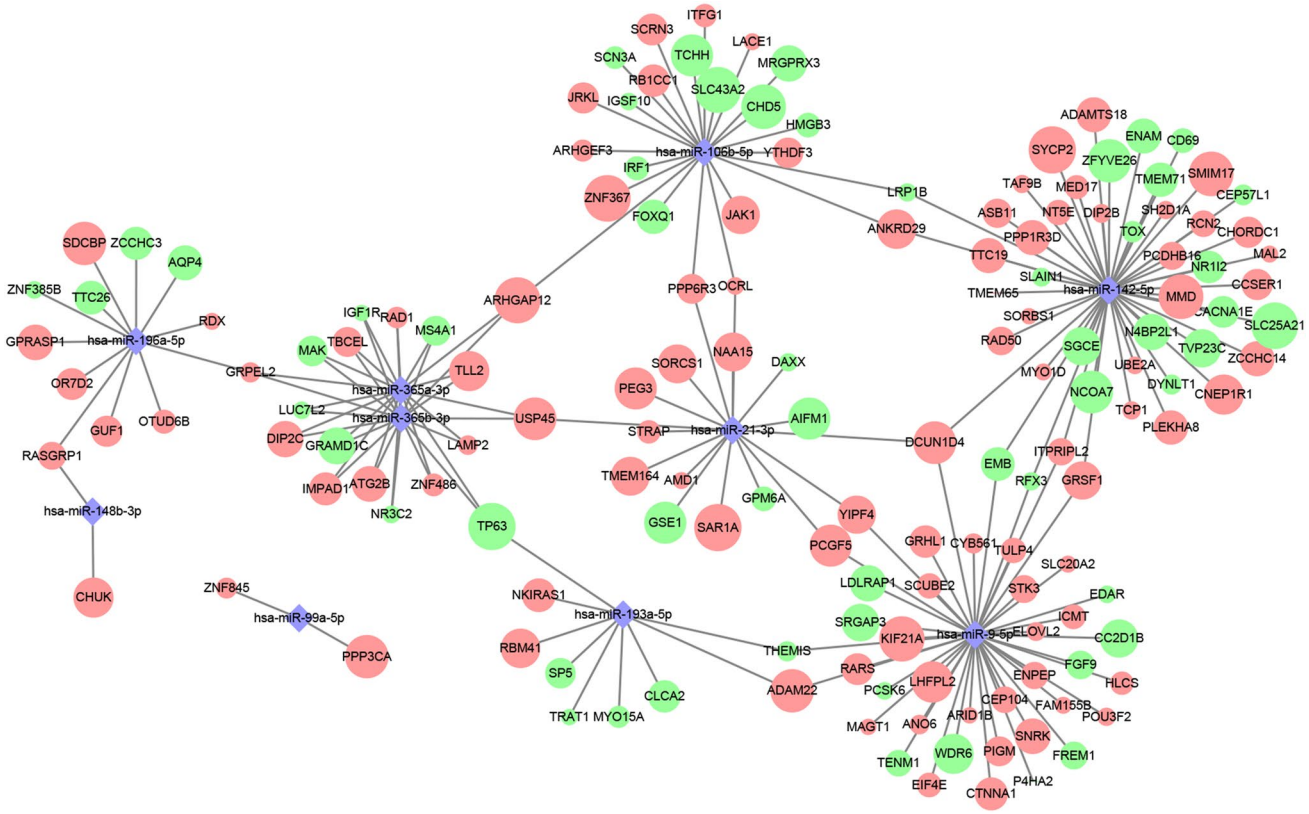


Fig. 4 Network of the ten miRNAs and the 155 targets associated with overall survival of breast cancer patients. The size of the circle represents the *P* value for overall survival. Red circles, median survival time of patients with low-expression levels is longer than those with high-expression levels. Green circles, median survival time of patients with high-expression levels is longer than those with low-expression levels

Table 2 Spearman's correlation of miRNAs and their targets with an inverse correlation of at least 0.25

miRNAs	gene symbols	*P* value of mRNA for OS	*P* value for Spearman's correlation	Spearman'scorrelation
hsa-miR-9-5p	SCUBE2	0.015	4.078E−45	−0.412
hsa-miR-106b-5p	SCRN3	0.025	5.826E−28	−0.326
hsa-miR-106b-5p	YTHDF3	0.023	1.458E−26	−0.318
hsa-miR-106b-5p	ITFG1	0.012	6.004E−21	−0.281
hsa-miR-9-5p	ITPRIPL2	0.018	1.316E−19	−0.272
hsa-miR-106b-5p	JAK1	0.035	1.659E−17	−0.256

OS overall survival

predicted poorer overall survival of breast cancer patients [7]. MiR-9-5p had a higher expression level in breast tumors than in normal breast tissues and associated with a worse prognosis of patients with luminal breast cancer [21]. MiR-196a-5p could enhance estrogen-induced breast cancer cell proliferation, migration and invasion [22]. MiR-99a, a well-studied tumor suppressor, was showed to exert its antitumor activity by targeting the mTOR signaling pathway in human breast cancer cells [23, 24]. MiR-106b could suppress migration and invasion of breast cancer cells by targeting MMP2, thus inhibiting bone metastasis [25]. These results all support the finding in our study.

We identified six miRNA/mRNA interactions with a significant inverse correlation. SCUBE2 was showed to be a tumor suppressor in breast cancer and could be a useful prognostic marker [26, 27]. SCUBE2 may exert its anti-carcinogenic role by antagonizing activity of bone morphogenetic protein (BMP), inhibiting the β-catenin signaling pathway, and driving the reversal of epithelial–mesenchymal transition [26–28]. In addition,

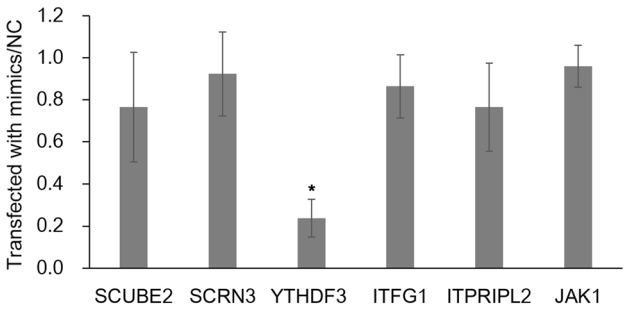


Fig. 5 Relative expression levels of the six targets in cells transfected with miR-9-5p mimics or miR-106b-5p mimics compared with those transfected with negative control of mimics (NC). SCUBE2 and ITPRIPL2 was detected in cells transfected with miR-9-5p mimics; and SCRN3, YTHDF3, ITFG1 and JAK1 was detected in cells transfected with miR-106b-5p mimics. *$P < 0.01$

SCUBE2 was mainly expressed in vascular endothelium and epithelial cells in normal breast tissue [28], suggesting SCUBE2 may serve as natural barriers against metastasis of breast cancer. In our results, we found that median survival time of patients with low-expression SCUBE2 was slightly longer than those with high-expression levels; however, patients with low-expression SCUBE2 had a much shorter 10-year survival (Figure S1). It should be noted that the patients with a survival time longer than 10 years may not die from breast cancer. ITFG1 was showed to interact with RUVBL1, thus contributing to the invasion in breast cancer cells [29]. Elevated JAK/STAT signaling has been reported in a variety of cancers [30]. JAK1/STAT3 signaling pathway has been a therapeutic target for breast cancer [31, 32]. The other three genes, SCRN3, YTHDF3 and ITPRIPL2 have not been individually studied. Among the six targets, only mRNA of YTHDF3 was showed to inversely correlate with miR-106b. Since miRNAs can regulate gene expression by suppressing translation or inducing mRNA degradation [4], whether these two miRNAs could regulate the expression of the targets at protein level should be confirmed further using luciferase reporter assay. There is a need for further study of the roles of these genes in breast cancer.

In conclusion, we identified a 10-miRNA signature associated with prognosis of breast cancer patients. Further analyses of the targets of these miRNAs have identified six potential targets that may be associated with the overall survival of breast cancer patients. This is the first study to formulate a survival risk score for breast cancer which consists of miRNAs.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interest.

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