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

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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 tumorspecific 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. Log₂|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 \(|\log_{2}\text{FC}| \geq 2\) 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 log₂FC 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 | 9.05E-21 | 3.58E-21   |
| hsa-miR-1269b | 6.175296 | 3.58E-16 | 1.14E-16   |
| hsa-miR-767 | 6.047708 | 5.80E-21 | 1.14E-16   |
| hsa-miR-105-2 | 5.999491 | 2.16E-23 | 8.38E-20   |
| hsa-miR-105-1 | 5.985519 | 6.23E-21 | 1.53E-21   |
| hsa-miR-449a | 5.736712 | 1.98E-26 | 3.68E-27   |
| hsa-miR-3156-1 | 4.752563 | 7.39E-18 | 2.21E-18   |
| hsa-miR-592 | 4.52489 | 6.23E-21 | 9.63E-81   |
| hsa-miR-449c | 4.442171 | 2.70E-18 | 4.72E-36   |
| hsa-miR-3156-3 | 4.419179 | 6.37E-17 | 3.85E-17   |
| hsa-miR-184 | 4.393167 | 2.35E-23 | 5.11E-24   |
| hsa-miR-449b | 4.357878 | 1.17E-18 | 3.68E-27   |
| hsa-miR-4724 | 4.221601 | 7.91E-35 | 9.71E-36   |
| hsa-miR-3156-2 | 4.198612 | 2.17E-14 | 7.71E-15   |
| hsa-miR-522 | 3.581905 | 1.06E-11 | 4.43E-12   |
| hsa-miR-4501 | 3.540763 | 6.30E-23 | 1.41E-23   |
| hsa-miR-190b | 3.524598 | 3.45E-46 | 2.84E-47   |
| hsa-miR-196a-1 | 3.428353 | 1.13E-41 | 1.03E-42   |
| hsa-miR-96 | 3.397427 | 3.64E-107 | 9.58E-109 |
| hsa-miR-1251 | 3.344711 | 9.18E-13 | 3.58E-13   |
| hsa-miR-196a-2 | 3.284369 | 5.54E-40 | 5.45E-41   |
| hsa-miR-4652 | 3.264641 | 2.75E-14 | 9.98E-15   |
| hsa-miR-187 | 3.260218 | 1.22E-23 | 2.57E-24   |
| hsa-miR-519a-1 | 3.230734 | 4.43E-12 | 1.78E-12   |
| hsa-miR-210 | 3.191708 | 5.40E-51 | 3.98E-52   |
| hsa-miR-183 | 3.073046 | 1.32E-100 | 4.16E-102 |
| hsa-miR-7-3 | 3.013492 | 1.47E-24 | 2.96E-25   |
| hsa-miR-301b | 2.993863 | 6.53E-38 | 7.22E-39   |
| hsa-miR-7705 | 2.975935 | 2.18E-47 | 1.72E-48   |
| **Downregulated** |        |            |            |
| hsa-miR-133b | 6.64828 | 1.12E-185 | 1.77E-187 |
| hsa-miR-1-2 | 5.66393 | 3.78E-259 | 3.31E-261 |

DEmiRNAs, expressed miRNAs; miRNA, MicroRNAs.

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 | 0.082     | 0.082    | 0.082 | 0.125     | 0.023    | 0.135 |
| ≥60                |      | 494       | 250      | 244   | 223       | 271      | 234   | 260       |          |       |
| <60                |      | 568       | 281      | 287   | 308       | 260      | 297   | 271       |          |       |
| Gender             |      | 0.082     | 0.082    | 0.082 | 0.457     | 0.590    | 0.860 |
| Male               |      | 12        | 9        | 3     | 9         | 3        | 3     | 9         |          |       |
| Female             |      | 1050      | 522      | 528   | 522       | 528      | 528   | 522       |          |       |
| Metastasis         |      | 0.125     | 0.023    | 0.135 | 0.457     | 0.590    | 0.860 |
| 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 | 0.397     | 0.153    | 0.002 |
| 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 | 0.546     | 0.361    | 0.564 |
| 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 | 0.074     | 0.003    | 0.444 |
| 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| <0.001    | <0.001   | <0.001| <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| <0.001    | <0.001   | <0.001| <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 | 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 frizzled 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.

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,
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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