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Integrated analysis of the potential roles of miRNA-mRNA networks in triple negative breast cancer

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Abstract. Triple negative breast cancer (TNBC) is a type of breast cancer where the tumor cells are negative for the estrogen, progesterone and human epidermal growth factor 2 receptors. To date, expression profiling of microRNA (miRNA/miR) and mRNA sequences have been widely applied for the diagnosis of TNBC. In the present study, an integrated analysis of miRNA-mRNA profiling arrays was performed. A total of five dysregulated miRNAs in patients with TNBC were identified, including upregulated miR-558 expression and downregulated miR-320d-1, miR-548v, miR-99a and miR-21 expression. In addition, 49 potential target mRNA sequences were identified. Bioinformatics analyses were performed on the identified miRNAs and mRNAs, including gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes pathway and miRNA-mRNA network analyses. A total of 31 GO terms and three signaling pathways were identified. The results indicated that the differentially expressed miRNAs and their potential target mRNAs may affect the pathogenesis of TNBC, and may therefore be considered as promising biomarkers for the early diagnosis and targeted therapy of patients with TNBC.

Introduction

Among all breast cancer types, 15-20% are pathologically diagnosed as triple negative breast cancer (TNBC). A typical immunohistochemical feature of TNBC tumors is that they are negative for the estrogen, progesterone and human epidermal growth factor 2 receptors (1). TNBC is highly invasive with common symptoms including breast lumps and nipple discharge. Epidemiological studies indicate that TNBC generally develops among pre-menopausal women, and particularly among young African-American women (2). It has been reported that TNBC is frequently diagnosed at stage III, and is usually correlated with increased metastasis, disease recurrence and cancer cell proliferation and invasion (3). To date, no specific treatment guidelines have been developed for TNBC. At present, the common methods for the treatment of breast cancer include surgery, radiotherapy, chemotherapy and traditional Chinese medicine. Therefore, patients with TNBC are currently treated with methods common to all breast cancer types. However, the rapid development of metastases and high recurrence rates lead to poor patient prognoses and high mortality rates (4,5).

MicroRNAs (miRNA/miR) are endogenous non-coding small RNA sequences that are 20-24 nucleotides in length. miRNAs regulate tumor cell metabolism, proliferation, invasion and metastasis (3). Each miRNA is able to regulate multiple target genes, while different miRNAs are able to regulate the same target gene. It is thought that approximately one-third of human genes are regulated by miRNAs (6). Previous studies indicate that ~70% of miRNAs in mammalian cells are located in miRNA transcription units (7). In addition, the majority of miRNAs are located in introns, which are highly conserved in different species (8). The high conservation of miRNAs among different species suggests common and important functions. Therefore, investigating the mechanisms and functions of miRNAs may be useful for the diagnosis and treatment of human cancers. To date, a number of previous studies have attempted to elucidate the mechanisms and functions of miRNAs in the diagnosis and treatment of human cancer; however, only a limited number have focused on understanding the function of miRNAs in the pathogenesis of TNBC (9,10). In addition, the mechanisms and signaling pathways regulated by miRNAs and their associated target genes require further investigation. With the development of gene expression microarrays and bioinformatics analysis technologies, an integrated and detailed investigation of the aberrant expression of miRNA and mRNA sequences in patients with TNBC was performed in the current study. The aim was to identify differentially expressed miRNA and

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mRNA sequences in TNBC tissues compared with paired normal adjacent tissues using published data from the Gene Expression Omnibus (GEO). In addition, bioinformatics analysis was performed to investigate the function and signaling pathways of the identified miRNA and target mRNA sequences.

Materials and methods

Microarray data. Two expression microarrays, which included miRNAs and mRNAs, were obtained from the GEO database (www.ncbi.nlm.nih.gov/geo). The accession numbers of these arrays were GSE61723 and GSE61724, which were based on the GPL16686 [HuGene-2_0-st, Affymetrix Human Gene 2.0 ST Array, transcript (gene) version] and GPL6244 [HuGene-1_0-st, Affymetrix Human Gene 1.0 ST Array, transcript (gene) version] platforms, respectively. The GSE61723 test dataset consisted of 33 TNBC tissue samples and 17 normal adjacent tissue samples. The GSE61724 validation dataset consisted of 16 TNBC tissue samples and 4 normal adjacent tissue samples. More detailed information regarding the tumor samples have been reported in a previous study (11).

Data processing and differential expression analysis. Series matrix files were employed and analyzed in the present study. As all microarray data consisted of preprocessed normalized data, a fold-change of ≥2.0 and a p-value of ≤0.05 were used as the thresholds for screening differentially-expressed miRNAs and mRNAs, using R software (version 3.3.2; www.r-project.org) and the edgeR package. Hierarchical clustering analysis was performed using Multiple Experiment Viewer software (version, 4.90; www.mev.tm4.org), and the Pearson correlation distance metric and average linkage method.

Prediction of miRNA target genes. The target genes of differentially expressed miRNAs were predicted using TargetScan software (version 7.0; www.targetscan.org) (12). In order to reduce the probability of identifying incorrect target genes, potential target mRNAs were selected using the intersection set for the differentially expressed mRNAs identified from the GSE61723 dataset, and the predicted target genes were identified using TargetScan software. The intersection set signifies that the genes existed in multiple data sets. The miRNA-mRNA interaction regulatory network was constructed using Cytoscape software (version 3.3.0; www.cytoscape.org) (13).

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Enrichment analyses for GO processes and KEGG signaling pathways were performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID; version 6.8) software program (www.david.ncifcrf.gov) (14,15). Biological processes, cellular components and molecular functions were the main GO terms used for functional analysis of the potential targets of miRNAs (16). GO terms and KEGG pathways were selected using a threshold of P≤0.05 and a count of ≥2.

Table I. miRNAs displaying significantly altered expression levels in triple negative breast cancer samples from GSE61723.

A, Downregulated		
miRNA	Fold change	P-value
hsa-miR-320d-1	-2.78	$9.66x10^{-4}$
hsa-miR-548v	-2.28	$3.80x10^{-3}$
hsa-miR-99a	-2.22	$1.41x10^{-6}$
.hsa-miR-21	-2.07	$6.26x10^{-4}$
B, Upregulated		
hsa-miR-558	2.01	$1.47x10^{-3}$

miRNA/miR, microRNA.

Table II. mRNAs displaying significantly altered expression patterns in triple negative breast cancer samples from GSE61723.

A, Upregulated		
mRNA	Fold change	P-value
SKA3	2.00	$8.80x10^{-6}$
PRDX1	2.00	$2.67x10^{-5}$
DEK	2.01	$2.76x10^{-4}$
SMC4	2.01	$1.56x10^{-5}$
SYNGR2	2.02	$7.77x10^{-4}$
SKIL	2.02	$9.45x10^{-5}$
RARRES1	2.02	$9.01x10^{-3}$
CXCL11	2.03	$6.14x10^{-5}$
GFPT1	2.03	$1.74x10^{-4}$
LRRC15	2.04	$1.22x10^{-4}$
C21orf91	2.05	$4.29x10^{-5}$
SNRPD1[a]	2.06	$1.18x10^{-4}$
DSC3	2.10	$6.24x10^{-3}$
MCUR1	2.11	$1.89x10^{-4}$
SRP9	2.11	$4.66x10^{-4}$
CHML	2.12	$2.98x10^{-5}$
ARNTL2	2.12	$2.27x10^{-4}$
STRAP	2.13	$3.37x10^{-5}$
MELK	2.13	$2.71x10^{-5}$
STIL	2.14	$7.21x10^{-6}$
NUSAP1	2.14	$7.03x10^{-7}$
TYMS	2.15	$4.99x10^{-3}$
GLYATL2	2.15	$9.02x10^{-3}$
HMGA1	2.17	$4.52x10^{-4}$
MKI67	2.18	$9.96x10^{-6}$
UBE2T	2.18	$2.82x10^{-5}$
CALU	2.19	$3.88x10^{-6}$
RAD51AP1	2.20	$7.80x10^{-6}$
RGS1	2.20	$1.13x10^{-4}$
RBM34	2.21	$7.94x10^{-7}$

Table II. Continued.

A, Upregulated

mRNA	Fold change	P-value
GPI	2.23	$9.43x10^{-6}$
ESRP1	2.23	$8.75x10^{-6}$
MYBL1	2.24	$7.28x10^{-4}$
STK38L	2.25	$3.86x10^{-6}$
PRR11	2.30	$1.58x10^{-6}$
AMD1	2.32	$3.92x10^{-4}$
DSC2	2.38	$9.00x10^{-4}$
KIF11	2.40	$1.92x10^{-6}$
NDC80	2.42	$8.30x10^{-7}$
TMSB15A	2.48	$8.05x10^{-4}$
BGN	2.48	$1.21x10^{-5}$
MT1H	2.51	$7.29x10^{-3}$
ECT2	2.52	$4.48x10^{-7}$
CCNA2	2.55	$1.79x10^{-6}$
S100A9	2.76	$9.01x10^{-4}$
LYZ	2.77	$1.03x10^{-3}$
TMEM65	2.79	$7.64x10^{-5}$
CD24	3.24	$1.45x10^{-5}$
HORMAD1	3.28	$4.46x10^{-6}$
TMSB10	3.31	$1.09x10^{-8}$
SULF1	3.38	$7.50x10^{-8}$
SPP1	3.40	$2.30x10^{-6}$
FN1	3.94	$1.26x10^{-6}$
TOP2A	4.13	$6.26x10^{-8}$
CKS2	4.20	$6.45x10^{-8}$
CXCL10	4.48	$1.19x10^{-5}$
CXCL9	5.14	$1.96x10^{-5}$
LOC100507381[a]	2.16	$4.06x10^{-4}$
UBE2C[a]	2.22	$1.53x10^{-7}$

B, Downregulated

PIP	-8.96	$1.08x10^{-6}$
APOD	-7.77	$9.23x10^{-9}$
ANKRD30A	-4.91	$9.08x10^{-12}$
OGN	-4.13	$7.52x10^{-11}$
DCN	-3.29	$8.40x10^{-10}$
PIGR	-3.19	$5.37x10^{-7}$
MUCL1	-3.17	$1.34x10^{-3}$
EGR1	-3.09	$3.01x10^{-8}$
AGR3	-3.01	$8.72x10^{-12}$
IGF1	-2.96	$1.21x10^{-8}$
CPE[a]	-2.74	$6.18x10^{-9}$
LIFR	-2.67	$3.25x10^{-7}$
SPARCL1	-2.65	$9.09x10^{-6}$
CXCL12	-2.60	$8.37x10^{-9}$
LEP	-2.53	$1.77x10^{-6}$
FGF7	-2.53	$1.47x10^{-4}$
CD36	-2.48	$4.12x10^{-7}$
DCLK1	-2.46	$1.27x10^{-8}$

Table II. Continued.

B, Downregulated

mRNA	Fold change	P-value
FGF10	-2.34	$7.78x10^{-11}$
PCDH18	-2.29	$6.78x10^{-6}$
TSHZ2	-2.28	$2.10x10^{-6}$
TAT	-2.27	$6.83x10^{-6}$
ANKRD30B	-2.24	$4.58x10^{-4}$
CHRDL1	-2.21	$3.71x10^{-7}$
FREM1	-2.18	$9.51x10^{-9}$
CCL28	-2.14	$9.17x10^{-5}$
KIT	-2.14	$1.47x10^{-2}$
EFEMP1	-2.13	$2.58x10^{-4}$
AGR2	-2.10	$3.56x10^{-6}$
JAM2	-2.08	$6.94x10^{-8}$
MME	-2.08	$6.00x10^{-5}$
CD34	-2.07	$1.14x10^{-6}$
PI15	-2.07	$1.23x10^{-4}$
AREG	-2.06	$2.80x10^{-6}$
AADACL2	-2.06	$3.20x10^{-6}$
KCTD14	-2.05	$4.39x10^{-3}$
TMEM144	-2.04	$5.22x10^{-7}$
C3orf62	-2.02	$4.69x10^{-6}$

[a]Genes which have been deleted from the NCBI database.

Results

Identification of differentially expressed miRNAs and mRNAs. In order to gain an improved understanding of the regulatory mechanisms underlying miRNA-mRNA interactions in TNBC, comprehensive microarray expression profiles were employed to identify differentially expressed miRNAs and mRNAs. By comparing TNBC tissues with normal adjacent tissues, five miRNAs (upregulated miR-558 and downregulated miR-320d-1, miR-548v, miR-99a and miR-21 expression) and 97 mRNAs were identified with a threshold of $P \leq 0.05$ and a fold-change of ≥2.0 (Tables I and II). According to the GenBank database (www.ncbi.nlm.nih.gov/genbank), LOC100507381 was removed due to inconsistencies with standard genome annotation processing. In addition, the gene UBE2C was permanently removed from the NCBI database, as there is not enough evidence to prove its presence in subsequent studies. Therefore, 95 differentially expressed mRNAs were identified between the 33 TNBC tissue and 17 normal adjacent tissue samples. Unsupervised hierarchical clustering analyses were performed using a fold-change threshold of ≥2.0. The results identified five differentially expressed miRNAs and 95 differentially expressed mRNAs (Figs. 1 and 2).

Validation of miRNA expression. To confirm the five differentially expressed miRNAs identified in TNBC samples from the GSE61723 array, microarray data from the GSE61724 array was obtained from the GEO database, which included

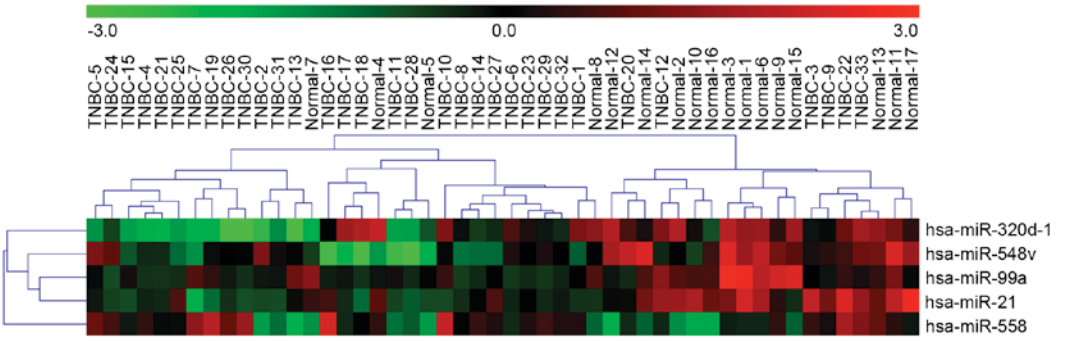

Figure 1. Unsupervised hierarchical clustering analysis of TNBC tissues and normal adjacent tissues from the GSE61723 array identified five differentially expressed miRNAs. TNBC, triple negative breast cancer; miR, microRNA.

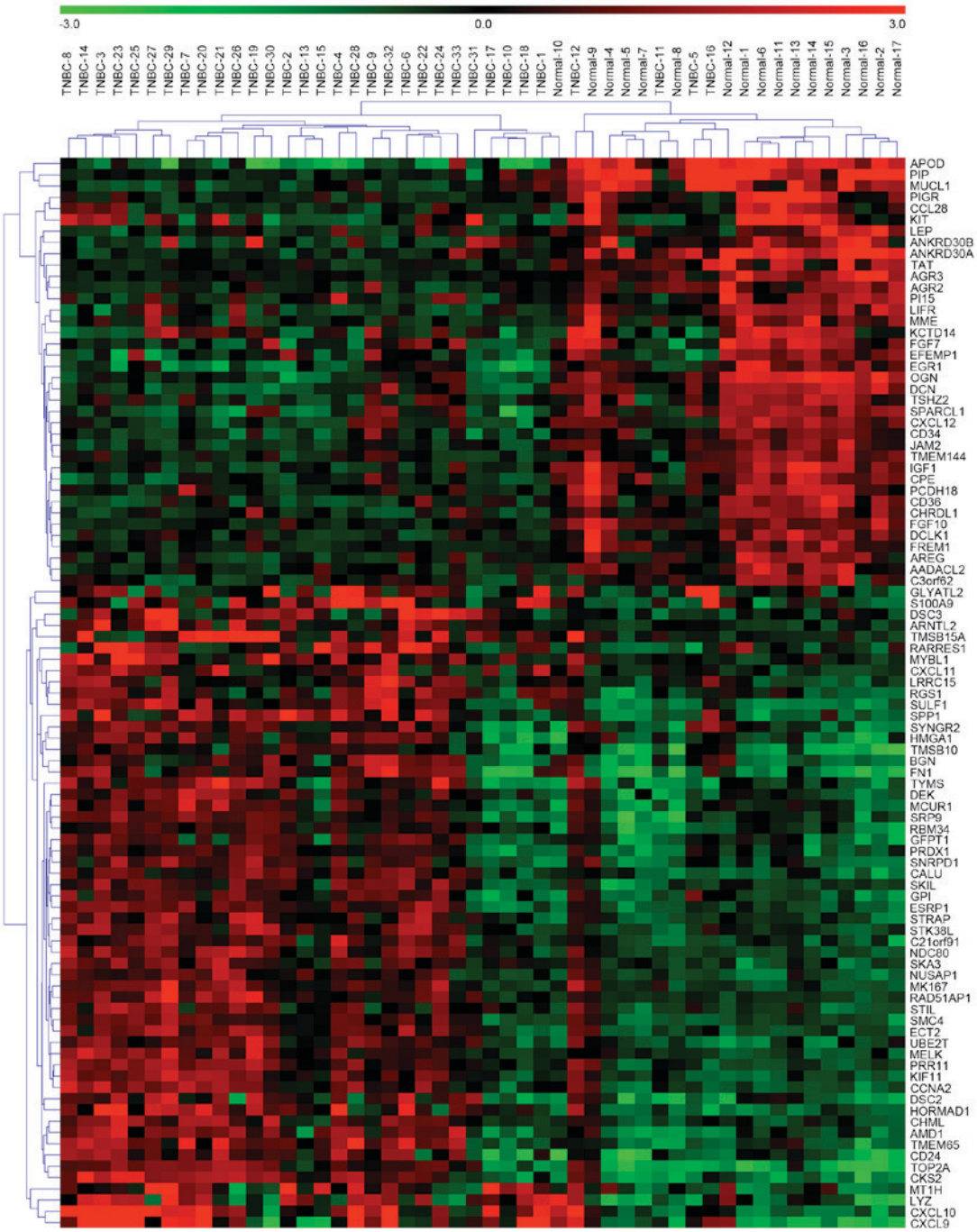

Figure 2. Unsupervised hierarchical clustering analysis of TNBC tissues and normal adjacent tissues from the GSE61723 array identified 95 differentially expressed mRNAs. TNBC, triple negative breast cancer.

Figure 3. miRNA-mRNA interaction regulatory network. The green color indicates downregulation and the red color indicates upregulation of miRNAs. Circular nodes represent mRNA, and the size of each node is proportional to the assumed functional connectivity of each miRNA according to the predicted target gene counts. miRNA, microRNA.

16 TNBC tissues and 4 normal adjacent tissue samples. Two miRNAs (miRNA-99a and miRNA-21) were selected for further validation from the five significantly dysregulated miRNAs. miRNA-99a ($P=1.91x10^{-6}$) and miRNA-21 ($P=1.84x10^{-4}$) were observed to be differentially expressed in TNBC samples when compared with normal adjacent samples in the GSE61723 array. Therefore, the results were consistent with the miRNA expression levels in the GSE61723 dataset, which demonstrated that the five dysregulated miRNAs were able to accurately distinguish between TNBC tissues and normal tissues. Data from GSE61723 and GSE61724 are not identical and certain miRNAs in GSE61723 were unable to be found in GSE61724; therefore, validation was only performed in two miRNAs.

Prediction of miRNA target genes and functional analysis. In order to determine the putative functions and target genes of the five dysregulated miRNAs identified in TNBC samples, the intersection set of sequences from the 95 differentially expressed mRNAs and the predicted target genes were identified using TargetScan software. A total of 49 target mRNAs were included for further analysis. The of miRNA-mRNA interaction regulatory network is shown in Fig. 3. The results indicated that miRNA-320d-1 targeted the smallest number of genes, whereas the remaining four miRNAs targeted at least 10 (Fig. 3). In addition, carboxypeptidase and the small nuclear ribonucleoprotein D1 polypeptide were identified as the hub nodes connected to three miRNAs, which suggests that they may serve a key role in cancer pathogenesis (Fig. 3).

Table III. Significantly enriched GO processes and KEGG pathways with P≤0.05 and a gene count of ≥2.

A, GO processes			
Category	Function	Gene count	P-value
GO:0005576	Extracellular region (CC)	18	$2.14x10^{-5}$
GO:0005615	Extracellular space (CC)	11	$2.20x10^{-5}$
GO:0044421	Extracellular region part (CC)	12	$7.38x10^{-5}$
GO:0005125	Cytokine activity (MF)	6	$2.44x10^{-4}$
GO:0008009	Chemokine activity (MF)	4	$3.22x10^{-4}$
GO:0042379	Chemokine receptor binding (MF)	4	$3.88x10^{-4}$
GO:0008083	Growth factor activity (MF)	5	$1.21x10^{-3}$
GO:0030335	Positive regulation of cell migration (BP)	4	$2.45x10^{-3}$
GO:0040017	Positive regulation of locomotion (BP)	4	$3.22x10^{-3}$
GO:0051272	Positive regulation of cell motion (BP)	4	$3.22x10^{-3}$
GO:0008284	Positive regulation of cell proliferation (BP)	6	$7.93x10^{-3}$
GO:0016477	Cell migration (BP)	5	$9.49x10^{-3}$
GO:0008283	Cell proliferation (BP)	6	$9.80x10^{-3}$
GO:0042330	Taxis (BP)	4	$1.25x10^{-2}$
GO:0006935	Chemotaxis (BP)	4	$1.25x10^{-2}$
GO:0051674	Localization of cell (BP)	5	$1.36x10^{-2}$
GO:0048870	Cell motility (BP)	5	$1.36x10^{-2}$
GO:0006928	Cell motion (BP)	6	$1.38x10^{-2}$
GO:0030334	Regulation of cell migration (BP)	4	$1.44x10^{-2}$
GO:0007155	Cell adhesion (BP)	7	$1.80x10^{-2}$
GO:0022610	Biological adhesion (BP)	7	$1.81x10^{-2}$
GO:0031099	Regeneration (BP)	3	$1.85x10^{-2}$
GO:0030246	Carbohydrate binding (MF)	5	$1.93x10^{-2}$
GO:0040012	Regulation of locomotion (BP)	4	$2.02x10^{-2}$
GO:0051270	Regulation of cell motion (BP)	4	$2.05x10^{-2}$
GO:0009611	Response to wounding (BP)	6	$2.12x10^{-2}$
GO:0000902	Cell morphogenesis (BP)	5	$2.22x10^{-2}$
GO:0035019	Somatic stem cell maintenance (BP)	2	$2.70x10^{-2}$
GO:0032989	Cellular component morphogenesis (BP)	5	$3.15x10^{-2}$
GO:0008354	Germ cell migration (BP)	2	$3.58x10^{-2}$
GO:0007626	Locomotory behavior (BP)	4	$4.98x10^{-2}$
B, KEGG pathways			
KEGG: 04060	Cytokine-cytokine receptor interaction	6	$2.86x10^{-2}$
KEGG: 04062	Chemokine signaling pathway	4	$3.52x10^{-2}$
KEGG: 04640	Hematopoietic cell lineage	3	$4.41x10^{-2}$

GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; CC, cellular components; BP, biological processes; MF, molecular functions.

Analysis of the GO processes and KEGG pathways involving the intersectional 49 target genes was performed using the DAVID tool, with thresholds of ≥2 gene counts and P-values of ≤0.05. The results identified 31 GO terms and three signaling pathways (Table III). The 10 most significant GO terms of the predicted target genes are indicated in Fig. 4. According to the biological function analysis of GO processes, a large proportion of the predicted targets of differentially expressed miRNAs encode cellular components in extracellular regions, and are involved in cell migration, proliferation, motion, adhesion and regeneration (Table III and Fig. 4). These biological functions are considered to be crucial for human tumorigenesis and metastasis (17). In addition, KEGG pathway analysis demonstrated that cytokine-cytokine receptor interactions, chemokine signaling and hematopoietic cell lineage pathways, which are associated with cell growth, proliferation and

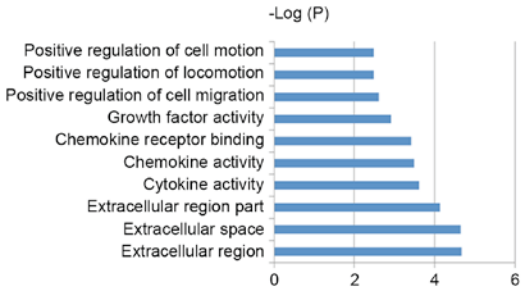

Figure 4. Ten most significant gene ontology terms of predicted microRNA target genes.

transformation, might be regulated by the predicted target genes (Table III).

Discussion

The mortality rates of patients with TNBC are high, as the vast majority of TNBC tumors are infiltrating ductal carcinomas, which often lead to visceral metastases (18). TNBC displays characteristic biological and clinical pathologic features, including rapid progression, poor prognosis and high recurrence rates (19). Surgery and chemotherapy are the major therapeutic strategies used to treat patients with TNBC. However, few studies have investigated the pathogenesis of TNBC. Therefore, there is an urgent need to identify novel therapeutic targets and treatments for patients with TNBC.

Expression microarrays have been widely applied for the diagnosis of various cancers (20,21). An important application of microarray experiments is the analysis of differentially expressed miRNAs or mRNAs between normal and tumor samples. The differentially expressed miRNA or mRNA sequences may be used for further functional, pathway or bioinformatics analyses. Microarray analysis is generally expensive; however, it produces a large quantity of data. Therefore, integrated mining of microarray data from public databases, such as the GEO database, is popular among researchers (22-24).

In the present study, five differentially expressed miRNAs (upregulated miR-558 and downregulated miR-320d-1, miR-548v, miR-99a and miR-21 expression), and 97 mRNAs were identified in TNBC tissue samples when compared with normal adjacent tissues using the GSE61723 array. GSE61724 microarray data was subsequently used to validate the five differentially expressed miRNAs, and the results demonstrated that two of the identified miRNA sequences (miRNA-99a and miRNA-21) were differentially expressed in the GSE61724 dataset. A total of 49 predicted target mRNAs of the 5 miRNAs were selected for further analysis according to the intersection set of the 97 differentially expressed mRNAs and the predicted target genes using TargetScan software. In addition, bioinformatics analyses were employed to investigate the functions and signaling pathways of the miRNA-mRNA network, in order to further investigate the possible mechanisms underlying the development of TNBC. This may provide relevant targets for the early diagnosis and treatment of patients with TNBC.

GO analysis is divided into three sections (molecular function, biological process and cellular component), which are widely used in the organizational and functional annotation of genes (25). Previous studies have primarily focused on applying GO analysis for miRNA or mRNA microarray results (26-28). In the current study, 31 GO terms were identified using the DAVID software tool with threshold values of ≥2 counts and P≤0.05. The results revealed that the predicted target genes encode regulatory factors and proteins located in extracellular regions, and may be involved in a number of biological processes, including regulation of cell migration, cell motion, cell proliferation, and cell adhesion. These processes are closely associated with the occurrence and metastasis of human tumor cells (29).

Signaling pathways are the way of facilitating the effect of extracellular signaling molecules within cells. In the present study, three main signaling pathways were demonstrated to be regulated by 49 target genes of the five differentially expressed miRNAs. These pathways included cytokine-cytokine receptor interaction, chemokine and hematopoietic cell lineage pathways. This suggests that these pathways may serve a significant role in tumor progression, prevention and survival rates (30-32). These identified signaling pathways, may provide promising novel therapeutic targets for the treatment of patients with TNBC.

The present study has a number of limitations. Firstly, due to the lack of microarray data comparing TNBC tumor tissues with normal adjacent control tissues, the results of only two GSE series were employed in the current study. Secondly, due to the small number of differentially expressed miRNAs identified, analysis of the associated functions and signaling pathways may be incomplete. In future studies, a more comprehensive analysis will be performed using a greater number of microarray datasets.

In conclusion, five differentially expressed miRNAs and 49 potential target genes were identified in TNBC samples when compared with normal adjacent control tissues. The results of integrated GO function and KEGG signaling pathway analyses suggested that the differentially expressed miRNAs and their potential target mRNAs may influence the pathogenesis of TNBC. In addition, the results suggest that these sequences may be promising biomarkers for the early diagnosis and targeted therapy of patients with TNBC.

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