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Clinical Value of miR-101-3p and Biological Analysis of its Prospective Targets in Breast Cancer: A Study Based on The Cancer Genome Atlas (TCGA) and Bioinformatics

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: MiR-101-3p can promote apoptosis and inhibit proliferation, invasion, and metastasis in breast cancer (BC) cells. However, its mechanisms in BC are not fully understood. Therefore, a comprehensive analysis of the target genes, pathways, and networks of miR-101-3p in BC is necessary.

Material/Methods: The miR-101 profiles for 781 patients with BC from The Cancer Genome Atlas (TCGA) were analyzed. Gene expression profiling of GSE31397 with miR-101-3p transfected MCF-7 cells and scramble control cells was downloaded from Gene Expression Omnibus (GEO), and the differentially expressed genes (DEGs) were identified. The potential genes targeted by miR-101-3p were also predicted. Gene Ontology (GO) and pathway and network analyses were constructed for the DEGs and predicted genes.

Results: In the TCGA data, a low level of miR-101-2 expression might represent a diagnostic (AUC: 0.63) marker, and the miR-101-1 was a prognostic (HR=1.79) marker. MiR-101-1 was linked to the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), and miR-101-2 was associated with the tumor (T), lymph node (N), and metastasis (M) stages of BC. Moreover, 427 genes were selected from the 921 DEGs in GEO and the 7924 potential target genes from the prediction databases. These genes were related to transcription, metabolism, biosynthesis, and proliferation. The results were also significantly enriched in the VEGF, mTOR, focal adhesion, Wnt, and chemokine signaling pathways.

Conclusions: MiR-101-1 and miR-101-2 may be prospective biomarkers for the prognosis and diagnosis of BC, respectively, and are associated with diverse clinical parameters. The target genes of miR-101-3p regulate the development and progression of BC. These results provide insight into the pathogenic mechanism and potential therapies for BC.

MeSH Keywords: Breast Neoplasms • Gene Expression Profiling • Gene Targeting • Information Systems • MicroRNAs

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Background

Breast cancer (BC), the most frequent carcinoma diagnosed and cause of death among women, affects 1,676,660 women worldwide and causes 521,900 mortalities every year [1]. The latest statistics have revealed that the morbidity and mortality rates associated with BC accounts for 29% and 14% of cancers, respectively, in the United States [2]. Similarly, the incidence of BC in China is also increasing annually [3]. Targeted drugs have been approved to treat HER2-positive BC, but resistance remains an inevitable outcome [4]. BC is a molecularly heterogeneous disease [5], and well-defined and effective molecular targets are still lacking. Therefore, the need to identify novel therapeutic targets for BC is urgent.

MicroRNAs (miRNAs) are highly conserved, small, noncoding, single-stranded RNAs with 19–24 nucleotides [6]. miRNAs transcriptionally or post-transcriptionally regulate gene expression through binding to targeted mRNAs and influence the degradation and translation of mRNA [6]. miRNAs regulate gene expression and the levels of proteins that act as oncogenes or cancer suppressors. Moreover, miRNAs are involved in various biological processes [7], and accumulating evidence suggests that aberrant levels of miRNAs are linked to proliferation, angiogenesis, and metastasis in various human malignancies [8]. With the development and application of molecular biology technology, a vital role for miRNAs in the diagnosis, prognosis, and therapy prediction of BC has been revealed [9–11]. The mature miRNA microRNA-101-3p (miR-101-3p, previously named miR-101) can be generated from miR-101-1 and miR-101-2 (the precursor miRNA, pre-miRNA). MiR-101-1 and miR-101-2 are located on different chromosomes and have different sequences, with diverse functions in the process of transcription [12]. Several studies have revealed that miR-101-3p is down-regulated in BC [13,14]. miR-101-3p inhibits proliferation, invasion, and metastasis via targeting Stathmin1 (STMN1) and CXCR7 [13,15] and promotes apoptosis by targeting JAK2 in BC cells [14]. Nevertheless, the precise mechanism of miR-101-3p in inhibiting neoplasia is still not entirely clear. Therefore, comprehensive analysis of the target gene networks and clinical value may help further clarify the function of miR-101-3p.

In recent years, the development of microarray technology has served as an effective measure to identify differentially expressed genes (DEGs) [16]. DEGs can be found through different experimental treatments, and their biological functions can be speculated via known information. Microarray technology has provided new insight into the alteration of gene expression during tumorigenesis [17]. Biomarkers associated with BC have been identified based on gene expression profiles. Several expression chips have confirmed the aberrant expression of miRNAs in BC and miRNAs influencing tumor behavior and progression [18,19]. A massive amount of complex biological information data has been generated and has greatly deepened our understanding of BC. Comprehensive analysis of gene expression patterns may aid in the prevention, treatment, and determination of prognosis in BC.

In this study, datasets of miR-101 in patients with BC, including 781 tumors and 87 adjacent non-tumor breast tissues from The Cancer Genome Atlas (TCGA), were explored. Furthermore, we analyzed the gene expression profile to identify DEGs between the miR-101-3p transfected group and the negative control group of BC cells. Bioinformatics analysis was carried out to predict targets of miR-101-3p. The target genes acquired from Gene Expression Omnibus (GEO) and prediction software were combined, and their potential roles were further explored with pathway, Gene Ontology (GO), and network analyses. The present study explored the comprehensive roles and prospective molecular mechanisms of miR-101-3p and might facilitate the discovery of potential novel biomarkers for future investigation of the mechanisms involved in BC.

Material and Methods

Patients in TCGA database

MiR-101 (miR-101-1 and miR-101-2) sequence data of BC were obtained from the TCGA dataset on 15 May 2016; the dataset included 781 patients with BC and 87 adjacent noncancerous breast tissues. In BC, the expression levels of $\log_2$-transformed miR-101-1 and miR-101-2 were analyzed, as was their correlation with survival data and clinical parameters.

Retrieval of BC gene expression microarray data

Microarray data for miR-101-3p transfected in BC cells were searched in Gene Expression Omnibus (GEO, *http://www.ncbi.nlm.nih.gov/geo/*). The array data for GSE31397 were retrieved as raw data files based on the GPL570 platform (Affymetrix Human Genome U133 Plus 2.0 Array; Affymetrix, Inc., Santa Clara, CA). The alterations in miR-101-3p transfected MCF-7 cells compared with scramble control transfected cells were included in the expression profiling.

Data preprocessing and DEG analysis

The Affymetrix package [20] in R language preprocessed the original data, and probe-level data were mapped to gene names. The DEGs between the miR-101-3p transfected cells and the control group were identified with the limma package of R language [21]. Down-regulated DEGs were determined with the criteria of fold change >2.5 and P value <0.05.

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Prediction of miRNA-3p target genes

Potential target genes of miR-101-3p were predicted by 13 online databases: DIANAmT, mirTarBase, RNA22, miRanda, PICTAR, miRDB, miRWalk, PolymiRTS, PITA, RNAhybrid, Targetscan, Targetminer, and TarBase. Targets were acquired from the DGEs in GEO and the predicted genes that were identified in more than 5 out of 13 programs. Moreover, the experimental validation of the targets using a luciferase reporter assay, WB, and RT-PCR was collected from TarBase, mirTarBase, and published studies.

GO, KEGG and network analysis

To discern the biological attributes of the putative target genes, GO and KEGG enrichment analyses were completed using DAVID (*https://david.ncifcrf.gov*, version 6.7) [22]. The functional network graph of the selected genes was further visualized with Cytoscape 3.3.0 [23].

Statistical analysis

SPSS 20.0 was used for data analysis; data are presented as the mean ± standard deviation (SD). Statistical significance was assessed with a 2-sample *t* test between the cancer samples and adjacent noncancerous tissues and the correlation between miR-101-3p and clinical features. Survival data were determined with the Kaplan-Meier method. $P<0.05$ represented statistical significance.

Results

Clinical significance of miR-101 (miR-101-1 and miR-101-2) in BC based on TCGA data

The miR-101-2 expression level was lower in BC than in the normal cells, but this difference was not statistically significant (Figure 1A, 1D). A receiver operating characteristic (ROC) curve was constructed to identify diagnostic value in the cases of BC compared with non-cancer breast tissues. The area under the curve (AUC) of miR-101-1 was 0.56 (95% CI: 0.50–0.62), with a sensitivity and specificity of 51.0% and 62.1%, respectively (Figure 1B). The AUC of miR-101-2 was 0.63 (95% CI: 0.58–0.68), with a sensitivity and specificity of 83.9% and 44.8%, respectively (Figure 1E). In the analysis of miR-101 and clinical parameters, miR-101-1 was prominently associated with the expression of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) in BC (Table 1). MiR-101-2 was significantly associated with tumor (T), lymph node (N), and metastasis (M) stages of BC (Table 2). Moreover, decreased miR-101-1 expression revealed a poor prognosis in BC (HR=1.79; 95% CI: 1.06–3.01; $P=0.028$) (Figure 1C). However, no significant prognostic value was identified for miR-101-2 in BC (HR=1.423, 95% CI: 0.84–2.41; $P=0.183$) (Figure 1F).

Potential targets of miR-101-3p in BC

As shown in Figure 2, GSE31397 array data were acquired, and 921 genes were selected as differentially expressed in miR-101-3p transfected MCF-7 cells compared with scramble control cells. In addition, the online prediction process was conducted with 13 databases to obtain potential targets of miR-101-3p, and 7924 genes were identified after duplicated genes were excluded. The genes predicted by 5 out of 13 programs were selected, and then 377 target genes were searched from the array data and predicted targets. Furthermore, 69 available genes that were identified as targets of miR-101-3p through TarBase, miTarBase and published literature were included (Table 3). Eventually, a total of 427 genes were identified for the gene-annotation enrichment analysis and KEGG pathway analysis.

Gene-annotation enrichment analysis and KEGG pathway analysis

Selecting *Homo sapiens* as the background of listed target genes in DAVID, we obtained the GO term annotations and KEGG pathway analysis through the functional annotation summaries. An EASE Score or modified Fisher's exact test was utilized for calculating the *p* value. The results of the GO analysis are summarized in Table 4 and Figure 3, and the top 10 enriched items are listed according to *p* values. The biological processes (BP) of the potential targets of miR-101-3p markedly focused on the gene expression, transcription, metabolism, biosynthesis, and proliferation processes ($p<0.001$). As for the cellular component (CC), the target genes were significantly located in insoluble, cell, and membrane fractions ($p<0.001$). Moreover, molecular function (MF) category was enriched in transcription factors and proteins involved in nucleotide binding ($p<0.001$). In terms of the KEGG pathway analysis, the results were notably enriched in pathways involved in cancer, renal cell carcinoma, and colorectal cancer. Moreover, results were highly enriched in the VEGF, mTOR, focal adhesion, Wnt, chemokine, ErbB, and p53 signaling pathways ($p<0.05$, Table 5 and Figure 4). The enrichment graphs for the potential target genes were generated with Cytoscape software. As shown in Figure 5, the clearly associated functional modules were metabolism, development, cellular, and biological processes in BP. Analysis of CC revealed the most correlated functions were membrane and intracellular fractions (Figure 6). In the MF network, protein and transcription factor binding were the top results (Figure 7).

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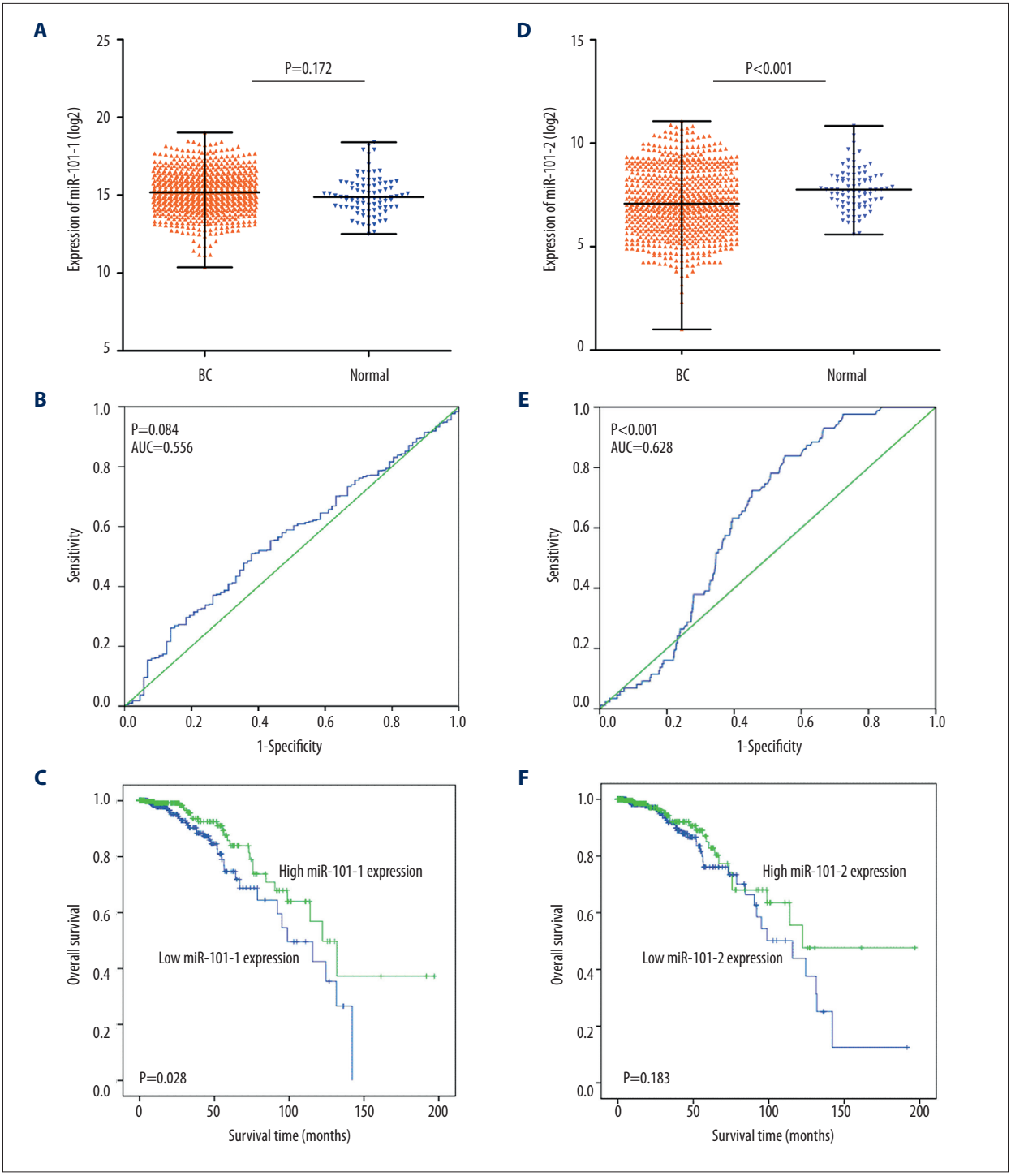

Figure 1. The clinical significance of miR-101 in BC in TCGA data. (**A**), miR-101-1 expression in BC compared with the normal group; (**B**), ROC curve analysis of miR-101-1 for discriminating BC from normal breast tissues; (**C**), Kaplan-Meier survival curves showed that lower miR-101-1 expression was associated with worse prognosis of patients with BC; (**D**), miR-101-2 expression in BC compared with the normal group; (**E**), ROC curve analysis of miR-101-2 for discriminating BC from normal breast tissues; (**F**), Kaplan-Meier survival curves revealed the connection between miR-101-2 and the prognosis of patients with BC.

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Table 1. Correlation of miR-101-1 expression with clinical parameters in BC of TCGA data.

Clinicopathological features		Cases	MiR-101-1 expression		*P* value
			Low	High	
Age	≥50	563	291	272	0.254
	<50	218	99	119	
Gender	Female	772	382	390	0.241
	Male	9	8	1	
T	T1–T2	657	338	319	0.086
	T3–T4	123	52	71	
N	N0	373	183	190	0.546
	N1–N3	398	205	193	
M	M0	610	325	285	0.298
	M1	9	2	7	
Stage	I–II	587	290	297	0.782
	III–IV	186	94	92	
ER	Positive	565	259	306	<0.001
	Negative	170	111	59	
PR	Positive	502	221	281	<0.001
	Negative	231	148	83	
HER2	Positive	99	57	42	0.037
	Negative	397	202	195	

T – tumor stage; N – lymph node stage; M – metastasis stage; ER – estrogen receptor; PR – progesterone receptor; HER2 – human epidermal growth factor receptor 2. The median was chosen as the cut-off value because of the skewed distribution of the expression data.

Table 2. Correlation between miR-101-2 expression and clinical parameters in BC from the TCGA data.

Clinicopathological features		Cases	MiR-101-2 expression		*P* value
			Low	High	
Age	≥50	563	287	276	0.150
	<50	218	104	114	
Gender	Female	772	385	387	0.485
	Male	9	6	3	
T	T1–T2	657	343	314	0.008
	T3–T4	123	48	75	
N	N0	373	200	173	0.029
	N1–N3	398	187	211	
M	M0	610	343	267	0.017
	M1	9	2	7	
Stage	I–II	587	298	289	0.129
	III–IV	186	88	98	
ER	Positive	565	275	290	0.058
	Negative	170	88	82	
PR	Positive	502	245	257	0.250
	Negative	231	118	113	
HER2	Positive	99	54	55	0.481
	Negative	397	207	190	

T – tumor stage; N – lymph node stage; M – metastasis stage; ER – estrogen receptor; PR – progesterone receptor; HER2 – human epidermal growth factor receptor 2. The median was chosen as the cut-off value because of the skewed distribution of the expression data.

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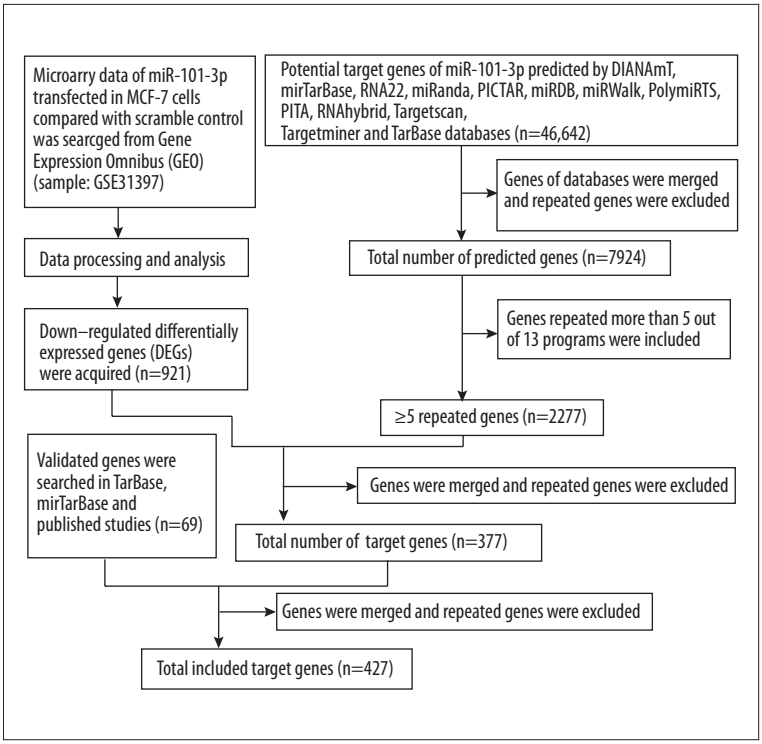

Figure 2. Flow chart showing target genes selection

Table 3. Validated targets of miR-101-3p in TarBase, miTarBase and published literatures.

	Genes					
All validated	*ABCA1*	*CFTR*	*EZB2*	*MCL1*	*PTGS2*	*SUZ12*
	AMPK	*c-Met*	*EZH2*	*Mcl-1*	*RAB5A*	*TET2*
	AP1	*COX2*	*FBN2*	*MEIS1*	*Rac1*	*TGFBR1*
	APP	*CPEB1*	*FMR1*	*MET*	*RAP1B*	*VEGF*
	ARID1A	*CXCL12*	*FOS*	*MITF*	*RLIP76*	*VEGFA*
	ATG4D	*CXCR7*	*HMGA2*	*MTOR*	*ROCK2*	*VEGF-C*
	ATM	*DNMT3A*	*JAK2*	*MYCN*	*RUNX1*	*VHL*
	ATP5B	*DUSP1*	*KLF6*	*NLK*	*SOCS2*	*ZEB1*
	ATXN1	*EED*	*Lin28B*	*PIK3CB*	*SOX9*	*ZEB2*
	CASP3	*EP4*	*MAGI2*	*Pim1*	*SphK1*	
	CDH5	*EYA1*	*MAPK1*	*PRDM16*	*STMN1*	
	CDK8	*EZB1*	*MARCH7*	*PTGER4*	*Stmnl*	
Validated in BC	*CXCR7*	*JAK2*	*MAPK*	*STMN1*		
	EYA1	*MAGI2*	*Mcl-1*	*VHL*		

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Table 4. GO functional annotation for the significant targets of miR-101-3p as determined by DAVID.

GO ID	GO term	Count (%)	*P* value	Gene symbol
Biological process				
GO: 0010629	Negative regulation of gene expression	31 (0.4)	6.89×10^{-6}	*GCLC, THRB, CBX4, ZEB2, ZEB1, SOX9, PRDM16, LIN28B etc.*
GO: 0006357	Regulation of transcription from RNA polymerase ii promoter	39 (0.5)	8.52×10^{-6}	*THRB, MITF, CBX4, ZEB1, PRDM16, SOX9, MED20, FOS etc.*
GO: 0010604	Positive regulation of macromolecule metabolic process	43 (0.6)	1.36×10^{-5}	*GCLC, THRB, MITF, ZEB1, PRDM16, SOX9, FOS, APP, MEIS2 etc.*
GO: 0009891	Positive regulation of biosynthetic process	37 (0.5)	1.83×10^{-5}	*THRB, MITF, ZEB1, ABCA1, PRDM16, SOX9, FOS, APP etc.*
GO: 0006325	Chromatin organization	25 (0.4)	2.10×10^{-5}	*EZH2, NAP1L1, CBX4, CDYL, H2AFV, SMARCD1 etc.*
GO: 0051173	Positive regulation of nitrogen compound metabolic process	35 (0.5)	2.15×10^{-5}	*THRB, MITF, ZEB1, ABCA1, PRDM16, SOX9, FOS, APP etc.*
GO: 0031328	Positive regulation of cellular biosynthetic process	36 (0.5)	3.20×10^{-5}	*THRB, MITF, ZEB1, ABCA1, PRDM16, SOX9, FOS, APP etc.*
GO: 0008284	Positive regulation of cell proliferation	26 (0.4)	3.24×10^{-5}	*NRP1, PTGS2, MARCKSL1, NAP1L1, CD47, AGGF1, TGFA etc.*
GO: 0016568	Chromatin modification	20 (0.3)	4.74×10^{-5}	*DNMT3A, AEBP2, UBE2A, EZH2, CBX4, ARID1A, RBBP7, UBE2B etc.*
GO: 0042127	Regulation of cell proliferation	39 (0.6)	4.94×10^{-5}	*NRP1, PTGS2, MARCKSL1, MITF, NAP1L1, ZEB1, SOX9, CDH5 etc.*
Cellular component				
GO: 0005626	Insoluble fraction	43 (0.6)	3.83×10^{-7}	*GALNT3, GNAI3, PTGS2, PLXNA1, HMGCR, SGPP1, ADCY6 etc.*
GO: 0000267	Cell fraction	49 (0.7)	1.78×10^{-6}	*RAB3GAP2, PLXNA1, PTGS2, HMGCR, SGPP1, ADCY6, SLC26A2 etc.*
GO: 0005624	Membrane fraction	40 (0.6)	2.65×10^{-6}	*GALNT3, GNAI3, PTGS2, PLXNA1, HMGCR, SGPP1, ADCY6 etc.*
GO: 0005694	Chromosome	28 (0.4)	3.14×10^{-6}	*HMGB3, CBX4, CDYL, SIN3A, H2AFV, PAFAH1B1, CHD6, TOP2B etc.*
GO: 0044427	Chromosomal part	25 (0.4)	4.29×10^{-6}	*CBX4, CDYL, SIN3A, H2AFV, PAFAH1B1, TOP2B, CHD6 etc.*
GO: 0000785	Chromatin	17 (0.2)	8.35×10^{-6}	*DNMT3A, UBE2A, PDS5B, CBX4, HMGA2, UBE2B, JUNB, MYCN etc.*
GO: 0009898	Internal side of plasma membrane	21 (0.3)	2.14×10^{-5}	*RAP2C, RAB39B, AP1G1, MAOB, PIP5K1C, SNAPIN, CTNNA1 etc.*
GO: 0019898	Extrinsic to membrane	27 (0.4)	3.26×10^{-5}	*PTGS2, CHMP5, ARHGAP17, MTMR2, DMXL2, PVRL1, RAC1 etc.*
GO: 0031974	Membrane-enclosed lumen	65 (0.9)	1.10×10^{-4}	*MRPL42, PTGS2, ZMAT3, ATP5B, EZH2, DNAJC10, ZEB1 etc.*
GO: 0043233	Organelle lumen	64 (0.9)	1.13×10^{-4}	*MRPL42, PTGS2, ZMAT3, ATP5B, EZH2, DNAJC10, ZEB1, WTAP etc.*
Molecular function				
GO: 0008134	Transcription factor binding	33 (0.5)	2.25×10^{-6}	*THRB, CBX4, ZEB1, PRDM16, CBFA2T2, RAB1A, TMF1 etc.*
GO: 0000166	Nucleotide binding	85 (1.2)	7.96×10^{-5}	*CPEB2, HMGCR, ATP5B, UBE2G1, ADCY6, PIP5K1C, HELZ etc.*

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Table 4 continued. GO functional annotation for the significant targets of miR-101-3p as determined by DAVID.

GO ID	GO term	Count (%)	*P* value	Gene symbol
GO: 0046332	SMAD binding	8 (0.1)	1.41×10^{-4}	*FOS, TGFBR1, ZEB2, SMAD2, SMAD1, PRDM16, PURB, PURA*
GO: 0003690	Double-stranded DNA binding	11 (0.2)	1.66×10^{-4}	*KLF6, FOS, ANKRD17, THRB, SMAD2, ZEB1, PURB etc.*
GO: 0030528	Transcription regulator activity	60 (0.9)	4.17×10^{-4}	*THRB, ELF5, EZH2, MITF, CBX4, ZEB2, ZEB1, LASS6, CBFA2T2 etc.*
GO: 0003682	Chromatin binding	13 (0.2)	4.22×10^{-4}	*ATRX, SUZ12, DNMT3A, CDYL, EZH2, MITF, SMARCA5, CBX4 etc.*
GO: 0003702	RNA polymerase II transcription factor activity	17 (0.2)	4.79×10^{-4}	*CEBPA, RXRB, MITF, TEAD3, SMAD1, SOX9, MED20, MEIS1 etc.*
GO: 0032553	Ribonucleotide binding	68 (1.0)	1.02×10^{-3}	*UBE2G1, ATP5B, ADCY6, PIP5K1C, HELZ, RAB1A, ANKRD17 etc.*
GO: 0032555	Purine ribonucleotide binding	68 (1.0)	1.02×10^{-3}	*UBE2G1, ATP5B, ADCY6, PIP5K1C, HELZ, RAB1A, ANKRD17 etc.*
GO: 0043566	Structure-specific DNA binding	12 (0.2)	1.13×10^{-3}	*KLF6, FOS, ANKRD17, THRB, SUB1, SMAD2, ZEB1, PURB etc.*

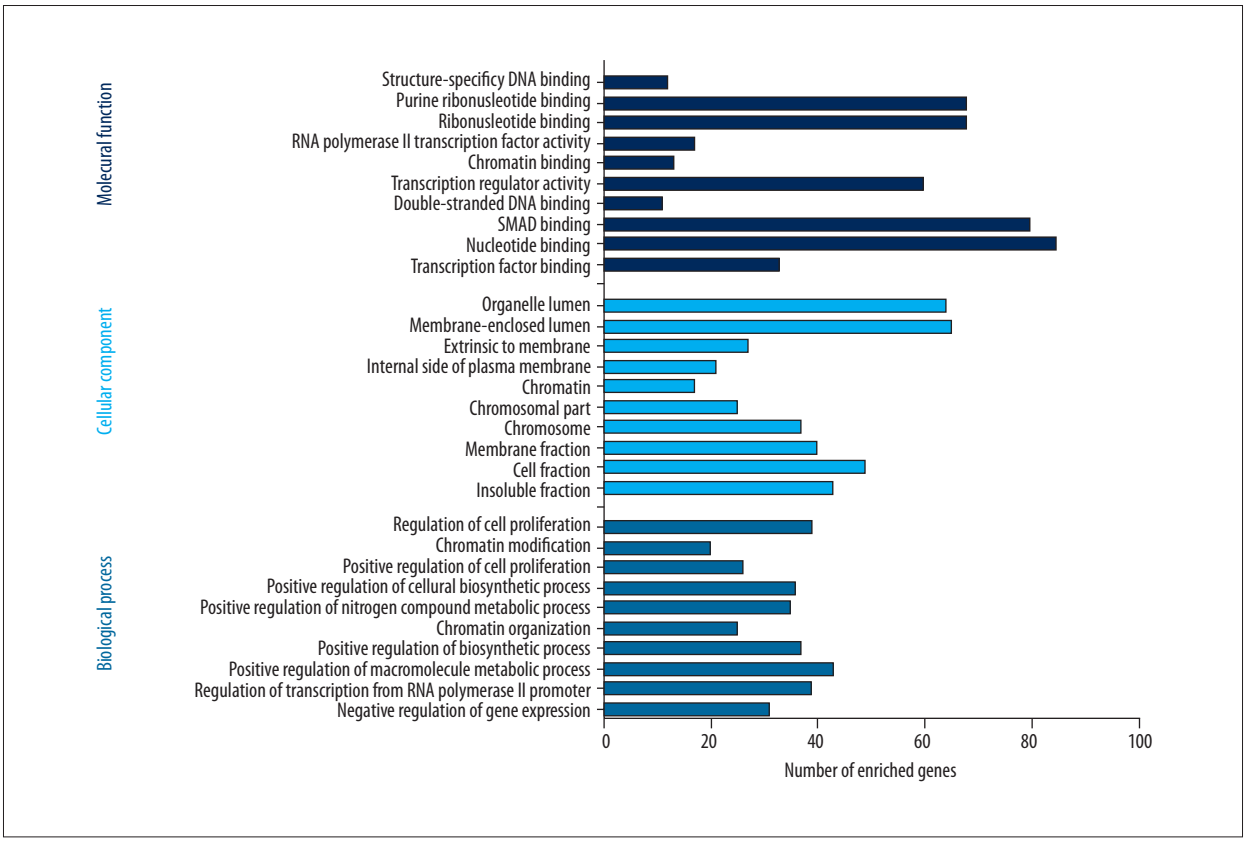

Figure 3. Functional annotation of the top 10 miR-101-3p targeted genes identified with GO using DAVID.

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Table 5. KEGG pathway enrichment analysis of miR-101-3p target genes by DAVID.

KEGG ID	KEGG term	Count (%)	*P* value	Gene symbol
hsa05200	Pathways in cancer	25 (0.4)	1.41×10^{-5}	*PTGS2, MITF, FOS, CASP3, RAC1, TGFA, RUNX1, CEBPA, RXRB, PIK3CB, VHL, TGFBR1, MET, ITGA2, SMAD2, CDK6, CTNNA1, STK4, FZD4, FZD6, NRAS, MAPK1, CDKN1A, VEGFA, MTOR*
hsa05211	Renal cell carcinoma	10 (0.1)	1.44×10^{-4}	*MAPK1, NRAS, VHL, PIK3CB, MET, VEGFA, GAB1, RAC1, TGFA, RAP1B*
hsa05210	Colorectal cancer	10 (0.1)	5.79×10^{-4}	*MAPK1, FOS, CASP3, PIK3CB, TGFBR1, MET, RAC1, SMAD2, FZD4, FZD6*
hsa04520	Adherens junction	9 (0.1)	1.44×10^{-3}	*PTPRJ, MAPK1, PVRL1, NLK, TGFBR1, MET, RAC1, SMAD2, CTNNA1*
hsa05223	Non-small cell lung cancer	7 (0.1)	4.05×10^{-3}	*MAPK1, NRAS, PIK3CB, RXRB, TGFA, CDK6, STK4*
hsa05212	Pancreatic cancer	8 (0.1)	4.15×10^{-3}	*MAPK1, PIK3CB, TGFBR1, VEGFA, RAC1, TGFA, CDK6, SMAD2*
hsa05221	Acute myeloid leukemia	7 (0.1)	5.79×10^{-3}	*CEBPA, MAPK1, NRAS, PIK3CB, PIM1, MTOR, RUNX1*
hsa05221	Glioma	7 (0.1)	8.65×10^{-3}	*MAPK1, NRAS, CDKN1A, PIK3CB, TGFA, CDK6, MTOR*
hsa04310	Wnt signaling pathway	11 (0.16)	1.06×10^{-2}	*PSEN1, CCND3, VANGL1, ROCK2, NLK, RAC1, SMAD2, PLCB1, FBXW11, FZD4, FZD6*
hsa05218	Melanoma	7 (0.1)	1.52×10^{-2}	*MAPK1, NRAS, CDKN1A, PIK3CB, MET, MITF, CDK6*
hsa04150	mTOR signaling pathway	6 (0.1)	1.56×10^{-2}	*MAPK1, PIK3CB, VEGFA, PRKAA1, MTOR, DDIT4*
hsa04120	Ubiquitin mediated proteolysis	10 (0.1)	1.57×10^{-2}	*UBE2D3, FBXW7, UBE2A, VHL, UBE2G1, UBE2F, CUL4B, UBE2D1, FBXW11, UBE2B*
hsa04062	Chemokine signaling pathway	12 (0.2)	1.71×10^{-2}	*MAPK1, NRAS, GNAI3, ROCK2, GNB1, PIK3CB, ADCY6, RAC1, RAP1B, JAK2, PLCB1, CXCL12*
hsa04670	Leukocyte transendothelial migration	9 (0.1)	1.88×10^{-2}	*F11R, GNAI3, ROCK2, PIK3CB, RAC1, RAP1B, CTNNA1, CXCL12, CDH5*
hsa04370	VEGF signaling pathway	7 (0.1)	1.94×10^{-2}	*MAPK1, NRAS, PTGS2, PIK3CB, VEGFA, SPHK1, RAC1*
hsa05220	Chronic myeloid leukemia	7 (0.1)	1.94×10^{-2}	*MAPK1, NRAS, CDKN1A, PIK3CB, TGFBR1, CDK6, RUNX1*
hsa04916	Melanogenesis	8 (0.1)	2.22×10^{-2}	*MAPK1, NRAS, GNAI3, MITF, ADCY6, PLCB1, FZD4, FZD6*
hsa04510	Focal adhesion	12 (0.2)	2.76×10^{-2}	*MAPK1, CCND3, ROCK2, PIK3CB, MET, VEGFA, RAC1, ITGA2, PIP5K1C, RAP1B, CAPN2, PARVA*
hsa04360	Axon guidance	9 (0.1)	3.02×10^{-2}	*MAPK1, NRAS, NRP1, GNAI3, PLXNA1, ROCK2, MET, RAC1, CXCL12*
hsa04012	ErbB signaling pathway	7 (0.1)	3.70×10^{-2}	*MAPK1, NRAS, CDKN1A, PIK3CB, GAB1, TGFA, MTOR*
hsa04115	p53 signaling pathway	6 (0.1)	4.36×10^{-2}	*CDKN1A, CASP3, CCND3, ZMAT3, CDK6, ATM*

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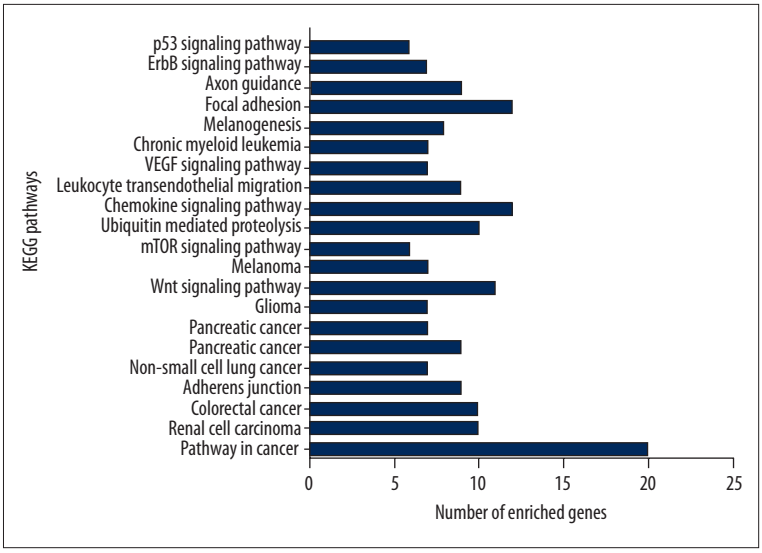

Figure 4. KEGG pathways enriched in miR-101-3p targeted genes as evaluated by DAVID.

Discussion

miRNAs play vital roles in BC, and an increasing amount of research has made important contributions to this field. MiRNAs, including miR-21/9/10b/27a/155, were overexpressed in BC, while miR-31/34a/125/205 were down-regulated during BC progression [24, 25]. Zhao et al. [26] demonstrated that miR-221 may be a predictive biomarker for BC. In the research of Eissa et al. [27], patients with BC positive for miR-10b had shorter relapse-free survival rates, and miR-10b was an independent prognostic factor of BC. MiR-451 influenced the drug resistances and miR-129-5p regulated radiosensitivity via accelerating the apoptosis of breast cancer cells [28,29]. Peptide nucleic acid (PNA) has been researched as a novel drug in miRNA therapeutics, but has still not been successfully used to treat BC [30,31]. Recently, decreased miR-101-3p levels were observed in tumors, including colon, gastric, lung, ovary, and prostate cancers [32]. miR-101-3p has important roles in the tumorigenesis of BC; however, the mechanisms and target genes of miR-101-3p are still unknown.

TCGA data demonstrated that miR-101-2 expression was lower in BC tissues than in normal tissues, while no statistical difference was shown for miR-101-1 expression. MiR-101-1 was also closely linked to ER, PR, and HER2, while miR-101-2 was associated with the T, N, and M stages of BC. This result revealed that miR-101-2 may have diagnostic value in BC to some extent. Down-regulated miR-101-1 was associated with poor prognosis in patients with BC, while no statistical significance was found for miR-101-2. miR-101 has 2 genomic *loci*, with miR-101-1 being located on chromosome 1p31.3 and miR-101-2 located on chromosome 9p24.1 [33]. This result revealed that the miR-101 transcripts on different chromosomes play diverse roles in the diagnosis, prognosis, and clinical outcome of BC. MiR-101-1 is processed into miR-101-3p and miR-101-5p, while miR-101-2 only produces mature miR-101-3p. In addition, the different sequences of miR-101-1 and miR-101-2 will promote or restrict different targets through participation in the translation process and reveal different biological functions [12]. However, it was difficult to extrapolate the mature miRNA levels (which are the final cellular effectors) based on these data. One precursor may be processed to 1 or 2 miRNAs; thus, the mature and precursor miRNA levels might not correlate, and this therefore will influence the clinical interpretation.

Putative miR-101-3p targets were derived from the expression profiling of miR-101-3p transfected in MCF-7 cells compared with scramble control cells, online prediction databases, validated targets, and published studies. Among the 427 putative target genes, the most predominant functions were transcription, metabolism, biosynthesis, proliferation, and transcription factor binding. This result indicated that candidate genes have a definitive impact on the pathogenesis of BC. In previous studies, 8 targets of miR-101-3p were validated in BC: AMP-activated protein kinase (AMPK), CX chemokine receptor 7 (CXCR7), eyes absent homolog 1 (EYA1), janus kinase 2 (JAK2), membrane-associated guanylate kinase 2 (MAGI2), myeloid cell leukemia 1 (Mcl-1), Stathmin1 (STMN1), and von Hippel-Lindau tumor suppressor (VHL). AMPK was found to regulate tumor metabolism and be targeted by mir-101-3p and was identified as a promising therapeutic target in triple-negative breast cancer (TNBC) [34]. miR-101-3p inhibited the development and lymph node metastasis of BC via targeting CXCR7 [15]. The latest research by Guan et al. [35] showed that miR-101-3p was down-regulated and inhibited cell proliferation and promoted apoptosis by targeting EYA1 in BC. JAK2 has been verified to participate in suppressing proliferation and promoting apoptosis in BC cells through miR-101-3p [14]. Sachdeva et al. [36] demonstrated that miR-101-3p reduced

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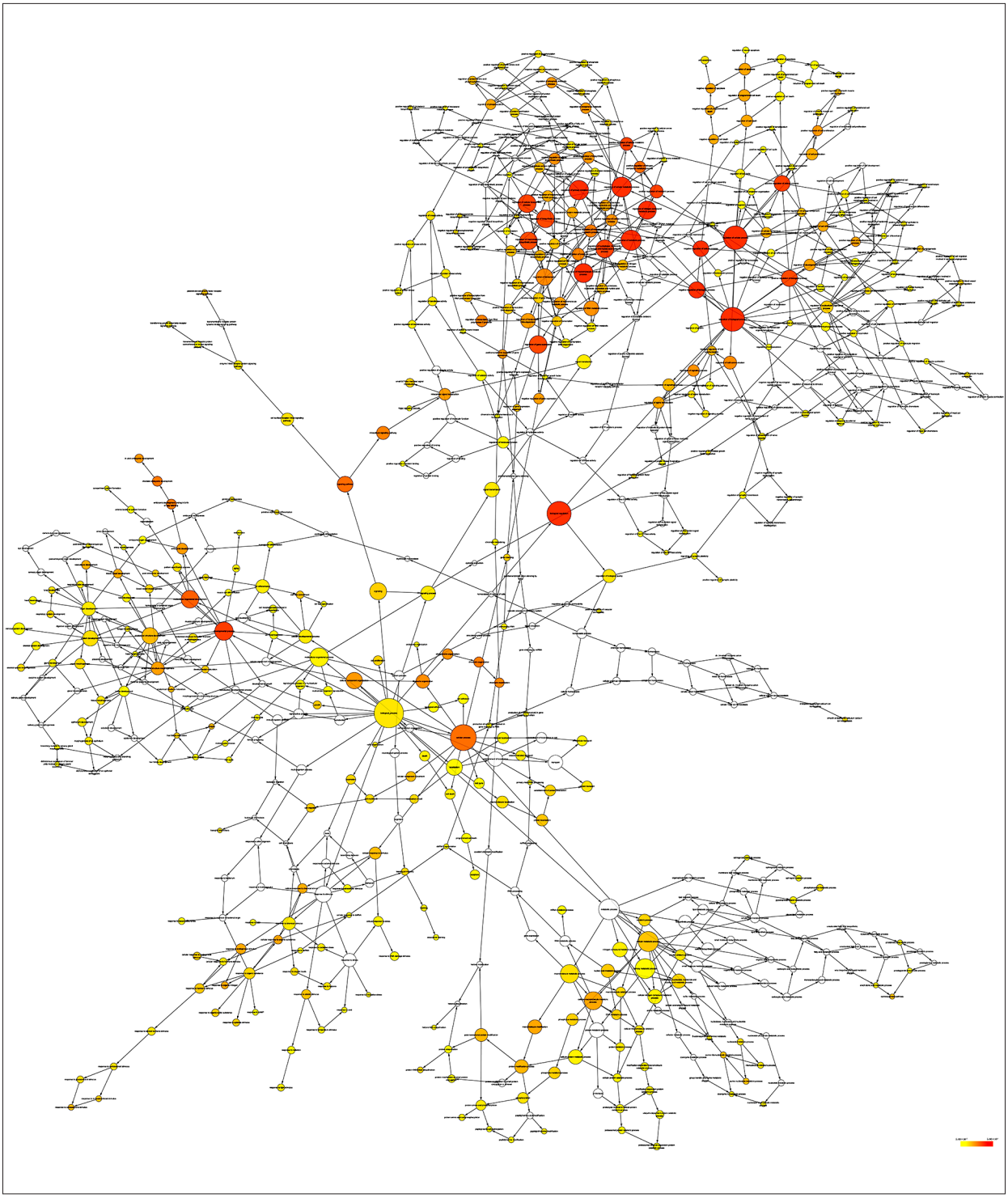

Figure 5. The biological process (BP) network of miR-101-3p targeted genes was constructed using Cytoscape. The color and size of the nodes indicate the significance of the interactions.

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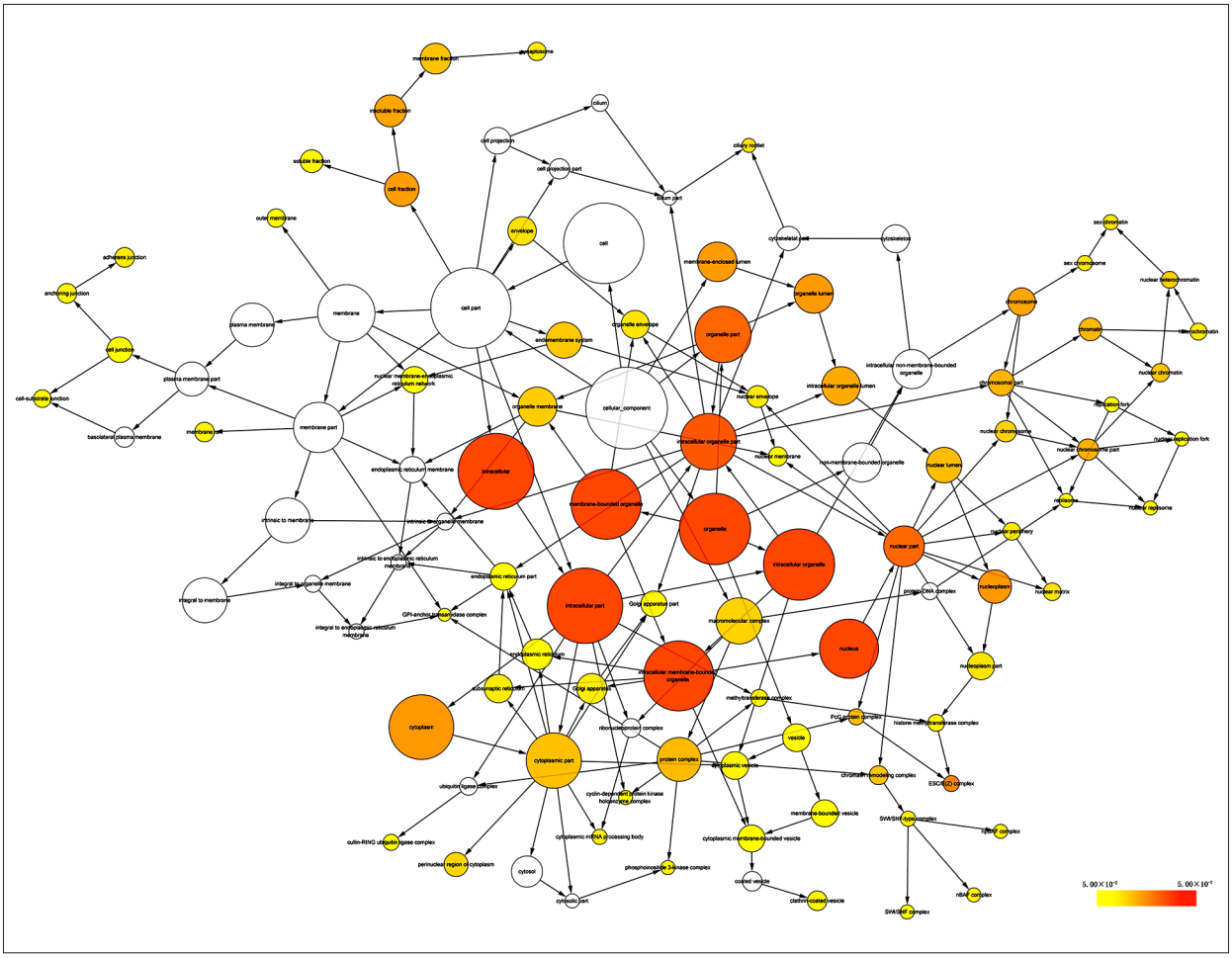

Figure 6. The cellular component (CC) network of miR-101-3p targeted genes was constructed using Cytoscape. The color and size of the nodes indicate the significance of the interactions.

phosphatase and tensin homolog (PTEN) activity by suppressing MAGI-2, leading to Akt activation. MiR-101-3p, which directly inhibited MCL-1, was reported to restrain cell progression and increase sensitivity to paclitaxel in TNBC [37]. In a study by Wang et al. [13], the down-regulation of miR-101-3p was confirmed to regulate STMN1, and was associated with cellular proliferation and invasiveness in different subtypes of BC tissues. Moreover, miR-101-3p enhanced apoptosis and cell cycle arrest by down-regulating VHL expression in normoxic conditions [38].

Several studies have reported the signaling pathways associated with miR-101-3p in BC. miR-101-3p inhibited CXCR7-STAT3 signaling and exerted tumor-suppressive effects in BC cells [15]. Down-regulated miR-101-3p suppressed cell proliferation through the Notch signaling pathway in BC [34]. Furthermore, estrogen deprivation enhanced the miR-101-3p-mediated activation of the Akt signaling pathway [36]. In this paper, a total of 26 bio-pathways were identified, and analysis of miR-101-3p targets revealed statistical significance for 21 pathways. In addition, the visualized network graph of miR-101-3p-mediated targets in Cytoscape showed biological functions similar to the DAVID analysis mentioned above. The highly connected targets are involved in important biological processes and molecular functions.

Among the pathways of the miR-101-3p targeted genes, the VEGF, mTOR, focal adhesion, Wnt, chemokine, ErbB, and p53 signaling pathways were highly enriched. The VEGF signaling pathway enhanced angiogenesis for the aggressive proliferation and malignant progression of BC [39]. The PI3K/Akt/mTOR signaling pathway plays a vital regulatory function in proliferation, apoptosis, metabolism, and migration, and the invasion of BC can be alleviated by miR-199a-5p via targeting the FAK/Src/Akt/mTOR signaling pathway [40]. Focal adhesion kinase (FAK) regulates cell motility, extracellular matrix integrin signaling, cell proliferation, and survival. MiR-7 inhibited cell transformation and the metastasis of BC via regulating FAK, which is correlated with a poor prognosis [41]. The chemokine signaling pathway mediates chemokines and promotes

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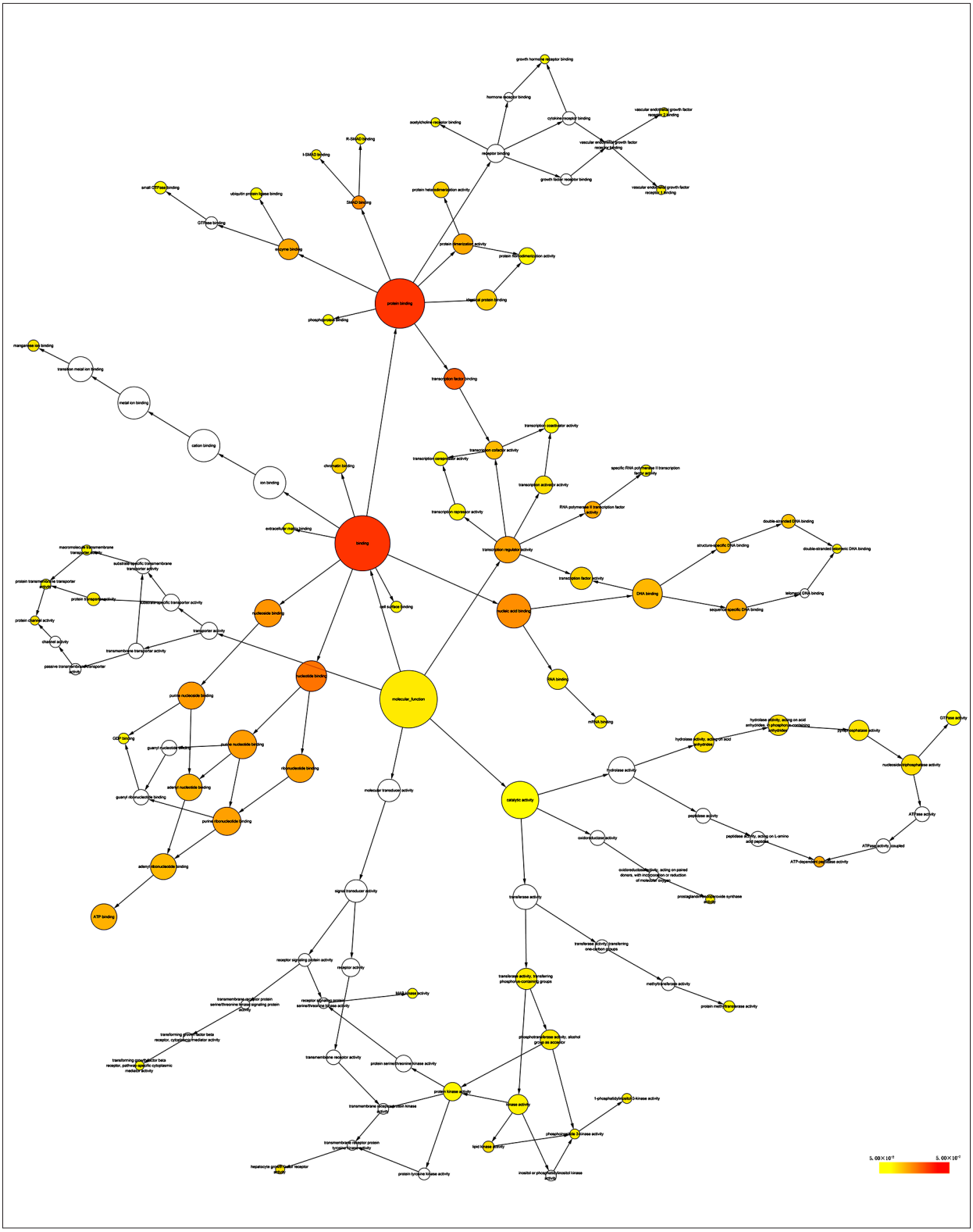

Figure 7. The molecular function (MF) network of miR-101-3p targeted genes was constructed using Cytoscape. The color and size of the nodes indicate the significance of the interactions.

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the chemotaxis, growth, and survival of BC cells [42]. miR-195 suppressed the Wnt signaling pathway, which promotes cell proliferation and the metastasis of TNBC [43]. Han et al. reported that the ErbB signaling pathway can be regulated by STAT1 in the tumorigenesis of BC [44]. Furthermore, scaffold/matrix-associated region-binding protein 1 (SMAR1) may increase radiosensitivity in the MCF-7 BC cell line by regulating the p53 signaling pathway [45]. Enrichment analysis of the potential pathways revealed that the Wnt signaling pathway probably regulates the cell cycle, metabolism, and phosphorylation by targeting CCND3 and ROCK. MAPK1, MTOR, and VEGFA may contribute to metabolism and biosynthesis, which are associated with the mTOR signaling pathway. The VEGF signaling pathway is involved in proliferation and apoptosis via targeting MAPK1, RAC1, NRAS, and VEGFA. The chemokine, focal adhesion, mTOR, VEGF, and ErbB signaling pathways might influence metabolism, phosphorylation, kinase activity, and intracellular signaling cascades by regulating PIK3CB. However, the interaction of target genes and their signaling pathways, as well as their molecular mechanisms, should be further explored.

Conclusions

This study is a comprehensive target analysis of the miR-101 using gene expression profiling, public databases, and prediction software. MiR-101 plays vital roles in BC, while the products derived from different locations on different chromosomes did not show the same functions. The genes targeted by miR-101-3p influence the progression of BC and might influence the biological functions performed by the VEGF, mTOR, focal adhesion, Wnt, and chemokine signaling pathways by targeting crucial genes. The mechanisms of novel molecular markers should be further verified with new techniques, and will contribute to the diagnosis and treatment of BC.

Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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