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MiR-4282 inhibits proliferation, invasion and metastasis of human breast cancer by targeting Myc

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Abstract. – OBJECTIVE: To investigate the expression and role of MicroRNA 4282 (miR-4282) in the development of breast cancer.

MATERIALS AND METHODS: *In situ* hybridization was performed to investigate the expression of miR-4282 in human breast cancer tissues. Cell lines stably over-expressed miR-4282 were established by lentivirus transfection. The effects of miR-4282 on the biological behavior of breast cancer cells were then explored *in vitro*.

RESULTS: The results showed that miR-4282 was down-regulated in human breast cancer tissues and was particularly in invasive and metastatic tumors. In addition, the expression of miR-4282 was related to the occurrence of metastasis and the clinical grade of breast cancer. Recovery of miR-4282 expression in the cell could inhibit the proliferation of breast cancer cells, blocked G1-S phase and promoted breast cancer cell apoptosis, inhibited breast cancer cell migration and invasion. Besides, miR-4282 also significantly enhanced the sensitivity of breast cancer cells to paclitaxel. The results of bioinformatics analysis combined with qRT-PCR and Western blot demonstrated that Myc might be the target gene of miR-4282 in breast cancer.

CONCLUSIONS: miR-4282 inhibited the occurrence and development of breast cancer by regulating Myc, which made it a new target for clinical diagnosis and treatment of breast cancer.

Key Words:
Myc, Breast Cancer, Proliferation, Invasion, Metastasis, MiR-4282.

Introduction

The incidence of breast cancer, one of the most common malignant tumors among women[1], has been increasing throughout the world. About 1.2 million females around the world develop breast cancer each year. The 5-year survival rate for early-stage breast cancer is approximately 90%, and this data is reduced to 15% in metastatic breast cancer[2, 3]. Although many early screening and treatment methods have been developed in recent years, the survival rate of breast cancer has not increased substantially in the past 20 years[1, 2]. Invasion and metastasis of tumor cells are the most common cause of treatment failure[4]. The mechanism of breast cancer metastasis is complex, involving cancer cell invasion, epithelial mesenchymal transition, degradation of extracellular matrix, angiogenesis, and changes in the tumor microenvironment[5-7]. The abnormal activation or inactivation of numerous oncogenes or tumor suppressor genes and tumor-related signaling molecules is regulated in transcriptional, post-transcriptional and translational levels. MicroRNA (miRNA) is a type of endogenous small-molecule non-coding RNA with a length of 19-24 nucleotides, which is involved in the regulation of gene expression[8,9]. MiRNAs functioned by completely or incompletely binding to the 3' untranslated region of mRNAs to directly degrade or inhibit the translation of target mRNA[10,11]. Researches[12] have reported that miR-4282 is downregulated in a few of malignant tumors such as colorectal cancer and gastric cancer, while its expression and underlying molecular mechanism in the development of breast cancer remain unclear. In order to investigate the molecular mechanism of miR-4282 in the development of breast cancer, a cell line steadily over-expressed miR-4282 was constructed by transfecting the lentivirus. The role of miR-4282 in the biological behavior of breast cancer cells and its possible regulation of target genes were studied.

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Materials and Methods

Reagents and Materials

The human breast cancer MCF-7 cell line and 293T cells were cultured in high glucose Dulbecco's modified eagle medium (DMEM, Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA). The lentivirus vector and the lentivirus packaging plasmids were purchased from Addgene (Cambridge, MA, USA). Taxol was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in phosphate-buffered saline (PBS). Myc rabbit monoclonal antibody was purchased from American Abcam (Cambridge, MA, USA). The digoxigenin-labeled hsa-miR-4282 oligonucleotide probe was purchased from Exiqon (Duesseldorf, Germany). Matrigel was purchased from BD (Franklin Lakes, NJ, USA).

Tissue In Situ Hybridization

Dig-labeled hsa-miR-4282 oligonucleotide probe was used to detect the expression level of miR-4282 in human breast cancer tissue microarray using *in situ* hybridization. The tissue microarray of human breast cancer contains breast cancer tissues and paracancerous tissues in 100 breast cancer patients. Among them, 40 cases were older than 60 years and 60 cases were less than 60 years old. In the degree of differentiation, there were 41 well-differentiated, 41 moderately differentiated and 18 poorly differentiated patients. There were 52 cases with lymph node metastasis and 48 cases without. There were 35 cases with distant metastasis and 65 cases without. The sections were deparaffinized, cleared, digested, fixed and acetylated. After that, the tissue samples were pre-hybridized using a pre-hybridization solution for 2 h. Subsquently, 500 ng/mL nucleic acid probe was added for hybridization at 37°C overnight. Hybridization was performed with diaminobenzidine (DAB) (Beyotime, Shanghai, China) and counterstained with hematoxylin. The sections were observed using a Nikon light microscope (Tokyo, Japan) and all results were interpreted by two pathologists using double-blind criteria.

Construction of a Stable Cell Line Expressing Mir-4282 By Lentivirus Transfection

The hsa-miR-4282 primer with restriction enzyme sites was synthesized and then ligated into the lentiviral expression vector pGIPZ. pGIPZ-miR-4282 (pGIPZ empty plasmid in control group), pCD/NL-BH*DDD and pLTR-G3-plasmids were transfected into 293T cells, respectively. Cells were then cultured in 1% FBS Opti-MEM for 48-72 hours. After the virus titers were measured, the MCF-7 cells were infected with lentivirus for 48-72 hours. The cells were then checked under an inverted fluorescence microscope and green fluorescence of MCF-7 cells was observed. Lentivirus-infected MCF-7 cells were digested to form suspensions of single cell, and GFP-positive cells were selected by flow cytometry. The expression of miR-4282 in MCF-7 cells after sorting was detected by quantitative Real-time-polymerase chain reaction (qRT-PCR).

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide) Assay

The cells were digested to make 2 x 10^4 cells/mL cell suspension and seeded into 96-well plates at 100 μL per well. After 20 μL MTT (Sigma-Aldrich, St. Louis, MO, USA) was added 24 h later, the culture was continued for 4 h in a 37°C incubator. The medium was then removed and dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) was added to each well. After shaking for 10 min, the enzyme was labeled. The absorbance of each well at a wavelength of 490 nm was recorded 4 consecutive days.

Transwell Cell Invasion Assay

Matrigel gel and serum-free medium were formulated into Matrigel at a ratio of 1:1. After mixing, 25 μL of Matrigel was added to each transwell chamber and incubated in a 37°C incubator for 2-3 h to solidify. The target cells were digested and diluted to 2.5 x 10^5 cells/mL cell suspension in DMEM containing 5% serum. A total of 200 μL of cell suspension were added to the upper chamber, and 800 μL of medium containing 20% serum were added to the lower chamber. After that, the cells were incubated at a 37°C, 5% CO_2 cell incubator for 48 h. After being fixed by 1% formaldehyde, cells were stained with 0.1% crystal violet. The matrigel and cells on the upper surface of the chamber were wiped off, photographed under an inverted microscope and counted.

Cell Cycle Detection

The cells were digested, pre-chilled with 70% ethanol, fixed at 4°C overnight, and then added with 1 mg/mL PI staining solution after washing with PBS. After incubation at room temperature

in the dark for 30 min, the percentage of cell cycle was measured by BD FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Apoptosis Detection

Cells at the logarithmic growth phase were digested to make a single cell suspension. After centrifugation, the cells were resuspended with 195 μL Annexin V-FITC solution, then added 5 μL Propidium Iodide (PI) solution and incubated at room temperature for 30 minutes in the dark. Apoptosis was detected by BD FACSCanto II flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

RT-PCR

Total RNA of MCF-7+pGIPZ-miR-4282 cells and MCF-7+pGIPZ cells was extracted and reversely transcribed into complementary Deoxyribose Nucleic Acid (cDNA) according to Fermentas reverse transcription kit instructions. GAPDH (glyceraldehyde 3-phosphate dehydrogenase), Myc, CDK4 and PGR primers were designed and synthesized for RT-PCR amplification. The results were calculated according to Ct value of the two groups of cell target gene expression differences. GAPDH was selected as an internal reference. The primer sequences used were as follows: upstream primer for CDK4: 5'-TCAGCACAGTTCGTGAGGTG-3', downstream primer: 5'-GTCCATCAGCCGGACAACAT-3'. Upstream primer for PGR: 5'-ATGTGGCAGATCCCACAGGAGTTT-3', and downstream primer: 5'-ACTGGGTTTGACTTCGTAGCCCTT-3'; Upstream Primer for Myc: 5'-GGCTCCTGGCAAAAGGTCA-3', Downstream Primer: 5'-CTGCGTAGTTGTGCTGATGT-3'

Western Blot

The total protein of MCF-7+pGIPZ-miR-4282 cells and MCF-7+pGIPZ cells was extracted and the loading amount was 50 μg. The proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) after sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis. After blocked with 5% skim milk at room temperature for 1 hour, the membrane was incubated with specific primary antibodies (Myc, 1:1000; GAPDH, 1:1000) at 4°C overnight. The membrane was then incubated with specific secondary antibodies at room temperature for 1 h the next day. After being washed for three times, the chemiluminescence solution was added dropwise to cool Charge-coupled Device (CCD) chemistry (Thermo Fisher Scientific, Waltham, MA, USA). The luminescence imaging system was used for imaging processing.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 16.0 statistical software (SPSS Inc., Chicago, IL, USA) were used for statistical analysis and *t*-test was used to compare the difference of measurement data between two groups. Kaplan-Meier curve was drawn for survival analysis and log-rank was used to verify the difference. $p < 0.05$ was considered statistically significant.

Results

Low Expression of miR-4282 in Human Breast Cancer

The results of *in situ* hybridization showed that the positive expression of miR-4282 in the normal tissue adjacent to the tumor was significantly higher than that in normal breast cancer and metastatic breast cancer ($p < 0.001$, Figure 1A-1C). Further analysis of the correlation between the expression of miR-4282 and the patient's clinical features (the number of cases was 100) showed that there was no significant association between miR-4282 and age, cancer differentiation or tumor size in breast cancer patients (Table I). However, in patients without metastasis, the positive expression rate of miR-4282 was 62.5%, which was dropped to 9.6% ($p = 0.001$, Table I) in breast cancer patients with invasive metastasis. In patients with advanced breast cancer (clinical stage III + IV), miR-4282 expression level also decreased significantly ($p = 0.009$, Table I), suggesting that the lack of expression of miR-4282 was closely related to the invasion of breast cancer cells. The survival of breast cancer patients included in the tissue microarray was further counted. The results showed that the median survival time of patients with low miR-4282 expression was 701 days, while the survival time of patients with high expression of miR-4282 was 1248 days ($p = 0.039$, Figure 1D). In conclusion, the expression of miR-4282 was positively associated with the prognosis of breast cancer patients.

Construction of Stable Cell Lines Over-Expressing miR-4282

Firstly, the pGIPZ-miR-4282 plasmid (control pGIPZ empty plasmid) was packaged in 293T

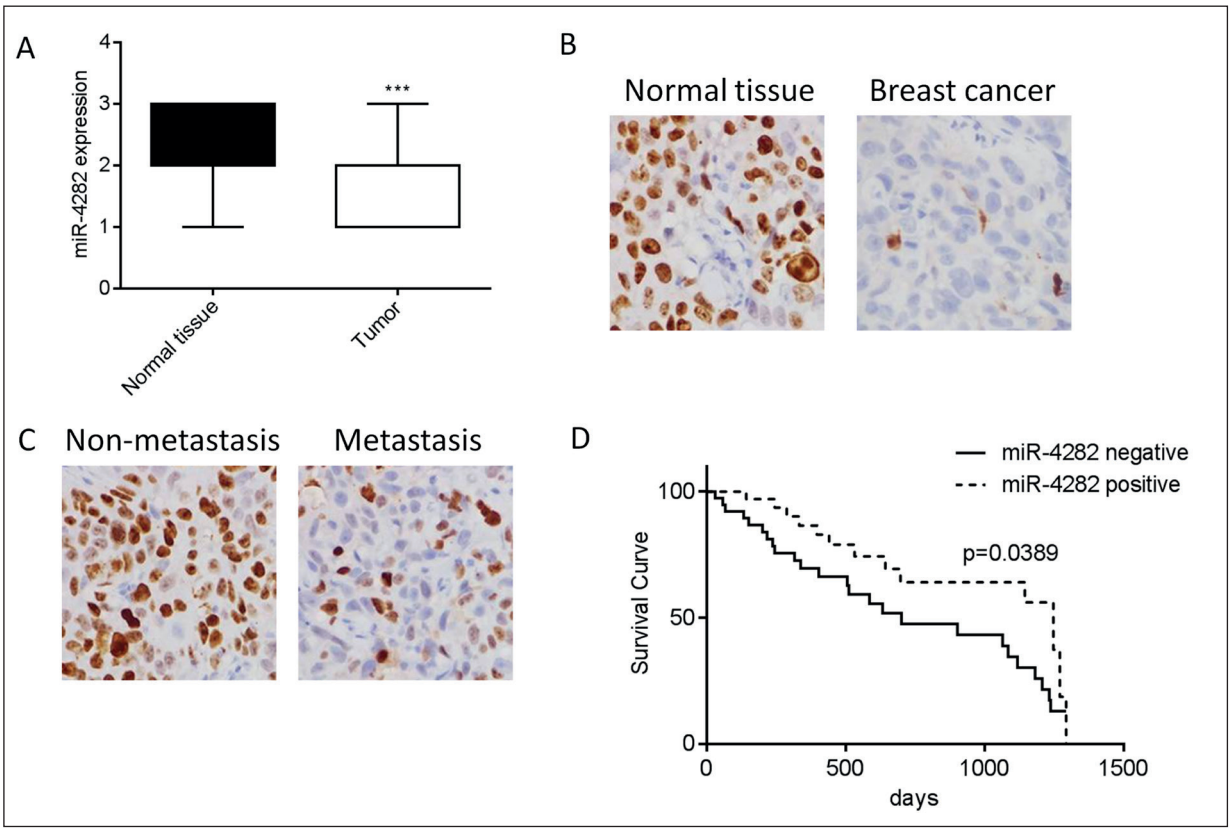

Figure 1. Expression of miR-4282 in human breast cancer tissues and adjacent normal tissues and its association with prognosis in breast cancer patients. ***A,*** Differential expression of miR-4282 in human breast cancer tissues and adjacent normal tissues. ***B,*** Expression of miR-4282 in human breast cancer tissues and adjacent normal tissues (20×). ***C,*** Schematic representation of miR-4282 expression in invasive and metastatic breast cancer and non-invasive metastasis groups (20×). ***D,*** Differences in survival between patients with miR-4282 negative expression and those with miR-4282 positive expression.

Table I. Correlation analysis of miR-4282 expression with clinicopathological features of breast cancer.

	All Cases	miR-4282 positive	miR-4282 negative	*p*-value
Age	100	52	48	0.253
< 60 years	60	34	26	
≥ 60 years	40	18	22	
Tumor differentiation				0.331
High	41	22	19	
Middle	41	20	21	
Low	18	10	8	
Tumor stage				0.009
I II	49	33	18	
III IV	51	19	30	
Tumor metastasis				0.001
Y	35	5	30	
N	65	47	18	
Lymph node metastasis				0.701
Y	52	28	24	
N	48	24	24	
Tumor size				0.356
≤ 5 cm	62	30	32	
> 5 cm	38	22	16	

cells. The virus titer reached 1 x 10^6 TU after 72 hours of culture. The fluorescence expression was good and the cell morphology had no significant change. The viral fluids of pGIPZ-miR-4282 recombinant plasmid and pGIPZ empty plasmid were then transfected into MCF-7 cells. After 72 hours of culture, significant green fluorescence was observed in MCF-7 cells (Figure 2A). The positive rate of GFP in MCF-7+pGIPZ-miR-4282 group and MCF-7+pGIPZ group was 30-40%, 40-50%, respectively. After GFP-positive cells were sorted by flow cytometry, the cells grew well (Figure 2B). The expression of miR-4282 in MCF-7 cells infected with lentivirus was detected by qRT-PCR, and the expression of miR-4282 in MCF-7+pGIPZ-miR-4282 group was found to be 13 times higher than that in MCF-7+pGIPZ group ($p < 0.001$), confirming the successful construction of the miR-4282 over-expressing breast cancer cell line (Figure 3B).

MiR-4282 Inhibits Proliferation of Breast Cancer Cells

The proliferation of MCF-7+pGIPZ-miR-4282 cells and MCF-7+pGIPZ cells was detected by MTT assay. The results showed that with the prolongation of culture time, the proliferation ability of cells overexpressed miR-4282 was significantly lower than that of the blank plasmid group, which suggested that miR-4282 inhibited breast cancer cell proliferation (Figure 2C). Flow cytometry was used to detect the cell cycle of the two groups of cells. We found that overexpression of miR-4282 led to G1 arrest in breast cancer cells (Figure 2D). Apoptosis of MCF-7 cells was detected by Annexin V-FITC staining and the apoptosis rate of blank plasmid group cells was (6.6 ± 2.5)%, which was significantly lower than that of miR-4282 overexpression group (23.9 ± 6.0)% (Figure 2E). The above results confirmed that miR-4282 inhibited the proliferation of breast cancer cells.

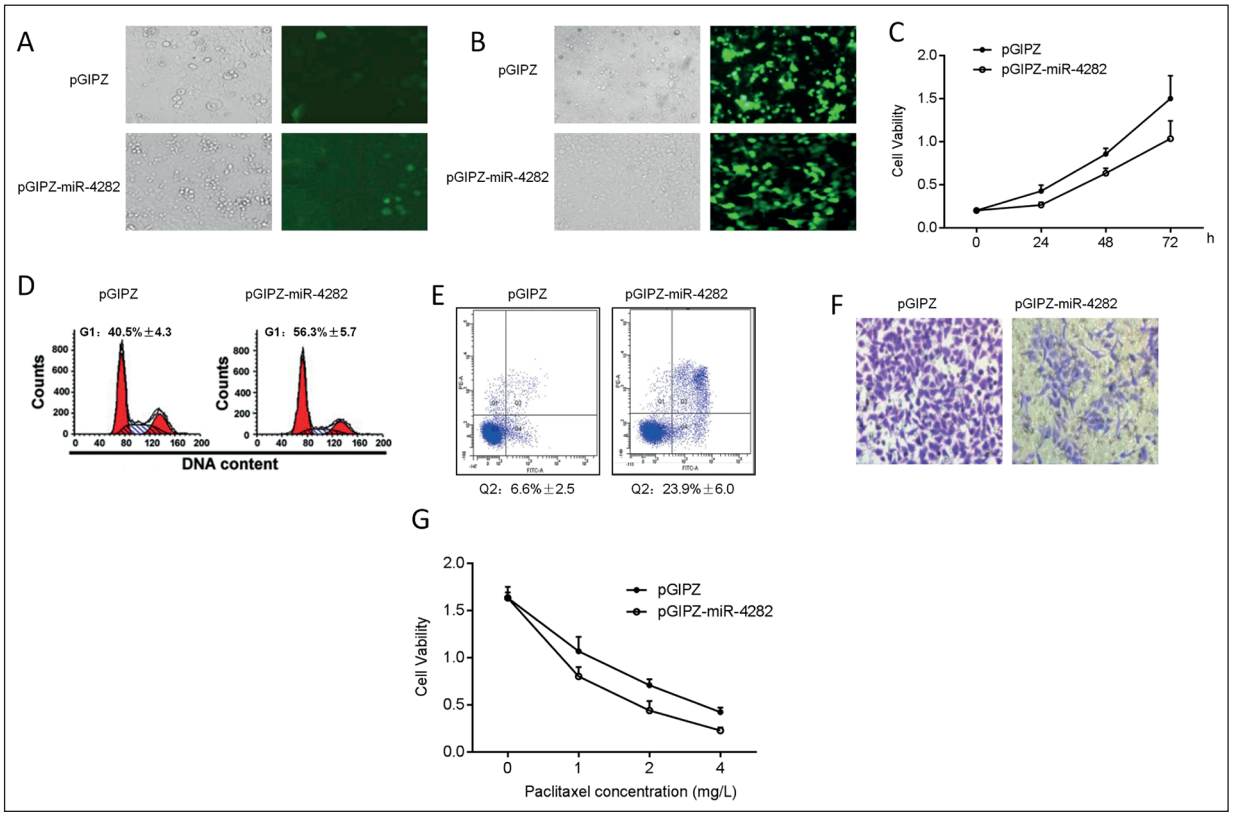

Figure 2. Effect of overexpression of miR-4282 on the biological function of breast cancer cells. ***A,*** Growth of cells (left) and GFP expression (right) after infection of MCF-7 cells with pGIPZ blank plasmid and pGIPZ-miR-4282 virus fluid. ***B,*** Growth (left) and fluorescence expression (right) of MCF-7+pGIPZ-miR-4282 cells and MCF-7+pGIPZ cells after sorting by flow cytometry. ***C,*** The effect of miR-4282 on the viability of MCF-7 cells was examined by MTT proliferation assay. Compared with pGIPZ, $p < 0.05$. ***D,*** Effect of miR-4282 on cell cycle of breast cancer cell line MCF-7. ***E,*** Overexpression of miR-4282 increased apoptosis in MCF-7 breast cancer cells. ***F,*** Matrigel transwell assay was used to examine the effect of miR-4282 on the invasive ability of MCF-7 cells. ***G,*** miR-4282 increased the sensitivity of breast cancer cell MCF-7 to paclitaxel.

MiR-4282 Attenuates Invasion and Metastasis of Breast Cancer Cells

The Matrigel Transwell assay was used to detect the effect of miR-4282 on cell migration and invasion. The results showed that cells in miR-4282 stably overexpressed group and the blank plasmid group migrated to the lower chamber after being cultured for 48 hours. The numbers were 45 ± 12 and 286 ± 32, respectively, and the differences were statistically significant ($p<0.05$, Figure 2F). Above data indicated that miR-4282 inhibited the invasion and metastasis of breast cancer cells.

MiR-4282 Increases Chemosensitivity of Breast Cancer Cells

After the cells were treated with different concentrations of paclitaxe, we found that MCF-7 cells in miR-4282 overexpression group was more sensitive to paclitaxel than cells in the blank plasmid group ($p < 0.05$, Figure 2G). This demonstrated that the recovery of miR-4282 in breast cancer cells increased the sensitivity of cells to chemotherapy drugs.

Prediction and Identification of Target Genes Regulated by miR-4282

Bioinformatics prediction of miR-4282 downstream target genes was performed using the TargetScan database. A total of 15 candidate genes with high scores were predicted in this study (Table II). Among them, the Myc gene was selected for further exploration because of its close relation to the development of tumors. qRT-PCR was used to detect Myc and its downstream genes CDK4 and PGR expression in MCF-7+p-GIPZ-miR-4282 and MCF-7+pGIPZ cells. The results showed that overexpression of miR-4282 downregulated the mRNA expression of Myc, CDK4 and PGR (Figure 3A). Further Western blot analysis was performed to detect the expression of Myc, CDK4 and PGR in cells of both groups. A consistent conclusion with the qRT-PCR results was obtained (Figure 3B). These results demonstrated that Myc might be involved in the invasion and metastasis of breast cancer as a miR-4282-regulated tumor associated molecule.

Discussion

Breast cancer is one of the most common malignancies in clinical practice and its incidence and mortality rate have risen in most countries and regions in the world, seriously threatening human life and health[13,14]. Invasion, metastasis and chemotherapy resistance of tumor cells are the main causes of cancer death[15]. The mechanism of invasion and metastasis of breast cancer cells is complex and involves the abnormal expression of various genes[16]. Abnormal miRNA expression also plays an important role in the development of cancers. Mature miRNAs are reported to regulate gene expression by completely or incompletely paired with the non-coding region (3'-UTR or intron region) of the target mRNAs, thus degrading mRNAs or inhibiting the translation of mRNA. MiRNAs participate in regulating cell differentiation, proliferation, apoptosis, tissue and organ development and oth-

Table II. List of 15 target genes with a higher probability of miR-4282 in the TargetScan database.

Target gene	Gene name
MYC	MYC proto-oncogene
DDX59	DEAD (Asp-Glu-Ala-Asp) box polypeptide 59
NUS1	Nuclear undecaprenyl pyrophosphate synthase 1 homolog
LEKR1	Leucine, glutamate and lysine rich 1
FBXO30	F-box protein 30
ZBTB37	Zinc finger and BTB domain containing 37
PSMG2	Proteasome (prosome, macropain) assembly chaperone 2
IGIP	IgA-inducing protein
C9orf41	Chromosome 9 open reading frame 41
TRPT1	tRNA phosphotransferase 1
C5orf51	Chromosome 5 open reading frame 51
C16orf91	Chromosome 16 open reading frame 91
VWC2	von Willebrand factor C domain containing 2
ACOX2	Acyl-CoA oxidase 2, branched chain
C10orf113	Chromosome 10 open reading frame 113

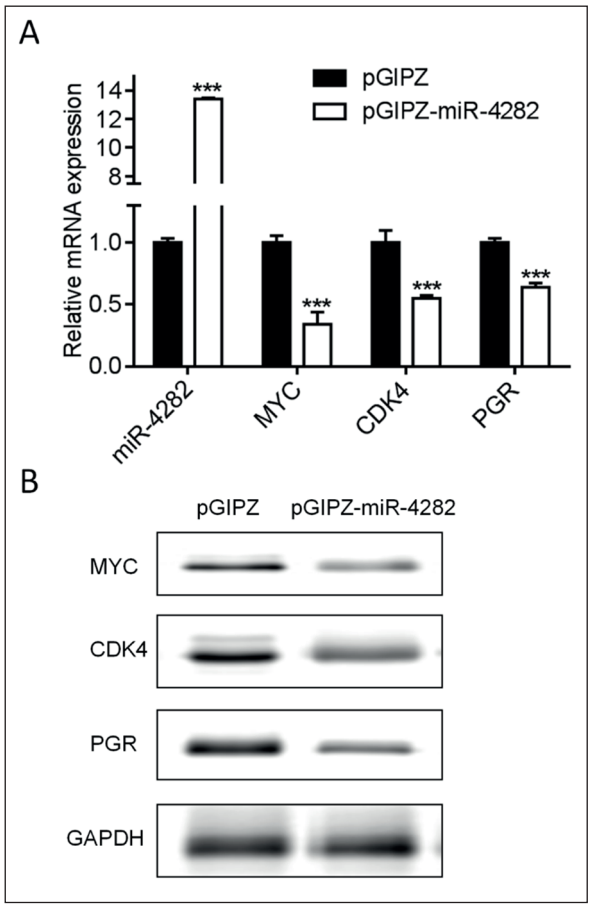

Figure 3. Effect of miR-4282 on target gene expression predicted by bioinformatics. ***A,*** qRT-PCR was performed to detect the expression level of Myc and its downstream gene expression. GAPDH was selected as an internal reference. Compared with the blank control group, ***$p < 0.001$. ***B,*** Western blot further validated that miR-4282 regulated the expression of Myc and its downstream genes.

er pathophysiological processes[17-19] Researches[20] have proved that miRNAs regulate the expression of at least 30% of human genes and exert a greater impact than protein-coding genes in breast cancer. In many human tumors, miRNAs are abnormally expressed due to gene translocation activation or gene locus mutations. Recent studies have found that miR-214 promotes tumorigenesis of nasopharyngeal carcinoma through targeted regulation of lacto transferrin[21,22]. MiR-101 affects the senescence of glioma cells by regulating the expression of CPEB1 gene[23]. These findings all show that miRNAs are closely related to the development of tumors.

MiR-4282 is located on chromosome 6 and functions to inhibit tumor metastasis. Down-regulation of miR-4282 is associated with many tumors, such as colorectal cancer[12]. We found that restoration of miR-4282 expression in breast cancer cells inhibited tumor growth and metastasis. The survival rate of patients with low expression of miR-4282 was significantly lower than that of high expression group. Kang et al[12] reported that the expression of miR-4282 was significantly down-regulated in colorectal cancer compared with matched normal tissues. Overexpression of miR-4282 could significantly induce cell cycle arrest of colorectal cancer cells and inhibit the invasion of colorectal cancer cells. However, the function and possible mechanism underlying miR-4282 in breast cancer remains to be elucidated. We found that miR-4282 was down-regulated in invasive and metastatic breast cancer cell lines. The results of *in situ* hybridization of tissue samples from patients with breast cancer also confirmed that miR-4282 expression was decreased in cancer tissues, especially in invasive and metastatic cancer tissues. The expression level of miR-4282 was also closely related to the prognosis of patients. In order to better understand the possible biological role of miR-4282 in human breast cancer, a stable cell line overexpressed miR-4282 was constructed by lentivirus transfection. The results showed that miR-4282 could significantly arrest cell cycle in G1 arrest, inhibit cell proliferation and promote apoptosis. Meanwhile, miR-4282 also reduced invasion and migration ability of breast cancer cell. The cells overexpressed miR-4282 showed a higher sensitivity to paclitaxel, a first-line chemotherapy drug for metastatic colorectal cancer, than that of cells in the blank plasmid group. The results of all above results showed that the function of miR-4282 as a tumor suppressor gene is consistent with previous research report. In order to further explore the possible mechanism of miR-4282 in breast cancer, we predicted Myc as a direct target gene of miR-4282 by bioinformatics. Myc was reported to up-regulated in breast cancer and promote tumor cell growth through signaling pathways such as CDK4[24,25]. CDK4 and PGR, as an important signal molecule in the downstream of Myc, are related to tumor cell cycle and cell invasion[26]. However, the specific mechanism by which miR422 regulates MYC in tumor development is not clear. Investigations[26-28] have reported that Myc, CDK4, and PGR are highly expressed in lung cancer, ovarian cancer and other tumors, and it has been confirmed that these genes are direct regulatory genes of many miRNAs in small cell lung cancer. Thus, miR-4282 might regulate

Myc expression in breast cancer, thereby exerting the effects of inhibiting tumor proliferation, invasion and metastasis, and enhancing the sensitivity of chemotherapeutic drugs.

Conclusions

We found that the expression of miR-4282 was decreased in breast cancer, and the down-regulation of miR-4282 expression is closely related to the invasion and metastasis of breast cancer. Besides, the expression level of miR-4282 expression exhibited a positive relation to the prognosis of the patients. *In vitro* experiments confirmed that miR-4282 attenuated the tumorigenesis by blocking the cell cycle, promoting apoptosis, inhibiting the migration and invasion ability of breast cancer cells. Mechanistically, miR-4282 might exert its tumor suppressor effect by regulating Myc expression in breast cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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