European Review for Medical and Pharmacological Sciences 2018; 22: 8374-8382

Increased expression of miR-1179 inhibits breast cancer cell metastasis by modulating Notch signaling pathway and correlates with favorable prognosis

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Abstract. – OBJECTIVE: MicroRNAs (miRNAs) play a key role in the regulation of gene expression. In this study, we aimed to identify the clinical values of miR-1179 and to investigate the potential molecular mechanisms in breast cancer (BC).

MATERIALS AND METHODS: RT-PCR was used to detect the expression levels of miR-1179 in both BC tissues and cell lines. We analyzed the association between the miR-1179 levels and clinicopathological factors and patient prognosis. The proliferation ability of miR-1179 on BC cells was assessed by MTT and colony formation assay. The role of miR-1179 in BC cells migration and invasion was measured by transwell assays. Western blot analysis was used to quantify the expression of the molecular biomarkers of the Notch signaling pathway.

RESULTS: Our results showed that miR-1179 expression was frequently downregulated in BC tissues and cell lines. Clinicopathologic analysis revealed that low miR-1179 expression is correlated with lymph node metastasis, advanced clinical stage and shorter overall survival. Multivariable Cox proportional hazards regression analysis suggested that increased miR-1179 expression was an independent prognostic factor of overall survival in BC patients. Gain-of-function assay indicated that the overexpression of miR-1179 significantly suppressed BC cells proliferation, migration and invasion. Mechanistically, miR-1179 up-regulation inhibited the expression of Notch 1, Notch 4 and Hes1, indicating that miR-1179 could suppress the activation of the Notch signaling pathway.

CONCLUSIONS: We showed that miR-1179 was a tumor suppressor that may serve as a novel potential prognostic biomarker or molecular therapeutic target for BC.

Key Words

MiR-1179, Breast cancer, Progression, Prognosis, Notch signaling pathway.

Introduction

Worldwide, breast cancer (BC) is the most common type of cancer and a leading cause of cancer-related death among women[1,2]. It is usually caused by the malignant proliferation of mammary gland cells or acinar cells and has a high frequency of occurrence[3]. The incidence rate of BC has been increasing every year, recently. Despite advances in operative treatment, chemotherapy and molecular targeting treatment, there are still some early cases in which appeared invasion and metastasis, which directly affected the prognosis of BC[4,5]. Although BC has been studied extensively for decades, the mechanisms of tumor progression have remained largely elusive. MiRNAs are a class of non-coding small RNA that comprised of about 18-23 nucleotides, which exist widely in the eukaryotic organisms[6]. They function as a negative regulator of the gene expression by binding to the 3'-untranslated region (3'-UTR) of target mRNA, thereby modulating mRNAs expression[7]. Growing studies[8,9] indicate that miRNAs play important roles in the development, differentiation, cell proliferation and apoptosis. On the other hand, increasing evidence has confirmed that ectopic miRNAs are key regulatory factors in various types of cancers[10-12]. For instance, miR-205 was reported to be highly expressed in human ovarian cancer and its overexpression promotes cell invasion by repressing TCF21[13]. MiR-381, a lowly expressed miRNA in gastric cancer, was found to inhibit the metastasis of gastric cancer cells by targeting TMEM16A expression[14]. In BC, it is also reported that miR-411-5p served as a tumor suppressor because its overexpression inhibited the proliferation and metastasis of BC cell by

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targeting GRB2[15]. These findings highlighted the importance of miRNAs in the treatment of various tumors, including BC. MiRNA-1179, located on chromosome 15q26.1, was a newly identified miRNA, which had been shown to be abnormally expressed in several tumors[16]. In colorectal cancer and esophageal squamous cell carcinoma, the expression of miRNA-1179 was up-regulated, while its down-regulation was found in papillary thyroid tumors, which suggests that miRNA-1179 may play different roles according to the types of tumors[17-19]. However, little is understood about the function of miR-1179 in BC. Based on this background, our present study firstly explored the expression pattern and biological function of miRNA-1179 in BC. Our results, for the first time, showed that miRNA-1179 is an important prognostic factor for BC patients and regulates BC cell proliferation, migration and invasion by modulating the Notch signaling pathway.

Patients and Methods

Patients and Clinical Specimens

Paired BC and adjacent non-tumor tissues were obtained from 161 patients who underwent primary surgical resection of BC at Cancer Hospital of China Medical University, Liaoning Cancer Hospital and Institute. The information of the hormonal status of all patients was shown as follows: triple negative (37), He^+ (96), P^+ (102). All samples were confirmed as BC by postoperative histopathological examination. Each case was given a unique identifier and linked to a database containing clinical-pathological data. Patients did not receive any anticancer treatment, including neo-adjuvant chemotherapy or radiotherapy, prior to the surgery. The histological grade of the cancer was determined according to the World Health Organization grading system. Overall survival was defined as the length of time between surgery and death or the last follow-up examination. For the use of clinical materials for research purposes, patient informed consent and approval from the Institutional Research Ethics Committee were obtained.

Cell Culture and Transfection

Human BC cell lines (MCF-7, T47D, ZR75-1, BT-474 and MDA-MB-453) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Human normal breast cell lines (MCF-10A) were obtained from Professor Zhang (The Fourth Affiliated Hospital of Harbin Medical University). All cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA), within a humidified atmosphere containing 5% CO_2 at 37°C. MiR-1179 mimic and miR-NC synthesized and purified by Hanheng (Pudong, Shanghai, China). For transfection, cells were seeded in 6-well plates and cultured overnight. Then, MCF-7 and T47D were transfected with miR-1179 and miR-NC using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. At 48 h after transfection, cells were harvested for further experiments.

RNA Preparation and Quantitative Real Time-PCR (qRT-PCR) Analysis

Total RNAs were isolated from cells or tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). To quantify human miR-1179 expressions, reverse transcription was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The expression levels of miR-1179 were quantified using qRT-PCR with the TaqMan® Micro-RNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and TaqMan® MicroRNA Assays (Applied Biosystems, Foster City, CA, USA). GAPDH was used as an internal control. The relative expression fold change of mRNAs was calculated by the $2^{-\Delta\Delta CT}$ method. The PCR primers used were as follows: 5'- GGTCTCCTCT-GACTTCAACA-3' (sense) and 5'- GTGAGG-GTCTCTCTCTTCCT-3' (antisense) for GAPDH; and 5'- GCGGAAGCATTCTTTCATT-3' (sense) and 5'- CAAGGGCTCGACTCCTGTTC-3' (antisense) for miR-1179.

Cell Proliferation Assays

The MCF-7 and T47D cells were briefly seeded in 96-well plates at 1×10^4 cells/well and cultured overnight. The plates were harvested for measurement at the indicated time points, and cell proliferation was assessed by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using an assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. Light absorbance of the solution was measured at 490 nm using a microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA).

Colony Formation Assay

MCF-7 and T47D cells were transfected with miR-NC and miR-1179 mimics and the cells were harvested after 24 hours. Cells were plated on 60-mm plates (0.5×10^3 cells/plate) and cultured for 10 days. Then, colonies were stained with 0.005% crystal violet and counted using an automated mammalian cell colony counter GelCount™ (Oxford Optronix, Abingdon, UK).

Cell Migration and Invasion Assays

MCF-7 and T47D cells were transfected with miR-1179 mimics or miR-NC. After 24 h, transfected cells were harvested. Then, the cells in serum-free media were seeded into the upper chamber for migration assays (8 μm pore size, Millipore, Billerica, MA, USA) and invasion assays with Matrigel (Sigma-Aldrich, St. Louis, MO, USA). The lower wells of 12-well plate were added with 700 μl medium supplemented with 10% fetal bovine serum. Twenty-four hours after incubation, the cells inside the upper chamber were removed with cotton swabs, while the cells on the lower membrane surface were fixed and then stained with 0.5% Crystal violet solution. Then, the stained cells were visualized under a microscope (high-power fields), counted in five random fields, and the average number was taken.

Western Blotting Analysis

Cultured cells were lysed using RIPA buffer in the presence of Protease Inhibitor Cocktail. Protein lysates were separated using 8% sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), then electro-transferred onto nitrocellulose filter membranes, which was subsequently blocked with Tris-Buffered Saline with Tween-20 containing 5% milk at room temperature for 3 h. After blocking with 2% bovine serum albumin, the membranes were probed with anti-Notch1 (Abcam, Cambridge, MA, USA), anti-Notch4 (Abcam, Cambridge, MA, USA), anti-Hes1 (Jackson Immunoresearch Laboratories, West Grove, PA, USA), and anti-β (Invitrogen, Carlsbad, CA, USA), followed by incubation with a horseradish peroxidase (HRP) conjugated secondary antibody (goat-anti-mouse IgG [1:2000] and goat-anti-rabbit IgG [1:3000]). The blots were visualized by enhanced chemiluminescence (ECL).

Statistical Analysis

All data analyses were performed using SPSS 19.0 software (SPSS, Armonk, NY, USA). The comparison of continuous data was analyzed using an independent *t*-test between the two groups, whereas categorical data were analyzed by the chi-square test. One-way ANOVA and Tukey post-hoc test were performed to analyze the difference among three or above groups. Survival curves were calculated by the Kaplan-Meier method and compared with log-rank tests. The significance of survival variables was evaluated using a univariate and multivariate Cox proportional hazards regression analysis. p-value <0.05 was considered statistically significant.

Results

MiR-1179 is Downregulated in BC and Is Associated With Advanced Tumor Stage

MiR-1179 expression levels were investigated in 161 paired BC samples and adjacent normal breast tissues using qPCR assays. As shown in Figure 1A, we found that the expression of

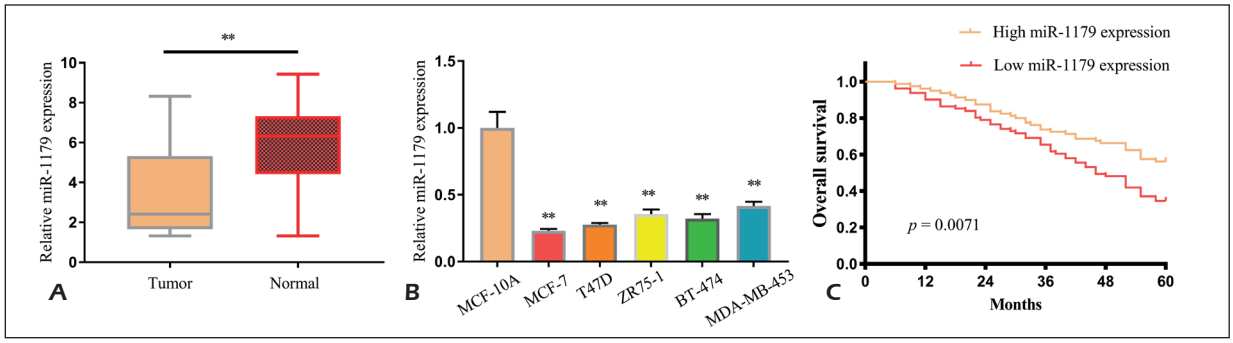

Figure 1. Expression of miR-1179 in BC tissues and cell lines and its clinical significance. **A**, MiR-1179 expression levels in BC tissues compared with corresponding non-tumor tissues were examined by qRT-PCR and normalized against GAPDH expression. **B**, MiR-1179 expression in difference BC cell lines and normal breast cells by qRT-PCR. C, Relationship between miR-1179 expression and survival time in BC by the Kaplan-Meier method and log-rank test. **p<0.01, *p<0.05.

miR-1179 was significantly down-regulated in BC tissues compared to matched normal tissues (p<0.01). We also detected its expression in several BC cell lines and normal breast cell lines, finding that the miR-1179 expression levels were lower in all five BC cell lines than in MCF-10A (p<0.01, Figure 1B). In addition, based the mean expression of miR-1179, all BC samples were classified into low miR-1179 expression group (n = 81) and high miR-1179 expression group (n = 80). The results of chi-square analysis indicated that low miR-1179 expression levels were closely correlated with lymph node metastasis (p=0.010) and advanced clinical stage (p=0.012). However, there was no association between miR-1179 expression and age, tumor size, differentiation grade, histological type, ER status, PR status and HER-2 status (p>0.05, Table I). Our finding revealed that the down-regulation of miR-1179 was involved in the development and progression of BC.

The Association Between MiR-1179 Expression and Overall Survival of Patients With BC

To further explore the prognostic value of miR-1179 in BC patients, we further analyzed the relationship between miR-1179 expression and the survival time of BC patients. As shown in Figure 1C, the result of the survival assay indicated that patients with higher miR-1179 expression have significantly longer and overall survival time compared to those with lower expression (p = 0.071), suggesting that miR-1179 may be used as a potential biomarker. Then, in univariate analysis, advanced lymph node metastasis (RR, 3.241; 95% CI, 1.445-5.763; p = 0.004), clinical stage (RR, 3.556; 95% CI, 1.574-5.236; p = 0.005) and low miR-1179 expression levels (HR, 3.784; 95% CI, 1.689-5.889; p = 0.001) were associated with worse prognosis of patients with BC (Table II). Moreover, the results of multivariate analyses showed that low

Table I. The correlations between miR-1179 expression and clinicopathological factors of BC patients.

Factors		No.Relative miR-1179 expression		p-value
		High	Low	
Age (years)				NS
≤ 50	72	32	40	
> 50	89	48	41	
Tumor size (cm)				NS
≤ 2.0	99	52	47	
> 2.0	62	28	34	
Differentiation grade				NS
G1-G2	93	50	43	
G3	68	30	38	
Histological type				NS
Ductal	72	34	38	
Lobular	89	46	43	
ER status				NS
Negative	49	24	25	
Positive	112	56	56	
PR status				NS
Negative	59	28	31	
Positive	102	52	50	
HER-2 status				NS
Negative	65	35	30	
Positive	96	45	31	
Lymph node metastasis				0.010
Absent	103	59	44	
Present	58	21	37	
Clinical stage				0.012
I + II	97	56	41	
III	64	24	40	

Table II. Univariate and multivariate analysis of overall survival in BC patients.

Variable	Univariate analysis			Multivariate analysis		
	Risk ratio	95% CI	*p*	Risk ratio	95% CI	*p*
Age (years) ≤50 vs. >50	1.342	0.534-1.894	0.342	–	–	–
Tumor size (cm) ≤2.0 vs. >2.0	1.458	0.774-2.351	0.135	–	–	–
Differentiation grade G1-G2 vs. G3	1.567	0.784-2.674	0.117	–	–	–
Histological type Ductal vs. Lobular	1.143	0.457-1.785	0.156	–	–	–
ER status Negative vs. Positive	1.679	0.894-2.788	0.192	–	–	–
PR status Negative vs. Positive	1.585	0.756-3.142	0.098	–	–	–
HER-2 status Negative vs. Positive	1.448	1.122-2.367	0.078	–	–	–
Lymph node metastasis Absent vs. Present	3.241	1.445-5.763	0.004	2.784	1.219-4.884	0.009
Clinical stage I + II vs. III	3.556	1.574-5.236	0.005	2.989	1.366-4.453	0.010
miR-1179 expression High vs. Low	3.784	1.689-5.889	0.001	3.136	1.277-4.984	0.005

miR-1179 expression levels (HR, 3.136; 95% CI, 1.277-4.984; $p = 0.005$), as well as lymph node metastasis and the clinical stage, are independent prognostic markers of overall survival of patients with BC.

MiR-1179 Overexpression Inhibited the Proliferation, Migration and Invasion of BC Cells In Vitro

The above results proved that the down-regulation of miR-1179 in BC tissue was closely correlated with poor prognosis and lower survival rate. We further explored whether miR-1179 could be involved in the proliferation of BC. MCF-7 and T47D cells were transfected with miR-1179 mimics or miR-NC. The transfection efficiency was assessed by qRT-PCR (Figure 2A). Then, we performed MTT assay and found that miR-1179 mimics significantly inhibited MCF-7 and T47D cell proliferation (Figure 2B, 2C). Consistent with this result, the overexpression of miR-1179 significantly inhibited colony formation in MCF-7 and T47D cells (Figure 2D). Clinical assay indicated that miR-1179 was associated with lymph node metastasis. Thus, we wondered whether miR-1179 may exhibit tumor suppressor role in the migration and invasion of BC cells. To demonstrate our idea, transwell assay was performed and the results showed that the up-regulation of miR-1179 significantly suppressed migration and invasion of MCF-7 and T47D cells (Figure 3A, 3B). Our findings revealed that miR-1179 exerted tumor suppressor role in BC cells.

MiR-1179 Regulated the Activation of Notch Signaling in BC Cells

Notch signaling plays an important role in BC. To investigate whether miR-1179 inhibits BC cell proliferation and metastasis by regulating Notch signaling, we chose three representative molecules and assessed their expression levels in MCF-7 and T47D cells after being transfected with miR-1179 mimics and miR-NC. As shown in Figure 4, the results of Western blot demonstrated that the overexpression of miR-1179 decreased the expression levels of Notch 1, Notch 4 and Hes1. These results suggest that miR-1179 may suppress proliferation, migration and invasion of BC by activating Notch signaling.

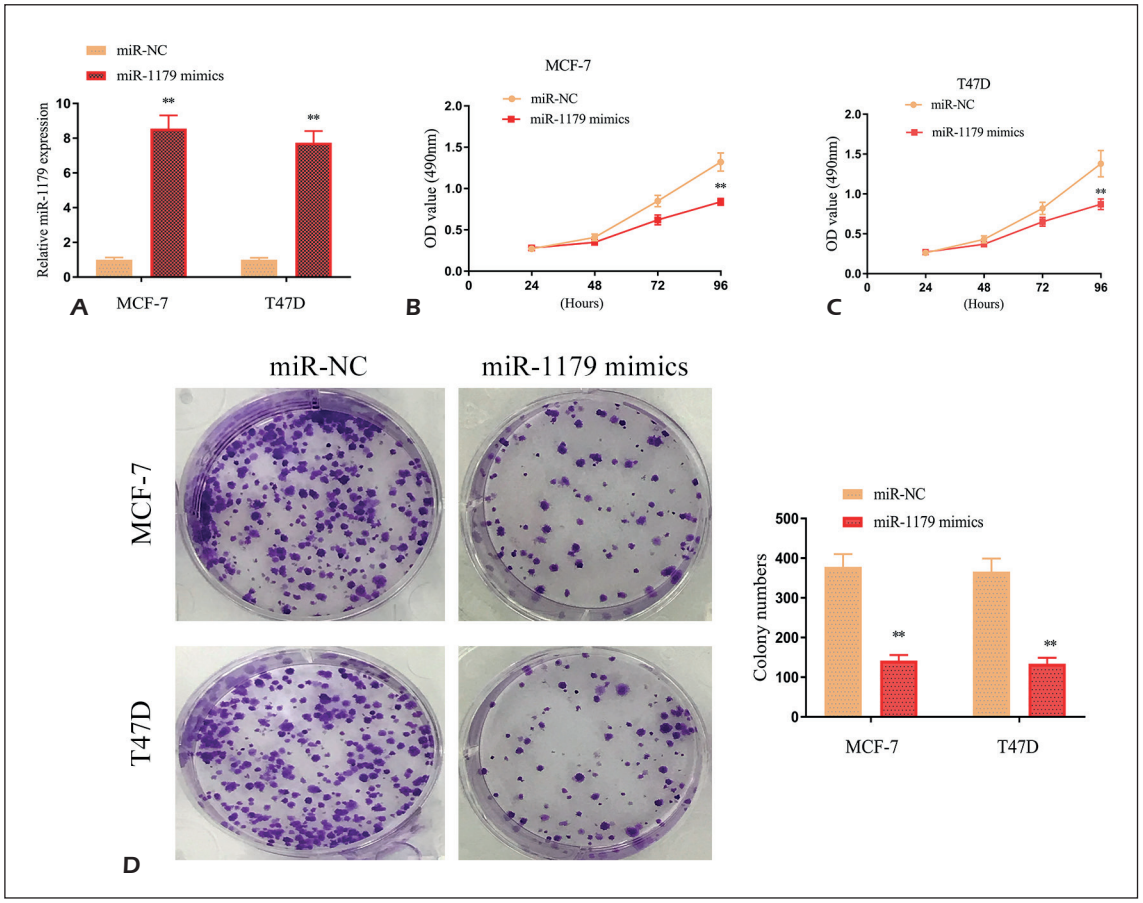

Figure 2. The overexpression of miR-1179 inhibits BC cell growth in vitro. **A**, qRT-PCR determined miR-1179 expression in MCF-7 and T47D cells transfected with miR-1179 mimic or miR-NC. **B-C**, MTT assay was performed to examine MCF-7 and T47D cell proliferation. The transfection of miR-1179 mimics significantly inhibited MCF-7 and T47D cell proliferation. **D**, Colony forming growth assay was performed to determine the proliferation in MCF-7 and T47D cells after transfection. $^{**}p<0.01$, $^{*}p<0.05$.

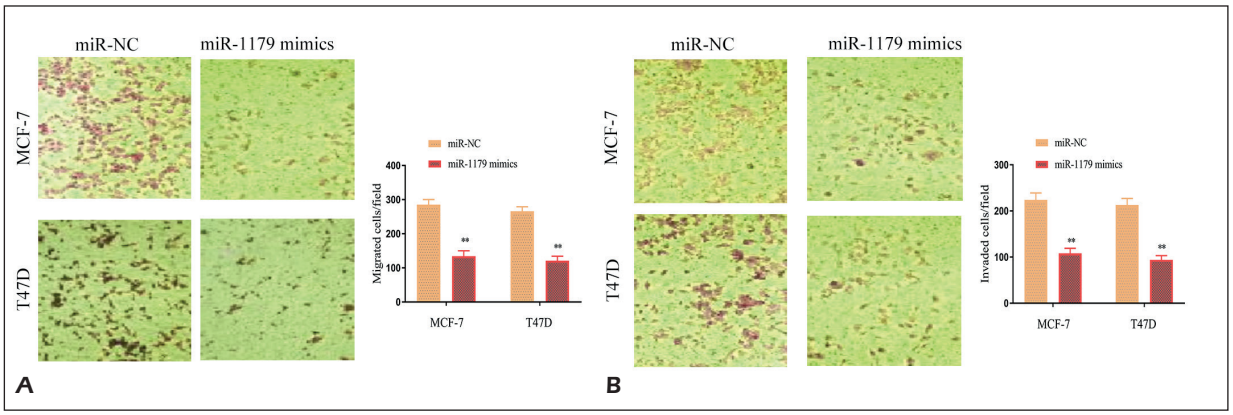

Figure 3. The up-regulation of miR-1179 suppresses BC cell proliferation, migration and invasion. **A**, The transwell migration assay was used to investigate the migration of miR-1179 mimics transfected MCF-7 and T47D cells. **B**, The transwell invasion assay was performed to investigate the invasion of miR-1179 mimics transfected MCF-7 and T47D cells. $^{**}p<0.01$, $^{*}p<0.05$.

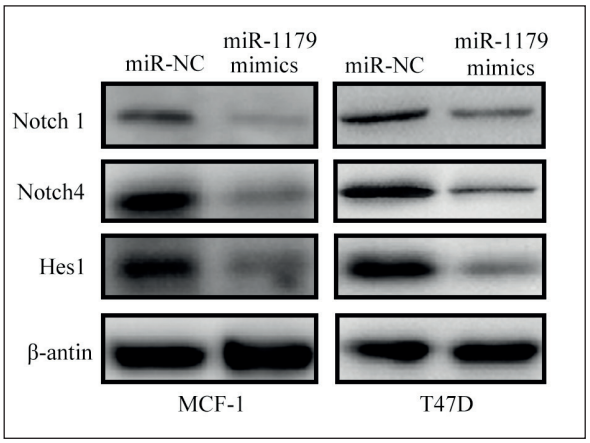

Figure 4. Expressions of Notch signaling pathway-related proteins in the miR-NC and miR-1179 mimics groups. The overexpression of miR-1179 suppressed the expression levels of Notch1, Notch4 and Hes1 proteins in MCF-7 and T47D cells.

Discussion

In China, BC has become one of the most common cancers and the second leading cause of death from cancer in women, with more than 1.6 million women being diagnosed and 1.2 million women dying each year[20]. Despite the progress in combined modality therapies, the long-term outcome of patients with BC remains poor[21]. Up to date, current TNM stage and several integrated models were not enough for BC patient outcome prediction[22]. It is of great significance to identify novel and efficient molecular markers accurately with high sensitivity and specificity to predict the patient prognosis and to identify the most suitable pathways to target using novel therapeutic agents. In this study, we firstly reported that the expression of miR-1179 was significantly down-regulated in BC tissues compared to matched normal breast tissues, indicating that miR-1179 may be involved in the progression of BC and could be used as a potential diagnostic biomarker. Then, we correlated decreased miR-1179 levels with aggressive clinicopathological features of BC tissues. In addition, Kaplan-Meier survival analysis indicated that the overall survival time of patients with lower miR-1179 expression levels was shorter than that of patients with higher miR-1179 expression levels. More importantly, in a multivariate Cox model, it was confirmed that miR-1179 expression was an independent poor prognostic factor for both overall survival of BC patients. Our results demonstrated that miR-1179 could serve as a prognostic biomarker for BC. Recently, several studies have reported that miR-1179 was dysregulated in several tumors. However, in different tumors, the expression trends were not identical, which suggested that miR-1179 may serve as a tumor suppressor or oncogene in different types of tumors. For instance, Xu et al[23] reported that miR-1179 expression was up-regulated in both glioblastoma tissues and cell lines, and was associated with the clinical prognosis of glioblastoma patients. Functionally, the overexpression of miR-1179 significantly inhibits glioblastoma cell proliferation and cell cycle progression by directly targeting E2F transcription factor 5. Lin et al[24] showed that miR-1179 was lowly expressed in human pancreatic cancer and its forced expression inhibits the proliferation, migration and invasion of human pancreatic cancer cells by targeting E2F5. However, Jiang et al[18] showed that miR-1179 was significantly overexpressed in esophageal squamous cell carcinoma and its knockdown could promote cell invasion through SLIT2/ROBO1 axis in esophageal squamous cell carcinoma. In addition, the down-regulation of miR-1179 was also reported in colorectal cancers although its function has not been demonstrated[17]. However, limited knowledge is available concerning the expression and function of miR-1179 in BC, which needs to be well documented. In this study, we used miR-1179 mimics to upregulate the expression of miR-1179 and perform several cells experiments, finding that the overexpression of miR-1179 significantly suppressed BC cells proliferation, migration and invasion, which suggested that miR-1179 served as a tumor suppressor in BC. Several signaling pathways, such as the PI3K/Akt signaling pathway, Wnt/β-catenin signaling pathway and Notch signaling pathway, have been demonstrated to be involved in the regulation of various cells progression, such as proliferation and metastasis[25-27]. Growing evidence showed that Notch signaling is critical for maintaining the balance between cell proliferation, differentiation and apoptosis and is also involved in metastasis of cancer cells[28,29]. The roles of Notch signaling have been reported in several tumors, including BC[30,31]. At the same time, many studies have reported that miRNAs could exhibit their biological function by modulating Notch signaling[32,33]. In this work, to study the potential mechanism by which miR-1179 regulated BC cells proliferation and metastasis, our attention forced on the association between miR-1179 and Notch signaling. We chose several important molecular biomarkers of Notch signaling, including Notch 1, Notch 4 and Hes1 to detect the

role of miR-1179 on the expression of these molecular biomarkers. We found that the overexpression of miR-1179 significantly reduced the level of Notch 1, Notch 4 and Hes1, suggesting that miR-1179 suppressed BC cells proliferation, migration and invasion by modulating Notch signaling.

Conclusions

On the basis of previous studies of miR-1179, we provided clinical and experimental evidence of the anti-oncogene role of miR-1179 and revealed a novel miR-1179/ Notch signaling axis in regulating BC cells proliferation and metastasis. Our work, for the first time, demonstrated that miR-1179 acts as a tumor suppressor in BC and might serve as a prognostic biomarker and molecular therapeutic target.

Conflict of Interests:

The authors declare that they have no conflict of interest

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