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Original Paper

MiR-212-5p Suppresses the Epithelial-Mesenchymal Transition in Triple-Negative Breast Cancer by Targeting Prrx2

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Key Words

Mir-212-5p • Triple-negative breast cancer • Prrx2 • Epithelial-to -mesenchymal transition

Abstract

Background/Aims: Triple-negative breast cancer (TNBC) is the most aggressive breast cancer subtype. Our study investigated the functional role of miR-212-5p in TNBC. ***Methods:*** Real-time PCR was used to quantify miR-212-5p expression levels in 30 paired TNBC samples and adjacent normal tissues. Wound healing and Transwell assays were used to evaluate the effects of miR-212-5p expression on the invasiveness of TNBC cells. Luciferase reporter and Western blot assays were used to verify whether the mRNA encoding Prrx2 is a major target of miR-212-5p. ***Results:*** MiR-212-5p was downregulated in TNBC, and its expression levels were related to tumor size, lymph node status and vascular invasion in breast cancer. We also observed that the miR-212-5p expression level was significantly correlated with a better prognosis in TNBC. Ectopic expression of miR-212-5p induced upregulation of E-cadherin expression and downregulation of vimentin expression. The expression of miR212-5p also suppressed the migration and invasion capacity of mesenchymal-like cancer cells accompanied by a morphological shift towards the epithelial phenotype. Moreover, our study observed that miR-212-5p overexpression significantly suppressed Prrx2 by targeting its 3′-untranslated region (3′-UTR) region, and Prrx2 overexpression partially abrogated miR-212-5p-mediated suppression. ***Conclusions:*** Our study demonstrated that miR-212-5p inhibits TNBC from acquiring the EMT phenotype by downregulating Prrx2, thereby inhibiting cell migration and invasion during cancer progression.

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Introduction

Triple-negative breast cancer (TNBC) is a highly aggressive tumor subtype associated with poor prognosis, in which mortality and morbidity arise from distant metastases, rather than from the primary tumor [1]. The epithelial-to-mesenchymal transition (EMT) is crucial in tumor progression, as it allows tumor cells to escape from the primary tumor to invade adjacent tissues and migrate to distant regions [2]. EMT is a key step in local invasion and distant metastasis and is important for enabling breast cancer to resist targeted treatment [3].

Aberrantly expressed microRNA (miRNA), including those regulating EMT and cancer metastasis, are common in many cancers and are important for tumorigenesis to develop and progress [4, 5]. MiR-212, located on chromosome 17p13.3, is deregulated in several human cancers [6, 7]. MiR-212 suppresses tumors in non-small cell lung cancer and gastric carcinoma [8]. However, other studies suggest that miR-212 exhibits oncogenic properties in colorectal, prostate and pancreatic cancers [9, 10]. Therefore, the biological functions of miR-212 are cancer-type specific, partly resulting from the different cellular contexts of various tumors. Importantly, the miR-212-5p expression level and its role in breast cancer remain undetermined.

Prrx2 is a newly identified EMT-inducer conferring migratory and invasive properties [11]. Prrx2 is encoded by the Prrx2 gene, which belongs to the paired class of homeobox genes [12]. Prrx2 and Prrx1, paired-related homeobox transcription factors, are highly expressed in undifferentiated mesenchymal cells during embryogenesis and are critical for normal morphogenesis in the craniofacial region, limbs and vascular system [13]. A recent study reported that Prrx2 works as a TGF-β-induced factor that enhances breast cancer invasion and migration [14]. Our previous study revealed that Prrx2 expression was associated with tumor size, lymph node metastasis, TNM stages and poor prognosis in breast cancer. Furthermore, silencing Prrx2 may suppress the proliferation, invasion and migration of breast cancer by reversing the EMT phenotypes by downregulating the Wnt/β-catenin signaling pathway [15]. Therefore, Prrx2 is an important contributor in breast cancer development. However, the regulatory mechanisms of Prrx2 expression in TNBC are poorly understood.

In this study, we provided experimental evidence that miR-212-5p is essential for the EMT and invasive phenotype of TNBC. Moreover, we revealed that a novel post-transcriptional regulatory mechanism of Prrx2 expression is mediated by miR-212-5p.

Materials and Methods

Clinical samples and data

Thirty pairs of TNBC and corresponding paired normal adjacent tissues were obtained from patients who underwent modified radical mastectomy at the Affiliated Hospital of Qingdao University. Tissues were diagnosed by histopathology. The matched non-cancerous adjacent tissues were harvested at least 5 cm away from the tumor site. No patients received radiotherapy or chemotherapy before surgery. Enrolled patients were divided into different subgroups based on miR-212-5p expression. Clinical features and survival information were compared between subgroups to determine the clinical significance and prognostic value of miR-212-5p.

Cell cultures and transfection

Three TNBC lines, MDA-MB 231 (PTEN wild-type, p53 mutant, and BRCA1 wild-type), MDA-MB-468 (PTEN null, p53 mutant, and BRCA1 wild-type), and HCC-1937 (PTEN null, p53 mutant, and BRCA1 mutant) and a normal mammary epithelial cell line (MCF-10A) were obtained from the Cancer Research Institute of Beijing, China. Cells were maintained per the supplier's instructions. Before the experiment, all cell lines were authenticated with short tandem repeat DNA profiling and were free of mycoplasma infection.

The miR-212-5p mimics were transfected at working concentrations using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). Full-length Prrx2 cDNA was purchased from GeneCopoeia™ (GeneCopoeia, Inc., Rockville, MD, USA) and subcloned into the eukaryotic expression vector, pcDNA3.1 (+).

Western blot analysis

Denatured protein was separated on an SDS-polyacrylamide gel, transferred to a Hybond membrane, and blocked overnight in 5% skim milk in TBST. For immunoblotting, the membrane was incubated overnight at 4°C with anti-Prrx2, anti-E-cadherin, and anti-vimentin antibodies (Santa Cruz, CA, USA; 1:500), followed by horseradish peroxidase-conjugated secondary antibody (Boster, Wuhan, China). Protein bands were visualized by ECL chemiluminescence (ECL New England Biolabs, USA),

MTT proliferation assay

Approximately, 5×10^3 cells were seeded into 96-well culture plates, incubated with 20 μL MTT (10 mg/ml) for 4 h at 37°C, and 200 μL of dimethyl sulfoxide (DMSO) was pipetted to solubilize the formazan product for 20 min at room temperature. Optical density was determined using a spectrophotometer at a wavelength of 570 nm.

Luciferase reporter assay

The luciferase reporter assay was performed as described previously [15]. Cell extracts were prepared 48 h after transfection, and luciferase activity was determined by the Dual-Luciferase Reporter Assay system (Promega, Madison, WI, USA).

Quantitative real-time PCR

Briefly, 10 ng of total RNA was reversed transcribed into cDNA using TaqMan miRNA hsa-miR-125b-specific primers (Applied Biosystems). Real-time PCR was performed using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). RNA U6 was used as an endogenous control in each sample.

Wound healing assay

A wound healing assay was performed to examine cell migration. Briefly, after the cells grew to 90% confluence in six-well plates, a single scratch wound was generated with a 200-μL disposable pipette tip. Scratch wounds were photographed over a 24 h period using a Nikon inverted microscope with an attached digital camera, and the wound widths were measured using Image software (Version 2.2.1, Nicon-BioImage Ltd., Japan).

Migration and invasion assays

Migration and invasion assays were performed in Boyden chambers as described previously [15]. Cancer cells were stained and counted microscopically. All experiments were performed at least twice in triplicate.

Mouse xenograft model

A total of 2×10^6 MDA-MB-231 cells infected with miR-212-5p or scramble viruses were propagated and inoculated subcutaneously into the dorsal flanks of female BALB/c nude mice (SLAC, Shanghai, China). Tumor size was measured every 7 days. After 42 days, the mice were sacrificed, necropsies were performed, and the tumors were weighed. The tumor volumes were determined by the following formula: $A\times B^2/2$, where A is the largest diameter and B is the diameter perpendicular to A.

Statistical analysis

Comparisons between groups were analyzed using *t*-tests and χ^2 tests. Overall survival curves and disease-free curves were plotted per the Kaplan-Meier method, and the log-rank test was used for comparison. Survival was counted from the date of surgery. Variables with $P<0.05$ by univariate analysis were used in a subsequent multivariate analysis based on the Cox proportional hazards model. Statistical analyses were performed using SPSS 18.0 software.

Results

miR-212-5p expression in TNBC tissues and its association with clinicopathologic features

To determine the expression status of miR-212-5p in TNBC, we initially compared miR-212-5p expression in 30 pairs of TNBC and adjacent non-tumor tissues. miR-212-5p expression in the TNBC tissues was significantly lower than in the matched tumor-adjacent tissues (P=0.003, Fig. 1A). Furthermore, we evaluated the relative expression of miR-212-5p in a normal breast epithelial cell line (MCF-10A) and three basal-like breast cancer cell lines (MDA-MB-468, HCC-1937 and MDA-MB-231). Reduced expression of miR-212-5p was observed in all three basal-like breast cancer cell lines compared to MCF-10A (P<0.01, Fig. 1B). Notably, miR-212-5p expression in HCC-1937 and MDA-MB-231 cells was lower than in MDA-MB-468 cells. We then determined the potential clinicopathological implications of altered miR-212-5p expression in 125 patients. The clinical samples were divided into low- and high-expression groups based on whether the miR-212-5p expression scores were greater or lower than the median. We analyzed the relationship between miR-212-5p expression and patient clinicopathological parameters. The results showed that low miR-212-5p expression was closely related to TNBC pathological parameters, including tumor size, lymph node status and vascular invasion (Table 1).

Table 1. Correlation of miR-212-5p expression with patient's clinical and pathological characteristics (*P<0.05)

Clinicopathological variables	Cases	miR-212-5p expression level		P value
		No. of low expression	No. of high expression	
Age (years)				0.871
≤45	45	26	19	
>45	80	48	32	
Tumor size (cm)				0.019*
≤2	46	21	25	
> 2	79	53	26	
Differentiation grade				0.925
Well-moderate	57	34	23	
Poor-undifferentiation	68	40	28	
Vascular invasion				0.012*
Negative	57	25	32	
Positive	68	49	19	
Lymph node status				0.015*
Negative	62	30	32	
Positive	63	44	19	
Ki67				0.824
≤20%	75	45	30	
>20%	50	29	21	

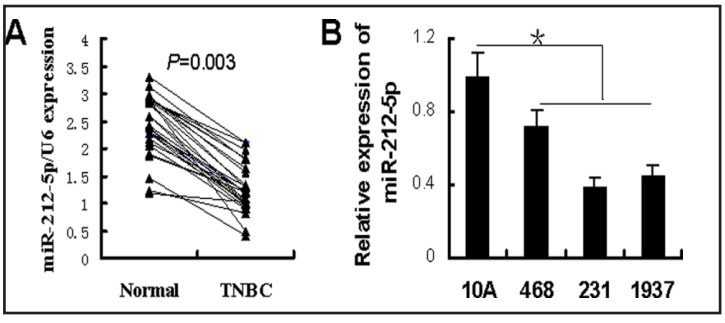

Fig. 1. miR-212-5p expression levels are frequently downregulated in TNBC. (A) Expression levels of miR-212-5p in 30 paired TNBC specimens and their corresponding paired adjacent normal tissues. (B) Expression levels of miR-212-5p determined by qRT-PCR in three basal-like breast cancer cell lines (HCC-1937, MDA-MB-468, and MDA-MB-231) and a normal epithelial cell line (MCF-10A). miR-212-5p expression was normalized based on U6 RNA expression. Error bars represent the standard deviations from triplicates of one representative experiment. *P <0.05.

Decreased miR-212-5p levels are correlated with poor clinical outcomes

To further analyze the significance of miR-212-5p on clinical prognosis, a Kaplan-Meier survival analysis was performed using the patient disease-free survival and overall survival. The results demonstrated that the 10-year disease-free survival and overall survival of 125 patients were 70.8% and 75.7%, respectively. When the patients were stratified by miR-212-5p expression status, the 10-year disease-free survival rates for high miR-212-5p expression and low miR-212-5p expression patients were 78.5% and 54.9%, respectively (P=0.001). Overall survival rates for high miR-212-5p expression and low miR-212-5p-expression patients were 81.9% and 62.3%, respectively (P=0.002) (Fig. 2). Univariate and multivariate survival analysis showed that higher histological stage, positive lymph node status, and low miR-212-5p expression were significantly associated with reduced disease-free survival and overall survival in the 125 patients (Table 2).

Table 2. Univariate and multivariate survival analysis of miR-212-5p expression in breast cancer (*P<0.05)

	Univariate analyses		Multivariate analyses	
	HR (95% CI)	P value	HR (95% CI)	P value
Age	0.915(0.530-1.582)	0.752	1.126(0.579-2.190)	0.727
Menopausal	0.815(0.471-1.409)	0.464	1.380(0.614-3.099)	0.435
Tumor size	1.373(0.795-2.371)	0.256	1.630(0.868-3.062)	0.129
Lymph node status	2.330(2.356-4.005)	0.002*	2.181(1.212-3.925)	0.009*
Histological grade	1.715(1.013-2.906)	0.045*	1.879(1.031-3.427)	0.027*
miR-212-5p	0.484(0.228-2.026)	0.049*	0.344(0.132-0.884)	0.027*

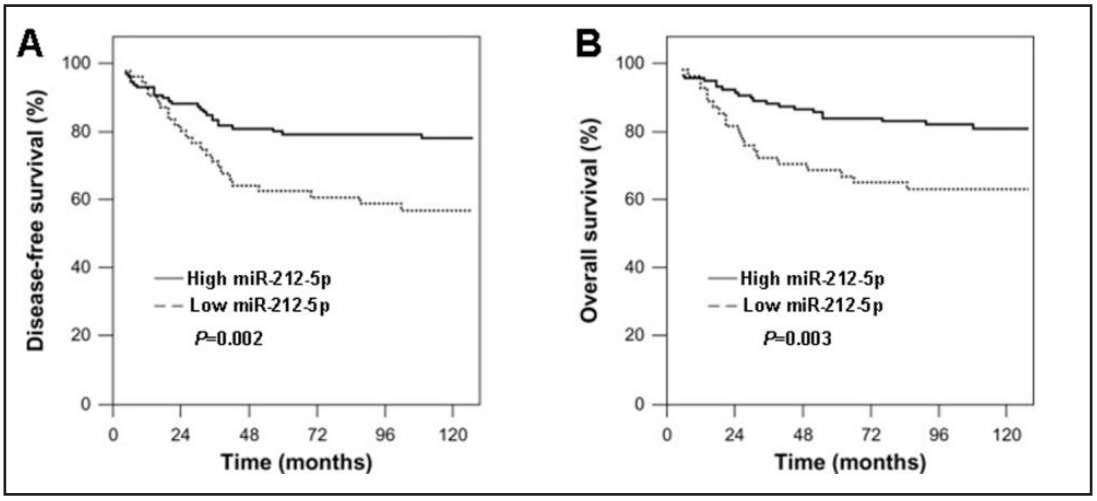

Fig. 2. Prognostic value of miR-212-5p for TNBC patients assessed by Kaplan-Meier analysis. Low miR-212-5p expression is associated with poor prognosis in TNBC patients. Disease-free survival (A) and overall survival (B) per the miR-212-5p expression level in 125 patients.

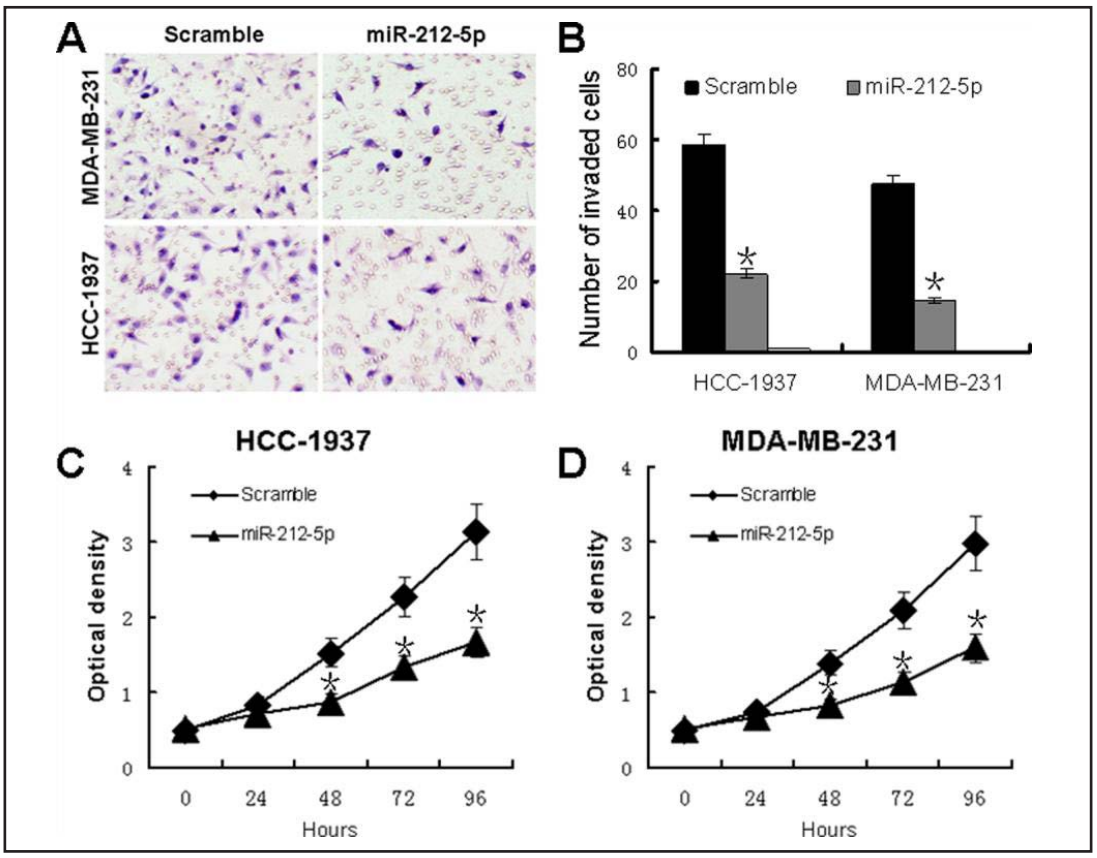

Fig. 3. miR-212-5p overexpression inhibits cell proliferation and invasion in vitro. MDA-MB-231 and HCC-1937 cells infected with miR-212-5p or scramble lentivirus. (A) The invasion assay was measured through Transwell assays with Matrigel. (B) The results from three separate assays were averaged and graphed. The growth of HCC-1937 (C) and MDA-MB-231 (D) cells infected with miR-212-5p or the scramble lentivirus was assayed. The results from three separate assays were averaged and graphed. *P<0.05.

Ectopic expression of miR-212-5p suppressed proliferation and invasion ability of breast cancer in vitro

To determine whether miR-212-5p functionally behaved as a tumor suppressor, we demonstrated that upregulated miR-212-5p expression inhibited the invasive capacity of breast cancer cells (Fig. 3A, B). We also demonstrated that overexpression of miR-212-5p significantly suppressed breast cancer cell proliferation (Fig. 3C, D).

miR-212-5p suppressed tumorigenesis and metastasis in vivo

To directly evaluate the role of miR-212-5p in tumor formation and growth *in vivo*, a xenograft model of human TNBC cells in nude mice was adopted. Briefly, MDA-MB-231 cells infected with miR-212-5p or the scramble lentivirus were injected subcutaneously into each flank of the nude mice. After the cells were injected, the tumor volume was monitored every 7 days, and tumor growth curves were plotted accordingly. All mice were then sacrificed to harvest the xenograft. The mean volume and weight of the tumors generated from the miR-212-5p overexpression group were significantly lower than the control group (P<0.05) (Fig. 4A, B, C). We then investigated the effect of miR-212-5p on tumor metastasis *in vivo*. MDA-MB-231 cells infected with miR-212-5p or the scramble lentivirus were transplanted into the nude mice via tail vein injection. After 60 days, the mice were anesthetized, and their lungs were dissected. As shown in Fig. 4D, significantly fewer macroscopic lung metastases were observed in the mice that received cells infected with miR-212-5p (P<0.05).

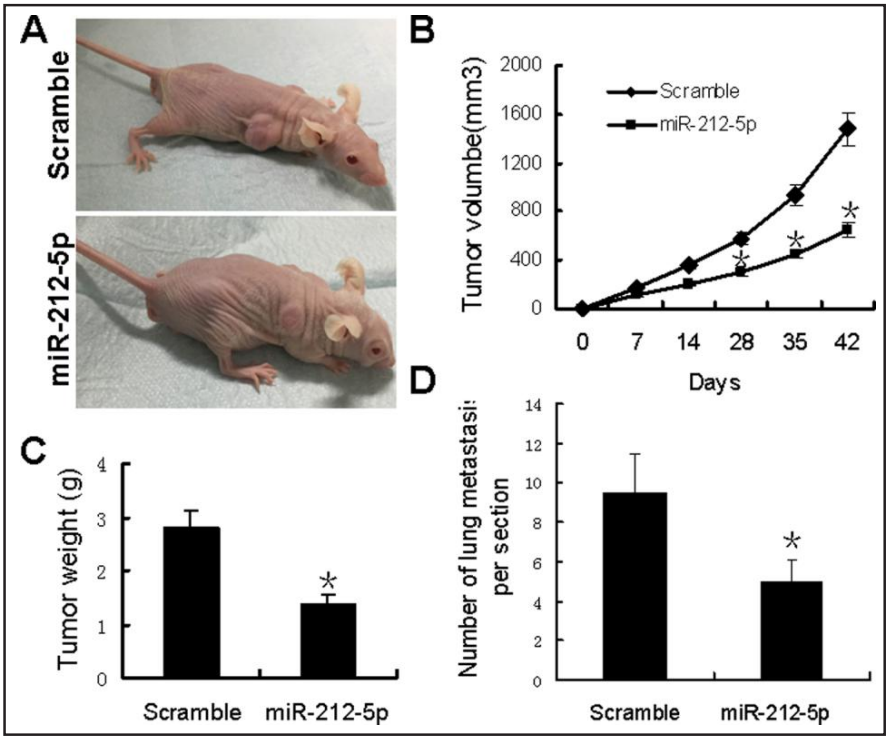

Fig. 4. miR-212-5p inhibits TNBC growth and metastasis in vivo. Tumor growth in mouse xenograft models. (A) MDA-MB-231 cells infected with miR-212-5p lentivirus or scramble were injected subcutaneously into nude mice. (B) Tumor volume was measured every 7 days. (C) After 42 days, the mice were sacrificed, necropsies were performed, and the tumors were weighed. Tumor metastasis in mouse xenograft models. MDA-MB-231 cells overexpressing miR-212-5p or scramble were injected into the tail vein of nude mice. After 60 days, the mice were sacrificed. (D) The disseminated nodules were evaluated. Each group had eight mice. *P<0.05.

miR-212-5p inhibited the EMT process of TNBC

To investigate the effects of miR-212-5p up-regulation on the EMT phenotype in breast cancer cells, we measured the relative protein expression of EMT markers by Western blot analysis. Upregulated miR-212-5p expression in MDA-MB-231 cells led to significantly increased E-cadherin expression and decreased vimentin expression in the cells (Fig. 5A, B). Additionally, cells with overexpressed miR-212-5p exhibited epithelial morphology (Fig. 5C).

miR-212-5p directly targeted the Prrx2 3′ UTR

We performed a bioinformatics analysis using TargetScan and Pictar, which predicted that miR-212-5p may target the Prrx2 3′ UTR region. Perfect base pairing was observed between the seed sequence of mature miR-212-5p and the 3′ UTR of Prrx2 mRNA, and these seed sequences were conserved across species (Fig. 6A). To determine whether the 3′ UTR of Prrx2 mRNA is a functional target of miR-212-5p in breast cancer cells, either the target sequence of Prrx2 3′ UTR (wt 3′ UTR) or the mutant sequence (mt 3′ UTR) were cloned into a luciferase reporter vector (Fig. 6B). Thereafter, HEK293 cells were transfected with a wt or mt 3′ UTR vector and miR-212-5p mimics. Luciferase activity decreased significantly compared with the control. As shown in Fig. 6C, ectopic overexpression of miR-212-5p inhibited Prrx2 expression (P=0.002), while a miR-212-5p inhibitor restored Prrx2 expression (P=0.010).

Exogenous Prrx2 reversed the inhibitory effects of miR-212-5p on breast cancer cells in vitro

We examined whether miR-212-5p regulates cell behaviors by downregulating Prrx2. We transfected pcDNA3.1-Prrx2 into the miR-212-5p-overexpressed breast cancer cell line, MDA-MB-231. Western blot analysis showed that Prrx2 reversed the expression of EMT-related genes caused by overexpressed miR-212-5p (Fig. 7A). We then tested whether

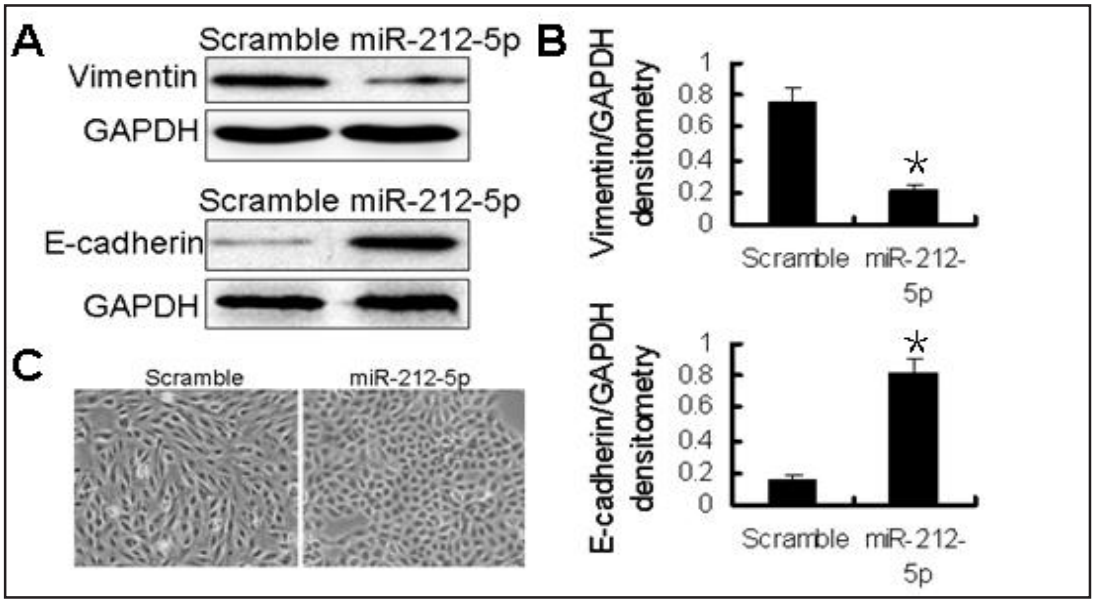

Fig. 5. Upregulation of miR-212-5p altered the EMT phenotype in breast cancer cells. Breast cancer cells (MDA-MB-231) were transfected with miR-212-5p mimics or negative control mimics for 48 h. (A) Western blot analysis of E-cadherin and vimentin expression in MDA-MB-231 cells. (B) Data were expressed as the mean ± SD of three independent experiments relative to GAPDH. (C) Morphological changes in the breast cancer cells were observed by phase-contrast microscopy (100×). *P<0.05.

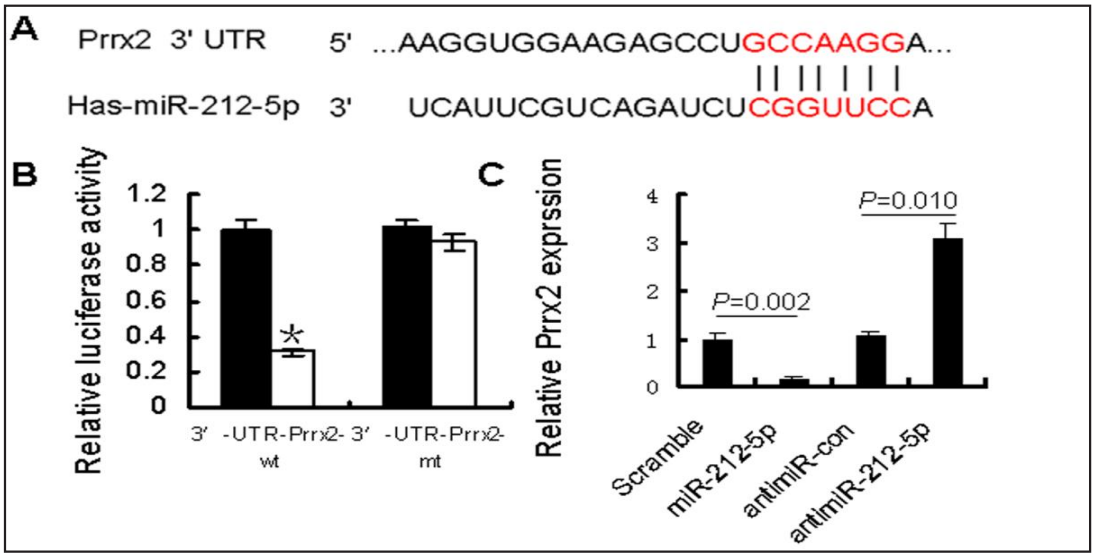

Fig. 6. Oncogene Prrx2 was specifically targeted by miR-212-5p. (A) The predicted binding sequences for miR-212-5p within the Prrx2 3′ UTR. Seed sequences are highlighted. (B) Luciferase activity assays using a luciferase reporter with wild-type or mutant human Prrx2 3′ UTR were performed after co-transfection of miR-212-5p mimics or control into HEK293 cells. mt 3′ UTR increased significantly compared with wt 3′ UTR. (C) Prrx2 expression was determined in MDA-MB-231 that stably overexpressed miR-212-5p and cells transfected with miR-212-5p inhibitors or anti-miR-control by Western blot analysis. *P<0.05.

restoring Prrx2 could reverse the miR-212-5p-mediated inhibition of breast cancer cell invasion. As shown in Figs. 7B, C, and D, Prrx2 over-expression using cDNA without a 3′ UTR partially abrogated the miR-212-5p-mediated suppression of breast cancer cell migration and invasion (P<0.05).

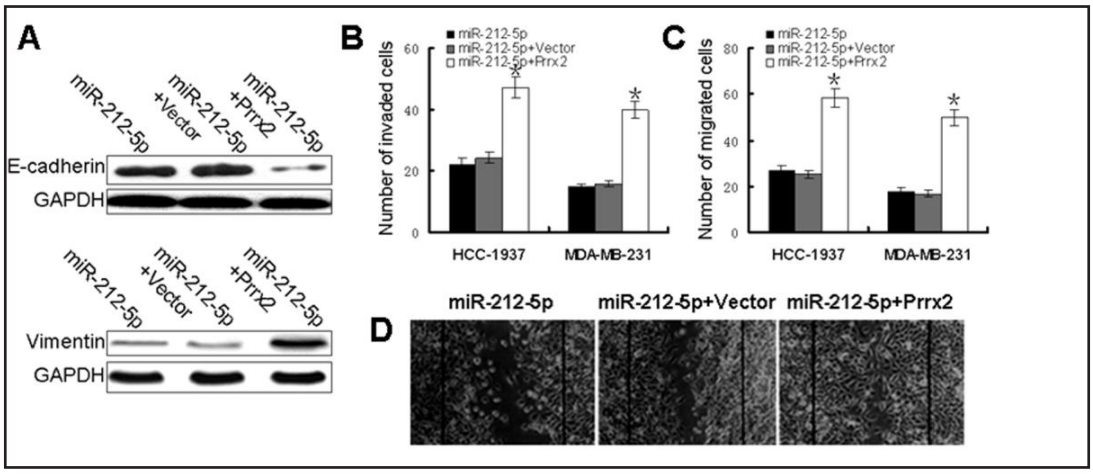

Fig. 7. Overexpressing Prrx2 reverses the inhibitory effects of miR-212-5p on breast cancer cells. (A) Western blot was used to analyze the Prrx2 and EMT-related genes, E-cadherin and vimentin, in miR-212-5p-vector co-transfected cells or miR-212-5p Prrx2 co-transfected cells compared with the control group. Transwell assay revealed that the reduced migration (B) and invasion (C) caused by overexpressing miR-212-5p could be reversed by introducing Prrx2. (D) A wound healing assay indicated that the reduced migration caused by overexpressing miR-212-5p could be reversed by introducing Prrx2 (40×). *P<0.05.

Discussion

miRNA regulates up to 30% of all human genes and controls many cellular processes [16, 17]. Recent studies have shown that miRNA is deregulated in various cancers, and miRNA expression is relevant to the diagnosis and prognosis of many diverse tumors [18, 19]. Various publications have associated miRNA with cancer; however, the relationship between miR-212-5p and TNBC remains unknown. In this study, we demonstrated that miR-212-5p is significantly downregulated in TNBC tissues. We also found that altered miR-212-5p expression levels are associated with tumor size, lymph node status and vascular invasion in breast cancer. We demonstrated a significant correlation between higher miR-212-5p expression and better prognosis in TNBC. Using wound healing and Transwell assays, we revealed that upregulating miR-212-5p in HCC-1937 and MDA-MB-231 cells suppressed the migration and invasion of these TNBC strains. Moreover, Kaplan-Meier survival analysis revealed that breast cancer patients whose primary tumors displayed low miR-212-5p expression had a shorter disease-free and overall survival. The observed aberrant expression of miR-212-5p suggested that miR-212-5p suppresses TNBC tumors.

New evidence suggests that the EMT is important for tumor metastasis and recurrence [20, 21]. Understanding the molecular mechanisms that regulate the EMT process is crucial for improving breast carcinoma treatment [22, 23]. Our study showed that ectopic expression of miR-212-5p in TNBC cells impaired their invasion, proliferation and growth. High expression of miR-212-5p resulted in increased E-cadherin expression and decreased vimentin expression. Moreover, *in vivo* data demonstrated that miR-212-5p inhibited tumor growth and metastasis. These *in vitro* and *in vivo* data further indicate that miR-212-5p inhibits cell migration, invasion and the EMT phenotype in TNBC. However, miR-212-5p mechanisms in the EMT and their therapeutic potential remain unclear. Various studies have shown that fatty acid synthase (FAS) and stearoyl-CoA desaturase-1 (SCD1) are potential downstream target genes of miR-212-5p [24, 25]. Interestingly, we characterized the role of miR-212-5p in regulating Prrx2 in TNBC. We performed a bioinformatics analysis using TargetScan and Pictar and predicted that miR-212-5p may target the Prrx2 3′ UTR region. Prrx2 is a transcription coactivator, enhancing the DNA-binding activity of serum response factors. A recent study reported that Prrx2 works as a TGF-β-induced factor that enhances invasion and migration in breast cancer. More importantly, our previous study showed that

silencing Prrx2 may suppress the proliferation, invasion and migration of breast cancer cells by reversing the EMT phenotypes by repressing the Wnt/β-catenin signaling pathway [15, 26]. Here, Prrx2 was identified as an important downstream target of miR-212-5p. Using a luciferase reporter assay, MiR-212-5p bound directly to the 3′ UTR of Prrx2, containing a miR-212-5p-binding site. Upregulating miR-212-5p significantly reduced the Prrx2 protein level in MDA-MB-231. More importantly, we showed that overexpressing Prrx2 partially abrogated miR-212-5p-mediated suppression. These data suggest that miR-212-5p may inhibit MDA-MB-231 proliferation and metastasis by regulating Prrx2.

Conclusion

We revealed that miR-212-5p was downregulated in TNBC, and its expression level was associated with tumor size, lymph node status and vascular invasion in breast cancer. Furthermore, miR-212-5p inhibited EMT and metastasis *in vitro* and *in vivo* by downregulating Prrx2 in TNBC. These findings suggest that miR-212-5p suppresses tumors in TNBC development, and miR-212-5p can serve as a prognostic marker for breast cancer.

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Disclosure Statement

The authors declare that they have no competing interests exist.

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