

Original Article

MiR-630 inhibits cells migration and invasion by targeting SOX4 in triple-negative breast cancer

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Abstract: Objective: To verify whether miR-630 could inhibit Triple-negative Breast Cancer (TNBC) cell line MDA-MB-231 cells migration and invasion by targeting SOX4 in TNBC. Methods: Normal breast tissue and breast cancer tissue (44 case of TNBC, 115 case of NTNBC) from patients undergoing breast cancer resection were collected. RT-PCR was used to test the expression of miR-630, miR-21, miR-134, miR-200a, miR-381, miR-1228 and SOX4 mRNA in the tissues; Western blot were used to test the expression of MMP-2, MMP-9, COL1A1, COL1A5 and SOX4 in the tissues. In vitro experiment, after miR-630 mimics was transfected into MDA-MB-231 cells, wound healing assay were employed to test the migratory ability of MDA-MB-231 cells, transwell chambers were used to test the invasion ability of cells, Western blotting were used to investigate the expressions of COL1A1, COL1A5, MMP-2, MMP-9 and SOX4. Luciferase assay was used to confirmed whether SOX4-3'-UTR the target gene of miR-630. SOX4 over-expression plasmid was transfected to further confirm miR-630 played its role by down-regulation of SOX4. Results: Compared with normal breast tissue, the expression of miR-630 was decreased in the TNBC tissue ($P<0.01$); meanwhile COL1A1, COL1A5, MMP-2, MMP-9 and SOX4 was significantly increased ($P<0.01$); the relative expression of miR-630 level was negatively correlated with SOX4 mRNA ($P<0.01$). In vitro experiment, compared with the mimic control, the migration and invasion activity of MDA-MB-231 cells was decreased after transfection of miR-630 mimics ($P<0.01$); meanwhile, miR-630 mimic also decreased the expression of SOX4 in MDA-MB-231 cells ($P<0.00$). The Luciferase activity of the SOX4-3'-UTR plasmid was significantly suppressed by miR-630 ($P<0.00$). Over expression of SOX4 could partly abrogated miR-630 mediated inhibition of MDA-MB-231 migration and invasion. Conclusion: In TNBC tissue, the expression of miR-630 decreased; miR-630 inhibits MDA-MB-231 cells migration and invasion by targeting SOX4-3'-UTR.

Keywords: Triple-negative breast cancer, miR-630, sex determining region Y-box 4, migration, invasion

Introduction

Breast cancer remains to be the most common malignant tumor of women and causes 400,000 deaths annually worldwide. Benefiting from the advances in the early diagnosis and adjuvant treatment of breast cancer, the rate of five years disease-free survival in patients was increasing in these decades [1]. Compared with other subtypes of breast cancer, triple negative breast cancer (triple-negative breast cancer, TNBC) was poorly differentiated, with high histological grade, prone to relapse and metastasis. Meanwhile because of low expression of estrogen receptor (ER), human epidermal growth

th factor receptor 2 (HER2) and progesterone receptor (PR), TNBC patients are unable to benefit from endocrine therapy or targeted therapy, and the mortality rate was much higher than NTNBC patients [2].

Metastasis is the first reason for the mortality of patients with most of the cancers including TNBC. Tumor metastasis is a complex process which includes migratory tumor cells leaving the primary position by invasion, disseminating throughout the body via the circulation, and colonizing at distant organs eventually [3]. It has already been proved that tumor cells acquiring the ability of migration and invasion to

depart from the original locality is the prerequisite for metastasis. Therefore, it is important to find an effective anti-TNBC cell migration and invasion way in order to improve the prognosis of patients with TNBC.

MicroRNAs (miRNAs) are small non-coding RNA molecules that bind to the 3'-untranslated region (UTR) of target mRNAs, resulting in translation repression or mRNA degradation [4]. Mounting evidence have revealed that deregulation of miRNAs expression is related with variety of human disease such as cardiovascular disease, metabolic disorders, and particular cancer [5]. Recently, some studies found that miRNAs could play important roles in breast cancer initiation and progression. For example, Ming et al. demonstrated that miR-381 and miR-200a could suppress the expression of Cx43 in breast cancer cells [6, 7]. Zhang et al. reported that miR-21 expression is increased in breast cancer and plays an important role as a tumor gene by targeting STAT3 [8]. O'Brien et al. showed that miR-134 in extracellular vesicles reduced TNBC aggression and increases drug sensitivity [2]. Liu et al. found that miR-1228 served as an oncogene to promote breast cancer proliferation, invasion and migration [9]. Corcoran et al. demonstrated miR-630 as a diagnostic and a predictive biomarker for response to HER-targeted drugs in breast cancer [10]. However, functions of these miRNAs in human TNBC are still less illustrated.

The SOX4 (sex-determining region Y-box 4) gene is a member of the SOX family, which has been shown to have important roles in the cell fate decision. In tumor genesis, lots of studied showed that SOX4 was significantly elevated in multiple human cancers like esophageal cancer, breast cancer, lung cancer, and cervical cancer [11-14]. In addition, its overexpression was closely correlated with tumor progression and metastasis [14, 15].

In this study, we found that miR-630 was significantly decreased in the TNBC tissues and further revealed that over-expression of miR-630 inhibited TNBC cells migration and invasion. More importantly, we identified SOX4 as a direct target gene of miR-630 in TNBC cells. Restoration of SOX4 partly reversed miR-630 induced inhibition of TNBC cells migration and invasion.

Materials and methods

Patients and samples

Human breast cancer and their corresponding non-tumor tissues were collected at the time of surgical resection from 159 patients with breast cancer from 2011 to 2014 at the Department of Oncology, People's Hospital of Shannxi Province. All specimens were confirmed pathologically. 44 patients were TNBC and 115 patients were NTNBC. Human tissues were immediately frozen in liquid nitrogen and stored at -80°C refrigerator. Informed consent was signed by all patients and the study was approved by the Ethics Committee of People's Hospital of Shannxi Province.

Cell culture and transfection

The human TNBC cell line (MDA-MB-231) was obtained from American Type Culture Collection (ATCC, Rockville, Maryland, USA) and kept in RPMI 1640 medium with 10% fetal bovine serum (FBS) (Gibco, CA, USA), 1% of 100 U/ml penicillin and 1% of 100 mg/ml streptomycin sulfates. The cells were incubated in humidified incubators with 5% CO₂ at 37°C.

MiR-630 mimics and mimics control were synthesized chemically in Suzhou GenePharma Co., Ltd. (Suzhou, China). The sequences were as follows: 5'-AGUUAUCUGUACCAGGGAAGGU-3' (mimics); 5'-AGUUAUCUGUAGGAGGGAAGCU-3' (mimics control). Human SOX4 gene was constructed into pcDNA3.1+HA vector by Life Technologies (Invitrogen, CA, USA), and the empty vector was served as the negative control. For transfection, after the cells were cultured to 70-80% confluence, miR-630 mimics or mimics control and pcDNA3.1+HA-SOX4 or pcDNA3.1+HA empty vector were transfected by using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions.

Quantitative real-time PCR

Total RNA was extracted from tissues or cells by using trizol reagent (Invitrogen, USA) and then miRNA were reverse transcribed to cDNA by using reverse transcription kit (Takara, Japan). Quantitative real-time PCR (qRT-PCR) were performed by using SYBR Green PCR Kit on ABI 7500 Fast Real-Time PCR system according to the manufacturer's recommendation.

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Table 1. Clinical characteristics of 159 patients and the expression of miR-630 in BC tissues

Parameters	Case	MiR-630 Median (range)	P
Age (years)			0.637
<52	89	1.64 (0.15-2.77)	
≥52	70	1.99 (0.26-2.98)	
Tumor size			0.036
<2 cm	41	0.89 (0.15-2.23)	
≥2 cm	118	2.02 (0.66-2.98)	
Lymph node metastasis			0.048
Negative	55	1.64 (0.15-7.46)	
Positive	104	2.05 (0.42-2.98)	
Clinical stage			0.012
I	49	0.92 (0.15-2.15)	
II,III	110	1.98 (0.78-2.98)	
ER			0.031
Negative	41	0.78(0.15-2.04)	
Positive	118	1.48 (0.26-2.98)	
Her-2			0.43
Negative	76	1.22 (0.15-2.85)	
Positive	83	1.44 (0.21-2.98)	
P53			0.017
Negative	116	0.89 (0.15-2.33)	
Positive	43	1.77 (0.35-2.98)	

ER: estrogen receptor; PR: progesterone receptor; Her-2: epidermal growth factor receptor-2.

The expression of miR-630, miR-21, miR-134, miR-200a, miR-381 and miR-1228 was normalized to U6. All experiments were done triplicate. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of genes.

Western blotting

Protein from tissues or cells was extracted with RIPA lysis buffer (Biyuntian, China). Protein lysates were then separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes (PVDF, Millipore, MA, USA). After blocking with 5% non-fat milk for 2 h at room temperature, the membranes were incubated with the primary antibodies: COL1A1 (1:1000, Abcam, USA), COL1A5 (1:1000, Abcam, USA), MMP-2 (1:1000, Abcam, USA), MMP-9 (1:2000, Abcam, USA), SOX4 (1:1000, Abcam, USA) and β -actin (1:1000, Abcam, USA) over-night at 4°C. Then the membranes were incubated in HRP-linked secondary antibodies (Santa Cruz Biotechnology, USA) for 2 h. Western blotting signals were detected using the

ECL plus Kit (Biyuntian, China). Each experiment was repeated three times independently.

Cell migration assay

MDA-MB-231 cells were transfected with miR-630 mimics or mimics control and pcDNA3.1+HA-SOX4 or pcDNA3.1+HA empty vector following to the manufacture's information. After 6 h of transfection, the cell scratch spatula was used to scratch the cells layer when cells was reached approximately 90% confluency. After being washed with warm PBS for three times the cells was continue to be incubated at 37°C for 24 h. Digital camera system (Olympus Corp., Tokyo, Japan) was used to acquire Images of the scratches of the cells after incubating for 0 and 24 h.

Cell invasion assay

Transwell chamber was used to examine cell invasion capability. MDA-MB-231 cells were transfected with miR-630 mimics or mimics control and pcDNA3.1+HA-SOX4 or pcDNA3.1+HA empty vector following to the manufacture's information. After 6 h of transfection, transfected cells were trypsinized and resuspended, 2.0×10^4 cells in 200 μ L RPMI 1640 medium were placed into the upper chambers (8-mm pore size; Corning, St. Lowell, MA). The lower chambers were filled with 600 μ L complete medium with 10% FBS. After incubation for 12 h at 37°C, the cells on the upper side of the inserts were softly scraped off. Cells that migrated to the lower side of the inserts were fixed with 4% paraformaldehyde and stained with crystal violet (1 μ g/ml), and then the cells from five independent, randomly chosen visual fields were counted under an immunofluorescence microscope ($\times 100$ magnification) for quantification of cells.

Dual-luciferase reporter assay

The potential miR-630 binding sites of SOX4 were predicted by three computer-aided algorithms including TargetScan, PicTar and miRanda. The mRNA 3'-UTR sequences of SOX4 (WT) was PCR amplified and inserted into the psi-

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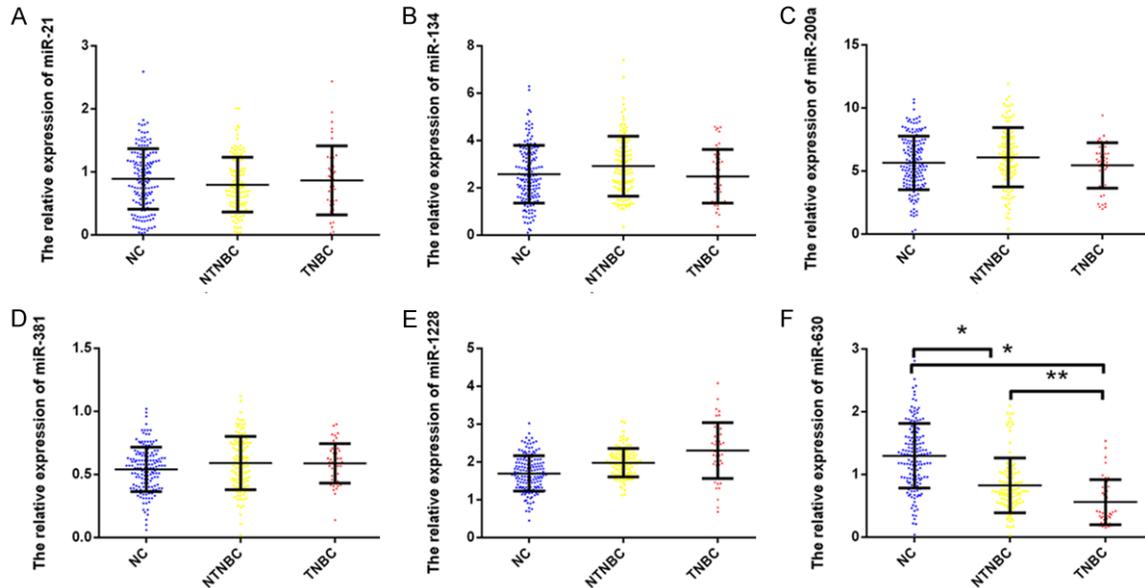


Figure 1. Expressions of breast cancer-related miRNAs and proteins in the breast cancer Analysis of the expressions of six miRNAs (miR-630, miR-21, miR-134, miR-200a, miR-381 and miR-1228) in normal breast tissue and breast cancer tissue (44 case of TNBC, 115 case of NTNBC) by qRT-PCR. U6 was used as an internal reference. The expression of miR-630 was significantly down-regulated in TNBC. NC: normal control; TNBC: triple-negative breast cancer; NTNBC: none triple-negative breast cancer. * $p < 0.01$ compared with NC, ** $p < 0.01$ compared with NTNBC.

CHECK-2 luciferase vector (Promega, Madison, WI, USA). The forward primer sequences for the mRNA 3'-UTR of SOX4 was 5'-CTTGACATGATTAGCTGGCATGATT-3' and reverse primer sequences was 5'-CCTGTGCAATATGCCGTGTAGA-3'. SOX4 mRNA 3'-UTR contained sequences with mutations (MUT) in the putative binding sites of miR-630 was chemically synthesized by Suzhou GenePharma Co., Ltd. (Suzhou, China). For luciferase reporter assay, MDA-MB-231 cells were seeded in 24-well plates, co-transfected with the WT or MUT constructed reporter plasmids and miR-630 mimics or mimics control by using Lipofectamine 2000. After 24 h transfection, the GloMax-Multi Jr Single Tube Multimode Reader (Promega, Madison, WI, USA) was used to detect the Firefly and Renilla luciferase activities. Firefly luciferase activity was used as an internal control to normalize the transfection efficiency.

Statistical analysis

All statistical analyses were performed using SPSS 20.0. Data were presented as mean \pm SD. Differences between groups were analyzed by using student's t-test or one-way ANOVA analysis. All experiments were all repeated at

least three times. The value of $P < 0.05$ was considered to be statistically significant.

Results

miR-630 is down-regulated in human TNBC

Since miRNAs could play important roles in tumor function, we first selected six miRNAs (namely miR-630, miR-21, miR-134, miR-200a, miR-381 and miR-1228) which were reported to be related with breast cancer. The expressions of these miRNAs in 159 cases of breast cancer tissue (44 cases TNBC and 115 case of NTNBC) and normal control were investigated by qRT-PCR analysis. Correlations of miR-630 expression and the clinicopathological characteristics of breast cancer patients were shown in **Table 1**. The results revealed no significant difference in the expressions of miR-21, miR-134, miR-200a, miR-381 and miR-1228 between these three groups. However, the expression of miR-630 in TNBC and NTNBC showed significant down-regulation compared with normal control. In addition, compared with NTNBC, the expression of miR-630 was degraded in TNBC tissue (**Figure 1**, $P < 0.01$). These data prompted us to focus on miR-630 in the following study.

MiR-630 inhibits MDA-MB-231 cells migration and invasion

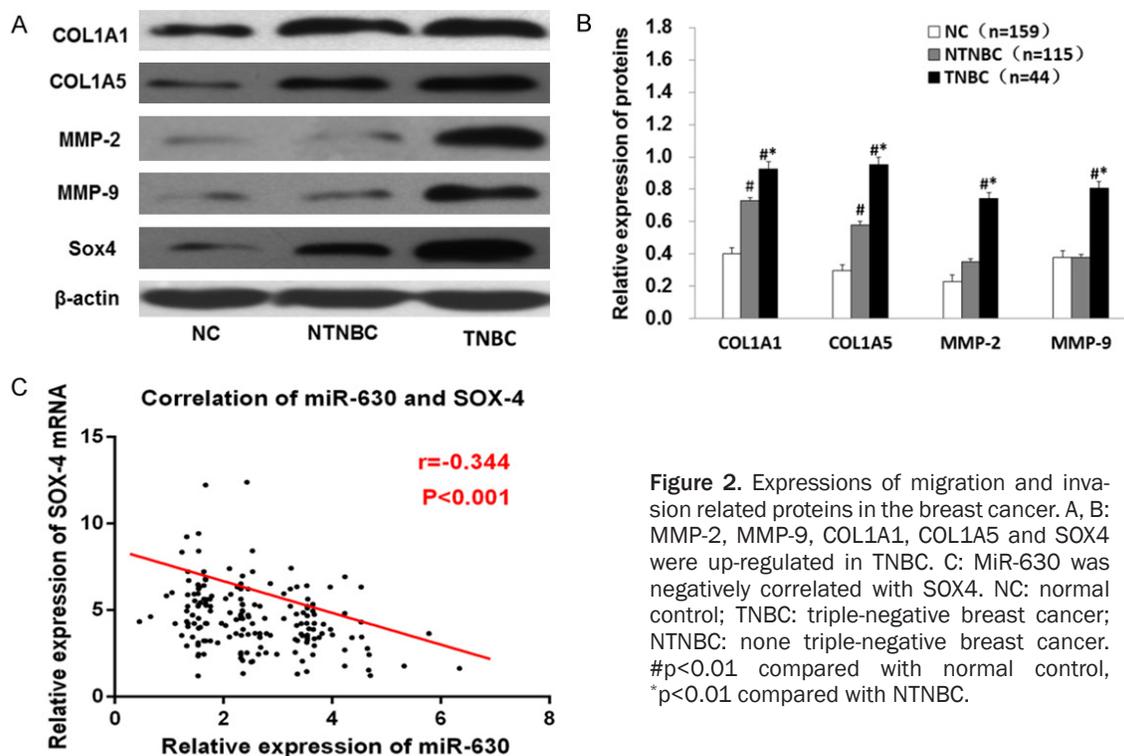


Figure 2. Expressions of migration and invasion related proteins in the breast cancer. A, B: MMP-2, MMP-9, COL1A1, COL1A5 and SOX4 were up-regulated in TNBC. C: MiR-630 was negatively correlated with SOX4. NC: normal control; TNBC: triple-negative breast cancer; NTNBC: none triple-negative breast cancer. # $p < 0.01$ compared with normal control, * $p < 0.01$ compared with NTNBC.

Then we tested the expression of COL1A1, COL5A1, MMP-2, MMP-9 and SOX4 in TNBC, NTNBC and normal breast tissue. We found that the expression of COL1A1, COL5A1, MMP-2, MMP-9 and SOX4 was increased in the TNBC compared with the normal control or NTNBC (Figure 2A, 2B, $P < 0.01$). What's more, the relative expression of miR-630 level was negatively correlated with SOX4 mRNA (Figure 2C, $P < 0.01$). These data reminded us the potential relationship between miR-630, SOX4 and TNBC cell migration and invasion.

miR-630 suppresses MDA-MB-231 cells migration and invasion

Images of the scratches were captured at 0 and 24 h after transfection, we found that over-expression of miR-630 markedly inhibited MDA-MB-231 cells migration after 24 h (Figure 3B, $P < 0.01$). In agreement, transwell chamber assay revealed that miR-630 overexpression decreased cell invasion ability after 12 h (Figure 3C). Meanwhile, restoration of miR-630 suppressed the expression of COL1A1, COL5A1, MMP-2 and MMP-9 (Figure 3D, 3E). Taken together, these results indicated that miR-630 was able to suppress the migration and invasion of MDA-MB-231 cells in vitro.

miR-630 directly targets SOX4 in MDA-MB-231 cells

The expression level of SOX4 was significantly down-regulated in miR-630 mimics transfectants compared with the mimics control (Figure 4A). In addition, bioinformatics analysis was performed to identify the potential targeted gene of miR-630. MiRanda algorithms revealed that there was a potential seed sequence of miR-630 in the 3'UTR of SOX4 (Figure 4B). Then, we generated a wide type (WT) recombinant reporter plasmid containing 945 nucleotides 3'-UTR of SOX4 mRNA to investigate whether SOX4 is a target gene of miR-630. In order to construct the mutant (MUT) recombinant reporter plasmid, we manually changed the potential binding sites by exchanging the G and U, A and C. The WT and MUT reporter plasmids were co-transfected with 100 nM miR-630 mimics or mimics control into MDA-MB-231 cells. The results demonstrated that the luciferase activity was inhibited by miR-630 mimics in the WT vector ($P < 0.01$), while the inhibitory effect of miR-630 mimics was vanished in the MUT vector (Figure 4C). Therefore, these data suggested that SOX4 might be a target of miR-630 in MDA-MB-231 cells.

MiR-630 inhibits MDA-MB-231 cells migration and invasion

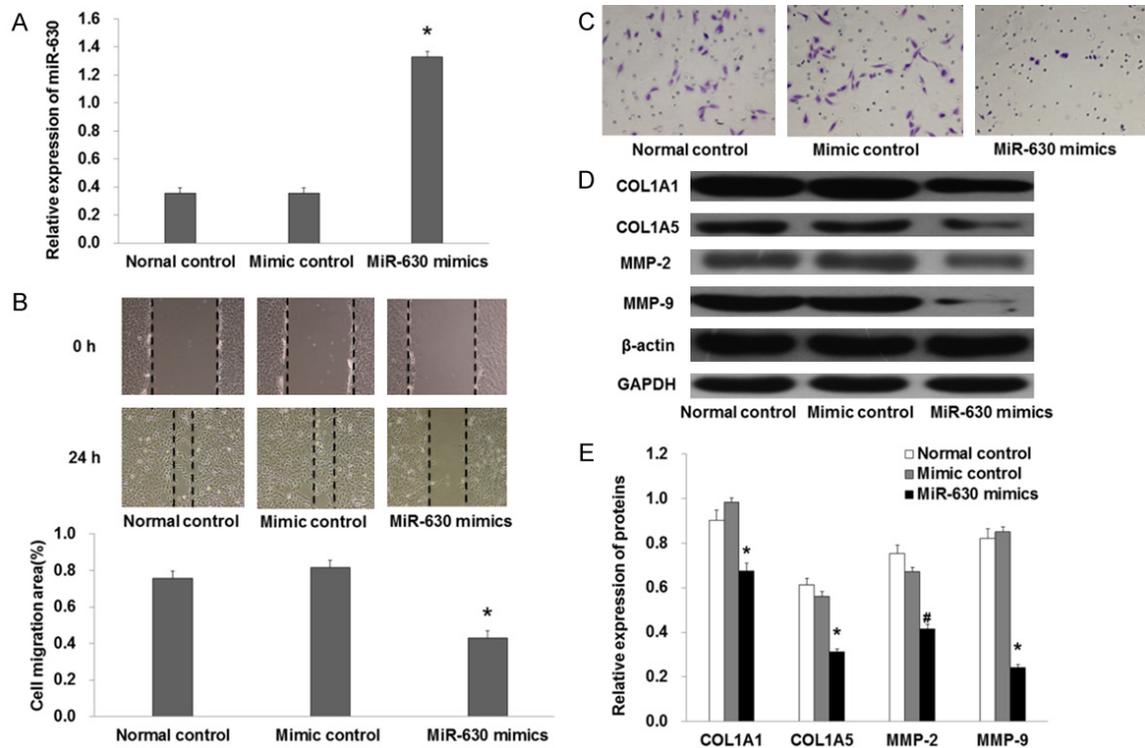


Figure 3. Restoration of miR-630 suppressed MDA-MB-231 cells migration and invasion in vitro. A: Relative expression of miR-630 in each group. B: Wound-healing assay showed that miR-630 inhibited MDA-MB-231 cells migration. C: Transwell chamber assay showed that miR-630 inhibited MDA-MB-231 cells invasion. D, E: The expression of COL1A1, COL5A1, MMP-2 and MMP-9 were measure by Western blotting in MDA-MB-231 cells. * $p < 0.01$ compared with mimic control.

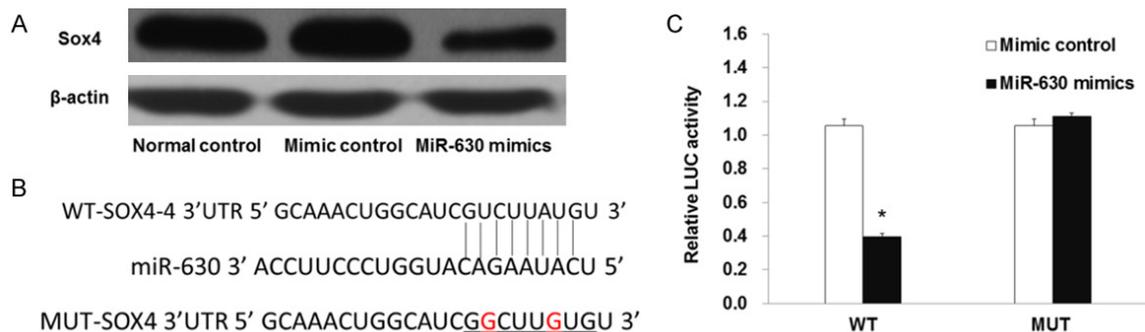


Figure 4. SOX4 was a direct target gene of miR-630 in MDA-MB-231 cells. A: Western blotting analyzes were used to detect the expression levels of SOX4 after miR-630 mimics or mimics control was transfected in MDA-MB-231 cells. B: The predictable binding site between miR-630 and SOX4 was shown. C: MiR-630 inhibited the luciferase activity in the SOX4 with WT binding sites 3'UTR, whereas, luciferase activity was not decreased in the MUT binding sites 3'UTR of SOX4 mRNA. WT: wild type; MUT: mutant. LUC: luciferase. * $p < 0.01$ vs mimic control.

SOX4 contributes to miR-630 suppressed migration and invasion of MDA-MB-231 cells

SOX4 was a directly target of miR-630 in MDA-MB-231 cells had been demonstrated by luciferase reporter assay, but the role of SOX4 in

miR-630-induced inhibition on MDA-MB-231 cells remains unknown. Over-expression of SOX4 could partly reverse the inhibitory effect of miR-630 on MDA-MB-231 cells migration and invasion (Figure 5). Taken together, these results strongly suggested that miR-630 played

MiR-630 inhibits MDA-MB-231 cells migration and invasion

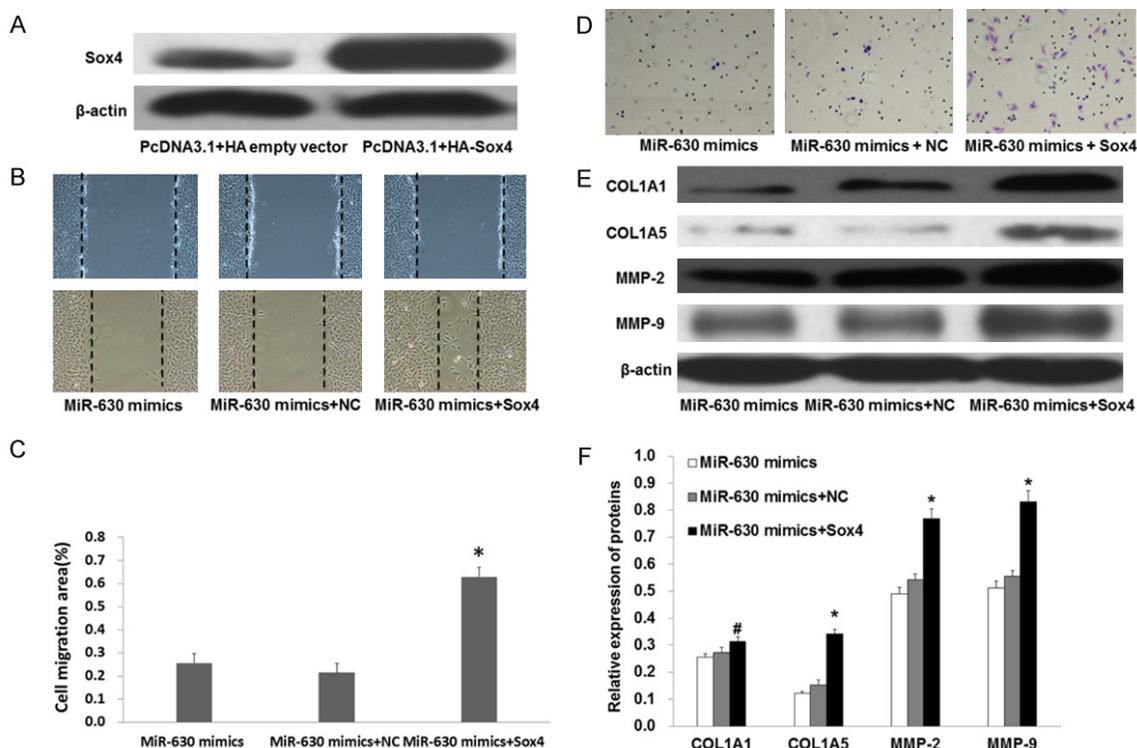


Figure 5. Over-expression of SOX4 partly abrogated miR-630 induced inhibitory effects on MDA-MB-231 cells. A: The expression of SOX4 was detected by western blotting analyzes. B, C: Cell migration ability was detected by wound-healing assay in MDA-MB-231 cells transfected with miR-630 mimics or miR-630 mimics+pcDNA3.1+HA-SOX4 or miR-630 mimics+pcDNA3.1+HA empty vector. D: Transwell chamber assay was performed in MDA-MB-231 cells. E, F: Western blotting analyzes were used to detect the expression of COL1A1, COL5A1, MMP-2 and MMP-9 in MDA-MB-231 cells. # $p < 0.05$ and * $p < 0.01$ compared with miR-630 mimics+pcDNA3.1+HA empty vector.

a crucial role in MDA-MB-231 cells migration and invasion by target SOX4 in TNBC.

Discussion

Advances in diagnostic techniques and therapeutic means have improved the early detection and reduced the mortality rate of breast cancer. However, it still is most common malignancy of women worldwide. Because of TNBC patients are unable to benefit from endocrine therapy or targeted therapy, it has higher mortality compared with NTNBC patients. The primary reason for its mortality and relapse is that TNBC cells have a powerful ability to metastasis [16]. In recent years, the great progresses has been made in the understanding of mechanisms of tumors, but tumor metastasis is a complex process including multiple sequential steps and the molecular mechanisms that regulate metastasis in TNBC cells are still poorly understood [17].

Since first discovered in 1993, miRNAs have been found to be involved in various processes

of many human diseases by regulating cell migration, invasion, proliferation, apoptosis and differentiation [1, 9, 18, 19]. Recently, it has been estimated that miRNAs could regulate at least 30% of all genes expression in human [20]. In this study, we found that miR-630 expression was down-regulated in TNBC and NTNBC patients compared to normal controls. What's more, COL1A1, COL5A1, MMP-2 and MMP-9 which had been reported as biomarkers involved in cell migration and invasion were also increased in the TNBC tissue [21-23]. So, we further investigated the roles of miR-630 on TNBC cells migration and invasion in vitro and found that overexpression of miR-630 significantly inhibited the migration and invasion ability of TNBC cells. These results suggested that miR-630 may be a tumor suppressor in the development and progression of TNBC.

At the molecular level, Song et al. reported that miR-630 targeted LIM domain only protein 3 (LMO3) to regulate cell growth and metastasis

in lung cancer [24]. Additionally, Cao et al. showed that MiR-630 could inhibit proliferation of human lung cancer A549 cells by targeting CDC7 kinase, but maintained the apoptotic balance by targeting multiple modulators [25]. Besides, in the field of breast cancer, Corcoran et al. found that miR-630 could regulate response to HER-targeting drugs by targeting IGF-1R in HER2 over-expressing breast cancer [10]. Recently Zhou et al. demonstrated that miR-630 suppressed breast cancer progression by targeting metadherin [26]. In this study, we found that over-expression of miR-630 significantly decreased the expression of SOX4 in MDA-MB-231 cells and further identified that SOX4 is a direct target of miR-630 in MDA-MB-231 cells by using bioinformatics and luciferase reporter gene assays.

SOX4 is an important transcription factor required for tissue development and differentiation in vertebrates [27]. Recently, overexpression of SOX4 was correlated with poor prognosis in patients with malignant tumor has been reported in many cancers including breast cancer [14, 15, 28, 29]. Hanieh et al. reported that down-regulation of SOX4 by aryl hydrocarbon receptor-microRNA-212/132 axis could suppresses metastasis in human breast cancer [13]. Additionally, Jin also found that MicroRNA-338-3p could function as tumor suppressor in breast cancer by down-regulation of SOX4 [30]. In our study, we found that over-expression of SOX4 could reverse miR-630 induced-migration and invasion of MDA-MB-231 cells. So we thought that SOX4 may be involved in miR-630 mediated TNBC metastasis.

In summary, in this study we demonstrated that miR-630 was down-regulated, while MMP-2, MMP-9, COL1A1, COL1A5 and SOX4 were up-regulated in TNBC tissue. We further investigated the role of miR-630 on MDA-MB-231 cells and found that miR-630 inhibited MDA-MB-231 cells migration and invasion by targeting SOX4. Our data provide new insight into the mechanism responsible for the development of human TNBC, which may also be benefit for the development of miRNA-directed diagnostic and therapeutic against TNBC.

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Disclosure of conflict of interest

None.

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