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MicroRNA miR-590-5p inhibits breast cancer cell stemness and metastasis by targeting SOX2

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Abstract. – OBJECTIVE: SOX2 (Sry-related high-mobility box SOX-2) is a transcription factor, which is essential for maintaining the cancer cell stemness. However, the role of microRNAs targeting SOX2 in cancer cell stemness remains unclear. We examined the effect of miR-590-5p, which targeted SOX2, on the breast cancer cell stemness and metastasis.

MATERIALS AND METHODS: We predicted and screened microRNA targeting SOX2, and further investigated the regulatory role of miR-590-5p on the level of SOX2 with Western blot, luciferase reporting assay and qRT-PCR analysis. Flow cytometry was performed to detect the effect of miR-590-5p on the breast cancer stem cell population with ALDEFLUOR Assay. We inoculated the breast cancer cells transfected with or without miR-590-5p to NOD/SCID mice to detect the tumorigenicity *in vivo*. Finally, forty-nine pairs of breast cancer samples and adjacent noncancerous tissues were obtained, and immunohistochemistry (IHC) with SOX2 antibody and qRT-PCR assay were used to quantify the expression of miR-590-5p in breast cancer samples.

RESULTS: miR-590-5p significantly downregulated the SOX2 protein expression, and inhibition of miR-590-5p increased SOX2 expression. The luciferase reporter assay indicated that miR-590-5p decreased the SOX2 3'UTR (3' untranslated region) reporter activity but not the luciferase activity of the mutant reporter, in which the binding sites for miR-590-5p were mutated. ALDEFLUOR Assay showed that miR-590-5p significantly decreased breast cancer stem cells population. NOD/SCID nude mice experiments indicated that miR-590-5p significantly inhibited tumorigenicity of breast cancer cells. IHC assay and qRT-PCR suggested that miR-590-5p expression was downregulated in breast cancer patients, and negatively correlated with SOX2.

CONCLUSIONS: miR-590-5p inhibited breast cancer cell stemness through targeting SOX2. Our study indicated that miR-590-5p might be a useful strategy for breast cancer treatment.

Key Words
miR-590-5p, SOX2, Cancer stem cell.

Introduction

Breast cancer is one of the most common cancers and the leading cause of death in women worldwide[1]. With the remarkable improvements in diagnosis and treatments, the mortality rate of breast cancer patients has significantly decreased. However, breast cancer recurrences and metastasis remain the most common cause of mortality even with the standard treatments[2,3]. Same as other solid tumor types, breast cancer has a small set of cancer stem cells (CSC), which have been suggested drive tumor initiation, progression and recurrences[4,5]. The CSC population is relatively resistant to radiation therapy and standard chemotherapeutics, thus pointing to the need for new-targeted treatments.

Sry-related high-mobility box (SOX)-2, an essential transcriptional factor in embryonic stem cell, is aberrantly expressed in a subset of breast cancer[6-8]. SOX2 has been reported to play an essential role in maintaining cancer stem cells[9]. The SOX2 expression has been found to positively correlate cancer cell stemness of solid tumors including breast cancer, and knockdown of SOX2 using siRNA was found to decrease invasiveness and cancer cell stemness[10-13]. MicroRNAs (miRNAs) are a class of small, non-coding RNAs that regulate protein synthesis through post-transcriptional suppression[14]. Deregulation of miRNA expression is involved in the development of cancers[15]. However, the miRNAs targets SOX2 to regulate breast cancer cell stemness are not reported. In this study, we predicted and screened the miRNAs targeting SOX2, and found miR-590-5p significantly downregulated the SOX2 protein expression, and inhibition of miR-590-5p increased SOX2 expression. The luciferase reporter assay indicated that miR-590-5p decreased the SOX2 3'UTR reporter activity but not lucife-

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rase activity of the mutant reporter in which the binding sites for miR-590-5p was mutated. ALDEFLUOR Assay showed that miR-590-5p significantly decreased breast cancer stem cells population. NOD/SCID nude mice experiments indicated that miR-590-5p significantly inhibited tumorigenicity of breast cancer cells. IHC assay suggested that miR-590-5p expression was downregulated in breast cancer patients, and negatively correlated with SOX2.

Materials and Methods

Plasmids, Cell Culture and Antibody

Expression plasmids of SOX2 was amplified by PCR using MCF-7 cDNA library as a template and cloned into pcDNA vector harboring FLAG epitope sequence. SOX2 3'UTR (3' untranslated region) luciferase reporter plasmid or its mutant were created by ligation of the wild-type and mutant miR-590-5p binding motif of SOX2 3'UTR (394 bp) into the pmirGLO Dual-luciferase miRNA target expression vector (Promega, Madison, WI, USA). Briefly, the 3'UTR of human SOX2 gene was obtained by PCR using the following primers: 5'-CGGAATTCATGGACCTTGTATAGATCTGG-3' and 5'-GCTCTAGAGATGGTTCGCCCGTCCTTG-3'. To introduce mutations into the seed sequences of the predicted miR-590-5p target sites within the SOX2 3'UTR, recombinant PCR was performed using the above-mentioned primers and the following primers: 5'-TCCATTGAAACGCCTAGGAGAATTTGC-3' (forward) and 5'- GCAAATTCTCCTAGGCGTTTCAATGGA-3' (reverse). Human breast cancer MCF-7 and ZR75-1 cell line were purchased from American Type Culture Collection (ATCC), and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Anti-SOX2 (ab97959) and anti-GAPDH (ab8245) were obtained from Abcam (Cambridge, MA, USA). miRNA miR-590-5p mimics and negative control (NC mimic), miR-590-5p inhibitors and negative controls were obtained from Genepharma (Shanghai, China)

Transfection of miR-590-5p Mimics and Inhibitor

MCF-7 and ZR75-1 cells were transfected 24 hr after being seeded in 24-well plates. miRNA mimics or inhibitor (50 nmol) was mixed with 5 μl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in 200 μl medium, and stood at room temperature for 20 min. The transfection solutions were then added to each well containing 500 μl of medium.

Extraction and qRT-PCR of miRNA

MiRNA of cell lines was extracted with the miRNeasy Mini kit (Qiagen, Hilden, Germany). Target miRNA was reverse transcribed to cDNA by a gene-specific RT primer using the miScript Reverse Transcription Kit (Qiagen, Hilden, Germany). MiRNA expression of cells was determined with miScript SYBR Green PCR Kit (Qiagen, Hilden, Germany) and performed on ABI7000 Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). The relative quantification value of the target, normalized to a control, was calculated by the comparative Ct methods.

SOX2-3'UTR Luciferase Assay

Cells were transfected with 0.2 μg of a SOX2-3'UTR reporter or mutant plasmids, 0.1 μg of pCMV-β-gal and where applicable, 50 nmol of miR-590-5p or control in 24-well plates. Following 48 h of incubation, cells were subjected to a luciferase reporter assay using Bright-Glo Luciferase Assay System (Promega, Madison, WI, USA). Luciferase activities were normalized by β-galactosidase activities. Each experiment was repeated at least three times in triplicate.

Quantitative Real-time RT-PCR (qRT-PCR)

qRT-PCR was performed to detect the SOX2 expression. After 48 hr 50 nmol miR-590-5p mimic or negative control transfection of ZR75-1 cells and MCF-7 cells, total RNA was extracted with TRIZOL (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription was performed by using a TaqMan Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). The expression of SOX2 was measured by qRT-PCR with SYBR Green PCR System (Qiagen, Hilden, Germany).

ALDEFLUOR Assay

ALDEFLUOR assay kit (StemCell Technologies, Vancouver, BC, Canada) was used to determine ALDH1 activity as described by the manufacturer[15]. Briefly, cells were suspended in ALDEFLUOR assay buffer containing an ALDH1 substrate and incubated for 1 h at 37°C. Diethylaminobenzaldehyde (DEAB), a specific inhibitor of ALDH1, was used as a negative control. Flow cytometry data were analyzed by FlowJo software (Ashland, OR, USA)

Tumorsphere Culture

For sphere-forming assays, 1,000 cells of MCF-7 and ZR75-1 cells were suspended in serum-free culture medium DMEM containing 1% N2 supplement, 2% B27 supplement, 1% antibiotic-antimycotic (Invitrogen, Carlsbad, CA, USA), 20 ng/ml human FGF-2 (Sigma-Aldrich, St. Louis, MO, USA), and 100 ng/ml EGF (Invitrogen, Carlsbad, CA, USA), and plated in ultra-negative attachment 6-well plates (Corning, Lowell, MA, USA). The number of spheroids larger than 50 μm was counted after 14 days under microscopy (Olympus, Tokyo, Japan).

Animal Model and In Vivo Tumor Formation Study

4-6 weeks old female NOD/SCID mice were purchased from Institute of Laboratory Animal of Chinese Academy of Medical Sciences (Beijing, China). All animal experiment protocols complied with National Health Guidelines of animal protection and use, which were approved by the IACUC Committee of Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Beijing, China).

ZR75-1 cells were transfected with miR-590-5p mimic or NC mimic for 24 hr. Cells were collected and inoculated into nude mice with 0.2 ml cell suspension of $1X10^6$ cells. The tumor sizes were measured using a caliper. On day 56, all tumors were collected to measure the tumor weights. The tumorigenicity is expressed using tumorigenicity ratio (the number of mice bearing tumors/the number of mice inoculated).

Immunohistochemistry

Fifty-two pairs of breast tumor samples and adjacent noncancerous tissues were obtained from the China PLA General Hospital with the informed consent of patients and with approval for experiments from the China PLA General Hospital. IHC analyses were performed as previously described. All of IHC staining was assessed by pathologists blinded to the origination of the samples. The widely accepted H-score system in considering the staining intensity and extent area was used. Briefly, H-score was generated by adding the percentage of strongly stained cells (3×), the percentage of moderately stained cells (2×), and the percentage of weakly stained cells (1×).

Statistical Analysis

Differences between variables were assessed by $\chi 2$ analysis, 2-tailed Student's *t*-test or Mann-Whitney U-test. Statistical calculations were performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). *p*-values of less than 0.05 were considered statistically significant.

Results

SOX2 is Regulated by miR-590-5p in Breast Cancer Cells

To investigate the role of SOX2-targeting miRNAs in breast cancer cell stemness, we used TargetScan and miRanda to screen for miRNAs that target SOX2. Our analysis predicted that miR-590-5p targeted SOX2. Western blot analysis showed that miR-590-5p inhibits SOX2 expression in MCF-7 and ZR75-1 breast cancer cells (Figure 1A). In contrast, inhibition of miR-590-5p increased SOX2 expression in the MCF-7 and ZR75-1 breast cancer cells (Figure 1B). However, miR-590-5p did not modulate the mRNA level of SOX2 (Figure 2C). To ascertain whether miR-590-5p directly regulates SOX2, we transfected MCF-7 cells with miR-590-5p and SOX2 3'UTR or 3'UTR mutated luciferase reporter. miR-590-5p decreased the SOX2 3'UTR reporter activity but not the mutant reporter in which the binding sites for miR-590-5p were mutated, suggesting that miR-590-5p specifically targets SOX2 and inhibits SOX2 expression by directly targeting its 3'UTR (Figure 1D).

miR-590-5p Regulates Breast Cancer Stem-like Cells

Since accumulating evidence showed that Sox-2 is essential to maintain the pluripotent stem cell phenotype, we examined the effect of miR-590-5p on ALDH1 expression, a common marker of breast cancer stem cells. As shows in Figure 2A, SOX2 overexpression increased ALDH1-positive cell population. In contrast, enforcing expression of miR-590-5p significantly decreased ALDH1-positive cell population. Furthermore, sphere growth assay indicated that ectopic expression of miR-590-5p decreased sphere growth in MCF-7 and ZR75-1 cells (Figure 2B).

Nod-SCID mice experiments showed that 1×10^6 ZR75-1 cells could form tumors with a tumor formation ratio of 5/6. In contrast, ZR75-1 cells transfected with miR-590-5p form tumors with a tumor formation ratio of 2/6. Our results suggested a potential role of miR-590-5p in breast cancer stem cells.

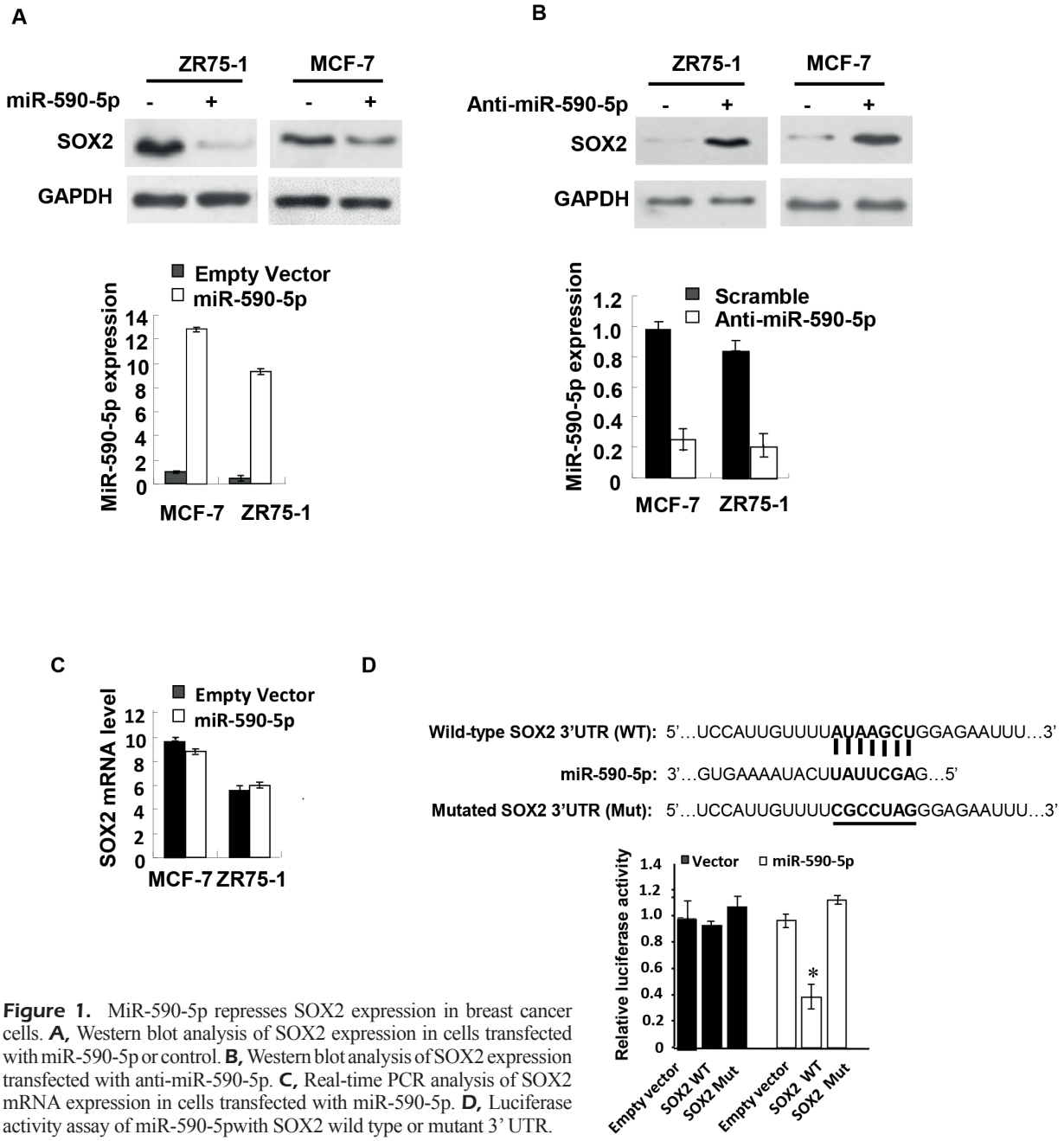

Figure 1. MiR-590-5p represses SOX2 expression in breast cancer cells. **A,** Western blot analysis of SOX2 expression in cells transfected with miR-590-5p or control. **B,** Western blot analysis of SOX2 expression transfected with anti-miR-590-5p. **C,** Real-time PCR analysis of SOX2 mRNA expression in cells transfected with miR-590-5p. **D,** Luciferase activity assay of miR-590-5pwith SOX2 wild type or mutant 3' UTR.

miR-590-5p Expression and Correlation with SOX2 i n Breast Cancer Patients

Firstly, we investigated the miR-590-5p expression in 49 pairs of breast cancer patients and their corresponding non-tumorous breast tissues by real-time RT-PCR. Compared with their corresponding non-tumorous counterparts, miR-590-5p expression was decreased in breast cancer tissues (p=5.01× 10^{-7}) (Figure 3A and B).

Then, we used immunohistochemistry (IHC) to detect SOX2 expression in 49 pairs of breast cancer tumors and matched non-tumor breast tissues, and found that SOX2 expression was up-regulated in breast cancer tissues (Figure 7C). Moreover, expression of miR-590-5p negatively correlated with SOX2 expression in breast cancer samples (p=2.3× 10^{-33}, r=-0.977) (Figure 7D). Together, these data show the important role of miR-590-5p and SOX2 in breast cancer.

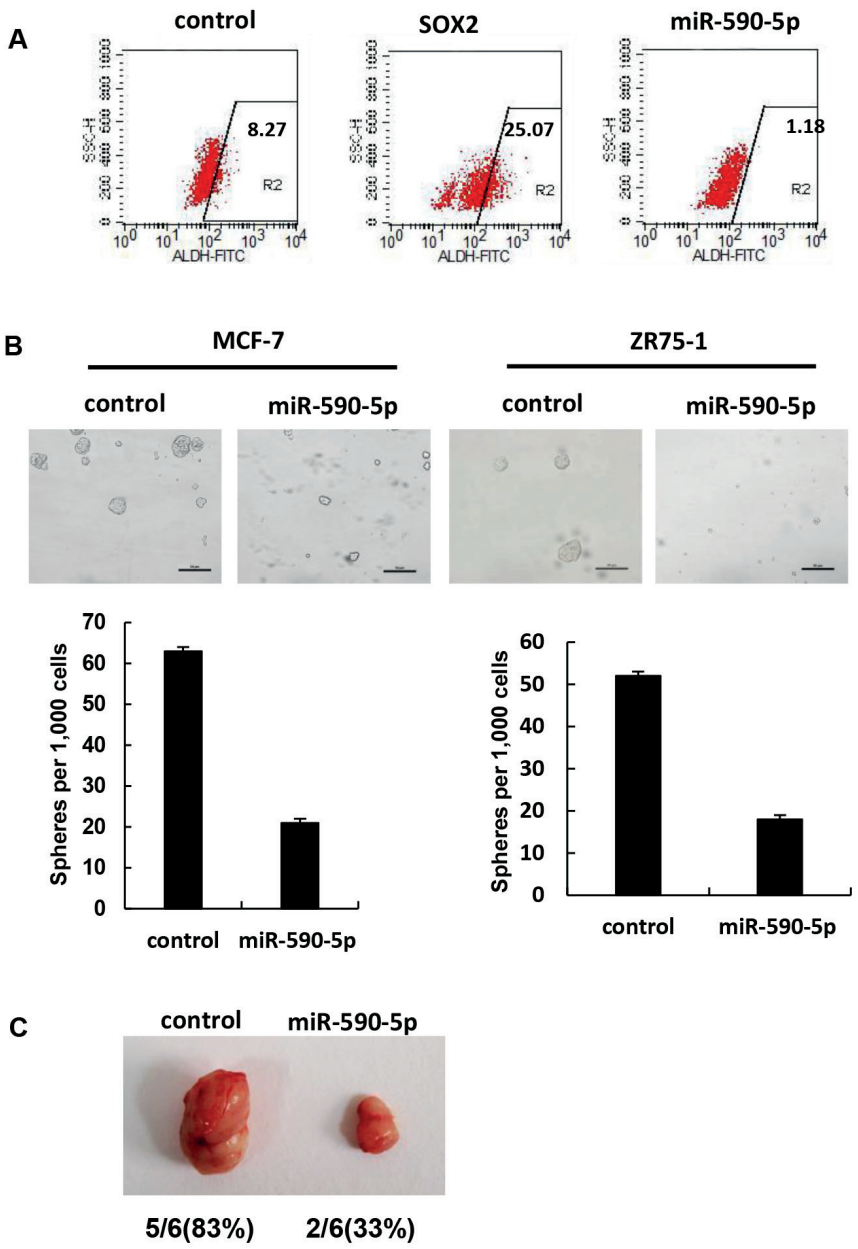

Figure 2. MiR-590-5p regulates breast cancer stem-like cells. **A,** Flow cytometry analysis of ALDH1 expression in cells transfected with miR-590-5p or control. **B,** Tumorsphere culture analysis of cells transfected with miR-590-5p or control. **C,** Tumor incidence of cells transfected with miR-590-5p or control by injection into Nod-SCID mice.

Discussion

A number of miRNAs have been reported to play an important role in cancer stem cell phenotype[17,18]. SOX2 plays an essential role in stem cell biology, which converts fibroblasts into inducible pluripotent stem cells[19,20]. SOX2 is a CSC-related transcription factor and is reported aberrantly expressed in breast cancer. However, the role of miRNAs targets SOX2 in breast cancer CSC properties remains unclear. In this study, we report that the miR-590-5p reduced SOX2 expression by directly targeting SOX2 3'UTR, which led to a decrease of breast CSC population. What's more, miR-590-5p significantly inhibited tumorigenicity of breast cancer cells. More importantly, miR-590-5p was downregulated in breast cancer patients, and negatively correlated with SOX2.

These miRNAs act as positive or negative regulators of cancer stem cell properties by targeting stem cell regulator. MicroRNA-191 increased CSC-like properties and was involved in neoplastic and metastatic properties of transformed human bronchial epithelial cells[21]. In contrast, miR-34a significantly inhibited CSC-phenotype and functionally reduced clonogenic and

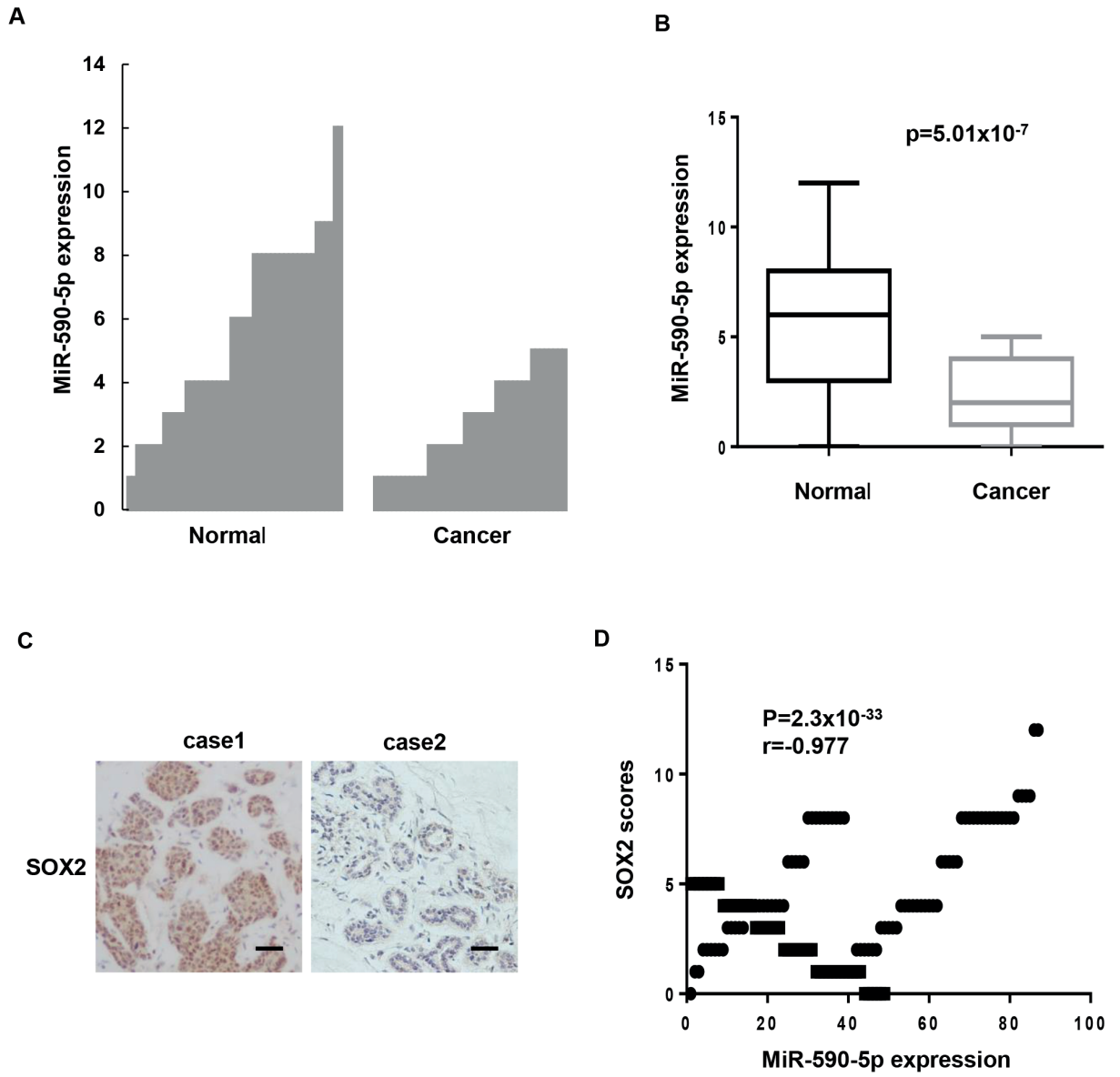

Figure 3. miR-590-5p expression and correlation with SOX2 in breast cancer patients. **A,** MiR-- 590-5p expression in human cancerous breast tissues and adjacent normal breast tissues was plotted using real-time RT-PCR. **B,** The Mann-Whitney U test was used for comparison of cancer tissues with normal tissues. B. Representative immunohistochemical staining of SOX2 in cancerous breast tissues and adjacent normal breast tissues. **C,** The relationship between miR--590-5p and SOX2 expression was detected by Spearman rank correlation analysis in breast samples.

invasive capacity in head and neck squamous cell carcinoma[22]. The importance of our finding was the observation that miR-590-5p acts as tumor stemness repressor in breast cancer. It is the first time that miR-590-5p if found, play an essential role breast cancer CSC phenotype.

Several targets of MicroRNA-590-5p had been found. MiR-590-5P inhibits S100A10 expression in HepG2 cells, and targets TGF-β RII to regulate hepatocellular carcinoma cells proliferation and invasion[23,24]. In endothelial cells, miR-590-5p mimics reduced LOX-1 expression and induced apoptosis. During osteoblast differentiation, microRNA-590-5p stabilizes Runx2 by targeting Smad7[25]. In this study, we find SOX2 is a novel target of microRNA-590-5p, and further study indicates that microRNA-590-5p/SOX2 axis regulates breast CSC property and inhibits tumorigenicity of breast cancer cells.

Recently, microRNAs have been found targeting SOX2. microRNA-21 and miR-371-5p were reported inhibited SOX2 expression through directly regulated SOX2 3'-UTR activity[26,27]. Here, we found microRNA-590-5p also targeted SOX2. The SOX2 expression has been associated with a stem cell state in human ovarian, cervical, pancreatic, head and neck squamous cell, and breast carcinoma. The SOX2 expression has been associated with tumors of high grade in several cancers. In this study, we found that miR-590-5p expression was downregulated in breast cancer patients and negatively correlated with SOX2, which suggested that miR-590-5p/SOX2 axis plays an important role in breast cancer development and progression. Therefore, miR-590-5p activation or SOX2 inhibition may be a useful strategy for breast cancer treatment.

Conclusions

Our study indicated that miR-590-5p inhibited breast CSC property through downregulating SOX2 expression. miR-590-5p was down-regulated and inversely correlated with SOX2 expression in breast cancer patients. Our investigation provides new insights for developing anti-breast cancer drugs based on miR-590-5p.

Conflicts of interest

The authors declare that no conflicts of interest exist.

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