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MiR-483 suppresses cell proliferation and promotes cell apoptosis by targeting SOX3 in breast cancer

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Abstract. – OBJECTIVE: To explore the mechanism underlying the effect of microRNA-483 (miR-483) in the progression of breast cancer (BC).

PATIENTS AND METHODS: MiR-483 expression was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) in both BC cells and tissue samples. The associations between miR-483 expression level and patients' overall survival rate were explored. Furthermore, cell proliferation assay and cell apoptosis assay were conducted, respectively. In addition, Western blot analysis and Luciferase assay were performed to explore the underlying mechanism.

RESULTS: The expression level of miR-483 was significantly decreased in tumor samples compared to that in adjacent tissues, which was also associated with patients' overall survival time. Moreover, cell growth was promoted, and cell apoptosis was inhibited after miR-483 was knocked down *in vitro*. Furthermore, SOX3 acted as a direct target of miR-483, and the expression of SOX3 was negatively correlated with the expression of miR-483 in tumor tissues.

CONCLUSIONS: These results suggested that miR-483 could suppress BC cell proliferation and promote BC cell apoptosis *via* targeting SOX3, which might be a potential therapeutic target in BC.

Key Words
Breast cancer, MiR-483, SOX3.

Introduction

Breast cancer (BC) is the most common carcinoma in female patients aging 40-60. The American Cancer Society indicated that about 232,340 cases were newly diagnosed in America in 2013, and almost 40,000 died of breast cancer. Moreover, about 12.5% of women will finally get breast cancer in her lifetime in the United States[1]. Chinese patients with breast cancer account for about 12.2% of all the newly discovered cases and 9.6% of mortality could be attributed to breast cancer[2]. Therefore, it is urgent to realize the mechanism underlying and develop new treatment strategies.

Micro-RNAs (miRNAs) were known as a class of small non-coding RNA with 18-22 nucleotides in length. MiRNAs were reported to interfere with protein synthesis and modulate the growth, differentiation, apoptosis and proliferation of cells either by inducing mRNA degradation or repressing translation[3]. Numerous researches have identified that miRNAs are closely related to various diseases these years. For instance, miR-497 was found to play an important role in diabetic nephropathy by regulating miR-497/ROCK signaling[4]. MiR-149-5p was remarkably up-regulated in non-small cell lung cancer cells and was also involved in gefitinib resistance[5]. MiR-199b-5p has finely regulated the development of medulloblastoma by targeting CD15, and the expression of miR-199b-5p was significantly decreased in patients with tumor metastasis[6]. By taking part in the Wnt/β-catenin signaling pathway, miR-34a was also proved to promote the progression of colon cancer[7].

Previous studies have suggested that a variety of miRNAs acted as tumor suppressors in breast cancer, such as miR-125, miR-205, miR-200 and miR-206 families[8]. MiR-483 played an important role in the development of several diseases, including alcoholic hepatitis, ovarian carcinoma, pancreatic cancer and diabetic cardiomyopathy[9-11]. However, the function of miR-483 in BC has not been revealed yet. We found that miR-483 expression was significantly down-regulated in BC tissues, and miR-483 repressed BC cell growth and induced cell apoptosis *in vitro*. Moreover, we also explored the mechanisms of miR-483 in the development of BC.

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Patients and Methods

Cell Lines and Clinical Samples

62 BC patients who received surgery at Liaocheng Infectious Disease Hospital were enrolled in this study. All the subjects signed an informed consent before the operation. None of the patients received radiotherapy or chemotherapy before the operation. The tissues obtained from the surgery were stored immediately at -80°C. All tissues were analyzed by an experienced pathologist. This study was approved by the Ethics Committee of Liaocheng Infectious Disease Hospital.

MCF-7, SKBR3, LCC2, MDA-MB-453, T-47D, LCC9 and normal human breast cell line (MCF-10A) were purchased from Shanghai Model Cell Bank (Shanghai, China). The culture medium consisted of 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), penicillin as well as Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY, USA). Besides, cells were cultured in an incubator at 5% CO_2 and 37°C.

Cell Transfection

MiR-483 mimics or miR-483 inhibitor (GenePharma Biotechnology Co., Ltd., Shanghai, China) were utilized to transfect BC cells. Non-specific siRNA was used as the negative control.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The total RNA was separated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, the total RNA was reverse-transcribed to cDNAs through reverse Transcription Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). Thermal cycle was as follows: 30 sec at 95°C, 5 sec for 40 cycles at 95°C, 35 sec at 60°C.

Western Blot Analysis

Reagent radioimmunoprecipitation (RIPA) (Beyotime, Shanghai, China) was utilized to extract protein from cells. Bicinchoninic acid (BCA) protein assay kit (TaKaRa, Dalian, China) was chosen for measuring the protein concentrations. The target proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and incubated with antibodies after transferring to the polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Cell Signaling Technology (CST, Danvers, MA, USA) provided us rabbit anti-GAPDH and rabbit anti-SOX3, as well as goat anti-rabbit secondary antibody. The chemiluminescent film was applied for the assessment of protein expression with Image J software.

Luciferase Assays

In our study, pGL3 vector (Promega, Madison, WI, USA) was used for carrying mutant 3′-UTR of SOX3 and wild-type (WT) 3′-UTR. Quick-change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) was used for the mutant of miR-483 binding site in SOX3 3′-UTR. Negative control or miR-483 mimics were co-transfected into BC cell with WT-3′-UTR or MUT-3′-UTR. The Luciferase activity was detected 48 h later by Dual-Luciferase reporter assay system (Promega, Madison, WI, USA).

Cell Proliferation Assay

The cell proliferation capacity was determined following the protocol of Cell Counting Kit-8 assay (CCK-8) assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). The absorbance of each well in the 96-well plates was measured every 24 h at 450 nm by using a microplate reader (Bio-Rad, Hercules, CA, USA).

Colony Formation Assay

The cells were placed in 6-well plates at a density of 5×10^2 per well and then cultured for 2 weeks. After being fixed in 70% ice-cold methanol for 10 min, the colonies were stained with 0.5% crystal violet for another 10 min. Then, the cells were washed three times by phosphate-buffered saline (PBS) before observation.

Cell Apoptosis Analysis

The apoptosis rate of osteoblast was estimated with Annexin V-APC/7-AAD Apoptosis Detection Kit II (KeyGEN Biotech Co., Ltd, Nanjing, China). The comparison was performed on Flow cytometry (FACScan, BD Biosciences, Franklin Lakes, NJ, USA) programmed with CellQuest software (BD Biosciences, Franklin Lakes, NJ, USA). All the experiments were repeated at least three times.

Statistical Analysis

Statistical Product and Service Solutions 20.0 (SPSS Inc., Chicago, IL, USA) was adopted to conduct the statistical analysis. Data were presented as mean ± SD. Chi-square test, Student *t*-test and Kaplan-Meier method were selected when appropriate. $p<0.05$ was considered statistically significant.

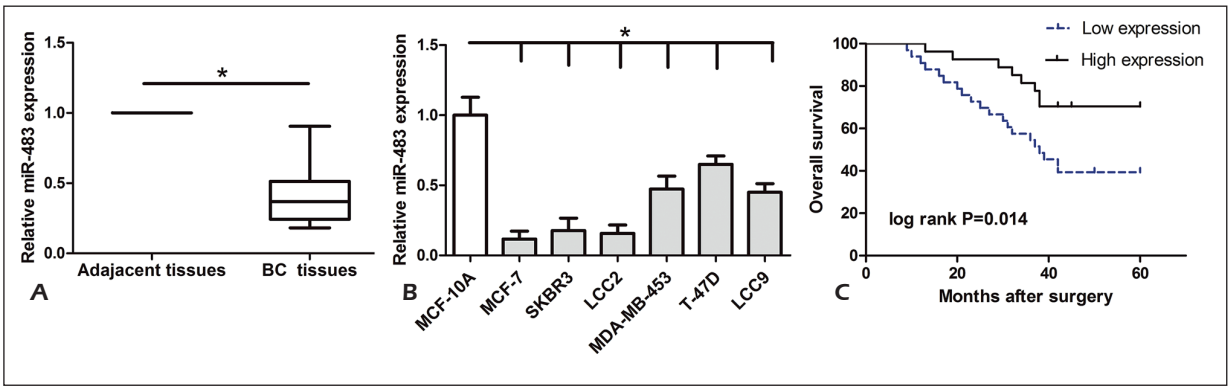

Figure 1. The expression level of miR-483 was decreased in BC tissues and cell lines and negatively associated with overall survival of BC patients. **A**, MiR-483 expression was significantly decreased in the BC tissues compared with adjacent tissues. **B**, Expression levels of miR-483 relative to β-actin were determined in the human BC cell lines and MCF-10A (normal human breast cell line) by RT-qPCR. **C**, High level of miR-483 was associated with better overall survival of BC patients. Data were presented as the mean ± standard error of the mean. *p<0.05.

Results

MiR-483 Expression Level in BC Tissues and Cells

First, qRT-PCR was conducted for detecting the miR-483 expression in 62 patients' tissues and 6 BC cell lines. We found that miR-483 was significantly down-regulated in tumor tissue samples (Figure 1A). The miR-483 level in BC cells was also markedly lower than that of normal human breast cell line (Figure 1B). Kaplan-Meier analysis showed that BC patients with higher miR-483 level had a better overall survival (Figure 1C).

Knockdown of MiR-483 Promoted Cell Growth in BC Cells

MCF-7 and T-47D BC cell lines were selected for the down-regulation of miR-483 in this study. The negative control and miR-483 inhibitor were synthesized and transfected into these two kinds of cells. Then qRT-PCR was utilized for detecting the miR-483 expression, which showed a remarkable decreased in miR-483 inhibitor-treated cells (Figure 2A). Moreover, the results of the CCK-8 assay revealed that after miR-483 was knocked down, the proliferation of BC cells was significantly enhanced (Figure 2B, 2C). The colony formation assay also indicated that after miR-483 was knocked down in BC cells, the number of colonies was remarkably increased (Figure 2D).

Knockdown of MiR-483 Suppressed Cell Apoptosis In Vitro

We then examined the effect of miR-483 on cell apoptosis. The results indicated that the apoptosis of both MCF-7 BC and T-47D BC cells was significantly repressed in the miR-483 inhibitor group compared with the negative control group (Figure 3A-B).

The Relation Between SOX3 and MiR-483 in BC

To further understand the mechanism underlying miR-483 in BC, we predicted SOX3 as a potential target gene of miR-483 via Starbase v2.0 (Figure 4A). QRT-PCR results showed that compared with the negative control group, the expression level of SOX3 in the miR-483 inhibitor group was significantly higher (Figure 4B). Western blot analysis revealed that the miR-483 inhibitor up-regulated the protein level of SOX3 (Figure 4C). Furthermore, the Luciferase assay demonstrated that the Luciferase activity of SOX3-WT cells transfected with miR-483 mimics was reduced, while no changes were observed in SOX3-MUT cells (Figure 4D). In addition, SOX3 expression in BC tissues was markedly higher than that in the adjacent tissues (Figure 4E). The correlation analysis also demonstrated that SOX3 expression level was negatively correlated to miR-483 expression in our breast cancer tissues (Figure 4F).

Discussion

Numerous studies have reported that miRNAs participated in the development of breast cancer by acting as post-transcriptional regulators of the expression of special genes. For example, miR-410 inhibited the development of Estrogen Receptor-Positive Breast Cancer by suppressing tumor cell growth and invasion[12]. MiR-519d-3p reduced the cell motility and proliferation of breast cancer cells by targeting LIM domain kinase1[13]. Also, the

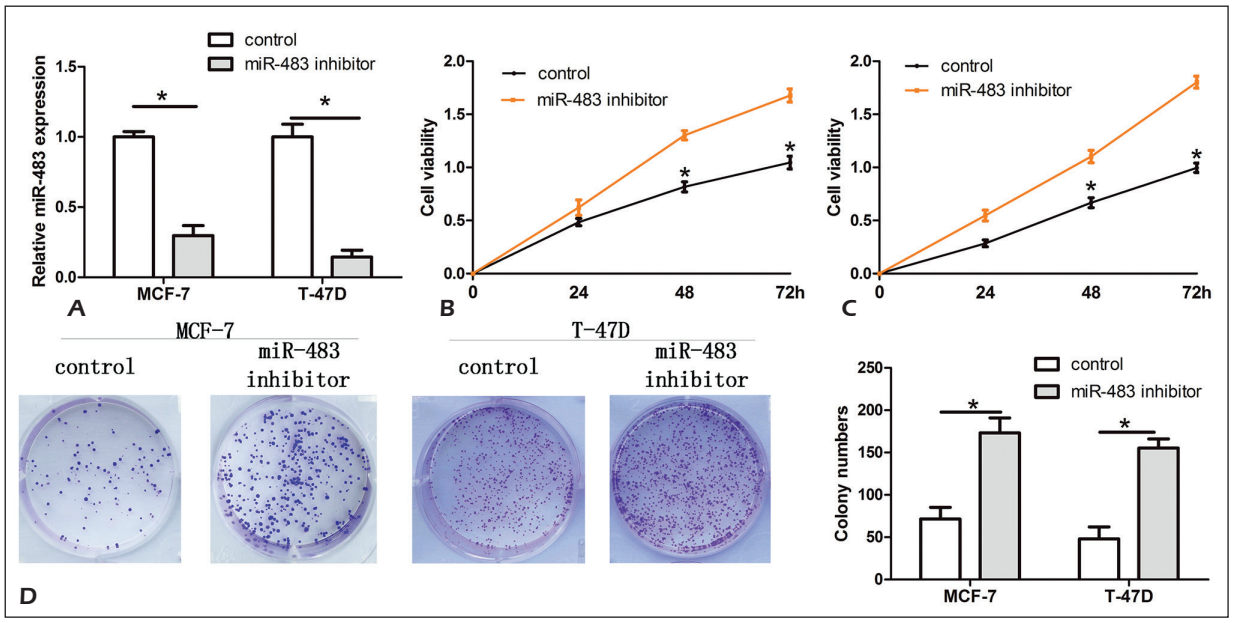

Figure 2. The knockdown of miR-483 increased BC cell proliferation. **A**, miR-483 expression in cancer cells transfected with negative control or miR-483 inhibitor was detected by qRT-PCR. β-actin was used as an internal control. **B**, CCK-8 assay showed that knockdown of miR-483 significantly increased cell proliferation in MCF-7 BC cells. **C**, CCK-8 assay showed that the knockdown of miR-483 significantly increased cell proliferation in T-47D BC cells. **D**, The colony formation assay showed that the knockdown of miR-483 significantly increased the number of colonies in MCF-7 and T-47D BC cells. The results represented the average of three independent experiments (mean ± standard error of the mean). *p<0.05, as compared with the control cells.

increased expression of miR-1207-5p promotes breast cancer cell proliferation *via* attenuating the expression of STAT6[14]. Moreover, by miR-424-5p was reported to target the doublecortin-like kinase 1 (DLK1), an oncogene to basal-like breast cancer, and regulate the proliferation, invasion and migration of cancer cell[15]. Accumulating evidence has proved that the mechanisms of breast cancer progression are closely related to multiple genes and microRNAs. Our study showed that miR-483 was down-regulated in BC samples and cells lines. Besides, a significant correlation was observed between patients' prognosis and the miR-483 expression level. Furthermore, after miR-483 was knocked down, BC cell growth was enhanced and BC cell apoptosis was reduced. The above results indicated that miR-483 repressed tumorigenesis of BC and might act as a tumor suppressor.

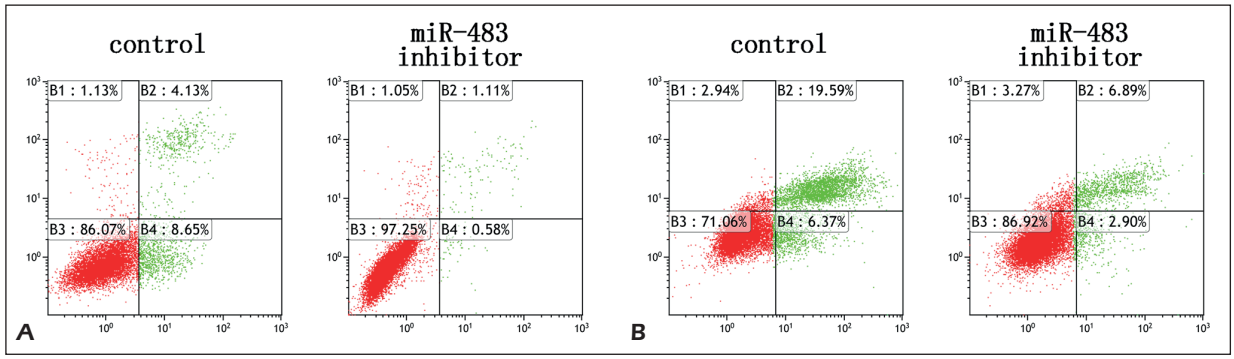

Figure 3. The knockdown of miR-483 repressed BC cell apoptosis. **A**, Cell apoptosis assay showed that MCF-7 BC cell apoptosis was markedly repressed in the miR-483 inhibitor group compared with that in the negative control group. **B**, Cell apoptosis assay showed that T-47D BC cell apoptosis was significantly repressed in the miR-483 inhibitor group compared with that in the negative control group. The results represented the average of three independent experiments (mean ± standard error of the mean). *p<0.05, as compared with the control cells.

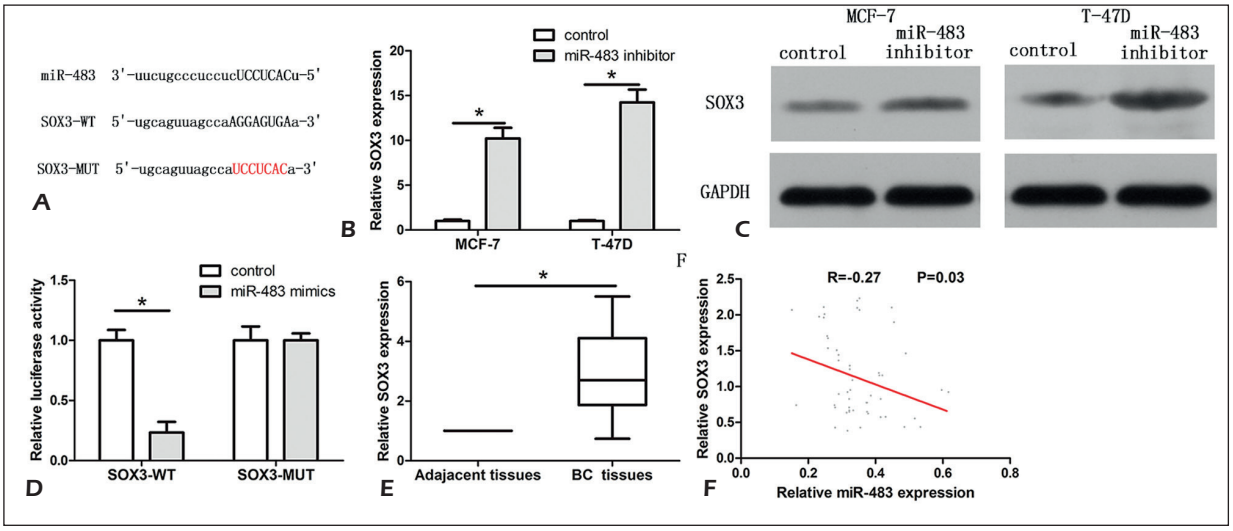

Figure 4. Relation between miR-483 and SOX3. **A**, The binding sites of miR-483 on SOX3. **B**, QRT-PCR results showed that SOX3 mRNA expression was increased in the miR-483 inhibitor group compared with that in the negative control group. **C**, Western blot analysis revealed that SOX3 protein expression was increased in the miR-483 inhibitor group compared with that in the negative control group. **D**, Co-transfection of miR-483 mimics and SOX3-WT in MCF-7 cells strongly decreased the Luciferase activity, while co-transfection of miR-483 mimics and SOX3-MUT did not change the Luciferase activity. **E**, SOX3 was significantly up-regulated in BC tissues compared with adjacent tissues. **F**, The linear correlation between the expression level of SOX3 and miR-483 in BC tissues. The results represented the average of three independent experiments. Data were presented as the mean ± standard error of the mean. *$p<0.05$.

Sox (Sry-like HMG box) protein is a kind of DNA-binding protein, which belongs to HMGb (high-mobility group box) protein superfamily[16]. Recent studies have shown that Sox3 was closely related to tumor development or incidence. For instance, the expression of Sox3 is significantly higher in small cell lung cancer (SCLC)[17]. Moreover, Sox3 was also found to promote lymph node metastasis in esophageal squamous cell carcinoma, thereby promoting the development of tumor[18]. In the present study, Luciferase assay revealed that SOX3 was directly bound to miR-483, and the SOX3 expression could be up-regulated after the knockdown of miR-483. Moreover, the SOX3 expression in BC tissues was negatively related to miR-483 expression. All the results above suggested that miR-483 might repress tumorigenesis of breast cancer *via* targeting SOX3.

Conclusions

We identified that miR-483 was remarkably down-regulated in breast cancer tissues and BC cells, and was also negatively related to overall survival of BC patients. Besides, miR-483 could suppress BC cell proliferation and promote BC cell apoptosis by targeting SOX3. These findings suggested that miR-483 might be a candidate target for the development of the therapy for BC.

Conflict of Interests

The authors declared that they have no conflict of interest.

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