Abstract. – OBJECTIVE: Breast cancer is one of the most common malignant tumors in women worldwide. Considering the poor therapeutic effect of breast cancer, we are supposed to dissect the functioning mode of miR-509-5p on breast cancer cell growth and metastasis, providing therapeutic targets for breast cancer.

PATIENTS AND METHODS: Quantitative Real-time PCR (qRT-PCR) assay was employed to detect miR-509-5p expression level. CCK8 assay and colony formation assay were incorporated to assess cell viability and proliferation capacities. Cell migration and invasion assay were performed to investigate metastasis capacity of breast cancer cells. Flow cytometry was used to identify cell apoptosis and cell cycle distribution. Protein levels were assessed by Western blotting assay. The target gene was predicted and verified by bioinformatics analysis and luciferase assay.

RESULTS: MiR-509-5p was obviously downregulated in breast cancer tissues when compared with pericarcinomatous tissues (n=76). Overexpressed miR-509-5p could attenuate breast cancer cell viability, proliferation, migration and invasion capacities, as well as promote cell apoptosis and induce cell cycle arrest at G0/G1 phase. Superoxide dismutase 2 (SOD2) was chosen as the target gene of miR-509-5p by bioinformatic analysis and Luciferase reporter assay. Moreover, restoration of SOD2 could rescue tumor suppression role of miR-509-5p on breast cancer tumorigenesis.

CONCLUSIONS: MiR-509-5p exerted tumor-suppressive effects on breast cancer progression and metastasis via targeting SOD2 in vitro, which provided an innovative and candidate target for diagnose and treatment of breast cancer.

Key Words: microRNAs, Proliferation, Metastasis, SOD2, Breast cancer.

Introduction

Breast cancer (BC) is one of the most common malignancies in women. Although the incidence of the disease in Euro-American developed countries tends to be stable and the mortality rate has declined, the incidence and mortality rate is increasing year by year worldwide, especially in China. According to the statistics of Chinese Academy of Medical Sciences, the number of new cases of BC was about 200,000 in 2010. The occurrence and development of BC is a complicated polygene-related process, the mechanism has not been fully identified. As a type of small non-coding RNA transcripts, microRNA is endogenous RNA with about 18 to 25 nucleotides in length. It suppresses gene expression through post-transcription in various biological processes. It can bind to the 3’UTR of their target genes to suppress protein translation. More and more studies discovered that microRNA could play an important role in various cell progressions. Therefore, well study of microRNA may be of great value in explaining the occurrence and development of tumors. MicroRNA-509-5p, a tumor-related miRNA, has been documented to be downregulated and plays an anti-oncogene role in several kinds of cancers, such as lung cancer, renal cell carcinoma, cervical cancer and hepatocellular carcinoma. Meanwhile, it has reported that miRNA participated in the process of BC tumorigenesis. However, the mechanism of action about miR-509-5p in development and progression of BC remains unknown. In the present study, we identified that miR-509-5p expression was decreased in BC tissues. Meanwhile, overexpressed miR-509-5p could suppress BC cell viability, proliferation, migration and invasion capacities in vitro, and prompted cell apoptosis and...
induced cell cycle arrest at G0/G1 phase. Further studies demonstrated that miR-509-5p could exert functions by modulating its target gene superoxide dismutase 2 (SOD2).

### Patients and Methods

#### Clinical Samples

76 pairs of BC tissues and matched pericarcinomatous tissues were obtained from patients undergoing routine surgery in the Affiliated Hospital of Qingdao University from 2015 to 2017. All surgical specimens were collected and then frozen immediately in liquid nitrogen until use. Tumor tissues were diagnosed and confirmed by pathological examination. This present research was approved by the Ethics Committee of Qingdao University. Written informed consents were signed from all participants before the study.

#### Cell Culture

The BC cell lines T-47D, SKBR3, LCC2, MCF-7, MDA-MB-453, DU4475, LCC9 and normal human breast cell line, MCF-10A, were purchased from Shanghai Model Cell Bank (Shanghai, China). Cells were cultured in media Roswell Park Memorial Institute 1640 (RPMI-1640) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. All cells were cultured in an incubator at 37°C with 5% CO2.

#### Plasmid and Transfection

For overexpression of miR-509-5p in BC cells, miR-509-5p mimics and corresponding negative control (miR-NC) were obtained from the Ribobio (Guangzhou, China). Transfections were performed using Lipofectamine 3000 Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocols. For overexpression of SOD2 for restoration, the SOD2 coding sequence was synthesized and inserted into vector pCDNA3.1 (Invitrogen, Carlsbad, CA, USA), and confirmed by sequencing. Empty pCDNA vector was used as control.

#### RNA Extraction and qRT-PCR

Total RNA was extracted from collected frozen BC tissues and matched paracarcinoma tissues using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s protocols. The relative expression level of miR-509-5p was determined using the mirVana™ qRT-PCR microRNA Detection kit (Ambion, Austin, TX, USA) according to the manufacturer’s protocols. U6 was used for normalization. Then we performed PCR reactions using the following primers: for miR-509-5p, forward, 5’- TAC TGC AGA CAG TGG CAA UCA -3’ and reverse, 5’- GTG CAG GGT CCG AGG T -3’; and for U6, forward, 5’- GCA CCT TAG GCT GAA CA-3’ and reverse, 5’-AGC TTA TGC CGA GCT CTT GT-3’. PrimeScript® RT reagent kit (TaKaRa, Dalian, China) was used to synthesize cDNAs. The relative mRNA expression level of SOD2 was measured by SYBR Green Real-time PCR and normalized to GAPDH using the following primers: for SOD2, forwards, 5’- ACC AGC ACT AGC AGC ATG T -3’ and reverse, 5’- CCT CCT TCT CAC CGG CAC AC -3’; and for GAPDH, forward, 5’-CGG AGT TGT TAT TCG TAT TC -3’ and reverse, 5’-TAC ATG ATG TGG ACG GCA TT-3’. QRT-PCR was carried out by utilizing the ABI 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, CA, USA).

#### Cell Counting kit-8 Assay

Cell viability was assessed by cell counting kit-8 (CCK8) assay (Promega, Madison, WI, USA) after the transfection according to the manufacturer’s protocols. The transfected cells were grown in 96-well plates (2000 cells/well), then 10 μL CCK8 solution was added into 90 μL Dulbecco’s Modified Eagle Medium (DMEM) and incubated for 3 h; the absorbance was measured at 490 nm.

#### Colony Formation Assay

Cell proliferation was detected by colony formation assay after the transfection. Cells were plated in 6-well plates at a density of 5×10^4 per well and were cultured for 2 weeks. The colonies were fixed in ice-cold 70% methanol for 10 min and stained with 0.5% crystal violet for another 10 min, then each well was washed 3 times with phosphate buffered saline (PBS).

#### Cell Apoptosis Analysis

Transfected cells suspension was prepared and double stained with 5 μL Annexin V-FITC and 1 μL propidium iodide (PI, 50 μg/mL). After the cells were incubated in the dark for 15 min, they were quantified by a flow cytometer equipped with CellQuest software (BD Bioscience, Rockville, MD, USA). The percentage of apoptotic cells was analyzed by Flowjo 7.6 (Version X; TreeStar, Ashland, OR, USA).
**Cell Cycle Analysis**
Transfected cells suspension were prepared and stained with propidium iodide using the BD CycleTest Plus DNA Reagent Kit (BD Biosciences, Rockville, MD, USA). The relative ratio of cells in G0/G1, S, or G2/M phase was analyzed by Flowjo 7.6 (Version X; TreeStar, Ashland, OR, USA).

**Wound Healing Assay**
Transfected cells were cultured in the 6-well plates marked by a horizontal line on the back. The cell was scratched by a pipette tip across the confluent cell layer. Next, the cells were washed gently and continued to culture with the serum-free medium for 24-48 h. Wound closure was captured using a light microscope (DFC500, Hanau, Germany).

**Transwell Assay**
Cells were cultured in the upper invasion chamber (BD, Rockville, MD, USA) coated with Matrigel. Serum-free medium was added into the upper chamber, whereas 10% FBS medium supplemented was added into the lower. After 48 h, the cells that were cultured on the upside of the filter, which did not invade through the chamber, were removed. The chamber was suspended by 100% precooling methanol, stained with 0.05% crystal violet and inspected with the microscope (Olympus, Tokyo, Japan). The values for the invasion cells were measured by counting five fields per membrane.

**Bioinformatics Analysis**
TargetScan (http://www.targetscan.org/vert_71/) and starBase v2.0 (http://starbase.sysu.edu.cn/index.php) were utilized to forecast the target genes. As shown in the database, SOD2 was the candidate gene we chose. The result of bioinformatic software indicated that 3'-UTR of SOD2 binds to miR-509-5p. Then qRT-PCR was performed to detect whether SOD2 was really inversely correlated with miR-509-5p expression in BC cells. Besides, Kaplan Meier-plotter (http://kmplot.com/analysis/index.php?p=service) database were utilized to forecast the prognosis of BC patients with ectopic expression of SOD2.

**Luciferase Reporter Assay**
SOD2 3'-UTR vector for luciferase reporter was purchased from Genechem (Montreal, Quebec, Canada). Cells were transfected with pGL3 luciferase expression construct containing the 3'UTR of SOD2 pRL-TK Renilla luciferase vector (Promega, Madison, WI, USA), and miR-509-5p mimics or miR-NC (Promega, Madison, WI, USA). Duo-Glo luciferase assay kit (Promega, Madison, WI, USA) was used to detect the luciferase signal following the manufacturer’s instructions. Meanwhile, we mutated the predicted miR-509-5p binding site on 3'UTR of SOD2, and examined whether this mutation could abrogate the decrease in luciferase activity by miR-509-5p mimics.

**Western Blot Analysis**
To investigate relative protein expression level, cells were lysed and the concentration of collected protein was measured using a protein assay kit purchased from Beyotime (Shanghai, China). The extracted protein (sum of 20 μg) was degenerated and chilled on ice. 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate protein and it was shifted to polyvinylidene fluoride (PVDF) membranes purchased from Millipore (Billerica, MA, USA). 5% fat-free milk was used to block non-specific protein interactions in tris buffered saline-tween (TBST) which contains Tris-HCl (50 mM), NaCl (150 mM) and Tween 20 (0.05%) at 4°C for 1 h. The membranes loaded with proteins were incubated at 4°C within the fat-free milk overnight with the following primary antibody against SOD2 (Absci, Nanjing, China). TBST buffer was used to wash the unbound antibody (10 min each time for three times). Then, secondary antibody was employed to incubate the membranes conjugated with horseradish peroxide (HRP) 1 h at room temperature. After these membranes were washed three times in TBST buffer, we developed the membranes using ECL (Millipore, Billerica, NY, USA) following the instructions.

**Statistical Analysis**
SPSS 18.0 software (IBM, Armonk, NY, USA) was used for statistical analysis, statistical data was presented with Graph PAD prism software (San Diego, CA, USA), and quantitative data was presented as mean ± SD. The regression and correlation analysis were analyzed using the Spearman X^2 test. The relative expression of mRNA was measured using the method of 2^ΔΔCT. p < 0.05 was seemed as statistically significant.
### Results

**MiR-509-5p Expression was Decreased in BC Tissues and Cell Lines**

The expression of miR-509-5p was detected in 76 pairs of BC tissues and pericarcinomatous tissues by qRT-PCR. The result indicated that miR-509-5p expression was remarkably decreased in BC tissues compared with the paired pericarcinomatous tissues on mRNA level (Figure 1A). This evidence implied that miR-509-5p might participate in BC tumorigenesis. Then, we investigated expression of miR-509-5p in several BC cell lines and normal breast cell line with qRT-RCR. It showed that, compared with MCF-10A cell line, all these BC cell lines expressed a relatively lower level of miR-509-5p, in which MCF-7 expressed the relatively lowest (Figure 1B). To identify the mode of action of miR-509-5p in BC tumorigenesis in vitro, MCF-7 cell line was transfected with miR-509-5p mimics and miR-NC for overexpression of miR-509-5p (Figure 1C).

**MiR-509-5p suppressed BC Cell Viability and Proliferation**

As shown in CCK8 assay, the viability of MCF-7 cells was significantly inhibited after transfected with miR-509-5p mimics when compared with miR-NC, in a time-dependent manner (Figure 2A). Meanwhile, we carried out colony formation assay to further explore cell proliferation capacity. It showed that there were fewer formed colonies of MCF-7 cells transfected with miR-509-5p mimics when compared with miR-NC (Figure 2B). Collectively, all these results showed that miR-509-5p could inhibit BC proliferation and viability.

**MiR-509-5p Promoted Cell Apoptosis and Induced Cell Cycle Arrest at G0/G1 Phase**

Since miR-509-5p could regulate cell proliferation capacity, then we were wondering whether cell apoptosis and cell cycle were also affected by miR-509-5p. As shown in flow cytometric analysis, the apoptotic rate of MCF-7 cells transfected with miR-509-5p mimics increased remarkably compared with miR-NC (Figure 2C). Moreover, the percentage of MCF-7 cells transfected with miR-509-5p mimics increased in G0/G1 phase while decreased in S phase obviously when compared with miR-NC (Figure 2D). The results indicated that miR-509-5p impaired BC cell proliferation capacity by promoting cell apoptosis and inducing cell cycle arrest at G0/G1 phase.

**MiR-509-5p Suppressed BC Cell Migration and Invasion**

We next evaluated the action role of miR-509-5p in BC cell metastasis in vitro. As shown in wound-healing assay, overexpressed miR-509-5p could suppress BC cell migration when compared with miR-NC (Figure 2E). Meanwhile, influence of miR-509-5p on cell invasion measured by using transwell assay was the same as the former

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**Figure 1.** MiR-509-5p expression was decreased in BC tissues and cell lines. **A:** analysis of miR-509-5p expression in paracarcinoma tissues (P) and tumor tissues (T); **B:** analysis of miR-509-5p expression in several BC cell lines and normal cell line; **C:** analysis of transfection efficiency in MCF-7 cells transfected with miR-509-5p mimics and miR-NC. Total RNA was detected by qRT-PCR and GAPDH was used as an internal control. Data are presented as the mean ± SD of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001.
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To better understand the mechanism about how miR-509-5p participated in these biological processes, we selected SOD2 as the potential downstream of miR-509-5p via using TargetScan and starBase database (Figure 3A). According to the consequence of prediction, miR-509-5p was transfected with SOD2 3’UTR luciferase reporter gene into MCF-7 cell. The results suggested that the luciferase activity was declined in BC cell transfected with wild-type SOD2 and miR-509-5p mimics when compared with cells transfected with mutated wild-type SOD2+ miR-NC (Figure 3B), indicating that SOD2 was a target of miR-509-5p. Meanwhile, we further detected expression level of SOD2 in transfected BC cells. The results indicated that SOD2 was downregulated in MCF-7 cells transfected with miR-509-5p mimics on mRNA level and protein level when compared with miR-NC (Figure 3C-D). All these results indicated that SOD2 was directly targeted by miR-509-5p.

Restoration of SOD2 Rescued Tumor Suppression Role by miR-509-5p

To further identify the interaction relationship between miR-509-5p and SOD2, we firstly measured the expression level of SOD2 in BC tissues. The results indicated that SOD2 was obviously overexpressed in BC tissues when compared with the paraneoplastic tissues on the mRNA level (Figure 4A), and the expression level of SOD2 was inversely correlated with miR-509-5p in BC tissues (Figure 4B). Moreover, we identified that overexpression of SOD2 led to a potential trend to reduce the survival rate of BC patients using Kaplan Meier-plotter database analysis (Figure 4C). Secondly, we explored whether SOD2 is responsible for the functional effects of miR-509-5p in BC tumorigenesis. We overexpressed SOD2 expression by transfected with pCDNA3.1-SOD2 in miR-509-5p-overexpressing MCF-7 cells (Figure 4D). SOD2 restoration not only increased the proliferation capacity of miR-509-5p-transfected cells (Figure 4E), attenuated cell apoptosis and induced cell cycle distribution at G0/G1 phase (Figure 4F-G), but also increased cell migration and invasion capacities partially, when compared with...
The results implied that miR-509-5p suppressed BC tumorigenesis by regulating SOD2.

**Discussion**

Increasing studies suggest that microRNAs play a crucial role in carcinogenesis and cancer progression of various types of tumors. For example, Xia et al.\(^\text{13}\) revealed that miR-22 could suppress the development and differentiation of colorectal cancer cells and miR-22/Spl/PTEN/AKT axis might represent a potential therapeutic target for colorectal cancer. Karimi et al.\(^\text{14}\) found that resveratrol could inhibit apoptosis, invasion, and switch EMT to MET phenotype through upregulation of miR-200c in colorectal cancer. Xiao et al.\(^\text{15}\) demonstrated that miR-100 might suppress osteosarcoma cell growth and decrease osteosarcoma cell chemo-resistance by targeting ZNRF2. Du et al.\(^\text{16}\) reported that miR-
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543 promoted prostate cancer cell growth and metastasis via targeting RKIP. BC has always been the serious threat to women in the world. In recent years, increasing studies implied that microRNAs played an important role in the genesis and development of BC. For example, Li et al.\textsuperscript{17} revealed that miR-148a functioned as a pivotal regulator in BC through targeting BCL-2; Wang et al.\textsuperscript{18} demonstrated that miR-766 could function as a novel tumor suppressor by enhancing p53 signal pathway. Jones et al.\textsuperscript{19} found that miR-200c could serve as a regulator of mesenchymal tumor cell growth via regulating expression of Flt1 and Vegfc. To date, there has not been any study of the relationship between miR-509-5p and BC tumorigenesis. In our present study, we demonstrated that miR-766 could function as a novel tumor suppressor by enhancing p53 signal pathway. Jones et al.\textsuperscript{19} found that miR-200c could serve as a regulator of mesenchymal tumor cell growth via regulating expression of Flt1 and Vegfc.

**Figure 4.** Restoration of SOD2 rescued tumor suppression role by miR-509-5p. A: analysis of SOD2 expression level in BC tissues (T) and matched paracarcinoma tissues (N), n=76; B: correlation between miR-509-5p and SOD2 expression in BC tissues (n=76); C: relationship between SOD2 and prognosis of patients with BC; D: analysis of transfection efficiency in MCF-7 cells transfected with miR-509-5p negative control (NC), mimics and/or pCDNA3.1-SOD2; E: overexpressed SOD2 rescued suppressed cell proliferation by miR-509-5p; F: overexpressed SOD2 attenuated cell apoptosis; G: overexpressed SOD2 attenuated cell cycle distribution at G0/G1 phase; H: overexpressed SOD2 increased cell migration of miR-509-5p-transfected cells; I: overexpressed SOD2 increased cell invasion of miR-509-5p-transfected cells. Data are presented as the mean ± SD of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

**Conclusions**

Our present study demonstrated that miR-509-5p had tumor-suppressive effect on BC progression and metastasis via targeting SOD2 in vitro. Our findings may help elucidating the molecular mechanisms underlying BC progression and provide miR-509-5p as an innovative and candidate target for diagnose and treatment of BC.

**Conflict of Interest**

The Authors declare that they have no conflict of interests.


