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MicroRNA-9501 inhibits breast cancer proliferation and metastasis through regulating Wnt/β-catenin pathway

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Abstract. – OBJECTIVE: This research was designed to explore the expression characteristics of microRNA-9501 in breast cancer (BCa), and to further explore whether it can influence the development of BCa through the regulation of Wnt/β-Catenin pathway.

PATIENTS AND METHODS: QPCR was carried out to examine microRNA-9501 level in tumor tissue samples and paracancerous ones collected from 42 BCa patients, and the interplay between microRNA-9501 expression and the clinical indicators, as well as the prognosis of BCa patients were analyzed. In addition, we detected microRNA-9501 expression in BCa cell lines by qPCR. Subsequently, microRNA-9501 overexpression model was constructed in BCa cell lines MCF-7 and MDA-MB-231. Then, CCK-8, EdU, cell wound healing, as well as transwell assays, were carried out to evaluate the impact of microRNA-9501 on the biological functions of BCa cells. Finally, the Dual-Luciferase reporting test and tumor formation experiment in nude mice were conducted to further clarify the potential molecular mechanism.

RESULTS: QPCR results indicated that microRNA-9501 level in tumor tissue specimens of BCa patients was remarkably higher than that in adjacent ones, and the difference was statistically significant. Compared with patients with high expression of microRNA-9501, patients with lowly-expressed microRNA-9501 had higher tumor stage, higher incidence of lymph node or distant metastasis, and lower overall survival rate. In addition, compared with control group, cells in microRNA-9501 overexpression group showed a significant decrease in proliferation rate, invasiveness, and migration ability. Meanwhile, luciferase reporting assay revealed that overexpression of β-Catenin remarkably attenuated the luciferase activity of the vector containing wild-type microRNA-9501 sequences, further demonstrating that microRNA-9501 can be targeted by β-Catenin. Meanwhile, qPCR revealed a negative association between β-Catenin and microRNA-9501 in BCa tissues. Finally, tumor-bearing experiments in nude mice also demonstrated that microRNA-9501 may suppress the malignant growth of breast tumor.

CONCLUSIONS: MicroRNA-9501 expression was found remarkably decreased in BCa tissues and cell lines, which was closely relevant to the pathological stage, metastasis incidence, and prognosis of BCa patients. In addition, microRNA-9501 may suppress the malignant progression of BCa via modulating Wnt/β-Catenin path-way.

Key Words:
MicroRNA-9501, Wnt/β-Catenin pathway, BCa, Proliferation, Metastasis.

Introduction

Breast cancer (BCa) is a common female malignant tumor, which seriously endangers women's health all over the world[1-3]. In China, BCa ranks third in female malignant tumor. Recently, due to the advancement of treatment and prevention methods, the incidence of BCa is on the decline, but on a global scale, there are still about 200 new cases of BCa growth annually. For the Western developed countries, incidence of BCa in female malignant tumor diseases ranks first[4,5]. Due to various factors, the long-term survival rate and prognosis of BCa patients are still not ideal[6,7]. So far, the patients' 5-year survival rate in advanced stage is still less than 10% since the tumor cells have begun to metastasize or have undergone extensive spread[8,9]. In the study of BCa, the discovery of a new early genetic marker can greatly promote the early diagnosis of BCa and improve the cure rate, which has important research value[10,11]. Briefly, the study on the pathogenesis and development process of BCa is of great significance to improve the therapeutic effect of BCa and improve the survival rate of patients.

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MicroRNAs can modulate genes by complementary pairing with the 3'-UTR of target gene mRNAs and lead to their degradation[12-14], or inhibit protein translation when the base is not completely paired with the target gene[15]. They play a pivotal role in cell proliferation, morphology, apoptosis, differentiation, and other aspects and have the characteristics of tissue specificity. In addition, miRNA has been confirmed to play a vital part in the process of tumor progression[16-18]. The abnormal expression of miRNA can regulate the biological behavior of tumor cells by regulating the mRNA of target gene and changing its expression[18]. At the present stage, researchers have carried out multiple attempts starting from targeted therapy and individualized therapy of tumors, and some achievements have been achieved[19-21]. Hence, the search for new molecular targets is of great significance for the diagnosis of BCa patients and the improvement of patients' prognosis. MicroRNA-9501 has been discovered for a long time, but its biological function has only just begun to be studied. Xi et al[22] have shown that microRNA-9501 level is decreased in many tumors, and microRNA-9501 plays a role in tumor inhibition and participates in several physiological and pathological processes.

The core of functional research on miRNA is the research on miRNA target genes, which clarifies how miRNA participates in regulating cell life activities by regulating target genes and influence cell biological behaviors[23,24]. The focus and difficulty of miRNA research has been to find and clarify the target genes regulated by miRNA and the signaling pathways involved[25]. In our research, through bioinformatics analysis, we found that microRNA-9501 may mediate the proliferation and metastasis of BCa through the regulation of β-Catenin. Therefore, we detected the expression of microRNA-9501 and its target gene β-Catenin in breast cancer tissues by qPCR and analyzed the interplay between microRNA-9501 and certain clinical parameters of BCa patients.

Patients and Methods

Patients and BCa Samples

56 pairs of BCa tissue samples and their corresponding adjacent ones were selected from surgically treated BCa cases and then collected at –80°C. The collection of clinical specimens was approved by the local Ethics Monitoring Committee, and patients and their families had been fully informed that their specimens would be used for scientific research, and all participating patients had signed informed consent.

Cell Lines and Reagents

The human BCa cell lines and normal mammary epithelial cells were provided by American Type Culture Collection (ATCC; Manassas, VA, USA). MCF-7 and MDA-MB-231 were treated with Dulbecco's Modified Eagle's Medium (DMEM) high glucose medium containing 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL). SKBR3 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium containing 10% fetal bovine serum (FBS) and antibiotics. All cells were cultured in a 37°C, 5% CO_2 incubator. When the cells were grown to 80%-90% confluence, they were digested with 1×trypsin+ EDTA (ethylenediaminetetraacetic acid).

Transfection

Control group (NC mimics) and the vector containing the microRNA-9501 sequence (microRNA-9501 mimics) were purchased from Shanghai Jima Company (Shanghai, China). Cells were plated in 6-well plates and grown to a cell density of 40-60%. Then, transfection was performed according to the manufacturer's instructions. After 48 h, cells were collected for qPCR and Western Blot analysis and cell function experiments.

CCK-8 Assay

Cells were collected and plated into 96-well plates at 2000 cells per well. After cultured for 24 h, 48 h, 72 h, and 96 h respectively, and CCK-8 (Dojindo Laboratories, Kumamoto, Japan) reagent was added. After incubation for 2 h, the OD value was measured in the microplate reader at 490 nm absorption wavelength.

5-Ethynyl-2'-Deoxyuridine (EdU) Proliferation Assay

To detect BCa cell proliferation, the EDU proliferation assay (RiboBio, Guangzhou, China) was performed. Cells transfected for 24 h were incubated with 50 μm EDU for 2 h, and then stained with Apollo and 4',6-Diamidino-2-Phenylindole (DAPI), and the number of EDU-positive cells was then detected by fluorescence microscopy. The display rate of EDU positive was shown as the ratio of the number of EDU positive cells to the total DAPI chromogenic cells (blue cells).

Transwell Assay

The cells after transfection for 48 h were digested, centrifuged, and resuspended in serum-free medium to adjust the density to 5 x 10^5 cells/mL. According to the different migration abilities of each cell line, cells were put back into the incubator and continued to culture for a specific time. The transwell chamber was taken out, washed 3 times with phosphate-buffered saline (PBS), and placed in methanol for 15 min cell fixation. Cells were observed under the microscope, and 10 fields of view were randomly selected for counting and statistical analysis was performed.

Cell Wound Healing

The cells after transfection for 48 h were digested, centrifuged, and resuspended in medium without FBS to adjust the density to 5 x 10^5 cells/mL. The density of the plated cells was determined according to the size of the cells (the majority of the number of cells plated was set to 50000 cells/well), and the confluency of the cells reached 90% or more the next day. After the stroke, cells were rinsed gently with PBS for 2-3 times and observed again after 24 h incubation with low-concentration serum medium (such as 1% FBS).

QPCR

RNA was extracted and reverse transcribed into cDNA using Primescript RT Reagent. QPCR reactions were performed using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). The primers were: microRNA-9501: F: 5'-CTAGGGGACTGGGCTGCT-3', R: 5'-AGGGTCTTAGGTTCCAGGCA-3'; U6: F: 5'-ATTGGAACGATACAGAGAAGATT-3', R: 5'- GGAACGCTTCACGAATTTG-3'; β-Catenin: F: 5'-CATCTACACAGTTTGATGCTGCT-3', R: 5'-GCAGTTTTGTCAGTTCAGGGA-3'; β-actin: F: 5'-CTCCATCCTGGCCTCGCTGT-3', R: 5'-GCTGTCACCTTCACCGTTCC- 3'. The relative expression levels of mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method.

Western Blot

Cells were lysed using cell lysis buffer, shaken on ice for 30 min, and centrifuged at 14,000 x g for 15 min at 4°C. The extracted proteins were separated using a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane. Immunoblotting was performed according to standard procedures.

Dual-Luciferase Reporting Assay

A reporter plasmid was constructed in which a specific fragment of the target promoter was inserted in front of the luciferase expression sequence. The transcription factor expression plasmid to be detected was co-transfected with the reporter gene plasmid into the MCF-7 and MDA-MB-231 cell lines. The activity of the luciferase can be determined by detecting the intensity of the fluorescence.

In Vivo Xenograft Model

The tumor-forming experiment in nude mice was approved by the local Animal Ethics and Use Committee. Ten 8-week-old male nude mice were obtained from the animal center and randomly divided into 2 groups (5 in each group). The MCF-7 cell line stably overexpressed microRNA-9501 was injected subcutaneously into the axilla of mice. Tumor size was monitored every 5 days; then, after 5 weeks, the mice were sacrificed. The volume of all samples was calculated using the following formula: tumor volume = (width 2 x length)/2.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 V5.01 software. Independent experiments were repeated at least three times for each experiment. $p<0.05$ was statistically significant.

Results

MicroRNA-9501 Was Lowly Expressed in BCa Tissue Specimens and Cell Lines

To determine the role of microRNA-9501 in BCa, a total of 56 pairs of tumor tissue specimens and paracancerous ones of BCa patients were collected, and microRNA-9501 level was examined using qPCR. The results indicated that in tumor tissues, microRNA-9501 level was found significantly lower than that in adjacent ones (Figure 1A), suggesting that microRNA-9501 may play a role as a tumor suppressor gene in BCa. At the same time, microRNA-9501 level in commonly used BCa cell lines was examined; among them, microRNA-9501 showed the lowest expression in MCF-7 and MDA-MB-231 cells, which were therefore used for subsequent experiments (Figure 1B). In addition, to explore the interplay between microRNA-9501 level and the prognosis of BCa patients, relevant follow-up data were collected,

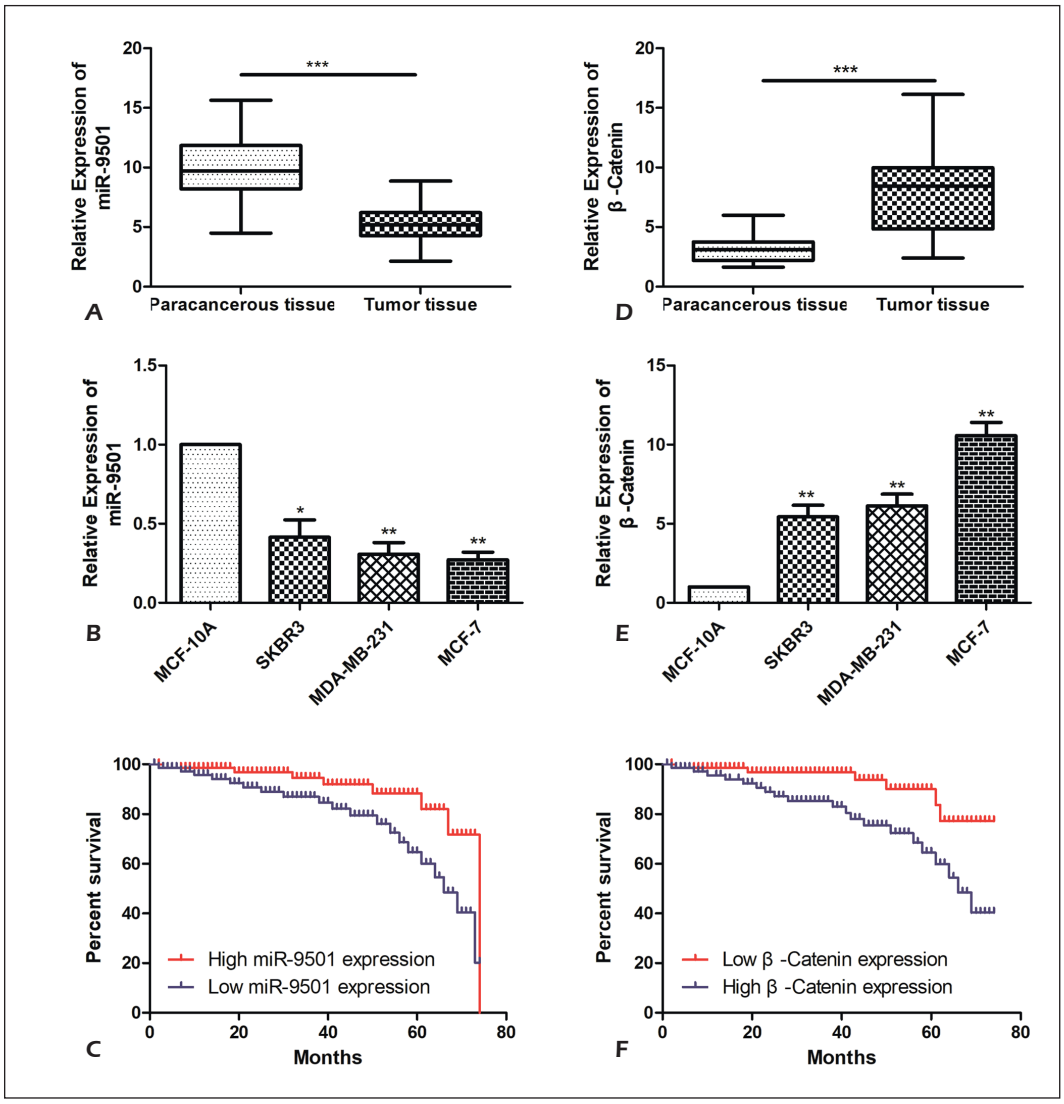

Figure 1. Low and high expression of miR-9501 and β-Catenin in breast cancer tissues and cell lines, respectively. **A**, QRT-PCR was used to detect the differential expression of miR-9501 in breast cancer tissues and adjacent tissues; **B**, QRT-PCR was used to detect the expression level of miR-9501 in breast cancer cell lines; **C**, Kaplan Meier survival curve of breast cancer patients based on miR-9501 expression showed that the prognosis of patients with low expression was significantly worse than that of high expression group; **D**, QRT-PCR was used to detect the differential expression of β-Catenin in breast cancer tissues and adjacent tissues; **E**, QRT-PCR was used to detect the expression level of β-Catenin in breast cancer cell lines; **F**, Kaplan Meier survival curve of breast cancer patients based on β-Catenin expression showed that the prognosis of patients with low expression was significantly worse than that of high expression group. Data are mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

and Kaplan-Meier survival curves revealed that low expression of microRNA-9501 was closely relevant to the poor prognosis of BCa (p<0.05; Figure 1C). These results suggested that microRNA-9501 may act as a tumor suppressor gene in BCa.

β-Catenin Was Highly Expressed in BCa Tissue samples As Well As in Cell Lines

It was found by qPCR that β-Catenin was increased in tumor tissues compared with adjacent ones in BCa patients (Figure 1D), suggesting that β-Catenin may act as a cancer-promoting gene in BCa. At the same time, β-Catenin was found highly expressed in BCa cells (Figure 1E). To clarify the interplay between β-Catenin expression and the prognosis of BCa patients, we collected relevant follow-up data and demonstrated through Kaplan-Meier survival curve that the highly-expressed β-Catenin was significantly related to BCa patients' poor prognosis, which was, the higher the expression level of β-Catenin, the worse their prognosis (p<0.05; Figure 1F).

Table I. Association of miR-548c-5p expression with clinicopathologic characteristics of breast cancer.

Parameters	No. of cases	MiR-9501 expression		*p*-value
		High (%)	Low (%)	
Age (years)				0.258
<60	22	15	7	
≥60	34	18	16	
T stage				**0.006**
T1-T2	33	25	8	
T3-T4	23	9	14	
Lymph node metastasis				**0.014**
No	35	25	10	
Yes	21	8	13	
Distance metastasis				**0.039**
No	40	27	13	
Yes	16	6	10	
PR expression				0.937
Negative	24	14	10	
Positive	32	19	13	
ER expression				0.492
Negative	19	10	9	
Positive	37	23	14	
HER-2 expression				0.114
Negative	20	9	11	
Positive	36	24	12	

Abbreviations: PR, progesterone receptor; ER, estrogen receptor.

MicroRNA-9501 Expression Was Relevant to Tumor Stage and Metastasis of BCa Patients

According to microRNA-9501 level detected by qPCR, we divided the above tissue samples into two groups, high and low expression group. Chi-square test was used to analyze the interplay between microRNA-9501 expression and age, pathological stage, lymph node, distant metastasis or hormonal pattern of BCa patients. As shown in Table I, lowly expressed microRNA-9501 was positively correlated with pathological stage, lymph node or distant metastasis of BCa patients.

Over-Expression of MicroRNA-9501 Inhibited BCa Cell Proliferation, Migration, and Invasion

To study the influence of microRNA-9501 on the function of BCa cells, we transfected microRNA-9501 mimics into BCa cell lines, and verified the transfection efficiency by qPCR (Figure 2A). Subsequently, CCK-8 test revealed that overexpression of microRNA-9501 remarkably decreased the proliferation rate of BCa cells (Figure 2B); meanwhile, similar results were obtained in EDU assay (Figure 2C), indicating that microRNA-9501 could remarkably inhibit BCa cell proliferation rate. Subsequently, transwell invasion and cell wound healing assays were carried out to examine the migration ability, as well as invasiveness of BCa cells. As shown in Figure 3A and 3B, overexpression of microRNA-9501 markedly weakened the migration and invasive ability of BCa cells. The above observations suggested that upregulation of microRNA-9501 could suppress BCa cell proliferation, migration, and invasive abilities.

MicroRNA-9501 Bound to β-Catenin

To explore the mechanism of microRNA-9501 in BCa, bioinformatics analysis was performed and demonstrated that microRNA-9501 may specifically bind to β-Catenin. Luciferase reporter gene experiments showed that overexpression of β-Catenin remarkably attenuated the luciferase activity of the wild-type microRNA-9501 vector, further demonstrating that microRNA-9501 can be targeted by β-Catenin (Figure 4A). Furthermore, qPCR validation in BCa tissues indicated a negative correlation between microRNA-9501 and β-Catenin expression levels

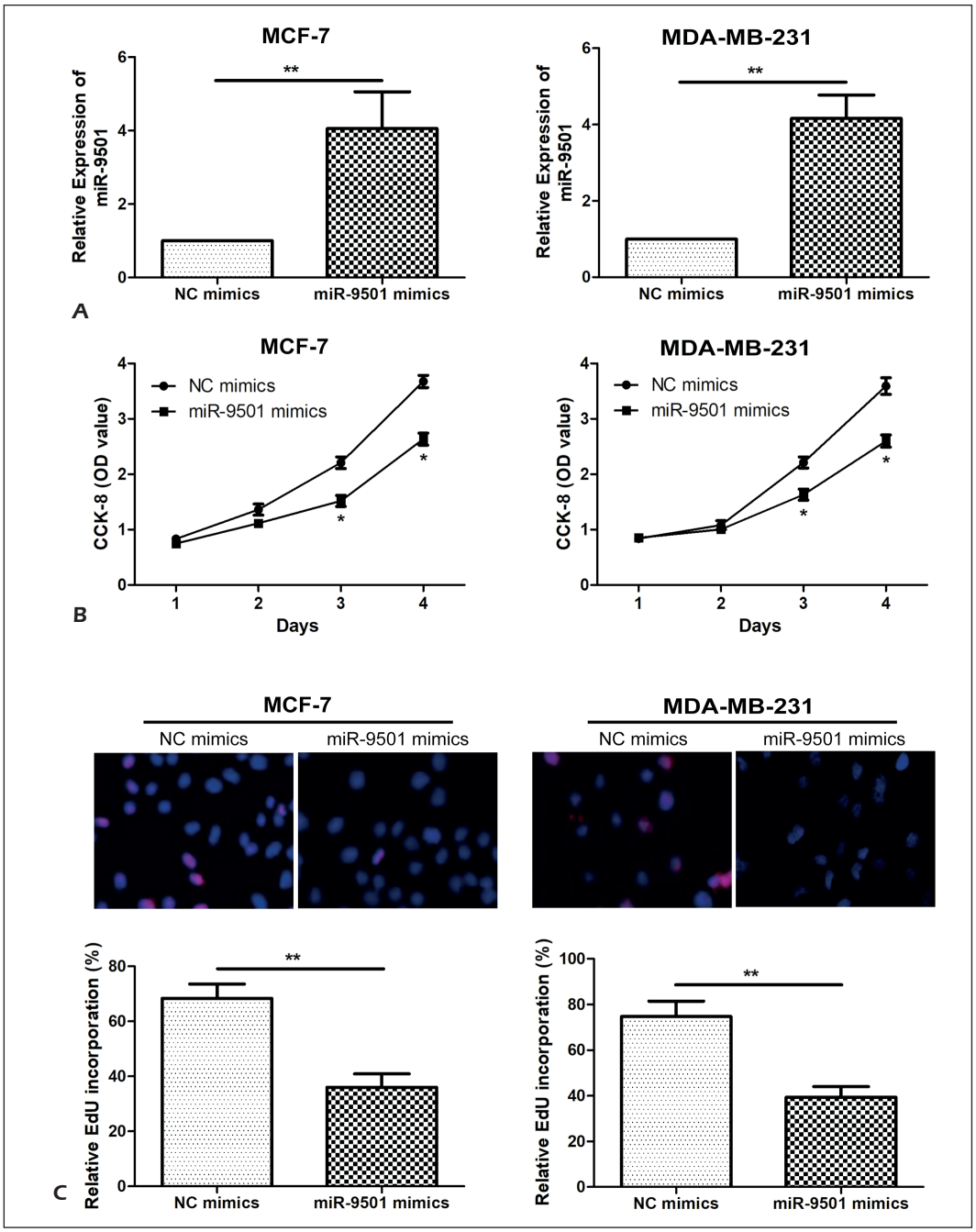

Figure 2. Overexpression of miR-9501 inhibits breast cancer cell proliferation. **A**, QRT-PCR verified the transfection efficiency of miR-9501 overexpression vector in MCF-7 and MDA-MB-231 cell lines; **B**, CCK-8 assay detected the effect of overexpression of miR-9501 on proliferation of breast cancer cells in MCF-7 and MDA-MB-231 cell lines; **C**, EDU assay was performed to evaluate the proliferation of breast cancer cells after overexpression of miR-9501 in MCF-7 and MDA-MB-231 cell lines (magnification: 20×). Data are mean ± SD, *p<0.05, **p<0.01.

(Figure 4B). Subsequently, qPCR and Western blotting were conducted to measure the changes of the key molecules including wnt 5a, β-Catenin, and GSK-3β in the Wnt/β-Catenin pathway after overexpression of microRNA-9501, and the results revealed that the above molecules were significantly down-regulated, indicating that microRNA-9501 can down-regulate the Wnt/β-Catenin pathway, thereby inhibiting the malignant progression of BCa.

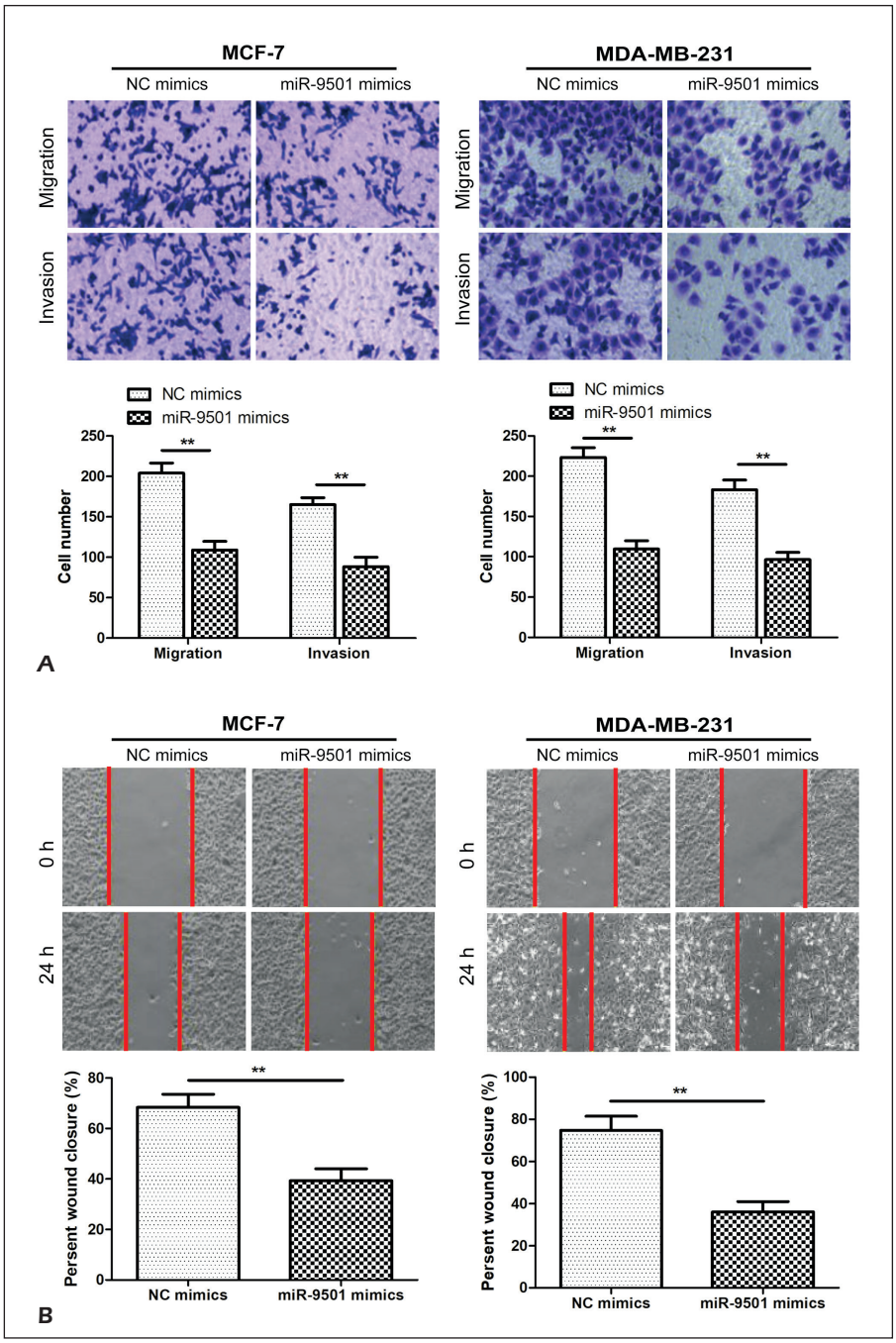

Figure 3. Overexpression of miR-9501 inhibits breast cancer cell metastasis. **A**, Transwell experiments detected the invasion and migration ability of breast cancer cells after transfection of miR-9501 overexpression vector in MCF-7 and MDA-MB-231 cell lines (magnification: 20×); **B**, Cell wound healing assay detected the ability of breast cancer cells to crawl after transfection of the miR-9501 overexpression vector in MCF-7 and MDA-MB-231 cell lines (magnification: 20x). Data are average ± SD, $^{**}p<0.01$.

Over-Expression of MicroRNA-9501 Inhibited BCa Progression In Vivo

In vivo, the transfected MCF-7 cell line was inoculated *in situ* into each nude mouse and injected in the left axilla. As we expected, either the tumor volume or the weight of nude mice inoculated with the microRNA-9501 overexpression vector was reduced when comparing to NC mimics group (Figure 5A-5C). Next, the RNA of the tumor-forming tissues of nude mice was extracted to detect

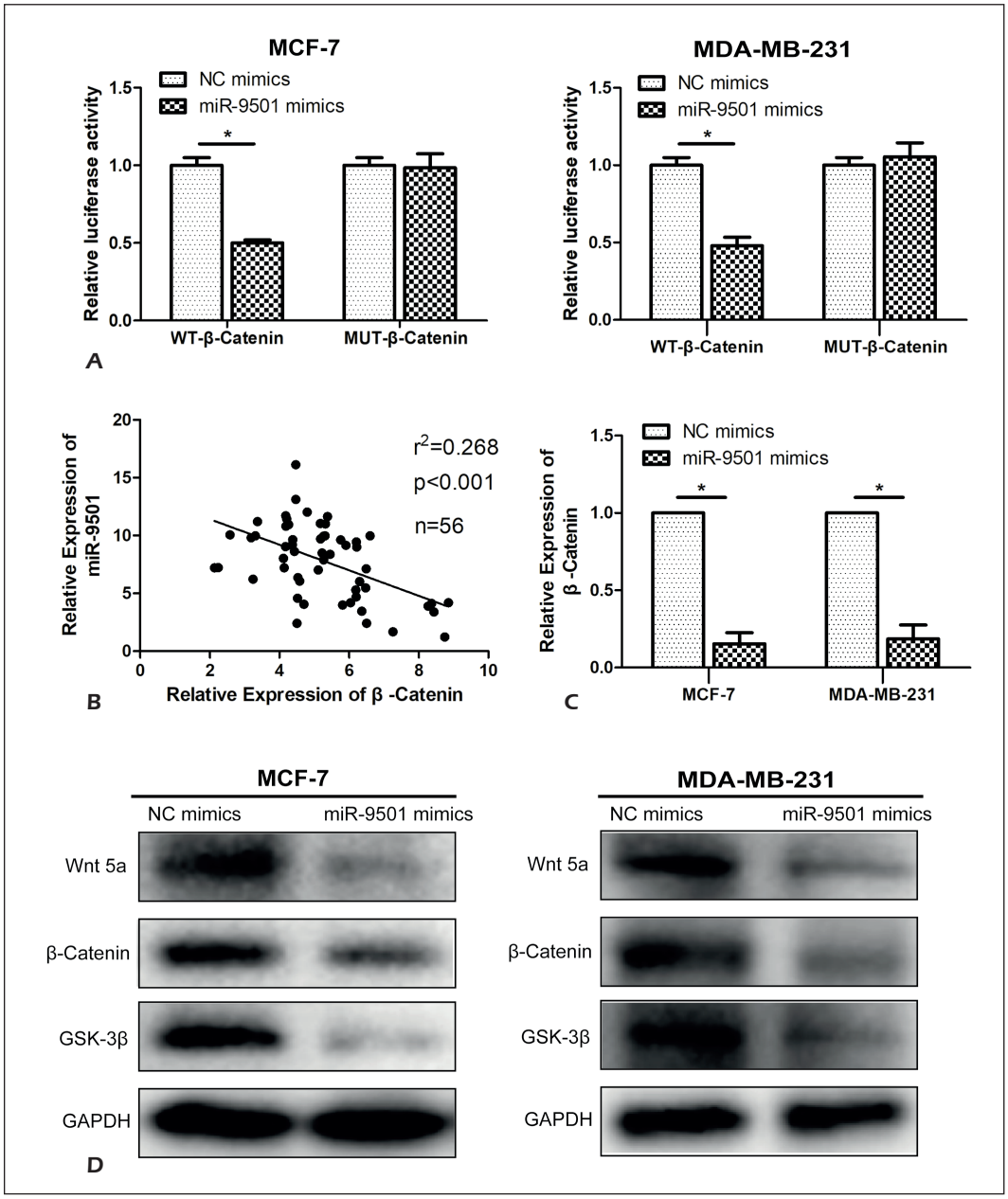

Figure 4. miR-9501 regulates the expression of β-Catenin. **A**, Dual-Luciferase reporter gene assay verified the direct targeting of miR-9501 and β-Catenin; **B**, There was a significant negative correlation between miR-9501 and β-Catenin expression in breast cancer tissues; **C**, QRT-PCR verified the expression level of β-Catenin after transfection of the miR-9501 vector in MCF-7 and MDA-MB-231 cell lines; **D**, Western blotting verified the expression levels of wnt/β-Catenin pathway-related molecules after transfection of the miR-9501 vector in MCF-7 and MDA-MB-231 cell lines. Data are mean ± SD, *$p<0.05$.

microRNA-9501 and β-Catenin using qPCR. As a result, inoculation of microRNA-9501 overexpression vector enhanced microRNA-9501 expression (Figure 5D) while reduced β-Catenin level in the resected breast tumor (Figure 5E), suggesting that microRNA-9501 may be able to suppress breast tumor formation in nude mice *in vivo*.

Discussion

Breast cancer is the most common malignant tumor in women, mainly occurring in women and occasionally in men[1-3]. In developed countries such as Europe and America, the incidence of BCa ranks first among female malignant tumors,

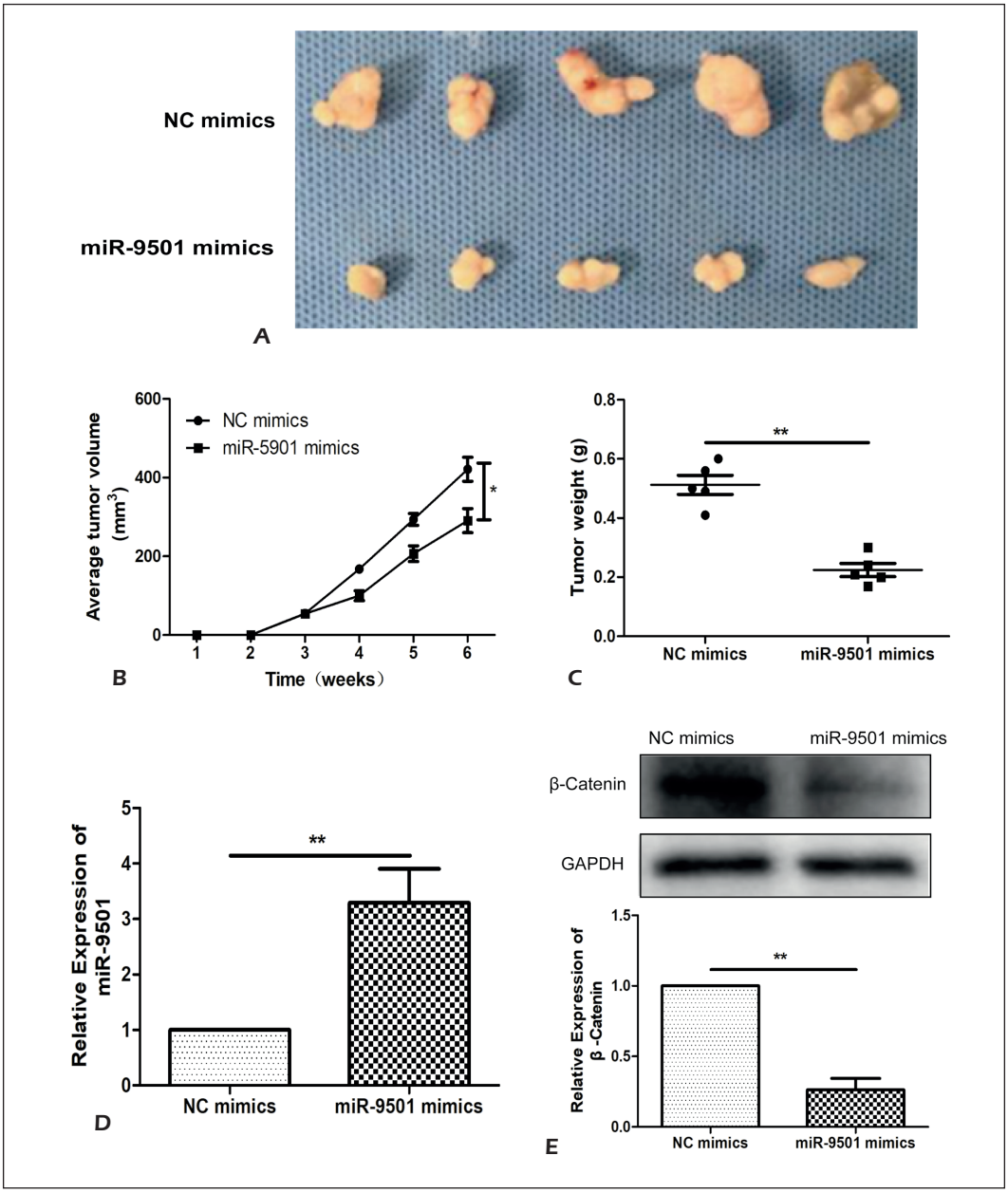

Figure 5. Overexpression of miR-9501 inhibits tumorigenicity in breast cancer nude mice. **A**, Gross images of different nude mice after injection of NC mimics and miR-9501 mimics, respectively; **B**, Tumor volume growth curves were calculated after injection of NC mimics and miR-9501 mimics, respectively; **C**, Tumor weight growth curves were calculated in different nude mice after injection of NC mimics and miR-9501 mimics; **D**, QRT-PCR was used to detect miR-9501 levels in hepatocellular carcinoma nude mice; **E**, QRT-PCR and Western blotting were used to detect the level of β-Catenin in the tumor-forming tissues of hepatocellular carcinoma in nude mice. Data are mean ± SD, *p<0.05, **p<0.01.

and ranks first among female cancer incidence in China, and the incidence rate is increasing year by year[4,5]. BCa has become the number one killer threatening women's physical and mental health and endangering their lives[1-5]. Recently, as the early diagnosis of BCa and the constant improvement of the health screening, individualized treatment design has become the main treatment strategy of BCa. Reasonably integrated application of surgery, radiotherapy, chemotherapy, biological target therapy, endocrine therapy, and other treatments have significantly improved the overall survival rate of patients with BCa[6-8]. Tumor is formed by the complex biological process of multi-factor, multi-step, multi-gene, and multi-signal pathway influencing and inter-

acting together[7,8]. There are multiple risk factors for BCa, including age of menarche and menopause, fertility factors, breastfeeding, genetics, lifestyle, endocrine status, mood, and ionizing radiation, which are all related to the incidence of BCa[9]. With the development of molecular biology, genomics, and other science and technology, interdisciplinary studies have triggered a fever to discuss the pathogenesis of tumors from the perspectives of molecules and genes[10,11].

In recent years, the emerging research on the regulatory expression of non-coding RNA (non-coding RNA), especially microRNA (miRNA) genes, provides a new means for understanding molecular events in the occurrence and development of BCa[12-14]. MicroRNAs are a class of about 22 nucleotides long noncoding single small RNAs that mainly exists in eukaryotes. It can promote the degradation or inhibit the translation of its target gene mRNA, thus playing a role in cell growth, development, proliferation, differentiation and apoptosis, and other process[15-17]. MiRNA plays an important role as a new potential key regulator of normal cell development, and the abnormal expression of miRNA may play a key role in tumorigenesis[18,19]. Xi et al[22] have shown that microRNA-9501 has a decreased expression and anti-tumor function in non-small cell lung cancer. In this research, we first examined microRNA-9501 level in BCa tissues, and found that microRNA-9501 level in BCa tissue was remarkably lower than that in adjacent tissues, and was positively correlated with the pathological stage, lymph node metastasis, distant metastasis, and poor prognosis of BCa, suggesting that microRNA-9501 may play an anti-cancer role in BCa. Later, we conducted CCK-8, EDU, transwell invasion and migration, and cell scratch experiments after the overexpression of microRNA-9501 in BCa cell lines. The results showed that microRNA-9501 had an anti-cancer effect in BCa and could remarkably reduce the proliferation and metastasis ability of BCa cells.

The study of miRNA target genes is the core of miRNA function research. MiRNA participates in the regulation of life activities by regulating target genes[23,24]. Therefore, the focus and difficulty of miRNA research has been to find and clarify the target genes regulated by miRNA and the signaling pathways involved[20,21]. Currently, the identification of target genes is based on the combination of miRNA and target gene 3'UTR, and the prediction of miRNA target genes by bioinformatics[25]. The following websites are commonly used to predict target genes: miRanda, TargetScan, PicTar, etc[24,25]. The above analysis results suggested that β-Catenin may interact with microRNA-9501. In addition, the Dual-Luciferase reporter gene experiment verified the direct binding of microRNA-9501 to downstream β-Catenin. Meanwhile, the results of qPCR in this experiment showed that β-Catenin was more highly expressed in tumor tissues of BCa patients than in normal BCa tissues, and that β-Catenin could promote cell proliferation and metastasis in BCa cells. To explore the regulatory role of microRNA-9501 and β-Catenin in the expression of breast cancer cell lines, it was found by qPCR and Western Blotting that overexpression of microRNA-9501 would lead to the downregulation of β-Catenin. This evidence suggests that the transcriptional activity of the gene loci in which microRNA-9501 is located may be regulated by the involvement of β-Catenin, which also coincides with the Dual-Luciferase reporter gene assay results that microRNA-9501 regulates the activity of the reporter gene of the β-Catenin promoter.

Conclusions

MicroRNA-9501 showed a significant low expression in BCa tissues and cell lines, and was significantly correlated with pathological staging, lymph node or distant metastasis of BCa patients. Additionally, microRNA-9501 may inhibit the proliferation and metastasis of BCa *via* regulating the Wnt/ β-catenin pathway.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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