

Original Article

LncRNA DDX11-AS1 promotes cell growth, migration and invasion through regulating epithelial-mesenchymal transition in breast cancer

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Article Info

Abstract



Article history:

Received: October 24, 2023

Accepted: February 28, 2024

Published: April 30, 2024

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This study aimed to investigate the expression of long non-coding ribonucleic acid (lncRNA) DDX11 antisense RNA 1 (DDX11-AS1) in breast cancer (BC) tissues and cells and investigate its biological function and potential molecular mechanism through *in vitro* experiments. Tissue specimens were obtained from 44 BC patients. TRIzol method was used to extract RNAs from the tissues. The relative expression of DDX11-AS1 in BC tissues and the expression of DDX11-AS1 in BC cells were detected *via* quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The effect of DDX11-AS1 on the proliferation ability of BC cells was detected *via* cell counting kit-8 (CCK-8) assay. Flow cytometry was adopted to study the effect of DDX11-AS1 on the distribution of BC cell cycle. Transwell assays were performed to analyze the effects of DDX11-AS1 on the migration and invasion abilities of BC cells. Finally, after interfering with the expression of DDX11-AS1 in BC cells, changes in the expressions of molecular markers for epithelial-mesenchymal transition (EMT) were detected *via* Western blotting. According to the results of qRT-PCR, the expression of DDX11-AS1 was up-regulated in 38 out of 44 cases of BC tissues compared with that in the para-carcinoma tissues, and the expression of DDX11-AS1 in BC cells was up-regulated as well. After interference with the expression of DDX11-AS1 in BC cells, it was found *via* CCK-8 assay that the proliferation ability of BC cells was restrained, flow cytometry results showed that the BC cell cycle was arrested at G1/G0 phase, and the results of transwell assays revealed that the cell invasion and migration abilities were suppressed in experimental group compared with those in control group. According to the results of Western blotting, after interfering with the expression of DDX11-AS1 in BC cells, there were changes in the expressions of molecular markers for EMT. In BC, the expression of lncRNA DDX11-AS1 is up-regulated, which promotes the proliferation, migration and invasion of BC cells by regulating EMT.

Keywords: Breast cancer, lncRNA, DDX11-AS1, Biological function, EMT

1. Introduction

Breast cancer is very common among women. To prevent breast cancer, one should be aware of its symptoms. But what are the symptoms of breast cancer? How can it be recognized? What are the treatment methods for breast cancer? [1,2].

Breast cancer is one of the types of cancer that occurs in breast tissue cells. This disease is the most common invasive cancer in women. Among women, it is the second leading cause of death from cancer after lung cancer. Breast cancer can occur in both men and women. But in general, it is more common in women. The lumps in breast cancer are called breast tumors, which are sometimes benign and sometimes malignant and cancerous. [1,3].

Since 1989, advances in breast cancer screening tests and treatments have dramatically increased survival rates. Also, due to early diagnosis and new approaches to treatment and understanding of the disease, the rate of deaths related to this disease has decreased. Malignant and be-

nign breast cancer is diagnosed through different methods. Many breast lumps are benign and do not pose much danger to a person. But if the breast cancer is malignant, the treatment steps should be done according to the oncology doctor's opinion [1-3].

It is interesting to know that 1% of breast cancer patients are men. Statistically, 12.5% of 85-year-old women are expected to have breast cancer during their lifetime. 2/3 of affected women are over 50 years old and most of the remaining people are 39 to 49 years old. 90% of women with breast cancer have a chance to live at least 5 years after diagnosis [1,3].

Breast cancer (BC) is a type of malignant tumor in the epithelial tissue of mammary glands. About 99% of all BCs are diagnosed in women, so it is a severe cancer threatening the life and health of women [1]. Although the incidence rate of BC in China is not high compared with world level, it has risen rapidly in recent years, and the situation is not optimistic. According to the Cancer Statistics

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in China 2017 released by the National Cancer Center, the number of new cancer cases has been rising continuously at a rate of 3%, accounting for 1/4 of the new cancer cases worldwide [2-4].

Many factors are involved in the occurrence and development of BC, and there is a complicated regulatory network. After many years of research, great progress has been made in understanding this regulatory network, based on which the treatment strategies of BC have achieved good results. However, due to the complexity of this regulatory network, many of its details are unknown, which restricts the further improvement of the comprehensive treatment effect on BC [5-7].

Long non-coding ribonucleic acid (lncRNA) DDX11-AS1, also known as DDX11 antisense RNA 1, with a transcript length of 2031 bp, is an antisense RNA that does not encode protein. According to studies, the expression of DDX11-AS1 is up-regulated in hepatocellular carcinoma, gastric cancer and colorectal cancer, and DDX11-AS1 is also involved in processes such as tumor development and drug resistance [8-10]. However, there have been no reports about its relation with BC. In this study, it was found that the expression of DDX11-AS1 in BC tissues was significantly higher than that in normal tissues, and the effect of the expression level of DDX11-AS1 on the invasion ability of BC cells and its mechanism was explored, so as to provide references for making new treatment strategies of BC.

2. Materials and methods

2.1. Tissue specimens

A total of 44 patients pathologically diagnosed with BC were selected. None of patients underwent chemoradiotherapy, endocrine therapy and targeted therapy before operation, and they were pathologically diagnosed with BC after operation. Those with a history of malignant tumors or metastatic BC were excluded. 44 cases of BC tissues (pathological type: primary invasive cancer) and paired para-carcinoma tissues (normal breast tissues >5 cm away from the cancer tissues) were collected from patients, quickly frozen in liquid nitrogen and stored in a refrigerator at -80°C. This study was approved by Ethics Committee of People's Hospital of Ganzhou, and the patients signed the informed consent.

2.2. Cell culture

Human normal breast epithelial cells MCF-10A and BC cells MCF-7, T47D, BT549, MDAMB-231 and SKBR3 were all kept in our laboratory. BC cells were inoculated into 1640/DMEM (dulbecco's modified eagle medium) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and cultured in an incubator with 5% CO₂ at 37°C. Upon reaching 80-90% confluence, the cells were passaged.

2.3. Cell transfection

Three lncRNA DDX11-AS1 siRNA fragments were designed and synthesized (Sangon, Shanghai, China). After digestion, the cells in the culture flask were inoculated into the culture plate. Upon reaching about 60% confluence, the cells were transfected with lncRNA DDX11-AS1 siRNA using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). The transfection efficiency was detected *via* quantitative real-time polymerase chain reaction (qRT-PCR).

2.4. RNA extraction

The cells were lysed with TRIzol (Invitrogen, Carlsbad, CA, USA) at room temperature for 5 min, extracted with chloroform, and placed for 10 min, followed by centrifugation at 12,000 g for 10 min. Then the aqueous phase was taken, added with isopropanol, and placed at room temperature for 10 min, followed by centrifugation at 12,000 g for 10 min. After the liquid waste was discarded, the precipitate was washed once with 70% ethanol, and centrifuged at 12,000 g for 5 min. After the liquid waste was discarded, the precipitate was air dried, added with 30 µL of nuclease-free water and stored at -80°C for later use.

2.5. Reverse transcription

The reverse transcription system was prepared according to the instructions of Fermentas reverse transcription kit (Shanghai Yubo Biotechnology Co., Ltd., Shanghai, China): 2 µg of RNA, 1 µL of Random Primers, and water added till the total volume of 12 µL (65°C for 5 min, and placed on ice for 2 min). Then 4 µL of reaction buffer, 1 µL of RNase inhibitor, 2 µL of dNTP and 1 µL of reverse transcriptase were added (42°C for 1 h, 70°C for 5 min). The cDNA was stored at -20°C.

2.6. QRT-PCR

The qRT-PCR system was prepared according to the instructions of kit (Fermentas). The reaction was performed using a fluorescence quantitative PCR instrument (Bio-Rad, Hercules, CA, USA) under the following conditions: 50°C for 2 min, 95°C for 2 min, 95°C for 15 s, 60°C for 30 s, a total of 40 cycles. The relative expression was detected using 2^{-ΔΔCt}. The primers are as follows: DDX11-AS1 F: 5'-CTGTGTAGCTCTAGAGAAA-3' R: 5'-GGCCTTAAGTTTAGAGCAA-3', glyceraldehyde 3-phosphate dehydrogenase (GAPDH) F: 5'-ATT CCATG GCACCGTCAAGGCTGA-3', R: 5'-TTCTCCATGGTG-GTGAAGACGCCA-3'.

2.7. Cell counting kit-8 (CCK-8) assay

The cells were digested, transfected and inoculated into a 96-well plate. CCK-8 reagent (Dojindo, Kumamoto, Japan) was added into the plate for incubation for 2 h. Then the optical density (OD) value was measured at 450 nm using a microplate reader. CCK-8 assay was repeated for 3 times independently.

2.8. Flow cytometry

At 48 h after transfection, the cells were digested with trypsin, and the single-cell suspension was collected, washed twice with cold phosphate buffered saline (PBS) and slowly added with 3 mL of pre-cooled 70% ethanol in a refrigerator at 4°C overnight. Then 5×10⁶ cells were taken, centrifuged in a 15 mL centrifuge tube for 10 min, resuspended with 500 µL of PBS, and added with 5 µL of RNase A solution, followed by water bath at 37°C for 30 min and centrifugation for 10 min. After the supernatant was discarded, the cells were collected, resuspended with 400 µL of Propidium Iodide (PI) dye and incubated at 4°C in the dark for 45 min, followed by detection.

2.9. Transwell assay

Invasion assay was performed using the transwell chamber (Corning, Corning, NY, USA) (8 µm pore size). 5×10⁴ cells in 150 µL of serum-free DMEM were added

into the upper chamber containing 50 μ g of Matrigel (BD, Bedford, MA, USA), while 600 μ L of 10% FBS + DMEM/1640 was added into the lower chamber. 48 h later, the upper surface of the chamber was immersed in pre-cooled methanol for 10 min, and stained with crystal violet for 30 min. Finally, the number of migrating cells into the lower chamber was counted. In migration assay, Matrigel was not used, and the tumor cells migrated for 12 h.

2.10. Western blotting

The cells were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) and ultrasonically smashed, followed by centrifugation at 14,000 g for 10 min. The supernatant was collected to measure the protein concentration using bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). After sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the protein was transferred onto polyvinylidene fluoride (PVDF) membrane, sealed with 5% skim milk at room temperature for 2 h, incubated with primary antibodies (E-cadherin, Vimentin, ZEB1, CST, Danvers, MA, USA) at 4°C overnight, and incubated again with goat anti-rabbit secondary antibodies (CST, Danvers, MA, USA) at room temperature for 2 h. Finally, the protein expression was detected using electrochemiluminescence (ECL) solution (Thermo Scientific, Waltham, MA, USA).

2.11. Statistical analysis

Graphpad Prism 7.0 software (La Jolla, CA, USA) was used for statistical analysis. The data were expressed as mean \pm standard deviation. *t*-test was performed for the comparison between two groups, analysis of variance for comparison among groups, and Student-Newman-Keuls (SNK) test for further pairwise comparison. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Expression of lncRNA DDX11-AS1

First, 44 cases of tissue specimens were collected from BC patients, from which RNA was extracted and reversely transcribed into cDNA. The results of qRT-PCR showed that DDX11-AS1 was up-regulated in 38 out of 44 cases of BC tissues (Figure 1A). Next, the relative expression of DDX1-AS1 in BC cells was detected using qRT-PCR. The results revealed that DDX1-AS1 was up-regulated in BC cells compared with that in human normal breast epithelial cells (Figure 1B). To study the biological function of DDX1-AS1, the DDX1-AS1 specific interference sequence si-DDX11-AS1 was designed and transiently transfected into BC cells. 48 h later, the interference efficiency was detected using qRT-PCR (Figure 1C and 1D).

3.2. Effects of DDX11-AS1 on proliferation and cycle of BC cells

Si-DDX11-AS1 was transiently transfected into BC cells, and the changes in proliferation of BC cells were detected via CCK-8 assay at 0, 24, 48, 72 and 96 h. The results manifested that the cell proliferation was inhibited in the experimental group compared with that in control group (Figure 2A and 2B). The results of flow cytometry showed that the cell cycle was arrested at G1/G0 phase after interference with the expression of DDX11-AS1 in BC cells (Figure 2C and 2D).

3.3. DDX11-A11 regulated migration of BC cells through EMT

After interference with the expression of DDX11-AS1 in BC cells, the changes in cell migration and invasion were determined using transwell assay. It was found that cell migration and invasion were suppressed in the experimental group compared with those in control group (Figure 3A and 3B). The results of Western blotting manifested that after interference with the expression of DDX11-AS1, the

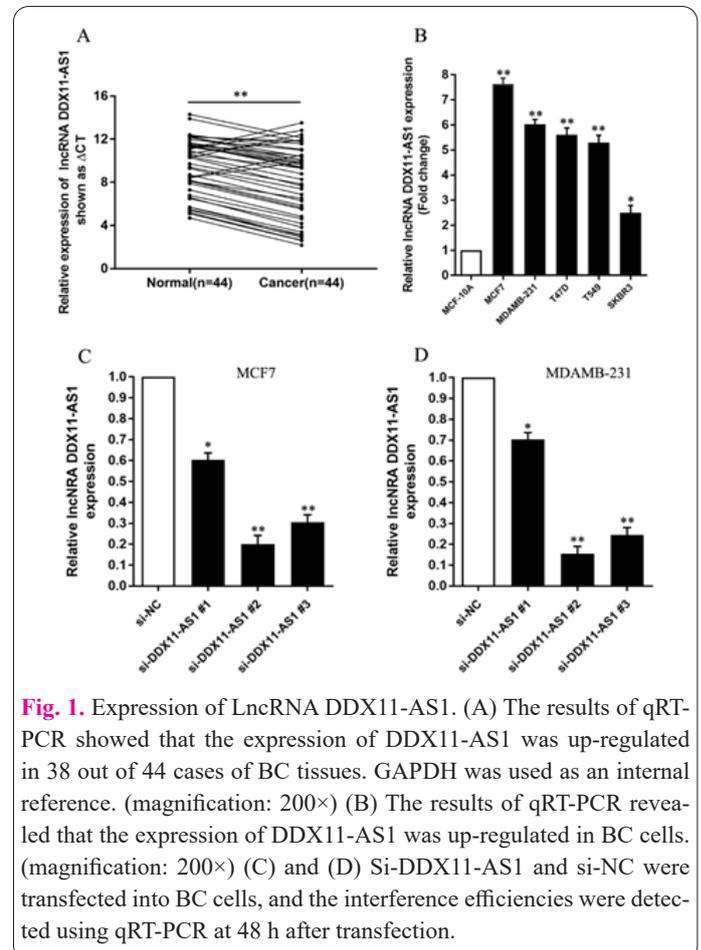


Fig. 1. Expression of lncRNA DDX11-AS1. (A) The results of qRT-PCR showed that the expression of DDX11-AS1 was up-regulated in 38 out of 44 cases of BC tissues. GAPDH was used as an internal reference. (magnification: 200 \times) (B) The results of qRT-PCR revealed that the expression of DDX11-AS1 was up-regulated in BC cells. (magnification: 200 \times) (C) and (D) Si-DDX11-AS1 and si-NC were transfected into BC cells, and the interference efficiencies were detected using qRT-PCR at 48 h after transfection.

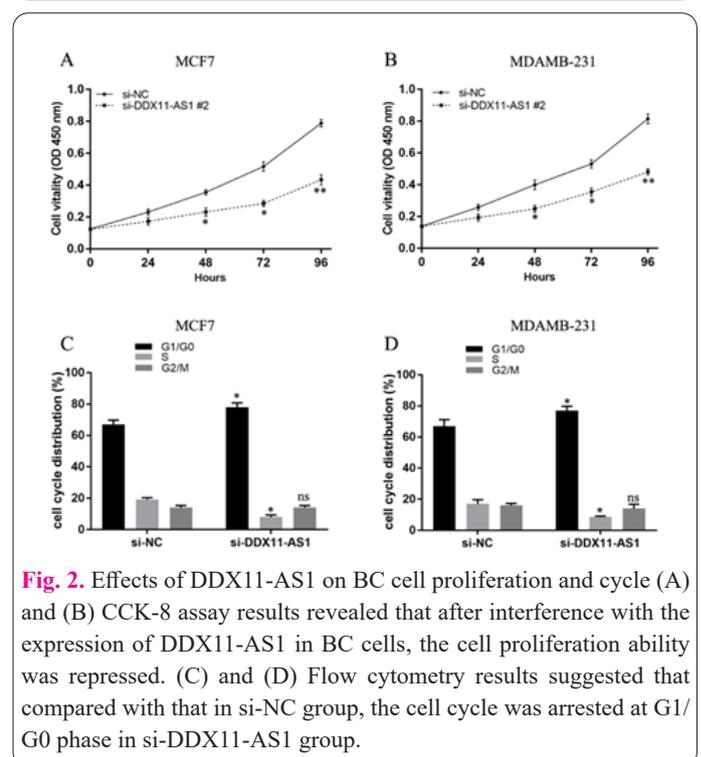


Fig. 2. Effects of DDX11-AS1 on BC cell proliferation and cycle (A) and (B) CCK-8 assay results revealed that after interference with the expression of DDX11-AS1 in BC cells, the cell proliferation ability was repressed. (C) and (D) Flow cytometry results suggested that compared with that in si-NC group, the cell cycle was arrested at G1/G0 phase in si-DDX11-AS1 group.

expressions of some molecular markers (Vimentin, ZEB1) for EMT were down-regulated, while some (E-cadherin) were up-regulated (Figure 3C and 3D).

4. Discussion

There are many types of lncRNAs, which are expressed continuously throughout cell process. Studies have manifested that lncRNAs can play extensive regulatory roles in almost every stage of gene expression. It was also revealed that lncRNAs play critical roles in regulating gene expression levels, maintaining genomic integrity, gene dose compensation, genomic imprinting, mRNA processing, cell differentiation and development and other processes [11,12]. Once lncRNAs are expressed aberrantly, many human diseases such as cancer, immunological diseases and neurological diseases will be induced [13,14].

So far, the functions of only a few lncRNA molecules are known, while the functions of most lncRNAs remain unclear. According to previous studies, many lncRNAs are dysregulated in human cancers. For example, the expression of lncRNA HOX transcript antisense intergenic RNA is remarkably up-regulated in primary and metastatic BC, which is 2,000 times higher than that in normal breast tissue. Moreover, the expression of HOX transcript antisense intergenic RNA is associated with tumor metastasis and prognosis [15]. lncRNA maternally expressed gene 3 is correlated with the pathogenesis and clinical progression of meningioma. Meanwhile, the loss of maternally expressed gene 3 is associated with tumor grade [16]. Therefore, understanding the generation of these lncRNAs and their regulatory mechanisms is of significant importance for the studies on their functions in tumorigenesis and tumor development.

E-Cadherin, an intercellular adhesion molecule in the epithelial tissue, plays an important role in maintaining the morphology and structure of epithelial cells and the function of epithelial tissues [17]. During the EMT process of tumor cells, the expression of E-cadherin is down-regulated and the intercellular adhesion is destroyed [18]. The transformed tumor cells are transferred to other organs *via*

lymph or blood circulation. According to studies, in breast invasive ductal carcinoma, patients with expression of E-cadherin show a lower lymph node metastasis rate, while those without E-cadherin expression exhibit a higher lymph node metastasis rate [19,20]. Vimentin, a type of intermediate filament protein in mesenchymal cells, regulates cytoskeleton and the interactions between proteins such as cell adhesion molecules, and participates in the adhesion, migration and cell signaling of tumor cells and tumor-related endothelial cells and macrophages. It was found that the expression of vimentin is significantly higher in BC tissues than in the adjacent tissues [21,22]. Zinc Finger E-Box Binding Homeobox 1 (ZEB1) is a transcriptional repressor, which has been identified as an inducer of EMT. In invasive solid tumor, ZEB1 is a key EMT inducer which works through repressing the E-cadherin transcriptional gene coding [23,24]. It is thus clear that all of the above proteins are associated with the migration and invasion of BC cells.

5. Conclusions

In this study, it was found through *in vitro* experiments that the expression of DDX11-AS1 was up-regulated both in BC tissues and BC cells. Besides, the results of *in vitro* experiments also confirmed that interfering with the expression of DDX11-AS1 can inhibit BC cell proliferation, migration and invasion, and promote BC cell cycle arrest at G1/G0 phase. Further studies showed that DDX11-AS1 enhances the invasion ability of BC cells by down-regulating the expression of E-cadherin and up-regulating the expressions of vimentin and ZEB1.

Conflict of Interest

The authors declared no conflict of interest.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

This study was approved by Ethics Committee of People's Hospital of Ganzhou.

Informed Consent

The patients signed the informed consent.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

Xun Xi and Huozhong Yuan designed the study and performed the experiments, Fulan Yang and Zhenluo Ding collected the data, Fulan Yang, Zhenluo Ding and Haoxiang Zhuang analyzed the data, Xun Xi and Huozhong Yuan prepared the manuscript. All authors read and approved the final manuscript.

Funding

None.

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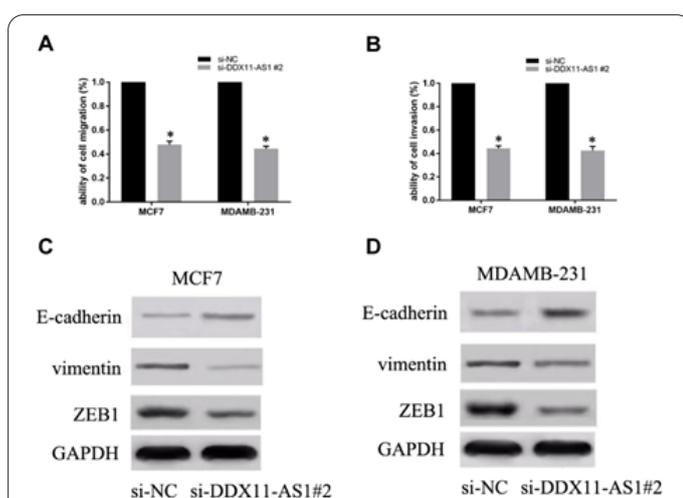


Fig. 3. DDX11-AS1 regulated migration of BC cells through EMT. (A) and (B) After interfering with the expression of DDX11-AS1, the results of transwell assays indicated that the migration and invasion abilities of BC cells were suppressed. (C) and (D) Western blotting results revealed that after interfering with the expression of DDX11-AS1, the expression of E-cadherin was up-regulated and the expressions of vimentin and ZEB1 were down-regulated.

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