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LncRNA BRE-AS1 acts as a tumor suppressor factor in bladder cancer *via* mediating STAT3

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Abstract. – OBJECTIVE: Long non-coding RNA (lncRNA) has been verified to regulate several cancers, including bladder cancer (BC). Our study aimed to elucidate the expression, function, and mechanism of lncRNA BRE-AS1 in BC.

PATIENTS AND METHODS: Relative expression of lncRNA BRE-AS1 in 77 BC tissues and adjacent normal tissues was determined using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Expression of lncRNA BRE-AS1 in T24 and EJ cells was up-regulated using lentivirus transfection. Cell counting kit-8 (CCK-8) assay and colony formation assay were used to assess the proliferation of T24 and EJ cells influenced by lncRNA BRE-AS1. Also, the influence of lncRNA BRE-AS1 on cell apoptosis and cell cycle was measured using flow cytometry. Western blot was employed to explore the downstream molecules for lncRNA BRE-AS1 in BC. *In vivo*, xenograft formation experiment was established in nude mice to study the function of lncRNA BRE-AS1 in BC.

RESULTS: LncRNA BRE-AS1 showed significantly decreased expression in BC tissues than the paired normal tissues. *In vitro* experiments demonstrated that over-expression of lncRNA BRE-AS1 inhibited cell proliferation but promoted cell apoptosis of EJ and T24 cells. STAT3 was determined as a target for lncRNA BRE-AS1. *In vivo*, up-regulation of lncRNA BRE-AS1 reduced cancer growth in nude mice bearing BC *via* repressing the phosphorylation of STAT3.

CONCLUSIONS: LncRNA BRE-AS1 was down-regulated in BC tissues. Over-expression of lncRNA BRE-AS1 inhibited BC cell proliferation *in vitro* and *in vivo via* repressing the phosphorylation of STAT3. This might provide a new sight for the understanding of BC progression and biotherapy.

Key Words:
LncRNA BRE-AS1, Bladder cancer, Suppressor, STAT3.

Introduction

Bladder cancer (BC) is one of the most common urinary system tumors with increasing morbidity and mortality in the world. It has become the 9th largest cancer and the 14th leading cause of death globally[1,2]. It has the highest incidence in Europe, North America, West Asia, and North Africa[3]. Although BC patients can be treated by radiation, surgery, and chemotherapy, the 5-year survival rate is still not satisfactory[4,5]. Therefore, it is important to reveal the molecular mechanism of BC development and progression.

It is well known that long non-coding RNA (lncRNA) is a RNA transcript of more than 200 nucleotides in length[6]. LncRNA plays a very important role in a series of biological processes and regulatory mechanisms. Biological processes regulated by lncRNAs include proliferation, DNA damage, angiogenesis, microRNA (miRNA) silencing, invasion, metastasis, and programmed cell death[7]. In addition, lncRNAs can also regulate embryonic development, immune cell development, and tumorigenesis. Many lncRNAs play replaceable roles in the occurrence and progression of BC[8]. So, lncRNA SPRY4-IT1 accelerates BC cell proliferation and metastasis by sponging miR-101-3p to up-regulate EZH2[9]. LncRNA H19 promotes BC metastasis by recruiting EZH2 to inhibit expression of E-cadherin[10]. LncRNA FOXD2-AS1 promotes BC recurrence *via* a feedback loop regulation of Akt and E2F1. LncRNA HCG22 suppresses growth and metastasis of BC cells by regulating PTBP1[11,12]. Also, high level of lncRNA DGCR5 indicates a better prognosis of BC and it facilitates expression of P21 to inhibit BC progression[13].

LncRNA BRE-AS1 is a non-coding single-stranded RNA of 1659 bp in length, located on 2p23.2[14]. The function of lncRNA BRE-AS1

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and its mechanism in BC development are temporarily unclear. In this paper, the expression of lncRNA BRE-AS1 was determined using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) in 77 BC tissues. Its regulation in the proliferation and apoptosis of EJ and T24 cells was verified by CCK-8 and flow cytometry, respectively. Furthermore, STAT3 was found to be a target for lncRNA BRE-AS1 in BC. In addition, the influence of lncRNA BRE-AS1 on BC cell growth *in vivo* was confirmed using xenograft assay in nude mice. This study might find a new target for the treatment of BC.

Patients and Methods

Clinical Tissues

BC tissue and adjacent normal tissue samples were collected from Jingmen No.2 People's Hospital. Tissues were surgically removed and immediately placed in liquid nitrogen for the next use. All the 77 patients signed the informed consent and the investigation was approved by the Ethics Committee of Jingmen No.2 People's Hospital.

Cell Lines and Transfection

BC-derived cell lines T24 and EJ were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). T24 and EJ cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Hyclone, South Logan, UT, USA) and 1% penicillin-streptomycin (Hyclone, South Logan, UT, USA). Cells were cultured in a 5% CO_2, 37°C incubator with humid air. For transfection, the lentivirus used for over-expression (LV-lncRNA BRE-AS1) and its control (Control) was synthesized by the Obio Biological Co., Ltd. (Shanghai China). After the cells were cultured to the logarithmic growth phase, transfection was done using polybrene (Obio, Shanghai China). After transfection, puromycin was used for screening, and finally transfection efficiency was tested by qRT-PCR.

RNA Isolation and QRT-PCR

The total RNA of 77 BC tissues and adjacent tissues was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). For real-time PCR, the cDNA was used as template, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal reference. The primers used were: lncRNA BRE-AS1: forward 5'-TGTGCACCGAGACTATTCCAG-3', reverse 5'-AGTAACTGGCCCCGGACTAA-3', GAPDH: forward 5'-GGGAGCCAAAAGGGTCATCA-3', reverse 5'-GCCAAATTCGTTGTCATACTTCT-3'. ABI 7600 HT (ABI, Applied Biosystems, Foster City, CA, USA) was employed for amplification at 95°C pre-denaturation for 3 min, followed by 39 cycles at 95°C denaturation for 5 s, 60°C annealing for 30 s, and 72°C extension for 30 s. The expression level of lncRNA BRE-AS1 was measured by the $2^{-\Delta\Delta Ct}$ method.

Cell Counting Kit 8 (CCK-8) Assay

CCK-8 (Dojindo Laboratories, Kumamoto, Japan) was purchased for the detection. A total of 100 μL of medium containing 3000 transfected EJ and T24 cells were plated in a 96-well plate. Once incubated for 24, 48, 72, and 96 h, 10 μL of CCK-8 solution was added into each well. The absorbance value at 470 nm was detected by a microplate reader, and three duplicate wells were set in each group.

Colony Formation Assay

After lentivirus infection, EJ and T24 cells were seeded to 6-well plates at 3000 per well, with 3 replicate wells per group. After the colonies were grown to the appropriate size, the supernatant was aspirated, and 4% paraformaldehyde was added to the wells. After fixation for 10 min at room temperature, each well was stained with crystal violet stain for 5 min. The crystal violet stain solution was aspirated and the 6-well plate was gently washed three times with phosphate-buffered saline (PBS). The number of clones containing more than 50 cells in each well was counted for data analysis.

Cell Apoptosis Analysis

Treated EJ and T24 cells were cultured for 48 h and harvested to a centrifuge tube. The FITC Annexin V/PI Apoptosis Detection Kit I (Ribobio, Guangzhou, China) was used for detection. After centrifugation at 1000 × g for 5 min, cells were immersed in 150 μL of binding buffer, mixing 5 μL of annexin V-FITC (Annexin V-FITC), and 10 μL of propidium iodide (PI). After incubation in the dark for a while, 200 μL of binding buffer was added into the tube. Apoptosis rate of EJ and T24

cells (Quadrant 2 and Quadrant 3) was detected by flow cytometry.

Cell Cycle Analysis

Experimental EJ and T 24 cells in the logarithmic phase were inoculated in a 6-well plate. After 40 h of culture, the single cell suspension was prepared and fixed with 950 μL of 75% ethanol for 24 h. After washing in the pre-cooled PBS, the supernatant was discarded, and 500 μL of PI (Ribobio, Guangzhou, China) staining solution of propyl iodide ingot was added. The cells were incubated at 37°C for 1 h, then placed on ice and detected within 24 h.

Western Blot

Proteins of treated EJ and T24 cells were isolated using radioimmunoprecipitation assay (RIPA) reagent (Beyotime, Shanghai, China). About 30 μg of isolated protein was added to each well of 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the protein was transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), and incubated in blocking solution (5% no-fat milk) at 37°C for 2 h. Then, the membrane was incubated overnight in primary antibodies (rabbit anti-GAPDH 1:1,500; rabbit anti-STAT3 1:1,000; rabbit anti p-STAT3; CST, Danvers, MA, USA) at 4°C, followed by incubation with goat anti-rabbit secondary antibody at 37°C for 2 h (1:3,000; CST, Danvers, MA, USA). Enhanced chemiluminescence (ECL) kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to detect the relative expression of proteins.

Xenograft Assay

Ten nude mice (Vitalriver, Beijing, China) were randomly divided into 2 groups of 5 rats each. BRE-AS1 and control group EJ cells were prepared into a suspension containing 5×10^7 cells per ml, and inoculated into the axillary space of the left side of nude mice by 200 μL per nude mouse. Five weeks later, mice were sacrificed by the spine disconnection method. The short diameter and long diameter of the tumors were recorded. The volume of the xenograft was calculated: Tumor volume = long diameter × short diameter^2 /2. The weight of the xenograft was weighed. The levels of p-STAT3 in the tissues were detected by IHC. This investigation was approved by the Animal Ethics Committee of Jingmen No. 2 People's Hospital Animal Center.

Immunohistochemistry (IHC)

After paraffin embedding, the tumorigenic tissues were sliced. IHC was done according to the manufacturers' instructions (Boster, Wuhan, China). A total of 5 random visions per sample were observed in the microscopic examination.

Statistical Analysis

Data analysis was performed using GraphPad Prism 5.0 (San Diego, CA, USA) and Statistical Product and Service Solutions (SPSS) 18.0 statistical software (SPSS Inc., Chicago, IL, USA). Differences between the two groups were analyzed by the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered as statistical significance.

Results

LncRNA BRE-AS1 Was Down-Regulated in BC Tissues

To evaluate the expression of lncRNA BRE-AS1 in BC tissues, we collected 77 BC tissue samples and paired normal tissues. Expression of lncRNA BRE-AS1 in BC tissues was significantly lower than that in adjacent normal tissues (Figure 1A). It is indicated that lncRNA BRE-AS1 might act as a tumor suppressor in BC. For exploring the influence of lncRNA BRE-AS1 on BC cells, we over-expressed lncRNA BRE-AS1 level in EJ and T24 cells by transfection of LV-lncRNA BRE-AS1. Comparing to each control group, EJ cells and T24 cells expressed significant elevated lncRNA BRE-AS1 level after transfection of LV-lncRNA BRE-AS1 (Figure 1B, 1C).

Up-Regulation of LncRNA BRE-AS1 Inhibited Cell Proliferation of BC

To verify the influence of lncRNA BRE-AS1 on BC progression, we detected the proliferation of established EJ and T24 cells with CCK-8 and colony formation assay. Clearly shown in Figure 2A and 2B, transfection of LV-lncRNA BRE-AS1 remarkably reduced cell proliferation of EJ and T24 cells compared with relative control group. Similarly, fewer colonies were observed in EJ cells and T24 cells overexpressing lncRNA BRE-AS1 (Figure 2C, 2D). It is indicated that lncRNA BRE-AS1 could inhibit the proliferation of BC cells.

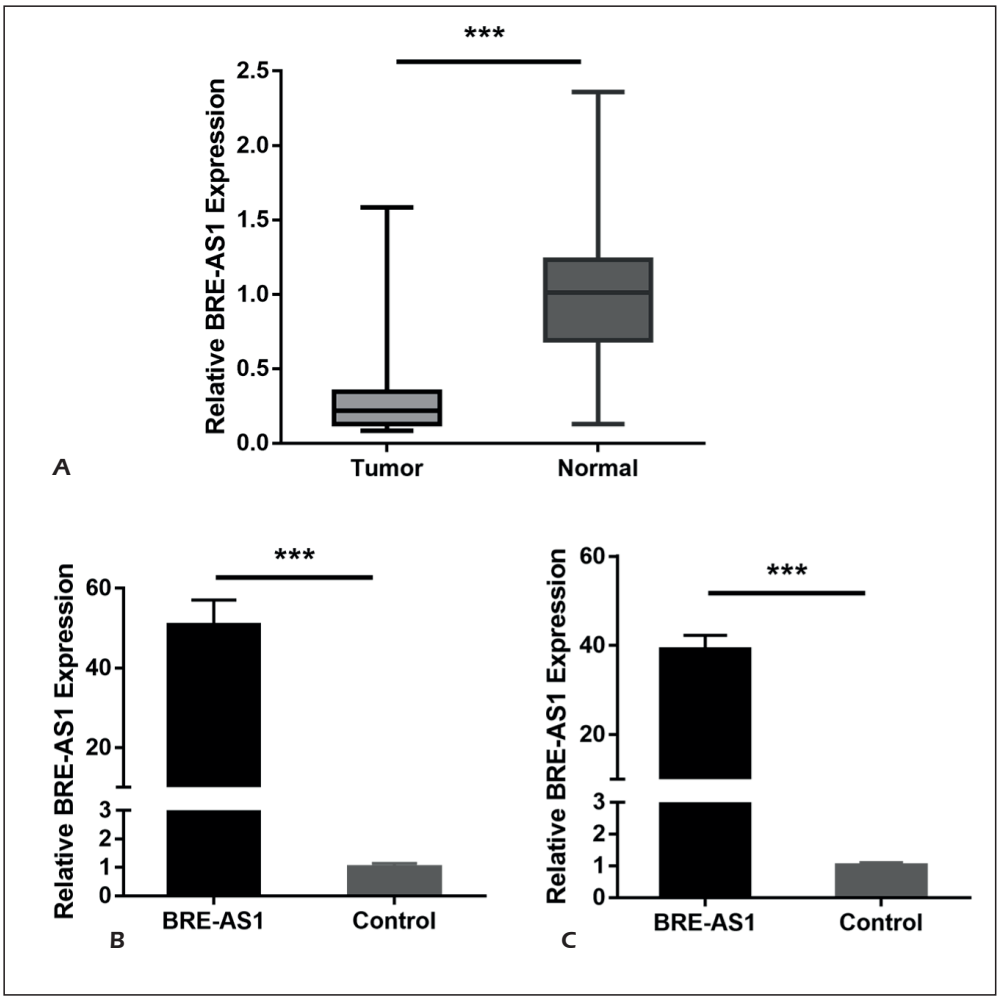

Figure 1. LncRNA BRE-AS1 was downregulated in bladder cancer (BC) tissues and cell lines. **A**, Analysis of the expression level of lncRNA BRE-AS1 in 77 pairs of BC tissue samples and adjacent normal tissue samples. **B**, Expression of lncRNA BRE-AS1 in EJ cells transfected with LV-lncRNA BRE-AS1 or negative control. **C**, Expression of lncRNA BRE-AS1 in T24 cells transfected with LV-lncRNA BRE-AS1 or negative control. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Ectopic LncRNA BRE-AS1 Affected Cell Apoptosis and Cell Cycle of BC Cells

We detected changes of cell apoptosis and cell cycle of experimental EJ and T24 cells. Overexpression of lncRNA BRE-AS1 significantly promoted cell apoptosis of EJ cells and T24 cells comparing with the control group (Figure 3A, 3B).

Furthermore, we found that lncRNA BRE-AS1 inhibited the cell cycle transition from G0/G1 phase to M phase, manifesting as elevated cell distribution in the G0/G1 phase, and decreased distribution in M phase after LV-lncRNA BRE-AS1 transfection (Figure 3C, 3D). These results suggested that lncRNA BRE-AS1 inhibited cell proliferation *via* repressing the transition from G0/G1 to M phase and promoting cell apoptosis.

LncRNA BRE-AS1 Inhibited Phosphorylation of STAT3

To further explore the underlying mechanism of lncRNA BRE-AS1 in BC, we searched several databases, including starBase and DIANA. STAT3 was found to be a potential target for lncRNA BRE-AS1. Next, we measured protein expressions of STAT3 and p-STAT3 in experimental EJ and T24 cells. Over-expression of lncRNA BRE-AS1 inhibited phosphorylation of STAT3, but had no effect on the expression of STAT3 in EJ and T24 cells when comparing with each control group (Figure 4A-4C). These data indicated lncRNA BRE-AS1 could inhibit the phosphorylation of STAT3 from suppressing the progression of BC cells.

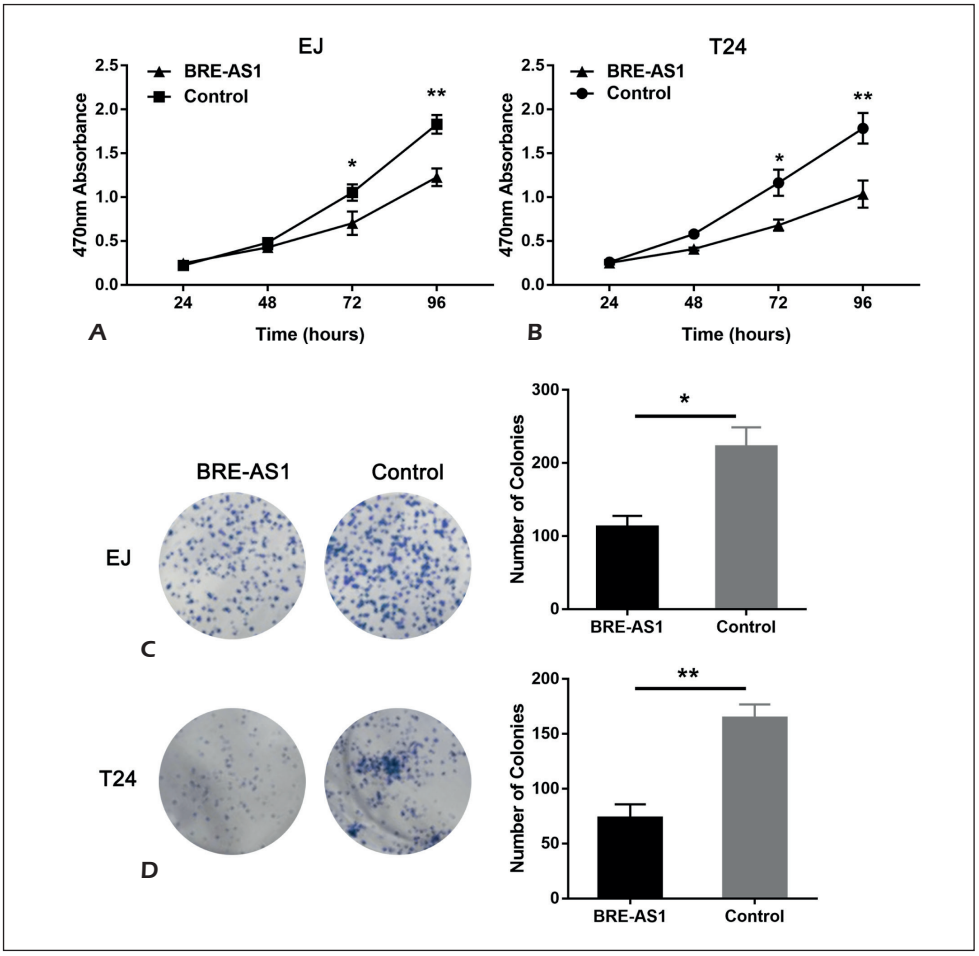

Figure 2. LncRNA BRE-AS1 effected the proliferation of BC cells. **A-B**, CCK-8 assay was performed to determine the proliferation of EJ (**A**) or T24 (**B**) cells transfected with LV-lncRNA BRE-AS1 compared to each negative control. C, D, Colony formation analysis was performed to determine the cell growth of EJ (**C**) or T24 (**D**) cells transfected with LV-lncRNA BRE-AS1, respectively (40×). *p<0.05, **p<0.01, ***p<0.001.

LncRNA BRE-AS1 Inhibited Cell Growth of BC In Vivo

As we have identified lncRNA BRE-AS1 could inhibit cell proliferation of BC cells *in vitro*, we further explored the function of lncRNA BRE-AS1 *in vivo* with nude mice. Nude mice were administrated with EJ cells transfected with LV-lncRNA BRE-AS1 or control. Five weeks later, we found that tumor weight of the lncRNA BRE-AS1 over-expression group was significantly lower than the control group (Figure 5A, 5B). Also, the growth curve of xenograft showed that lncRNA BRE-AS1 overexpression slowed down cancer growth rate of BC (Figure 5C). Next, we measured the expression of p-STAT3 in nude mice using IHC. Positive expression of p-STAT3 was markedly lower in mice with *in vivo* over-expression of lncRNA BRE-AS1p. These results suggested that lncRNA BRE-AS1 could inhibit the growth of BC cells *in vivo via* p-STAT3.

Discussion

Studies[4,15,16] on bladder cancer (BC) over the years have suggested that the occurrence and progression of BC involves a series of molecular biological changes, but the specific mechanism is far from understandings. Long non-coding RNA (lncRNA) has long been considered as 'transcriptional noisy' with rare biological functions. Later, their participation in various pathophysiological

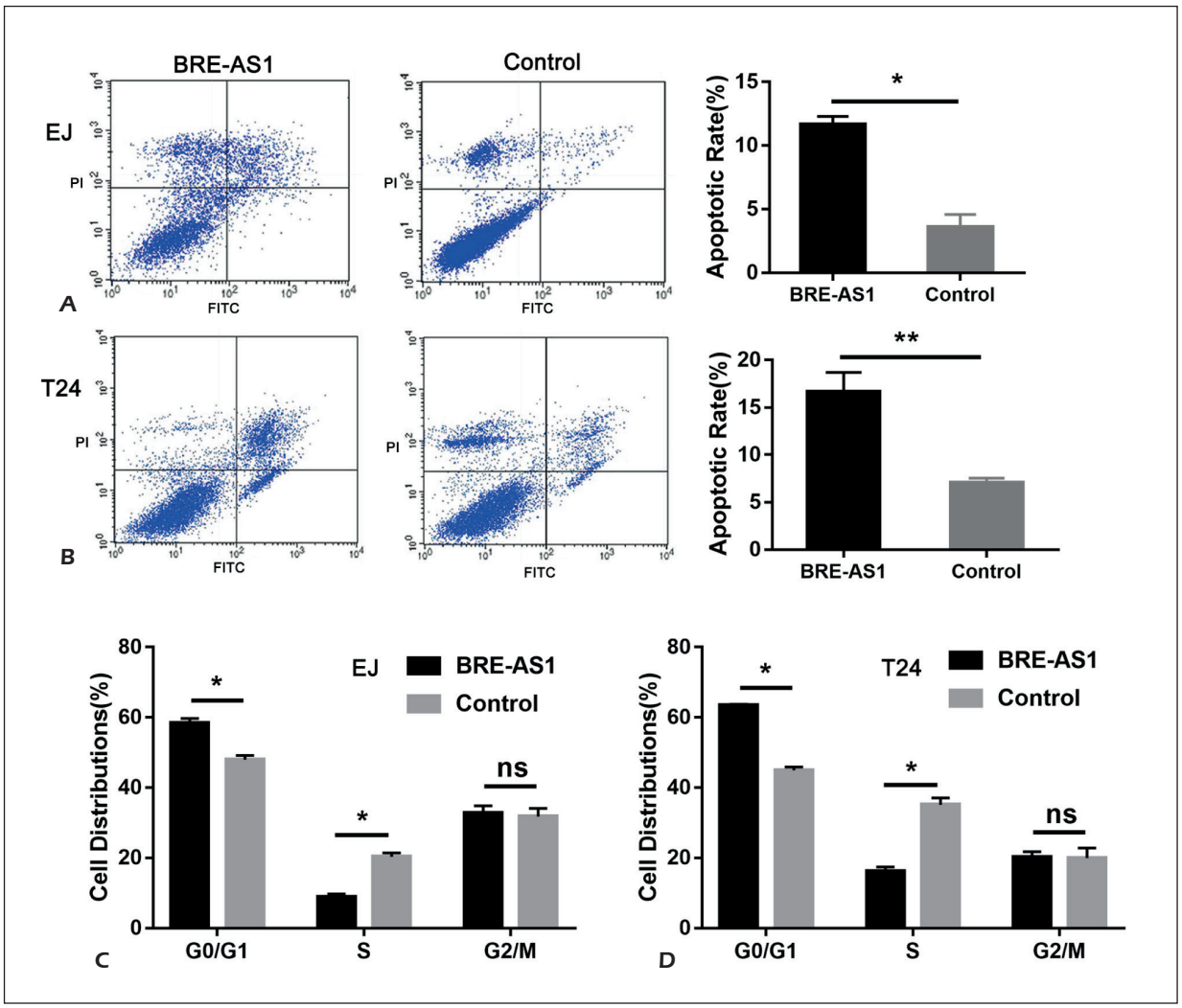

Figure 3. LncRNA BRE-AS1 affected the cell apoptosis and cell cycle of BC cells. **A-B**, Apoptosis assay performed by Flow cytometry to determine the apoptotic rate of EJ cells (**A**) or T24 cells (**B**), transfected with LV-lncRNA BRE-AS1 respectively. **C-D**, Flow cytometry performed to determine the cell distribution in cell cycle progression of EJ cells (**C**) or T24 cells (**D**) transfected with LV-lncRNA BRE-AS1. Data are presented as the mean ± SD of three independent experiments. *p<0.05, **p<0.01, ***p<0.001.

processes of cancers has been identified[7,817]. LncRNA PSTAR suppresses hepatocellular cancer by promoting p53 axis to inhibit deSUMOylation of hnRNP K[18]. In gastric cancer, lncRNA HOXC-AS1 regulates tumorigenesis *via* binding YBX1[19]. In addition, lncRNA LINC-PINT regulates a highly conserved sequence element to repress cancer cell invasion[20]. In triple-negative breast cancer, lncRNA LINP1 modulates the repair of DNA double-strand breaks[21].

LncRNA BRE-AS1 is reported to repress the proliferation and survival of non-small cell lung cancer through up-regulating NR4A3[14]. Also, it inhibits cell growth and promotes cell apoptosis of prostate cancer *via* interacting with miR-145-5p[22]. Moreover, it has a potential to be tumor-specific biomarker in chromophobe renal cell carcinoma[17]. However, no evidence has demonstrated the expression and function of lncRNA BRE-AS1 in BC. In our study we first detected the expression of lncRNA BRE-AS1 in 77 paired BC tissues and adjacent normal tissues. A significantly decreased level of lncRNA BRE-AS1 in BC tissues and cell lines indicated its potential role in BC development. Next, *in vitro* functional experiments identified that up-regulated lncRNA BRE-

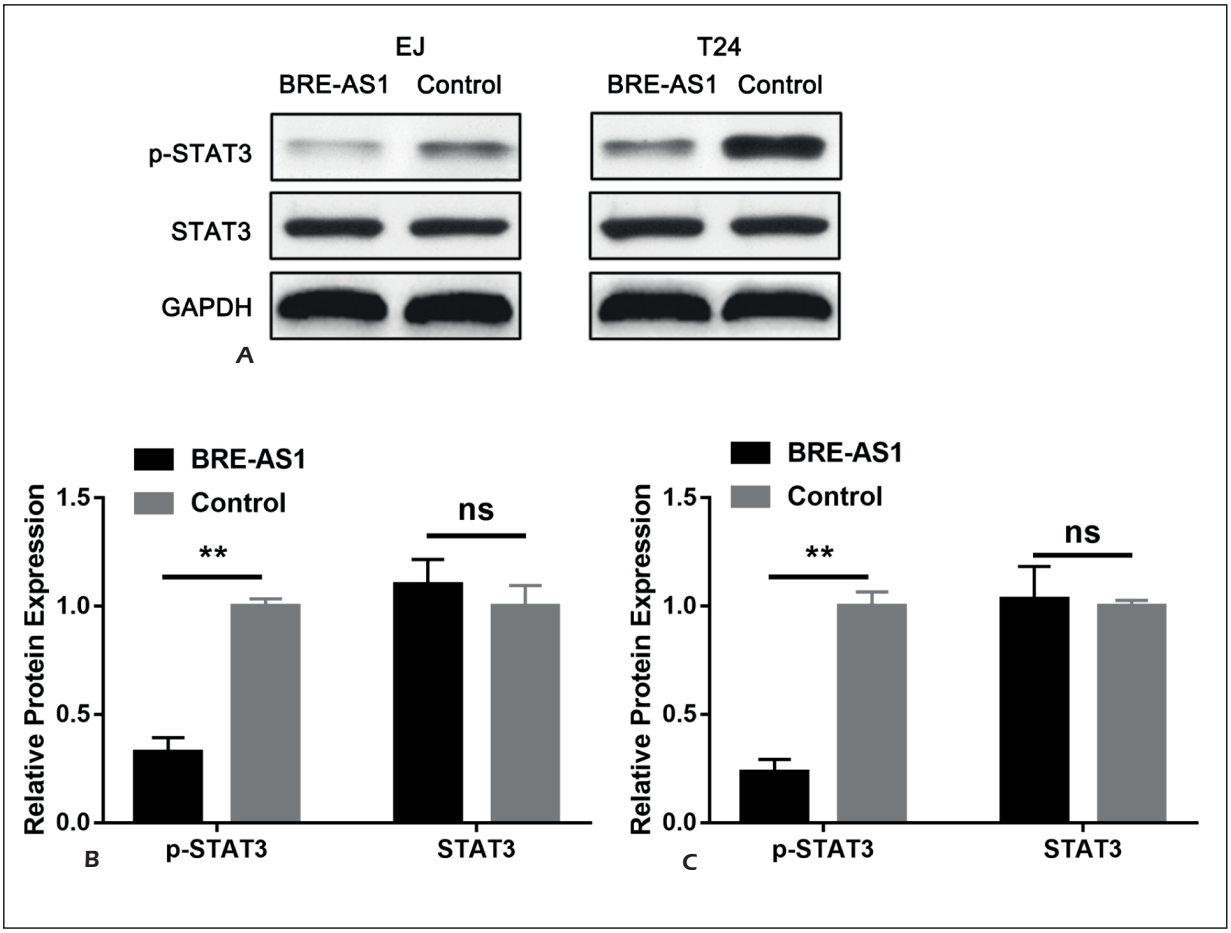

Figure 4. LncRNA BRE-AS1 inhibited phosphorylation of STAT3 in BC cells. **A**, Expressions of p-STAT3 and STAT3 in experimental cells, GAPDH was used as internal control. **B-C**, Relative protein levels of target genes. Data are presented as the mean ± SD of three independent experiments. *p<0.05, **p<0.01, ***p<0.001.

AS1 significantly reduced cell proliferation and cell cycle transition but promoted cell apoptosis. These verified lncRNA BRE-AS1 as a protective factor in BC progression.

Furthermore, we hypothesized STAT3 as a target for lncRNA BRE-AS1 in BC according to database analyses. In EJ and T24 cells transfected with LV-lncRNA BRE-AS1, phosphorylation of STAT3 was inhibited. STAT3 is indicated as an important molecule involved in several signal pathways which promoting BC development and progression[23]. Deactivation of STAT3 induced by Paeoniflorin reduces the growth of BC cells[23].

In addition, STAT3 acts as a target of ncRNAs, including DANCR, miR-124, and lncRNA SNHG16, to regulate the NC cell proliferation and metastasis[24-26]. We showed that phosphorylation of STAT3 might be downstream mechanism of lncRNA BRE-AS1 in BC.

Next, we verified that over-expression of lncRNA BRE-AS1 could inhibit *in vivo* growth of BC in nude mice. Similarly, the expression of p-STAT3 was reduced by up-regulation of lncRNA BRE-AS1 *in vivo*.

Conclusions

Taken together, our study demonstrated a new biological target lncRNA BRE-AS1 for BC. It was down-regulated in BC tissues and inhibited cell proliferation but accelerated cell apoptosis *via* STAT3 *in vitro* and *in vivo*. This might provide a novel target for the diagnosis and treatment of BC.

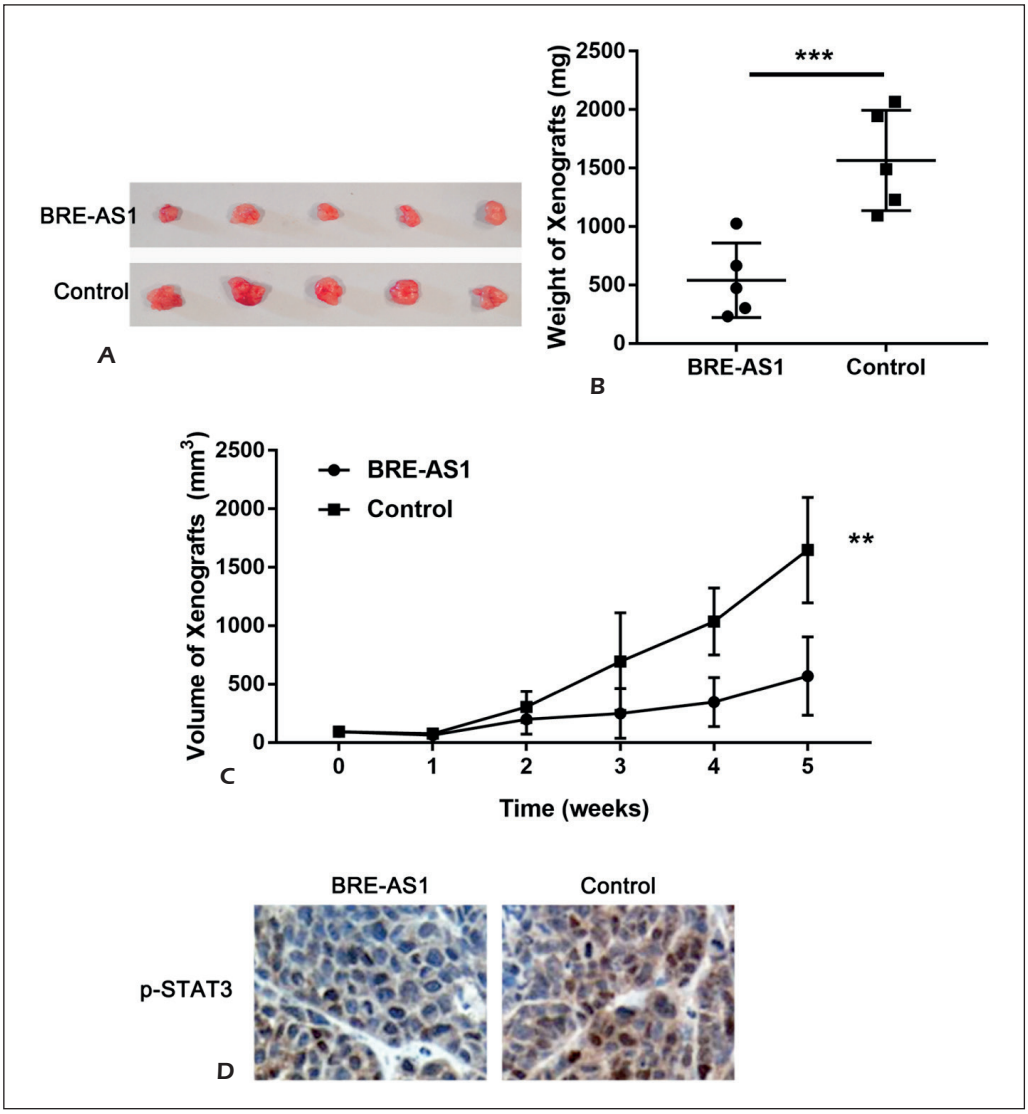

Figure 5. LncRNA BRE-AS1 inhibited BC cell growth *in vivo*. **A**, Xenografts of EJ cells transfected with LV-lncRNA BRE-AS1 or LV-Control. **B**, Analysis of the weight of xenografts. **C**, Growth curve of xenografts. **D**, IHC showed the expression of p-STAT3 protein in xenografts. (400×). Data are represented as the mean ± SD of three replicates. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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