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Upregulation of lncRNA CASC2 Suppresses Cell Proliferation and Metastasis of Breast Cancer via Inactivation of the TGF-β Signaling Pathway

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Breast cancer is one of the major malignancies with a mounting mortality rate in the world. Long noncoding RNA (lncRNA) cancer susceptibility candidate 2 (CASC2) has been identified to regulate the initiation and progression of multiple tumorous diseases according to previous studies. However, its biological role has been rarely reported in breast cancer. In the present study, lncRNA CASC2 was found to be significantly downregulated in breast cancer tissues and cell lines using real-time quantitative PCR. Furthermore, gain-of-function assays demonstrated that overexpression of lncRNA CASC2 significantly repressed breast cancer cell proliferation and metastasis. Moreover, CASC2 induced cell cycle arrest and much more early apoptosis of breast cancer. Additionally, based on the above research, we illustrated that inactivation of the TGF-β signaling pathway was involved in the function of lncRNA CASC2. Collectively, lncRNA CASC2 was a key factor in the tumorigenesis and malignancy of breast cancer, suggesting it may possibly be a potential therapy target for the treatment of breast cancer.

Key words: Long noncoding RNAs (lncRNAs); CASC2; Breast cancer; Transforming growth factor-β (TGF-β)

INTRODUCTION

Breast cancer (BC) is known as one of the most common malignant tumors in females[1,2]. Currently, the main treatments for BC patients are chemotherapy, surgery, and, sometimes, radiotherapy[3,4]. Despite many efforts that have been made to improve the treatment of BC, prognosis remains unsatisfactory. Although many tumor suppressor genes and carcinogens have been identified in accumulating studies[5–7] associated with the development and progression of BC, the detailed mechanisms are still unclear. Therefore, it is necessary to perform research on the molecular mechanisms to discover novel molecular biomarkers for the treatment of BC.

Long noncoding RNAs (lncRNAs), longer than 200 nucleotides (nt) in length, have been reported to be associated with many biological behaviors in the process of carcinogenesis. For example, in 2017, Jin et al. demonstrated that upregulated lncRNA PlncRNA-1 accelerated proliferation and induced epithelial–mesenchymal transition (EMT) in prostate cancer[8], while He et al. revealed that lncRNA UCA1 modulated the proliferation and migration of glioma through interacting with miR-182 to target iASPP[9]. The lncRNAs CCAT2, NEAT1, FOXO1, and PVT1 have been demonstrated to play crucial roles in the progression of BC[5,10–12]. Nevertheless, the knowledge about how lncRNAs exert functions in BC is insufficient. Cancer susceptibility candidate 2 (CASC2), a novel human lncRNA located at 10q26, has been initially identified to be a tumor suppressor in endometrial cancer[13]. Liao et al. concluded that CASC2 modulated the growth of glioma and its resistance to TMZ by interacting with miR-181a to regulate the phosphatase and tensin homolog (PTEN) pathway[14]. Low expression of lncRNA CASC2 caused by miR-21 stimulates the proliferation and migration of renal cell carcinoma cells[15]. The role of lncRNA CASC2 in BC is unclear, and so further investigation is urgently needed.

This work aims to explore the biological function of lncRNA CASC2 in BC. First, we measured the expression level of lncRNA CASC2 in tumor tissues and BC

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cell lines. We found that the expression level of CASC2 was obviously elevated compared with negative control (NC). Furthermore, gain-of-function assays and mechanism experiments were performed to determine the biological function and underlying mechanism of lncRNA CASC2 in BC cells. The results of the mechanism experiments revealed that overexpressed CASC2 curbed the proliferation and metastasis while inducing the apoptosis of BC cells via regulating the transforming growth factor-β (TGF-β) signaling pathway. These findings might be beneficial in determining potential therapeutic targets for gene therapy in BC.

MATERIALS AND METHODS

Histological and Intrinsic Subtypes of BC Samples

According to the St. Gallen consensus in 2013[16], BCs were mainly divided into three subtypes (luminal, HER2 positive, and triple negative). Among these subtypes, luminal was the most common type, and it was further differentiated into subtypes A and B. Luminal BC cell lines were characterized by positive estrogen receptor (ER) and/or progesterone receptor (PR) expression where PR positivity drove their luminal phenotype. HER2-positive cell lines were featured by ER negativity and HER2 positivity. They bridged the gap between luminal and basal cell lines and heterogeneously encompassed both luminal and basal features. Triple-negative BC cell lines, as represented by the name, were featured by low or no expression of all three markers (ER–PR–HER2). Considering the luminal type is the most common type of BC, tissues and samples used in our study are of the luminal type.

Human Tissues

BC tissues and their matched normal tissues were collected from BC patients who received treatment at The First Department of General Surgery, Hongqi Hospital, Mudanjiang College of Medicine from 2015 to 2016. This study was approved by the ethics committee of Hongi Hospital, Mudanjiang College of Medicine. The patients were divided into two groups: 28 with lymph node metastasis and 24 without lymph node metastasis. No patients were treated with preoperative radiotherapy, chemotherapy, or positive surgical margins in our study. Informed consent was obtained from all the patients. We acquired tumor tissues and paired healthy tissues from patients and immediately froze them with liquid nitrogen before storage at −80°C for future use.

Cell Culture and Treatment

The healthy human breast cell line HCC1937 and the BC cell lines LCC9, MDA-MB-231, and MCF-7 were obtained from the Chinese Academy of Sciences. All cells were cultured in RPMI-1640 medium (Hyclone, Logan, UT, USA) together with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and antibiotics. We conserved all the cells in the recommended culture conditions. The cells were cultured at 37°C in a humidified environment and 5% CO_2.

si-Smad2 and si-NC small interfering RNA (siRNA) sequences were bought from GenePharma (Shanghai, P.R. China) for knockdown of Smad2. To knock down TGF-β, a 10 mM stock solution of LY2109761 (Eli Lilly and Co., Indianapolis, IN, USA) in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) was prepared. The CASC2 sequence was synthesized by RiboBio (Guangzhou, P.R. China) and then subcloned into pcDNA 3.1. pcDNA-CASC2 and empty pcDNA vector (control) were transfected into BC cells by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Forty-eight hours later, the levels of CASC2 expression were detected by real-time quantitative polymerase chain reaction (RT-qPCR).

RT-qPCR

We conducted qPCR in accordance with previous studies[10,17]. The TRIzol Kit (Invitrogen) was employed to independently isolate all RNAs from tumor tissues and cell lines. With the help of an Omniscript RT Kit (Qiagen, Valencia, CA, USA), complementary DNA (cDNA) was subsequently synthesized from total RNAs under specified instructions. We carried out the RT-qPCR on the Mastercycler ep realplex (Eppendorf 2S, Hamburg, Germany). The 25-μl reaction mixture consisted of 1 μl of cDNA from samples, 12.5 μl of 2× Fast EvaGreen qPCR Master Mix, 1 μl of primers (10 mM), and 10.5 μl of RNase/DNase-free water. The Ct value was defined as the cycle number at which the fluorescence intensity reached a certain threshold where amplification of each target gene was within the linear region of the reaction amplification curves. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was regarded to be the internal control. The primers used in this experiment were as follows: CASC2, 5′-GCACATTGGACGGTGTTTCC-3′ (forward) and 5′-CCCAGTCCTTCACAGGTCAC-3′ (reverse); GAPDH, 5′-CGGAGTCAACGGATTTGGTC GTAT-3′ (forward) and 5′-AGCCTTCTCCATGGTGGT GAAGAC-3′ (reverse). We determined the relative mRNA expression of CASC2 by means of the $2^{-\Delta\Delta Ct}$ method.

Cell Viability

MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltrtrazolium bromide] assay was designed to define cell viability. Cells (5×10^3)/well were transfected with a vector and then sealed in a flat-bottomed 96-well plate for 24 h. Finally, the mixture was cultured in normal medium. At 0, 24, 48, 72, and 96 h following transfection, each well was supplemented with the MTT solution (5 mg/ml, 20 μl). After 4 h of culture, we discarded the media and

added 100 µl of DMSO to each well. We made use of the optical density (OD) of cell lysates at 560 nm to determine the relative number of surviving cells. The experiments were carried out three times independently.

Colony Formation Assay

Before being placed into six-well plates and then cultured at 37°C, cells (500 cells/well) were treated with a specified vector. After 14 days, the cells were fixed and dyed with 0.1% crystal violet for 15 min at room temperature. The number of visible colonies was manually counted.

Cell Migration and Invasion Assays

After treatment with the indicated expression vectors, LCC9 and MDA-MB-231, cells (3×10^5 cells/ml) were conserved. The Transwell assays (Corning, Corning, NY, USA) were employed to estimate cell metastasis and invasion. Treated cells were suspended in serum-free medium, followed by seeding 200 µl of cell suspensions into the upper chamber with or without Matrigel (BD Biosciences, San Diego, CA, USA). The cells in the bottom of the chambers were replenished with complete medium. After 48 h of migration and invasion, we determined the number of migrated or invaded cells by using the microscope from five different perspectives. Each sample was prepared three times.

Flow Cytometric Analyses of Apoptosis and Cell Cycle Distribution

The Annexin-V/FITC Apoptosis Detection Kits (BD Biosciences) were used to analyze apoptosis according to the manufacturer's instructions. To analyze the cell cycle, we gathered cells directly or 48 h after transfection, followed by washing with ice-cold phosphate-buffered saline (PBS) and fixation with 70% ethanol overnight at −20°C. The fixed cells were rehydrated in PBS for 10 min and incubated in RNase A (1 mg/ml) for 30 min at 37°C. The cells were then subjected to PI/RNase staining at normal temperature for 20 min, and then subjected to flow cytometric analysis with a FACScan instrument (Becton Dickinson, Mountain View, CA, USA) and the Cell Quest software (Becton Dickinson, San Jose, CA, USA).

Western Blot Analysis and Antibodies

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10%) was applied to isolate total protein lysates, followed by electrophoretic transfer to polyvinylidene difluoride membranes (Roche, Palo Alto, CA, USA). A mouse anti-GAPDH monoclonal antibody was used to confirm the protein loading. The membranes were stained with 10% skimmed milk in TBST for 2 h at room temperature, washed, and then probed with rabbit anti-human TGF-β (ab27969; 1:2,000 dilution), anti-human Smad2 (ab40855; 1:2,000 dilution), α-SMA (ab59526; 1:2,000 dilution), and GAPDH (ab8245; 1:3,000 dilution) at 4°C overnight. Finally, secondary antibody immunoglobulin G (IgG) (ab6785; 1:5,000; ZSGB-BIO, Beijing, P.R. China) conjugated to horseradish peroxidase was applied for 2 h at room temperature. The proteins were visualized with the help of an enhanced chemiluminescence system, followed by its exposure to x-ray film. The dilution medium used in this assay was the Human Detection Antibody Diluent (EPX-33333-000; Invitrogen). All the referred antibodies mentioned above were obtained from Abcam (Cambridge, MA, USA).

Statistical Analysis

Data were presented as the means±standard deviation from at least three different experiments. Statistical analyses were conducted using the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Student's *t*-test was applied to determine the difference between groups. Multiple-group comparisons were analyzed with one-way analysis of variance (ANOVA). A value of $p<0.05$ was considered to be statistically significant.

RESULTS

CASC2 Was Downexpressed in BC Tissues, Metastasis Tissues, and BC Cell Lines

In order to determine the role of CASC2 in BC, we assessed the relative expression level of CASC2 in BC tissues and corresponding normal tissues. The results in Figure 1A reveal that the level of CASC2 was markedly lower in cancer tissues than that in the matched normal tissues. Moreover, RT-qPCR was utilized to compare the level of CASC2 in the migratory cancer tissues and nonmigratory tissues. The results showed that the expression level of CASC2 in the tissues with metastasis was significantly decreased with respect to that in the tissues without metastasis (Fig. 1B). We also explored the internal relationship between CASC2 expression and the TNM stage of BC. The results showed that the expression level of CASC2 was much lower in the higher stage (Fig. 1C). Additionally, the expression level of CASC2 in three BC cell lines (LCC9, MDA-MB-231, and MCF-7) and the healthy breast cell line HCC1937 was detected by RT-qPCR. The results showed that CASC2 was relatively much lower in BC cell lines than in the normal HCC1937 cell line (Fig. 1D). These findings all indicated that CASC2 might suppress tumor formation in BC.

Overexpression of CASC2 Suppressed Cell Proliferation, Caused Cell Cycle Arrest, and Induced Apoptosis

On the basis of the above experiments, we found the lowest CASC2 expression in the MCF-7 and LCC9

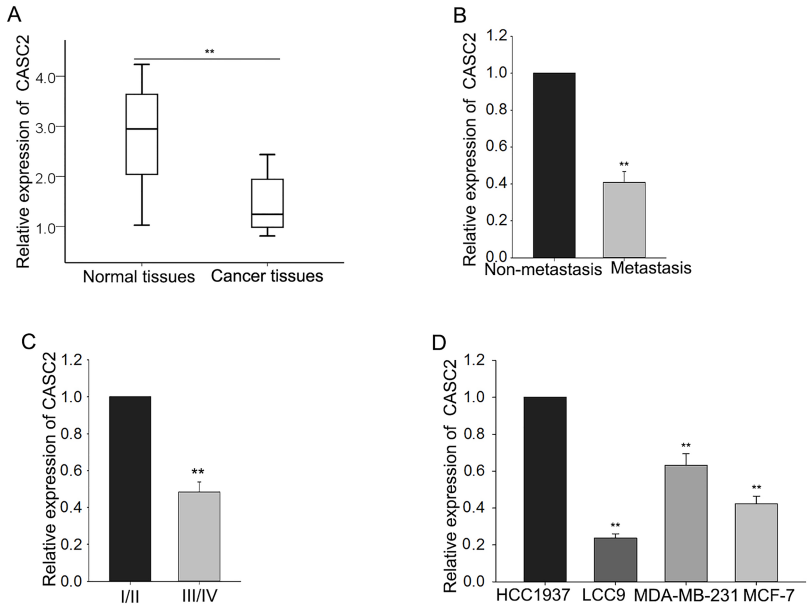

Figure 1. Cancer susceptibility candidate 2 (CASC2) was downexpressed in breast cancer (BC) tissues, metastasis tissues, and BC cell lines. (A) Downregulated CASC2 was defined in BC tissues and matched healthy tissues by real-time quantitative polymerase chain reaction (RT-qPCR). (B) RT-qPCR was used to determine the expression level of CASC2 in cancer tissues with or without metastasis. (C) The positive correlation between CASC2 expression and TNM stage was detected in BC. (D) The expression levels of CASC2 in three BC cell lines (LCC9, MDA-MB-231, and MCF-7) and a healthy breast cell line (HCC1937) were detected by RT-qPCR. Error bars represented the mean ± standard deviation (SD) of at least three independent experiments. $^{**}p < 0.01$ versus control group.

cell lines; hence, we chose to use them in the following experiments. To explore how CASC2 influenced cell proliferation in the LCC9 and MCF-7 cells transfected with pcDNA-CASC2, pcDNA was used as the NC. The appropriate transfection efficiency was detected after 48 h (Fig. 2A). The MTT experiment was employed to measure how CASC2 affected cell viability. As shown in Figure 2B, forced expression of CASC2 significantly decreased cell viability, compared with NCs. Consistent with the MTT assay, the results from the colony formation assay further confirmed the growth inhibition of CASC2 in BC cells (Fig. 2C). To comprehend the potential mechanism of CASC2-mediated growth inhibition, experiments exploring cell apoptosis and cell cycle distribution were performed by flow cytometry. Cell apoptosis analysis revealed that upregulation of CASC2 significantly increased the apoptosis rate of LCC9 cells and MCF-7 cells (Fig. 2D). As illustrated in Figure 2E, overexpressed CASC2 in LCC9 cells and MCF-7 cells obviously caused cell cycle arrest at the G_0/G_1 phase. These findings explained that CASC2 repressed the tumorigenesis in BC and could inhibit cell proliferation through influencing cell cycle and apoptosis.

Overexpressed CASC2 Inhibited the Migration of BC Cells

As shown in Figure 1B, the level of CASC2 expression was closely related with the metastasis in BC tissues, so we supposed that CASC2 could regulate migration and invasion in BC cells. To determine the regulatory principle of CASC2 on cell migration capacity, we performed the Transwell assay. It is shown in Figure 3A that CASC2 overexpression weakened the migration capacity of BC cells. Consistent with the migration result, the invasion capacity of BC cells was also decreased when CASC2 was overexpressed (Fig. 3B). These findings revealed that CASC2 could inhibit BC cell migration and invasion capacities.

CASC2 Is a Tumor Suppressor in BC Cells Through Inhibiting TGF-β Signaling

It has been verified that lncRNAs can affect tumorigenesis via modulating the TGF-β signaling pathway[10,18]. Similarly, CASC2 was able to modulate a certain signal pathway to exert its antioncogenic function in glioma[19]. In this study, we speculated that CASC2 could affect the malignant progression of BC via modulating the TGF-β signaling pathway. To investigate the underlying mechanism, we detected the levels of proteins associated with the TGF-β signaling pathway such as TGF-β, Smad2, and α-SMA in BC cells transfected with CASC2 expression vector. As shown in Figure 4A and B, highly expressed CASC2 reduced the increased mRNA and protein levels of TGF-β, Smad2, and α-SMA in two BC cells. Similarly, TGF-β was inhibited in BC cells by ly2109761. As illustrated in Figure 4C (left), the expression level of CASC2 was elevated by the knockdown of TGF-β. These data

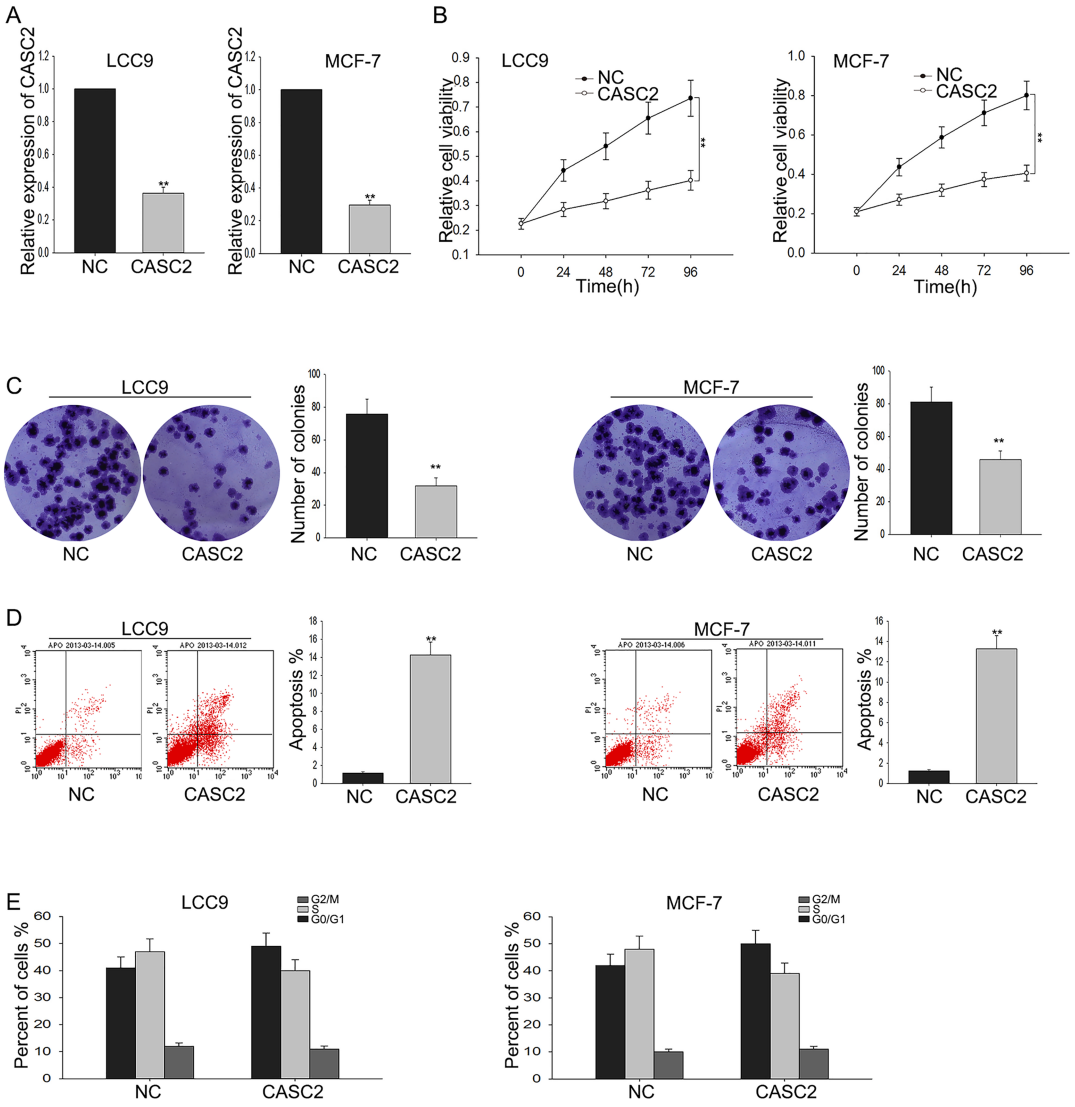

Figure 2. Overexpression of CASC2 suppressed proliferation, caused cell cycle arrest, and induced apoptosis. (A) LCC9 and MCF-7 cells were transfected with CASC2 expression vector, and satisfactory transfection efficiency was confirmed by RT-qPCR. We found that the CASC2 group had a much higher expression than the negative control (NC) group. The ability of CASC2 to affect cell proliferation was determined by (B) MTT and (C) colony formation experiments. The CASC2 group had lower proliferation ability than the NC group. Flow cytometry assays were applied to analyze how CASC2 influenced (D) cell apoptosis and (E) cell cycle. Compared with NC, we found that cell apoptosis was induced by transfecting with pcDNA-CASC2, while cell cycle was stagnated. Error bars represented the mean ± SD of at least three independent experiments. $^{**}p < 0.01$ versus the NC group.

showed that CASC2 exerted its function as a tumor suppressor in BC via regulating the TGF-β signaling pathway. Smad2 is known as a signal transduction protein that mediates signal transduction in human diseases[20,21]. Smad2 downregulation could negatively affect the activity of TGF-β in human cancers[22]. The result of the Western blot showed that Smad2 knockdown inhibited the TGF-β expression in BC cells (Fig. 4C, right). On the basis of the former experiment, we knew that overexpression of CASC2 repressed Smad2 expression. Together, we supposed that CASC2 promoted Smad2 expression to inactivate TGF-β signaling in BC cells. For further proof, Western blot analysis was conducted in both BC cells. The results suggested that TGF-β induced Smad2 phosphorylation, but the result was reversed by CASC2 overexpression (Fig. 4D). So we concluded that upregulation of CASC2 inactivated TGF-β signaling to repress BC cell proliferation and metastasis.

DISCUSSION

In recent years, researchers have fixed their eyes on lncRNAs that participate in various biological behaviors

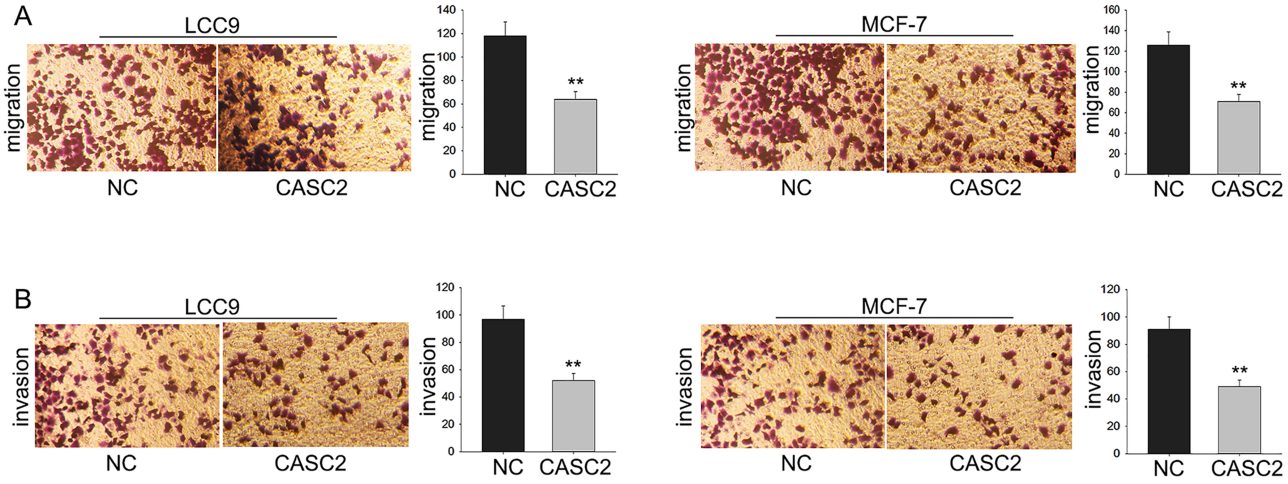

Figure 3. Overexpressed CASC2 inhibited the migration of BC cells. The Transwell assay helped us detect changes in the (A) migration and (B) invasion of BC cells. Compared with NC, we observed that both migration and invasion were suppressed after cells were transfected with pcDNA-CASC2. Error bars represented the mean±SD of at least three independent experiments. $^{**}p<0.01$ versus the NC group.

of different cancers. Accumulating research has revealed that many lncRNAs are involved in the tumorigenesis of various kinds of cancers. For example, lncRNA MEG3 was reported to be a tumor suppressor and to predict a better prognosis in BC[23], as well as induce hypermethylation in cervical cancer and trigger the epigenetic manipulation of EMT in lung cancer[24,25]. The lncRNA GAS5 could inhibit carcinogenesis and improve radiosensitivity through inhibiting the level of miR-135b in non-small cell lung cancer[26]. However, oncogenes like lncRNA CCAT1 were demonstrated to predict poor prognosis in hepatocellular carcinoma (HCC) and accelerate migration via modulating EMT mediated by Snail-2[27]. CCAT2 also boosted the proliferation and migration of BC through influencing the TGF-β signaling pathway[10].

CASC2, a recently identified lncRNA, has been demonstrated to take part in many types of cancers. For example, in 2017, Pei et al. reported that attenuated CASC2 facilitated the malignancy of bladder cancer through activating the Wnt/β-catenin signaling pathway[28]. Liao et al. demonstrated that CASC2 cooperated with miR-181a to mediate glioma growth and sensitivity to TMZ through the PTEN pathway[14]. In 2016, Li et al. revealed that CASC2 suppressed the growth of GC cells through affecting the mitogen-activated protein kinase (MAPK) signaling pathway[29], and Cao et al. depicted that CASC2 promoted the malignancy of renal cell carcinoma cells[30] and played critical roles in colorectal cancer[31], non-small cell lung cancer[32], and endometrial cancer[33]. CASC2 was reported to be a tumor suppressor in many articles. It could suppress tumorigenesis in lung adenocarcinoma[34], esophageal carcinoma[35], HCC[36], and gastric cancer[37]. The above findings all suggest that CASC2 has an antioncogenic function in various tumors. Therefore, we speculated that CASC2 could inhibit tumor progression in BC. This has still not been verified by other researchers; thus, we thought our study would be valuable and meaningful. Compared with a previous study, we investigated the antioncogenic function of CASC2 and overexpressed CASC2 in cancerous cells[38] to explore the effect of CASC2 overexpression on proliferation and metastasis. CASC2 has not been well elucidated in BC; thus, our study is novel and original. CASC2 was also discovered to be a tumor opponent via inhibition of the Wnt/β-catenin signaling[19]. Our study also revealed that CASC2 could be an opponent of BC progression via inactivating TGF-β signaling. Interaction between CASC2 and Wnt/β-catenin signaling in BC was firstly elucidated in our study. In terms of this article, it was discovered that CASC2 was obviously underexpressed in BC tissues and cell lines. Moreover, gain-of-function assays revealed that overexpression of CASC2 curbed the proliferation of BC cells in vitro. Flow cytometry suggested that CASC2 mediated growth inhibition through affecting cell cycle and apoptosis. Besides, the results of the Transwell assays revealed that highly expressed CASC2 suppressed BC metastasis and invasion.

The TGF-β signaling pathway can be activated/inactivated by lncRNAs, thus modulating progression of various cancers[10,18,39]. We confirmed the oncogenic function of this pathway. In this article, we elucidated the same function of TGF-β signaling in BC. According to the above references, the TGF-β signal was inactivated by antioncogenic lncRNAs. Similarly, we also observed that downregulation of CASC2 could be overexpressed to inactivate TGF-β signaling in BC. Fortified CASC2 remarkably lessened the mRNA and protein levels of TGF-β, Smad2, and α-SMA in BC cells. Therefore, the results illuminated that lncRNA CASC2 might perform

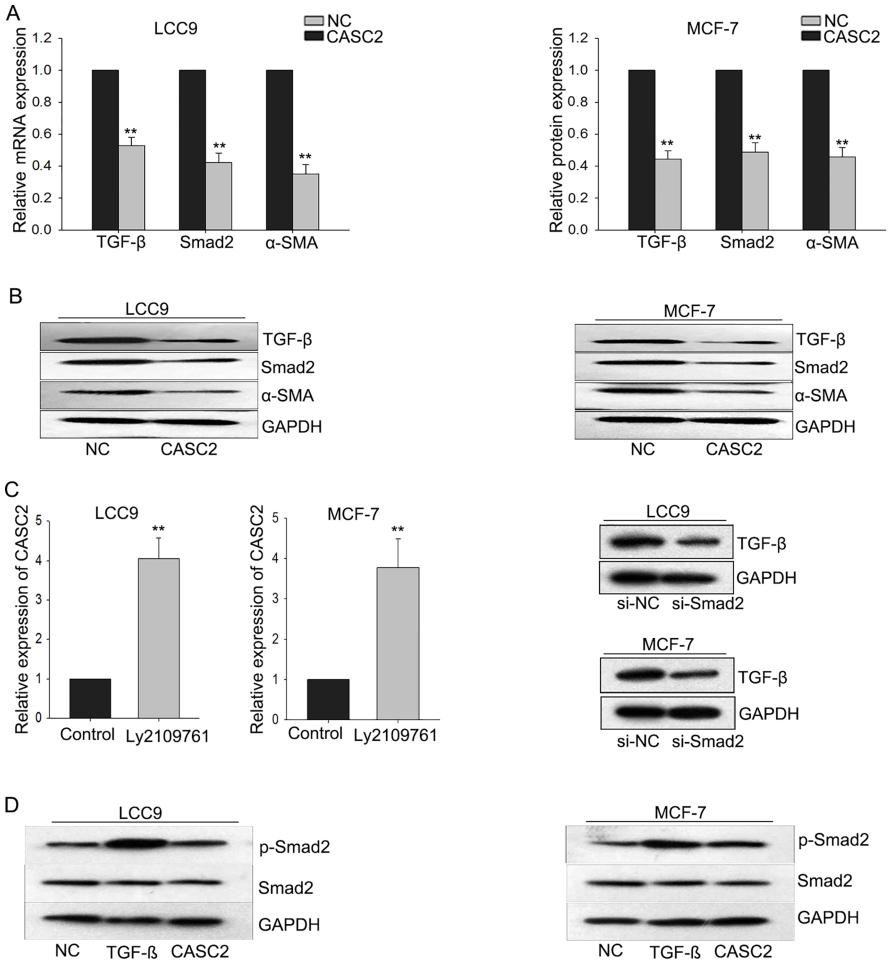

Figure 4. CASC2 was a tumor suppressor in BC cells through inhibiting transforming growth factor-β (TGF-β) signaling. The levels of TGF-β, Smad2, and α-SMA were defined through (A) RT-qPCR and (B) Western blot in response to the high level of CASC2. (C) Knockdown of TGF-β contributed to CASC2 upregulation (left). Downregulated Smad2 reduced the protein level of TGF-β in BC cells (right). (D) The result of the Western blot assay suggested that CASC2 could reduce the TGF-β-increased Smad2 phosphorylation in BC cells. Error bars represented the mean ± SD of at least three independent experiments. $^{**}p < 0.01$ versus the control or NC group.

as a tumor suppressor in BC progression via targeting the TGF-β signaling pathway. Although we did not detect the whole function of the TGF-β signaling pathway in BC, we will perform further studies to elucidate the effect of TGF-β on CASC2 expression in the future.

In general, our study indicated that CASC2 was strikingly downexpressed in BC tissues and cell lines. Overexpressed CASC2 could suppress BC cell proliferation through influencing the cell cycle, inducing cell apoptosis, and repressing cell metastasis and invasion. Moreover, the high level of CASC2 inactivated the TGF-β signaling pathway. Thus, CASC2 probably inhibited the progression of BC through inactivating the TGF-β signaling pathway. We did all these experiments in MCF-7 and LCC9 cells. Since the MDA-MB-231 cell line holds high metastatic ability, we will perform future experiments using it. All in all, our findings would be helpful in cancer research.

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