

Downregulation of BC200 in ovarian cancer contributes to cancer cell proliferation and chemoresistance to carboplatin

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Abstract. Previous studies have demonstrated that long non-coding RNAs (lncRNAs) serve an important role in carcinogenesis. BC200 is a lncRNA that is reportedly associated with ovarian cancer. The aim of the present study was to investigate this potential association between BC200 and ovarian cancer, and to subsequently analyze the biological function of BC200 in the disease. BC200 expression was compared in ovarian cancer tissue and normal ovarian tissue samples through the use of quantitative polymerase chain reaction. To allow the biological function of BC200 in ovarian cancer to be analyzed, small interfering RNA was used to knock down the expression of BC200 in SKOV3 and A2780 ovarian cancer cells. The proliferative, invasive and migratory abilities of the cells were identified by means of cell counting kits and Transwell assays. Carboplatin was also used to treat the ovarian cancer cells, and a luminescent cell viability assay was subsequently used to detect the sensitivity of the cells to the carboplatin. The results demonstrated that BC200 expression was reduced in ovarian cancer compared with normal ovarian tissue samples. In the SKOV3 and A2780 cells, BC200 exerted no effect on invasive or migratory ability, however, the inhibition of BC200 was demonstrated to promote cell proliferation. Additionally, it was observed that carboplatin induced BC200 expression in the cell

lines, and that the inhibition of BC200 decreased the sensitivity of the cells to the drug. BC200 is therefore likely to have a tumor suppressive function in ovarian cancer by affecting cell proliferation. Furthermore, BC200 appears to serve a role in the mediation of carboplatin-induced ovarian cancer cell death.

Introduction

Ovarian cancer is one of the most prevalent cancers observed in females and is the leading cause of mortality among gynecological malignancies (1). The 5-year survival rate of patients with ovarian cancer has remained relatively consistent over the past few decades, despite advances in surgical techniques and medical treatment (1). Increased efforts are required to achieve a greater understanding of the molecular mechanisms underlying ovarian cancer, and to aid the development of novel diagnostic and therapeutic strategies.

The human genome produces a large number of long non-coding RNA (lncRNA) transcripts that have structural, regulatory and other unknown functions (2). In recent years, lncRNAs have gained increasing attention from researchers. Different from the small non-coding RNAs (18-200 nucleotides), lncRNAs are generally defined as being ≥ 200 nucleotides in length (3). With the advance of high-throughput sequencing technologies, it has been predicted that thousands of human lncRNAs may exist (4). Despite only a handful of lncRNAs being functionally characterized to date, lncRNAs are believed to serve a role in almost all processes of cellular and molecular biology, including the initiation and progression of cancer (5).

BC200 RNA is a 200-nucleotide non-coding RNA that is specifically expressed in neurons of the human nervous system (6,7). As a translational modulator, BC200 is implicated in the regulation of local synaptodendritic protein synthesis in neurons through its interaction with certain translational machinery components (8). However, the neuron-specific control of BC200 expression is dysregulated during carcinogenesis in non-neuronal human tissues (9). It has been reported that BC200 RNA is expressed in carcinomas of the breast, cervix, esophagus, lungs, ovaries, parotid gland and tongue, but not in corresponding normal tissues (9). Similar to the

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Abbreviations: lncRNAs, long non-coding RNAs; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; NC, negative control; CCK-8, cell counting kit-8

Key words: long non-coding RNA, BC200, ovarian cancer, proliferation, carboplatin

majority of lncRNAs, the role of BC200 in cancer remains unclear.

In the present study, BC200 expression was detected in ovarian cancer tissues and normal ovary tissues through the use of quantitative polymerase chain reaction (qPCR). Furthermore, the biological functions of BC200 were evaluated using RNA interference in two ovarian cancer cell lines.

Materials and methods

Clinical samples. A total of 22 ovarian tissue samples, including 10 normal ovarian samples and 12 epithelial ovarian cancer samples, were obtained from 22 patients who had previously undergone surgery at the First and Third Affiliated Hospitals of Harbin Medical University (Harbin, China), between May 2012 and December 2014. Of the 12 epithelial ovarian cancer samples, 8 were serous, 2 endometrial and 2 mucinous. Normal ovarian samples were obtained from patients who had previously received an ovariectomy due to the presence of endometrial or cervical cancer. Therefore, the normal ovarian tissues and epithelial ovarian cancer tissues were not matched from the same patient. Patients in the two groups (normal and cancer) were selected from the same hospital, and were matched to cases in terms of the same age (± 5 years) and date of hospital admission. All samples were flash-frozen in liquid nitrogen and stored at -80°C until RNA extraction. The diagnosis of each sample was confirmed by at least two pathologists. No patient had received any therapy prior to surgery. The research was performed according to The Code of Ethics of the World Medical Association (Declaration of Helsinki). All patients provided informed consent prior to their inclusion in the study, and the study was initiated only after approval by the Ethical Committee of Harbin Medical University.

Cell lines. The human ovarian cancer cell lines, SKOV3 and A2780, used in the present study were purchased from the Cell Resource Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; HyClone; GE Healthcare Life Sciences, Logan, UT, USA), with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 $\mu\text{g}/\text{ml}$ streptomycin and 100 IU/ml penicillin at 37°C in a humidified atmosphere containing 5% CO_2 .

RNA extraction and qPCR. Total RNA from the frozen tissue samples and cell lines was extracted with TRIzol[®] (Invitrogen; Thermo Fisher Scientific, Inc.), following the manufacturer's protocol. The cDNA was synthesized from 1 μg of total RNA using EasyScript[™] Reverse Transcriptase (Beijing TransGen Biotech Co., Ltd., Beijing, China). BC200 levels were quantified by qPCR using SYBR Green PCR MasterMix (Beijing TransGen Biotech Co., Ltd.) and CFX96 Touch[™] Real-Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Primers (Shanghai GenePharma Co., Ltd., Shanghai, China) specific for BC200 were designed as follows: Forward, 5'-AGACCTGCCTGGGCAATATAGC-3' and reverse, 5'-GTTGTTGCTTTGAGGGGAAGTTACG-3'. BC200 levels were normalized to β -actin. Primers specific for

β -actin were designed as follows: Forward, 5'-CCCTGGCAC CCAGCAC-3' and reverse, 5'-GCCGATCCACACG-GAGTAC-3'. PCR conditions were as follows: Denaturation at 95°C for 5 min; and then 39 cycles of amplification at 95°C for 10 sec/cycle and 60°C for 30 sec. Melting curve analyses were performed using the PCR products and progressive heating from 65°C to 95°C .

Transfection of small interfering RNA (siRNA). Three siRNAs (siRNA1, siRNA2 and siRNA3) targeting BC200, and a scrambled siRNA used as negative control (NC), were purchased from Shanghai GenePharma Co., Ltd. siRNA oligonucleotides (50 nmol/l) were transfected into the SKOV3 and A2780 cells using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), following the manufacturer's protocol. Target sequences for BC200 siRNAs were as follows: siRNA1, 5'-AATAAGCGTAACTTCCCTCAAAG-3'; siRNA2, 5'-AACTTCCCTCAAAGCAACAACCC-3'; and siRNA3, 5'-AAGCGTAACTTCCCTCAAAGCAA-3'.

Cell counting kit-8 (CCK-8) assay. At 24 h post-transfection, the cells were seeded in a 96-well plate at a density of 1×10^4 cells/well, and subsequently incubated in DMEM supplemented with 10% FBS at 37°C for 3 days. During the 3-day incubation, cell proliferation was evaluated using the CCK-8 kit (Beyotime Institute of Biotechnology, Beijing, China) at 0 (after adherence to the wall for 4 h), 24, 48 and 72 h. The CCK-8 reagent was added to the cell culture medium at 10 $\mu\text{l}/\text{well}$. Following incubation for 2 h, absorbance was determined at a wavelength of 450 nm.

Luminescent cell viability assay. At 24 h post-transfection, the cells in the 96-well plate were treated with graded carboplatin (0, 50 and 100 $\mu\text{g}/\text{ml}$; Qilu Pharmaceutical Co., Ltd., Jinan, China) for 48 h. Cellular viability was measured using the CellTiter-Glo[®] Luminescent Cell Viability assay (Promega Corporation, Madison, WI, USA). The 96-well plate was briefly equilibrated to room temperature for ~ 30 min. The CellTiter-Glo[®] reagent (100 μl) was then added to each well. Following this, the media and reagent were mixed for 2 min on an orbital shaker and left to incubate at room temperature for 10 min prior to recording luminescence, using a SpectraMax[®] M5 microplate reader (Molecular Devices, Inc., Sunnyvale, CA, USA). Luminescence values were normalized to a dimethyl sulfoxide control, and final values were presented as a relative percentage.

Cell migration and invasion assays. A cell migration assay was performed using a Transwell chamber (Costar; Corning Incorporated, Corning, NY, USA), and an invasion assay was performed using the BioCoat Matrigel Invasion Chamber (BD Biosciences, Franklin Lakes, NJ), following the manufacturer's protocol. The diameter of the pore was 8.0 μm . Following transfection with siRNA for 24 h, 5×10^4 cells (SKOV3 and A2780, individually) were plated in the upper chamber in serum-free media. The bottom chamber contained DMEM with 10% FBS. The chamber was incubated at 37°C for 24 h to allow for cell migration and invasion, and subsequently the bottom of the chamber insert was fixed and stained with hematoxylin. Cells on the stained membrane were counted in

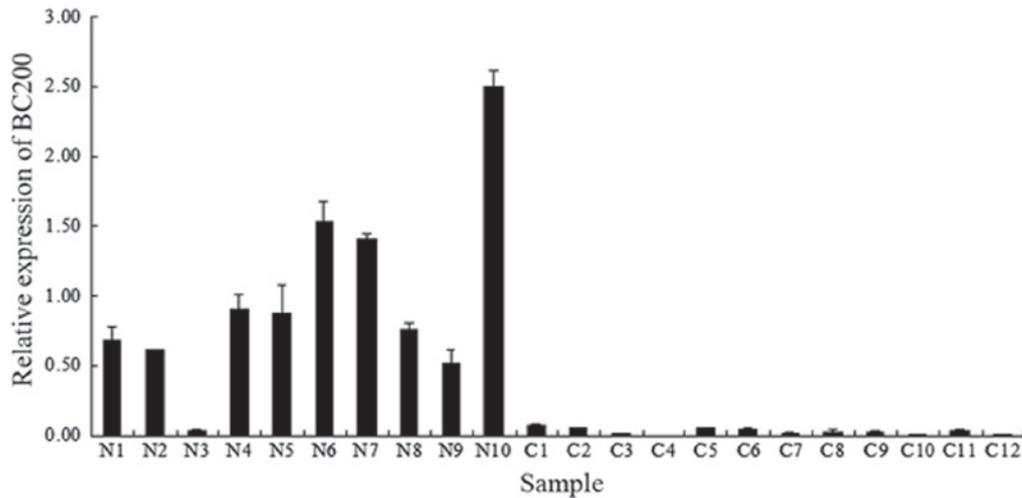


Figure 1. Level of BC200 in ovarian tissues, as determined by quantitative polymerase chain reaction. BC200 in the ovarian cancer samples was significantly downregulated compared with the normal ovarian samples. The relative expression of BC200 is presented by the value of $2^{-\Delta Cq}$. $\Delta Cq = BC200 Cq - Actin Cq$. N, normal ovarian sample; C, ovarian cancer sample. Cq, quantification cycle.

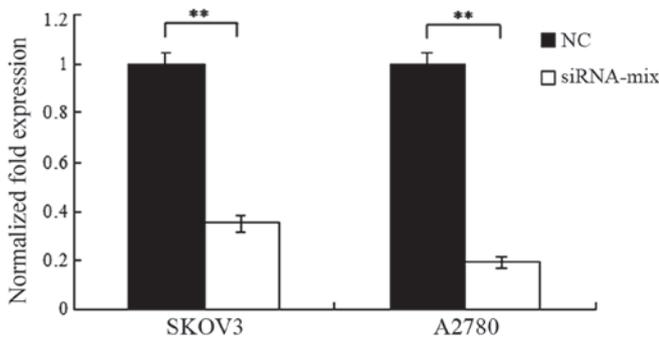


Figure 2. Efficiency of siRNAs targeting BC200. The mix of three siRNAs was most effective to knock down BC200 in the SKOV3 and A2780 cells. NC, negative control; siRNA, small interfering RNA. **P<0.01.

five randomly selected x200 magnification fields under light microscopy. The average cell number was calculated and used to represent the invasive and migratory ability.

Statistical analysis. All data are presented as the mean \pm standard deviation. Comparisons between groups were tested by Student's t-test or a one-way analysis of variance using GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

BC200 is downregulated in ovarian cancer. During the present study, the level of BC200 was compared between normal ovarian samples and ovarian cancer samples by means of qPCR. As presented in Fig. 1, the relative expression level of BC200 was greater in the majority of normal ovarian samples (9/10), whereas in the ovarian cancer samples, BC200 was significantly downregulated (12/12). The average level of BC200 in the normal tissues was >30 times higher than that observed in the ovarian cancer tissues. This downregulation

of BC200 may therefore be associated with the initiation and progression of ovarian cancer.

siRNA is effective in knocking down BC200 in SKOV3 and A2780 cells. To observe the efficiency of siRNAs targeting BC200, the level of BC200 was analyzed by qPCR at 48 h post-transfection with the addition of each siRNA. The results demonstrated that all three siRNAs, and the mixture of the three siRNAs, were effective to knock down BC200 in the two cell lines (P<0.01; Fig. 2). The siRNA-mix was the most effective and was thus used for the following experiments.

BC200 inhibits the proliferative ability of ovarian cancer cells. A CCK-8 assay was used to establish the proliferative ability of siRNA-transfected and NC SKOV3 and A2780 cells. In the two cell lines, the siRNA-transfected cells grew faster than the NC cells (Fig. 3). A significant difference was observed at 48 and 72 h in the SKOV3 cells and at 24, 48 and 72 h in the A2780 cells (P<0.01). Knockdown of BC200 therefore promotes the proliferative ability of ovarian cancer cells, indicating that BC200 may inhibit cell proliferation through an unknown pathway.

BC200 is associated with cell death induced by carboplatin. Initial experiments were performed to determine the range of carboplatin concentration that elicited growth inhibition and cell death. The SKOV3 and A2780 cells were treated with graded carboplatin (0, 50 and 100 μ g/ml) for 48 h, and following this, the level of BC200 was detected by qPCR. Notably, the level of BC200 was upregulated in the ovarian cancer cells when carboplatin was added to the medium in a dose-dependent manner (P<0.05 and P<0.01; Fig. 4A). Subsequently, a cell viability assay was performed to analyze the role of BC200 in sensitizing the cells to carboplatin treatment. In the ovarian cancer cells treated with carboplatin, the siRNA-transfected cells exhibited higher cell viability in comparison to the NC cells (P<0.05 and P<0.01; Fig. 4B),

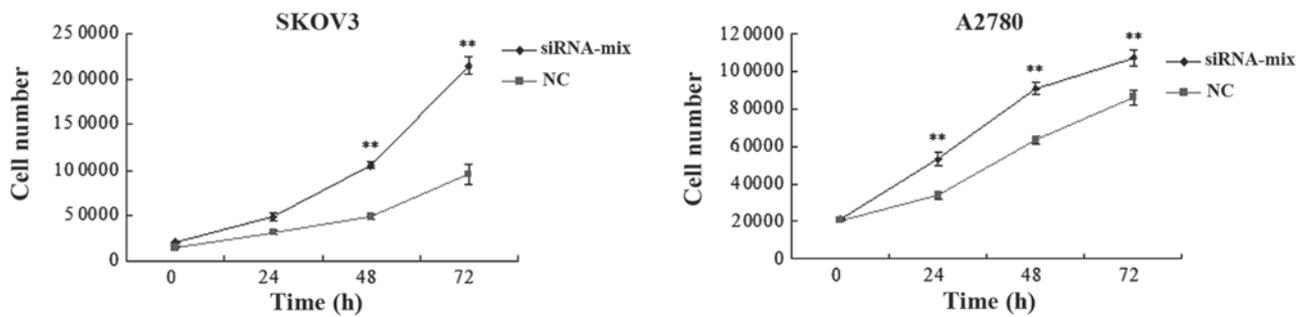


Figure 3. Cell growth curve, as determined by CCK assay. SKOV3 and A2780 cells transfected with the siRNA-mix grew faster than the respective NC cells. A significant difference can be observed at 48 and 72 h in the SKOV3 cells, and at 24, 48 and 72 h in the A2780 cells. siRNA, small interfering RNA; NC, negative control. ** $P<0.01$.

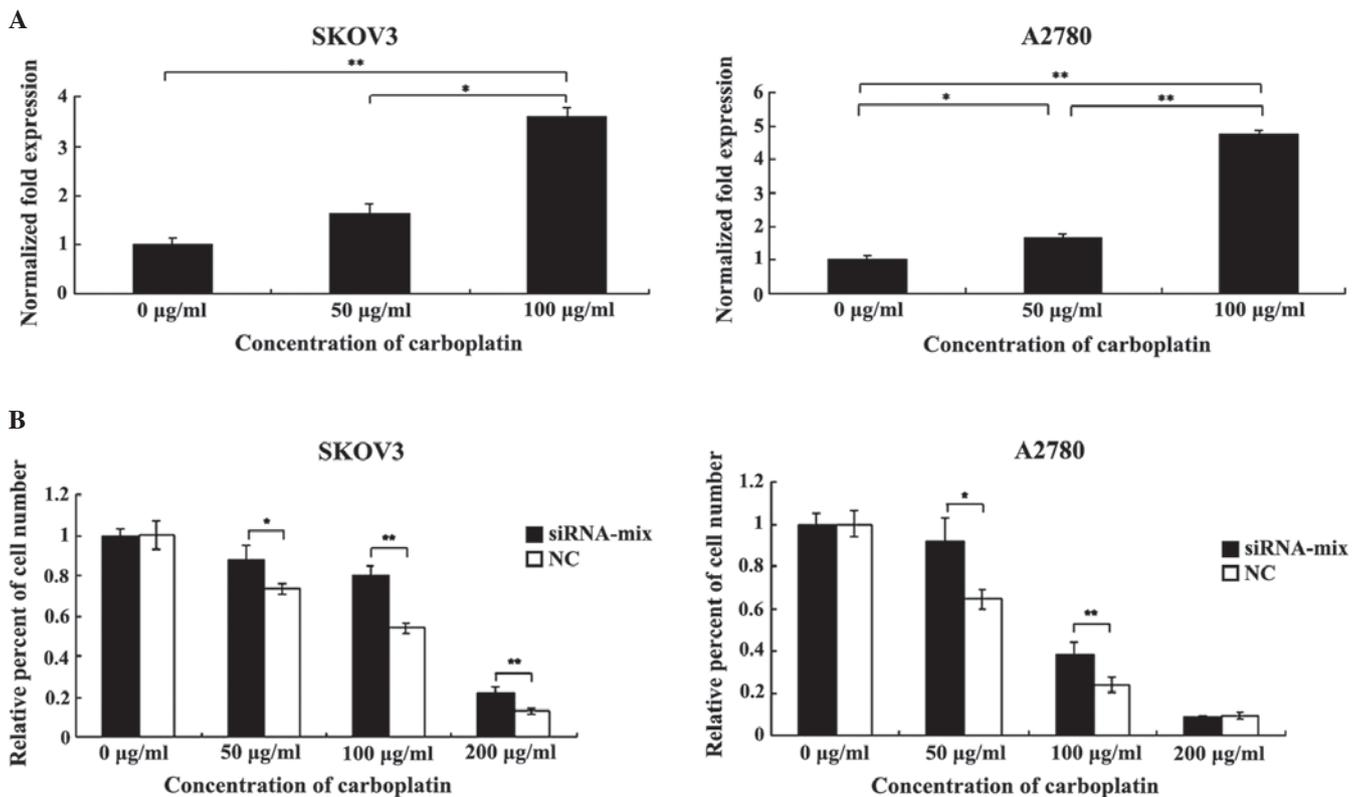


Figure 4. Association of BC200 and carboplatin treatment. (A) Quantitative polymerase chain reaction; BC200 expression increased with the increasing dose of carboplatin in the SKOV3 and A2780 cells. (B) Luminescent cell viability assay. Downregulation of BC200 contributed to the chemoresistance to carboplatin in the SKOV3 and A2780 cells. siRNA, small interfering RNA; NC, negative control. * $P<0.05$ and ** $P<0.01$.

indicating that the downregulation of BC200 contributed to the chemoresistance of the ovarian cancer cells to carboplatin.

BC200 has no effect on the invasive and migratory ability of ovarian cancer cells. To investigate the effect of BC200 on cell mobility, invasion and migration, assays were performed on the siRNA-transfected and NC SKOV3 and A2780 cells using Transwell chambers with and without Matrigel. The results demonstrated that there was no difference in the average number of cells observed on the stained membrane between the two groups for each cell line ($P>0.05$; Fig. 5), indicating that BC200 had no effect on the invasive and migratory ability of the ovarian cancer cells.

Discussion

In recent years, lncRNAs have gained increasing attention as a novel class of molecules demonstrating a functional role in carcinogenesis (10,11). Previous studies have demonstrated that the expression of lncRNAs is dysregulated in a variety of human cancers, and these aberrant lncRNAs may serve key roles during the initiation and progression of cancer (12-14). However, lncRNA research remains premature when compared to what is understood about protein-coding RNA and microRNA. In fact, the biological functions of the majority of lncRNAs are currently unknown. To date, several lncRNAs have been identified to be abnormally expressed in ovarian cancer, including imprinted maternally expressed transcript,

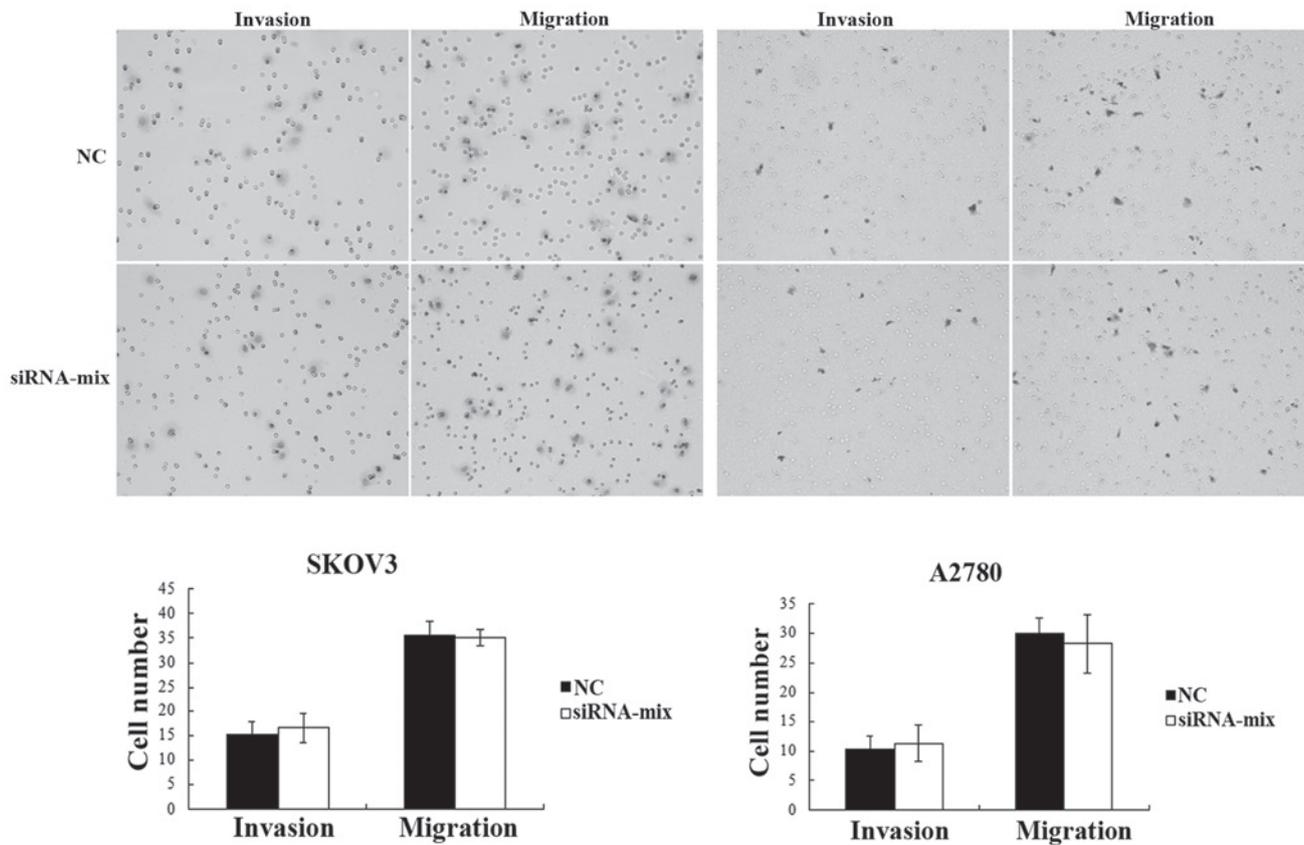


Figure 5. Invasion and migration assays. The average number of invasive and migratory SKOV3 and A2780 cells showed no difference between the siRNA-transfected and NC cells. The average number under a x200 magnification field is shown by the bar graph. NC, negative control; siRNA, small interfering RNA.

X-inactive specific transcript, long stress-induced non-coding transcript 5, Pvt1 oncogene, serum resistance-associated gene and host genes 2 (15-20). These lncRNAs are involved in various biological processes of ovarian cancer, including chemotherapy, proliferation, apoptosis and metastasis (21-24). Previous studies have suggested the potential role of lncRNAs in ovarian cancer to a certain extent (15-24), however, further investigation is required to explain their respective functions and mechanisms of action.

BC200 is an lncRNA that is reported to be associated with ovarian cancer. However, the function and mechanism of BC200 in cancer is not fully understood. In the present study, the expression of BC200 in ovarian cancer tissues and normal ovarian tissues was compared. The results demonstrated that BC200 was significantly downregulated in the ovarian cancer tissues, suggesting that the reduction of BC200 may be associated with the occurrence of ovarian cancer, and thus, BC200 may potentially be used as a diagnostic marker. However, Chen *et al* (9) reported that BC200 RNA was detected at low levels in ovarian cancer tissues and was undetectable in normal ovarian tissues. The possible causes of this conflict may have been due to the following reasons: Firstly, the study by Chen *et al* only detected BC200 in one pair of ovarian tissues from the same patient, whereas the results from the current study were based on detection in 10 normal ovarian samples and 12 ovarian cancer samples. Secondly, the study by Chen *et al* used matched normal and tumor tissues from the same patients, whereas the normal and tumor tissues in

the present study were obtained from different patients. Despite results from an internal control possibly holding more weight, one limitation is that it cannot be ensured that matched 'normal' tissue really is normal. Malignant tumors generally lack a clear boundary, and whether the tissue is normal or not cannot be determined by the naked eye. Thirdly, the study by Chen *et al* used northern blot hybridization to detect BC200, while the present study used qPCR. Additionally, high levels of BC200 in normal ovarian tissues highlight the uncertainty of the theory that BC200 expression is neuron-specific. In addition to the primate nervous system, BC200 is also expressed in germ cells, including oocytes, and in cultured immortal cell lines of non-neural origin (25,26).

Based on the results of the present study, we hypothesize that BC200 may function as a tumor suppressor in ovarian cancer. BC200 expression was significantly decreased in ovarian cancer when compared with normal ovarian tissues. Furthermore, the downregulation of BC200 contributed to ovarian cancer cell proliferation, and inhibited the sensitivity of ovarian cancer cells to carboplatin. The effect of BC200 on chemoresistance was supported by additional evidence. SKOV3 is a platinum-resistant cell line, whilst A2780 is a platinum-sensitive cell line. Notably, the level of BC200 was significantly higher in the A2780 cells than in the SKOV3 cells (data not shown). To date, lncRNAs, including maternally expressed 3 (MEG3) and papillary thyroid carcinoma susceptibility candidate 3 (PTCSC3), have been reported to function as tumor suppressors. Downregulation of MEG3 has been

observed in various types of human cancer, and the lncRNA has been demonstrated to inhibit cancer cell proliferation and induce apoptosis (27,28). PTCSC3 is a newly identified, thyroid-specific lncRNA that is significantly downregulated in thyroid cancer. PTCSC3 can induce growth inhibition, cell cycle arrest and increased apoptosis (29,30). BC200 was previously speculated to be associated with invasion and migration in breast cancer. It was reported that BC200 was expressed at high levels in invasive carcinomas of the breast, and may potentially serve as a molecular tool in the diagnosis and/or prognosis of breast cancer (31). However, the results of the present study demonstrated that BC200 has no effect on the migration and invasion ability of ovarian cancer cells.

In conclusion, the expression of BC200 was observed to be reduced in ovarian cancer tissues when compared with normal ovarian tissues, and the inhibition of BC200 was demonstrated to promote the proliferative ability of ovarian cancer cells. Furthermore, carboplatin induced the expression of BC200, and BC200 increased the sensitivity of ovarian cancer cells to carboplatin. The results of the current study identified a partial function of BC200 in ovarian cancer, and also provided a novel target for mechanistic and clinical treatment studies. Continued investigation is required to further expand our understanding of the function and mechanism of action of BC200 in ovarian cancer.

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