Effect of lncRNA-BC200 on proliferation and migration of liver cancer cells in vitro and in vivo

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Abstract. In recent years, the important role of long non-coding RNAs (lncRNAs) in the development of liver cancer has received increasing attention. The abnormal expression level of long non-coding RNAs has been associated with the occurrence and development of liver cancer. However, the role and molecular mechanisms of lncRNAs in the development and progression of liver cancer are not fully understood. The present study aimed to clarify the function and molecular mechanism of lncRNA brain cytoplasmic 200 (BC200) in liver cancer. In the present study, it was found that BC200 expression level was higher in hepatocellular carcinoma (HCC) tissues than that in adjacent tissues. Cell function was examined by constructing BC200 knockout (KO) and BC200-overexpression in vitro models. It was found that BC200 affected the proliferation and migration of HepG2 cells. Interestingly, it was found that BC200 affected the expression of c-Myc protein but did not affect the mRNA expression level of c‑MYC. BC200 KO cells exhibited a reduced protein expression level of Bax protein and an increased protein expression level of Bcl-xL. Conversely, BC200 overexpression reduced the expression of Bcl-xL protein and increased the expression of Bax protein. Importantly, it was found that BC200 affected the formation of subcutaneous tumors in nude mice. In conclusion, the present results suggested that lncRNA BC200 may play an important role in liver cancer.

Introduction

Liver cancer is one of the most common cancers and is a serious threat to human health. After surgery, the incidence of tumor recurrence and metastasis is high, and the prognosis is poor. Due to the development of chemotherapy and novel treatments, such as immune checkpoint inhibitors (1) and chimeric antigen receptor T cells (2), rates of recurrence and metastasis have decreased and the 5-year survival rate has improved. However, the mechanisms underlying the metastasis and recurrence of liver cancer have not been fully elucidated. Therefore, the discovery of new regulatory molecules to develop more effective liver cancer treatment strategies remains crucial.

Long non-coding RNAs (lncRNAs), non-coding RNAs with >200 nucleotides, are involved in the transcriptional and post-transcriptional regulation of various biological processes involved in the development of tumors, and are associated with tumor prognosis (3,4). Increasing studies have reported that lncRNAs are implicated in liver cancer.

In previous studies, various lncRNAs, such as the PVT1 oncogene (5), colorectal neoplasia differentially expressed (CRNDE) (6) and CDKN2B antisense RNA 1 (CDKN2B-AS1) (7) have been found to regulate HCC cell migration, invasion, proliferation, apoptosis and other important biological processes. DiGeorge syndrome critical region gene 5 (DGCR5) was found to play a role in HCC by inhibiting the progression of HCC through inactivation of the Wnt signaling pathway (8). DGCR5 was found to repress the development of HCC by targeting the miR-346/KLF14 axis (9). Hepatocellular carcinoma upregulated long non-coding RNA (HULC) was found to regulate the drug resistance of HCC by triggering autophagy by stabilizing Sirtuin 1 (10). Although thousands of lncRNAs have been identified to be associated with liver cancer, various aspects warrant further investigation and examination, including the regulatory mechanisms of lncRNAs in liver cancer, the underlying mechanisms of the association between lncRNAs and liver cancer, and novel molecular markers that may be discovered.

lncRNA-BC200, also known as BCYRN1, is a brain-specific small cytoplasmic RNA with a length of 200 nucleotides that is transcribed by RNA polymerase III. Human lncRNA-BC200 is located on chromosome 2p16. lncRNA-BC200 consists of a monomeric Alu, an A-rich central region with a unique C-rich region, which can be divided into three domains. The 5' end is homologous to the high copy number Alu repeater in the primate genome. The 3' end is unique and has no apparent similarity to known human DNA sequences, but is similar to...
several short elements of rodent BC1 RNA. IncRNA-BC200 has RHAU helicase activity and binds to RHAU, exerting regulatory functions by unwinding the four-chain cytosine-rich tetra-chain at the 3′ end (11). IncRNA-BC200 can also bind to a variety of proteins, such as poly(A)-binding protein, heterodimeric signal recognition particles, fragile X mental retardation protein and synaptic cytoplasmic interactions protein. Previous studies have shown that IncRNA-BC200 serves an important role in certain human diseases, such as asthma, Alzheimer’s disease, and various common tumors (12,13). Singh et al (14) showed that IncRNA BC200 is expressed to a higher degree in estrogen receptor-positive breast cancer compared with estrogen receptor-negative breast cancer, and low expression of BC200 can inhibit the proliferation of estrogen-dependent breast cancer tumor cells in vitro and in vivo, by promoting the protein expression of the apoptotic factor Bcl-xS. Zhao et al (15) showed that IncRNA BC200 is significantly increased in esophageal squamous cell carcinoma and is an independent risk factor for disease-free survival and overall survival in patients with esophageal squamous cell carcinoma. Notably, a high expression level of IncRNA BC200 may be associated with poor prognosis. Recent studies have shown that IncRNA BC200 is highly expressed in non-small cell lung cancer. In addition, c-MYC can bind to the promoter region of the IncRNA BC200 gene. In vitro cell transfer assays have shown that IncRNA BC200 regulates cell migration and invasion (16). Recent studies have shown that BC200 is highly expressed in hepatocellular carcinoma (HCC) and is an effective independent prognostic marker. In addition, T3/TR (thyroid hormone/its receptor) has a negative regulatory effect on the expression of BC200 (17).

Lin et al (17) silenced BC200 in J7 and SK-Hepl cells by shRNA technology, and overexpressed BC200 in Hep3B and Huh7 cells, and further studied the function of BC200 in vitro and in vivo. The results showed that BC200 promoted cell growth and transformation in vitro and in vivo. In the present study, we knocked out BC200 by using CRISPR/Cas9 technology and simultaneously knocked out and overexpressed BC200 in the same cell line, and investigated the expression, function and potential mechanism of BC200 in liver cancer in vitro and in vivo. According to a previous study, BC200 affects cancer cell survival and proliferation (13). BC200 IncRNA is considered to be a potential predictor of poor prognosis in esophageal squamous cell carcinoma (15). However, there are few reports on the effect of BC200 on cancer. In the present study, the expression, function and potential mechanisms of BC200 in liver cancer were investigated in vitro and in vivo.

Materials and methods

Collection of HCC tissue specimens. In total, 45 pairs of matched HCC tissues and adjacent tissues were obtained from patients at the Affiliated Tumor Hospital of Guangxi Medical University between December 2016 and March 2017 (Table I). All patients had no history of anticancer therapy. All tissues were maintained in liquid nitrogen. Informed consent was obtained by each patient. The present study was approved by The Ethics Committee of the Affiliated Tumor Hospital of Guangxi Medical University.

RNA extraction and reverse transcription-quantitative (RT-q) PCR. RNA was extracted from tissue or HepG2 cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). NanoDrop 2000 (Thermo Fisher Scientific, Inc.) instrument was used to determine the concentration and quality of RNA. The RNA was reverse transcribed using the Moloney murine leukemia virus reverse transcriptase (Vazyme). Subsequently, qPCR was performed using the ChamQ™SYBR®qPCR Master Mix (High ROX Premixed; Vazyme) according to the manufacturer's instructions. The relative gene expression was calculated using the 2^ΔΔCq method (18). The primers used in the present study were the following: GAPDH forward, AAC GATTTGTTGCATATTG and reverse, GGAAGATGTTGAGGGATT; c-MYC forward, TATCCCTAACTCTACATC AAC and reverse, TCAAAATCGCCTCCACTT; BC200 forward, GCCTGTAATCCAGCTCTCA and reverse, GTTCGGAGGGAGTTAGCT.

Cell culture. HepG2 cells were purchased from The Chinese Academy of Sciences Cell Bank. The culture conditions of the cells were: 10% FBS DMEM (BI, Biological Industries), 5% CO2 and 37°C incubator.

Plasmid transfection. Knockout (KO) of BC200 was performed using CRISPR/Cas9 technology as previously described (19). An empty plasmid (Plasmid-con) and the plasmid for BC200 KO (Plasmid-BC200 KO) were obtained from the University of Mississippi (Department of Pharmacology/Toxicology Cancer Institute University of Mississippi Medical Center, Jackson, MS, USA). The plasmid carries green fluorescence and puromycin resistance, and the plasmid construction process is shown in Fig. 1A. Plasmids were transfected into HepG2 cells with Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), and cells were divided into: i) Negative control (NC) group; ii) Plasmid-con group; and iii) Plasmid-BC200 KO group. Cells were transfected with the indicated plasmid, and 1-3 days after, stable cell lines were screened using 1 μg/ml puromycin.

Lentivirus (LV) infection. The lentiviral vectors were purchased from Shanghai Jikai Gene Co., Ltd. The target gene vector was (polyA-MCS-UBI) RV-SV40-EGFP-IRES-puromycin, and the negative control virus vector was Ubi-MCS-SV40-EGFP-IRES-puromycin (Fig. 1B). Vectors were infected into HepG2 cells according to the manufacturer's instructions. Cells were grouped into: i) NC group; ii) LV-con group; and iii) LV-BC200 group. After 1-3 days, stable cell lines were screened using 1 μg/ml puromycin.

Cell proliferation assay. Cells were seeded into 96-well plates at a density of 2,000 cells/well. The assay was performed every 24 h. Cell Counting Kit-8 [CCK-8; Multisciences (Lianke) Biotech Co., Ltd.] solution was added 1 h before testing. The plates were incubated for 5 days. Detection of absorbance at 450 nm was performed using a microplate reader.

Cell migration assay. Transwell chambers (Corning, Inc.) were used for Transwell migration assay. The cells were seeded at a density of 3x10^4/ml. In total, 200 μl of single-cell suspension was added to the upper Transwell chamber, and the lower chamber was filled with DMEM supplied with 10%
FBS, while the upper chamber contained serum-free DMEM. After 20 h, the cells were stained using 10% Giemsa staining for 30 min (Beijing Solarbio Science & Technology Co., Ltd.), and observed under a microscope (Olympus Corp.). A total of 10 high power fields of view were randomly-selected, cells were counted and the mean cell number was calculated.

**Western blot analysis.** RIPA buffer was used to lyse cells and extract cellular proteins. Electrophoresis was used for protein separation. Protein samples (150 µg) (including protein samples from HepG2 cells from the NC group, control group, BC200 KO group and LV-BC200 group) were transferred to a nitrocellulose membrane following separation by 10% SDS-PAGE. The membranes were incubated with various primary antibodies. Anti-Bax (cat. no. WL01637; rabbit mAb; Wanleibio Co., Ltd.), anti-c-Myc (D3N8F) (cat. no. 13987; rabbit mAb; Cell Signaling Technology, Inc.) and anti-Bcl-xL (54H6) (cat. no. 2764; rabbit mAb; Cell Signaling Technology, Inc.) GAPDH (cat. no. 5174; Cell Signaling Technology, Inc.) was used as the loading control. Secondary antibody was HRP-labeled goat anti-rabbit IgG (H+L) (cat. no. A0208; Beyotime Institute of Biotechnology). The dilutions were carried out using Primary Antibody dilution buffer (cat. no. A1810; Beijing Solarbio Science & Technology Co., Ltd.) and Secondary Antibody dilution buffer (cat. no. P0023D; Beyotime Institute Biotechnology). The primary or secondary antibodies were diluted at a ratio of 1:1000. Protein bands were analyzed using Gel Doc XR+ Analyzer Software (Bio-Rad Laboratories, Inc.).

**Experimental animals.** The Experimental Animal Ethics Committee of Guangxi Medical University approved the present study. In total, 18 male BALB/c nude mice (weight, 16-20 g; age, 4-6 weeks) were purchased from the Laboratory Animal Center of Guangxi Medical University. Animals were housed at the animal facility of the Laboratory Animal Center of Guangxi Medical University. The experimental conditions of the mice included: A specific pathogen-free environment, 12-h light/dark cycle, and free access to food and water. In order to establish a subcutaneous tumor model, cultured HepG2 cells were diluted using PBS at a final concentration of 2x10^6/ml. In total, 200 µl of the cell suspension was subcutaneously injected into the right leg of the mice. Nude mice were divided into: i) NC group (n=6); ii) BC200 KO group (n=6); and iii) LV-BC200 group (n=6). The weight of the nude mice and subcutaneous tumor volume were measured every other week. Mice were sacrificed by cervical dislocation on day 60. Subsequently, the subcutaneous solid tumor was collected for measuring the tumor weight. Tumor volume was calculated as follows: Tumor volume=length x (width)^2/2.

**Statement of authentication.** HepG2 cell line has been authenticated by STR profiling.

**Statistical analysis.** All data are presented as the mean ± SD. Differences between groups were evaluated by Student's t-test, one-way ANOVA or repeated measures ANOVA using SPSS 17.0 software. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**BC200 is upregulated in HCC.** To investigate the expression pattern of BC200 in human HCC, the mRNA expression level...
of BC200 was examined in 45 matched pairs of adjacent tissue and HCC tissue specimens using RT-qPCR. The levels of BC200 were measured and compared between HCC and healthy samples. A higher expression level of BC200 was detected in the HCC samples compared to the level noted in the normal adjacent liver tissues (Fig. 1C).

**Verification of transfection efficiency.** In order to downregulate the expression of BC200, a BC200 KO plasmid was transfected into HepG2 cells to further examine the functional role of BC200 in liver cancer. Fluorescence of HepG2 cells after plasmid transfection was assessed under a fluorescence microscope (Fig. 2A-D). The qPCR results showed a significant decrease in the expression level of BC200 in the BC200-KO (Plasmid-BC200 KO) group compared to the control (Plasmid-con) and NC groups (Fig. 2E). The RNA expression level of c-MYC did not change significantly among the NC, Plasmid-con and Plasmid-BC200-KO groups (Fig. 2E).

**Verification of infection efficiency.** Cells were observed after 72 h of lentiviral infection. It was found that >90% of cells expressed fluorescence and presented a normal morphology (Fig. 3A-D). The expression level of BC200 was detected by qPCR in HepG2 cells from the NC group, LV-control and LV-BC200 groups. In the LV-BC200 group, the expression level of BC200 was significantly higher than levels noted in the NC and LV-control groups (P<0.05; Fig. 3E). The mRNA expression level of c-MYC was not significantly altered among the NC, LV-con and LV-BC200 groups (Fig. 3E).

**Effect of BC200 KO on the migration and proliferation of HepG2 cells.** After transfection of plasmid-BC200 KO into HepG2 cells, the effect of BC200 KO on proliferation and migration was investigated. As shown in Fig. 4, Transwell assay demonstrated that the migration rate of HepG2 cells was significantly reduced after BC200 KO transfection (Fig. 4A and B). CCK-8 experiments showed that there were no significant differences in the proliferation of HepG2 cells among the BC200 KO, NC and control groups. The proliferation curve was drawn according to the optical density value (Fig. 4C).

**Effect of overexpression of BC200 on the migration and proliferation of HepG2 cells.** After infection of LV-BC200 into HepG2 cells, the influence of LV-BC200 on the proliferation and migration of HepG2 cells was investigated. As shown in Fig. 5, migration of HepG2 cells infected with LV-BC200 was significantly promoted at 20 h. Transwell experiments showed that the migration rate of HepG2 cells in the LV-BC00 group was significantly higher than that in the NC and control groups (Fig. 5A and B). The proliferation of HepG2 cells transfected with LV-BC200 was not significantly increased compared with the NC and control groups during the 5 day period. The proliferation curve was drawn according to the optical density value (Fig. 5C).

**Effect of BC200 on subcutaneous tumor formation in nude mice.** To examine the effect of BC200 on tumor growth *in vivo*, stable cell lines were transfected with a plasmid
overexpressing BC200 and BC200 KO and selected by puromycin resistance. The present results showed that the volume and weight of tumors in the BC200 KO group (0.53±0.45 cm³ and 0.70±0.61 g) were significantly decreased compared with the LV-BC200 group (1.85±0.42 cm³ and 2.0±0.16 g; P<0.05; Fig. 6A-D). The increase in the body weight of the mice in the LV-BC200 group was lower than that in the BC200 KO group (Fig. 6E).

**Discussion**

During the past two decades, the role of long non-coding RNAs in cancer has received increased attention (20). In total, more than 60,000 lncRNAs have been identified, accounting for ~60% of the transcriptome (21). In recent years, research on lncRNAs in liver cancer has increased. lncRNAs serve a variety of roles in the development of liver cancer, by acting as an anti-apoptotic factor (22), promoting HCC metastasis (23), acting as prognostic biomarkers (24), and promoting cell proliferation, migration and invasion (7). However, the mechanisms underlying the role of lncRNAs in liver cancer are not fully understood, and further investigation is required.

lncRNA-BC200 was found to be expressed in the cytoplasm, particularly in dendritic neuronal cells, and transcribed by RNA polymerase III (25-27). In addition, high expression of BC200 has been reported in various tumor types. Chen et al (28) used northern hybridization technology to detect the expression of BC200 RNA in various human tumor tissues and found that BC200 has a low expression level relative to GAPDH in the liver (13). The present results demonstrated that
BC200 expression level in HCC tissues was higher than that in adjacent tissues. However, the present data are in contrast with a previous study by Chen et al. (28) that suggested that BC200 RNA is not detectable in liver carcinoma tissues. However, the present study analyzed BC200 expression by qPCR in 45 matched pairs of HCC tissues and adjacent tissues. The study by Chen et al. (28) investigated four patients with liver carcinoma. Importantly, there were differences in the detection method and in the number of samples, which may have affected the experimental results. Lin et al. (17) used RT-qPCR and In situ hybridization (ISH) methods and found that BC200 expression was significantly upregulated in HCC tissues compared with that noted in benign and adjacent normal tissues. Moreover, Kaplan-Meier survival analysis showed the association of high BC200 expression with the poor overall survival (OS) rate of HCC patients. In the TCGA database (http://cancergenome.nih.gov/), patients with high expression of BC200 showed lower OS and disease-free survival than patients with low expression of BC200.

To the best of our knowledge, the present study is the first to detect the expression of BC200 in HepG2 cells. In addition, various cell models were established, including BC200 KO and BC200 overexpression cells, and RT-qPCR was used to verify that the in vitro models were successfully constructed. The present results showed that BC200 KO inhibited the migration of HepG2 cells, and high expression of BC200 promoted the migration of HepG2 cells. The present results are in line with a recent study indicating that knockdown of BC200 RNA expression reduces cell migration and invasion (29).

In the present study, BC200 was found to influence cell proliferation by CCK-8 assay. The present results showed that BC200 did not affect the proliferation of HepG2 cells, either following knockout of BC200 or overexpression of BC200. These results are in line with a recent study showing that the growth rate was not affected in HCT116 cells transfected with BC200 small interfering RNA (30). In addition, the present study investigated the role of BC200 in vivo, and overexpression of BC200 was found to promote the growth of subcutaneous tumors in nude mice, whereas the knockout of BC200 inhibited the growth of subcutaneous tumors. These results are in line with a previous study that showed that BC200 KO significantly reduced tumor growth in female nude mice.
Collectively, the present results indicated that under in vitro conditions, where the cell environment is relatively simple, changing the expression level of BC200 did not affect cell proliferation. However, in a complex in vivo environment, the expression level of BC200 affected the growth of tumors in nude mice, indicating that the regulation of cell proliferation by
BC200 is context-dependent. The environment of nude mice is more similar to the human body environment compared with in vitro conditions, and in vivo experiments can better reflect the role of BC200 in humans.

c-Myc protein was previously identified to interact with the BC200 promoter, and high expression of BC200 is due to the binding of c-Myc to its promoter (16). Research by Hu and Lu (16) provided new ideas for our research. Therefore, we conversely aimed to ascertain whether altering the expression of BC200 affects the expression of c-Myc. In the present study, the association between BC200 and c-MYC was investigated at the RNA and protein levels following knockout and overexpression of BC200 in HepG2 cells. The present results showed that knockout of BC200 or overexpression of BC200 had no significant effects on c-MYC mRNA levels, whereas c-Myc protein expression was significantly decreased in the BC200 KO group. Conversely, c-Myc protein was increased in the LV-BC200 group. This effect may have been caused by the lack of IncRNA-BC200 effects on the transcription level of the c-MYC gene. By contrast, IncRNA-BC200 may have an effect only on the translation rate of c-MYC mRNA. c-MYC is an oncogene that plays an important role in cell proliferation, differentiation, apoptosis and cell cycle. c-MYC is a transcription factor that requires dimerization with another protein, such as Myc-associated protein X, to become transcriptionally active. The mechanism by which c-MYC oncogene/oncoprotein participates in cell proliferation, apoptosis and cycle may vary depending on the context and is regulated by a variety of factors (31).

The Bcl-2 family is divided into two classes, pro-apoptotic proteins and anti-apoptotic proteins; Bcl-xL is an anti-apoptotic protein, whereas Bcl-xS and Bax are pro-apoptotic proteins (32,33). Singh et al (14) showed that knockout of BC200 increased Bcl-xS protein expression, whereas Bcl-xL protein was not altered significantly. The regulation of Bcl-xS by BC200 is of great significance for the pathogenesis of breast cancer. Gu et al (34) showed that BC200 can affect the expression of Bax and Bcl-2, suggesting that BC200 is involved in the apoptosis of colorectal cancer (CRC) cells. It has been previously reported that the ratio of Bax/Bcl-xL can be used

Figure 6. Effects of BC200 on tumor growth in nude mice. (A) Tumor sizes. (B) Gross morphology of nude mice. (C) Mean weight of the excised tumors. (D) Mean volume of the excised tumors. (E) NC cells, LV-BC200 cells or BC200 KO cells were injected into the right leg of nude mice. Nude mouse body weight was measured every other week, starting from 1 week after injection. *P<0.05, BC200 KO group vs. LV-BC200 group. BC200; lncRNA brain cytoplasmic 200; NC, negative control; LV, lentivirus; KO, knockout.
to measure the level of apoptosis (35). In the present study, following BC200 KO, Bax protein expression was decreased and Bcl-xL protein expression was increased. Conversely, in the LV-BC200 group, Bax protein expression was increased and Bcl-xL protein expression was decreased. Collectively, the present results suggested that lncRNA-BC200 may affect both apoptotic and anti-apoptotic proteins. However, due to the balancing effects of anti-apoptotic Bcl-xL and pro-apoptotic Bax, cell apoptotic rates may not be affected. Our study is the first to study the relationship between BC200 and Bcl-xL and Bax protein in liver cancer. The results of this study are inconsistent with the results in other cancers, probably due to other regulatory networks in liver cancer. However, there is no relevant research to prove our guess, and more research is needed to support it in the future. A review of the literature also found a corresponding example, in neuroblastoma cells. Inhibition of the expression of long noncoding RNA KCNQ1OT1, found that the expression of Bax was reduced (36). In diabetic retinopathy (DR), Bax expression was found to be increased by inhibiting the expression of long noncoding RNA KCNQ1OT1 (37). This shows that there are different regulatory effects in different cancers.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

NT, CO and BZ conceived and designed the experiment. NT and HS performed the experiments. YFT and JRW collected and analyzed the data. MF, CRL and ZQC collected the clinical samples and analyzed the clinical data. NT wrote the manuscript. NT, CO and HS reviewed/edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The patient study was approved by the Academic Committee of the Ethics Committee of the Affiliated Tumor Hospital of Guangxi Medical University. All patients and healthy volunteers...
provided written informed consent prior to their inclusion in the study. The animal study was approved by The Experimental Animal Ethics Committee of Guangxi Medical University.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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