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Long non-coding RNA BCAR4 accelerates cell proliferation and suppresses cell apoptosis in gastric cancer *via* regulating MAPK/ERK signaling

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Abstract. – OBJECTIVE: As the fourth most common malignant tumor with high mortality rate, gastric cancer (GC) seriously threatens people's health and life quality worldwide. The aim of this study was to explore the functional role of long non-coding RNA (lncRNA) BCAR4 in GC.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) assay was used to detect the expression level of lncRNA BCAR4 in GC cell lines and tissues. Subsequently, cell counting kit-8 (CCK-8) assay, colony formation assay, and flow cytometry were recruited to investigate the role of lncRNA BCAR4 in the proliferation and apoptosis of GC cells, respectively. Western blotting was used to detect the protein expression level of mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK) in GC. Besides, tumor formation assay was applied to examine the ability of lncRNA BCAR4 *in vivo*.

RESULTS: LncRNA BCAR4 was highly expressed in both GC tissues and cell lines. CCK-8 assay, colony formation assay, and flow cytometry results indicated that up-regulated lncRNA BCAR4 significantly promoted cell proliferation and suppressed cell apoptosis in GC. Besides, over-expression of lncRNA BCAR4 could activate the MAPK/ERK signaling pathways. Tumor xenograft formation assay demonstrated that over-expression of lncRNA BCAR4 promoted tumor formation *in vivo*.

CONCLUSIONS: LncRNA BCAR4 was proved significantly up-regulated in GC. Over-expression of lncRNA BCAR4 promoted cell proliferation and suppressed cell apoptosis *in vitro* and promoted tumor formation *in vivo*. Besides, Western blotting revealed that lncRNA BCAR4 played an oncogenic role in GC via regulating MAPK/ERK signaling.

Key Words:
LncRNA BCAR4, Gastric cancer (GC), Proliferation, Apoptosis, MAPK/ERK.

Introduction

As the fourth most common malignant tumor with high mortality rate[1], gastric cancer (GC) seriously affects people's health and life quality. Currently, the incidence of GC in China is relatively high, with a high mortality rate. Meanwhile, it has become the second leading cause of deaths in China[1]. About 80% of the patients were already at the advanced stage when first diagnosed, with five-year survival rate lower than 25%[2]. Delayed diagnosis has become the largest obstacle affecting the treatment and prognosis of GC. Currently, the combination of gastroscopy and biopsy is still the gold standard for the diagnosis of GC over the world[3]. Long non-coding RNAs (lncRNAs) can be directly obtained from tissues, blood, and saliva, which are more acceptable to patients. Therefore, lncRNAs can be used as biomarkers for GC.

LncRNAs have no protein-coding function, accounting for >90% of the human genome[4]. LncRNAs have been reported to be positively correlated with digestive system malignant tumors. The expression level of H19 in GC tissues is significantly up-regulated and, the expression level of H19 in the plasma of GC patients is significantly down-regulated after surgery. LINC00152 is stably overexpressed in GC tissues and plasma and significantly up-regulated in GC cell lines. More-

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over, LINC00152 can be used as a tumor marker for diagnosis, with high sensitivity and specificity[6]. Nevertheless, the role of other lncRNAs, i.e., BCAR4 in GC has not been fully elucidated. Therefore, the aim of this work was to investigate the physiological function of lncRNA BCAR4 in the GC progression.

In the present study, we first examined the expression level of lncRNA BCAR4 in GC by qRT-PCR. The results showed that lncRNA BCAR4 was significantly up-regulated in GC tissues and cell lines. CCK-8 assay and colony formation assay demonstrated that lncRNA BCAR4 promoted the proliferation of GC cells. Next, flow cytometry revealed that lncRNA BCAR4 accelerated cell cycle progression and inhibited cell apoptosis. Tumor formation assay indicated that lncRNA BCAR4 promoted tumor formation *in vivo*. To investigate the underlying mechanism of lncRNA BCAR4 function in GC, we validated that lncRNA BCAR4 might exert its physiological function *via* regulating mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK) signaling. Taken together, our findings elucidated that lncRNA BCAR4 accelerated cell proliferation and suppressed cell apoptosis in GC.

Patients and Methods

Tissue Samples

45 paired GC tissues and adjacent normal tissues were obtained from patients who received surgical treatment in Putuo People's Hospital of Tongji University between 2016-2017. Collected tissue samples were immediately preserved in liquid nitrogen. All tumor tissues were confirmed by pathological examination. This research was approved by the Ethics Committee of Putuo People's Hospital of Tongji University. Written informed consents were obtained from all participants before the study.

Cell Lines

3 GC cell lines (AGS, HGC27, and MGC803) and 1 normal gastric epithelial cell line (GES-1) were obtained from the Shanghai Cell Bank (Shanghai, China). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone; South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in an incubator at 37°C with 5% CO_2.

Cell Transfection

Selected cell lines were transfected with sh-BCAR4 (5′-GGGACTTGAGTTATGTTGGTG-GCTA-3′), which was synthesized by GenePharma (Shanghai, China). Over-expression of lncRNA BCAR4 was conducted by the transfection of pcDNA3-BCAR4 into cell lines according to the instructions of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Transfection efficiency was verified by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) assay.

Total RNA Extraction and QRT-PCR

Total RNA in tissue specimens and cell lines were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). All cDNAs were synthesized *via* Reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan) according to the standard protocol. LncRNA-LINP1 expression was assessed through SYBR Green real-time PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was taken as an internal reference. Primer sequences used in this study were as follows: BCAR4 forward 5'-3': ACAGCAGCTTGTTGCTCATCT-3′, reverse 5'-3': TTGCCTTGGGGACAGTTCAC, GAPDH forward 5'-3': TATCGGACGCCTGGTTAC, reverse 5'-3': TATCGGACGCCTGGTTAC.

Colony Formation Assay

Cells (1.0×10^3) were first seeded into 60 mm culture plates and cultured for 2 weeks. After washing with phosphate-buffered saline (PBS; Gibco, Rockville, MD, USA) twice, formed colonies were fixed in ice-cold 70% methanol for 15 min and stained with crystal violet staining solution (Beyotime, Shanghai, China). Finally, the colonies were observed under a microscope, and the number of colonies was counted.

Cell-Counting Kit-8 Assay (CCK-8)

CCK-8 assay was used to detect the proliferation of GC cells. Transfected cells were planted into 96-wells plates at a density of 6×10^3/well. Subsequently, CCK-8 solution (Beyotime, Shanghai, China; 10 μL/well) was added to each well, followed by incubation for 2 h at 37°C. The optical density (OD) value at 450 nm was finally evaluated by a micro-plate reader.

Flow Cytometry

For detecting cell apoptosis, Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Detection Kit (Vazyme, Nanjing, China) was performed according to the relevant

instructions. For cell cycle, transfected cells were immersed in 70% ethanol at –20°C overnight before staining with PI (Vazyme, Nanjing, China). Flow cytometry was conducted by BD FACSCanto II (BD Biosciences, Franklin Lakes, NJ, USA) flow cytometry.

Western Blotting

Total protein was isolated by radioimmunoprecipitation assay (RIPA) buffer (Thermo, Fisher Scientific, Waltham, MA, USA) containing phenylmethanesulfonyl fluoride (PMSF). Protein lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Then, the membranes were incubated with primary antibodies at 4°C overnight. On the next day, the membranes were incubated with corresponding secondary antibodies. Primary rabbit antibodies used in this study included: anti-Mek (Cell Signaling Technology; Danvers, MA, USA), anti-p-Mek (Cell Signaling Technology; Danvers, MA, USA), anti-Erk (Cell Signaling Technology; Danvers, MA, USA), and anti-p-Erk (Cell Signaling Technology; Danvers, MA, USA). Rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Cell Signaling Technology, Danvers, MA, USA) was taken as a loading control. Relative expression level of protein was determined by the Image Lab software.

Xenograft Model

This investigation was approved by the Animal Ethics Committee of Putuo People's Hospital of Tongji University Animal Center. Transfected AGS or MGC803 cells (7×10^5/mL) were injected into two flanks of nude mice (6 weeks old) subcutaneously. Tumor growth was monitored and recorded every week. Tumor volume was calculated by the formula: volume = length × width^2 × 1/2. Tumors were extracted after 4 weeks.

Statistical Analysis

All researches were repeated for at least three times independently. Experimental data were expressed as mean ± standard deviation (SD). Student's unpaired *t*-test was used for statistical analysis. $p<0.05$ was considered statistically significant.

Results

LncRNA BCAR4 Expression Was Up-Regulated in GC

QRT-PCR was first used to detect the expression level of lncRNA BCAR4 in GC. The expression level of lncRNA BCAR4 was significantly up-regulated in GC tissues (Figure 1A). Similarly, we examined the expression level of lncRNA BCAR4 in GC cell lines. The results showed that GC cells exhibited significantly higher expression level of lncRNA BCAR4 in comparison with human normal gastric epithelial cell line (Figure 1B). Transfection efficiency was verified by qRT-PCR. As shown in Figure 1C, lncRNA BCAR4 up-regulated group had relatively higher expression level of lncRNA BCAR4. However, opposite results were observed in sh-BCAR4 group.

LncRNA BCAR4 Over-Expression Promoted Cell Proliferation In Vitro

CCK-8 assay and colony formation assay were applied to examine cell proliferation. As shown in Figure 2A, lncRNA BCAR4 up-regulated group exerted markedly higher OD value when compared with control group. Similarly, the number of formed colonies in lncRNA BCAR4 up-regulated group was remarkably more than that of control group. However, lncRNA BCAR4 down-expression group had significantly less formed colonies in comparison with control group (Figure 2B). All these findings elucidated that up-regulated lncRNA BCAR4 promoted cell proliferation *in vitro*.

Up-Regulated LncRNA BCAR4 Accelerated Cell Cycle Progression and Suppressed Cell Apoptosis In Vitro

To detect the effect of lncRNA BCAR4 on cell cycle and apoptosis, flow cytometry was used. As shown in Figure 3A, lncRNA BCAR4 over-expression significantly increased cell distribution in S fraction and induced cell cycle progression. Moreover, flow cytometry results revealed that up-regulated lncRNA BCAR4 inhibited cell apoptosis *in vitro* (Figure 3B). Taken together, we concluded that lncRNA BCAR4 over-expression promoted cell cycle progression and suppressed cell apoptosis.

Over-Expressed LncRNA BCAR4 Promoted Tumor Formation In Vivo

Tumorigenicity assay was performed to explore the ability of lncRNA BCAR4 in tumor formation *in vivo*. As shown in Figure 4A, tumor

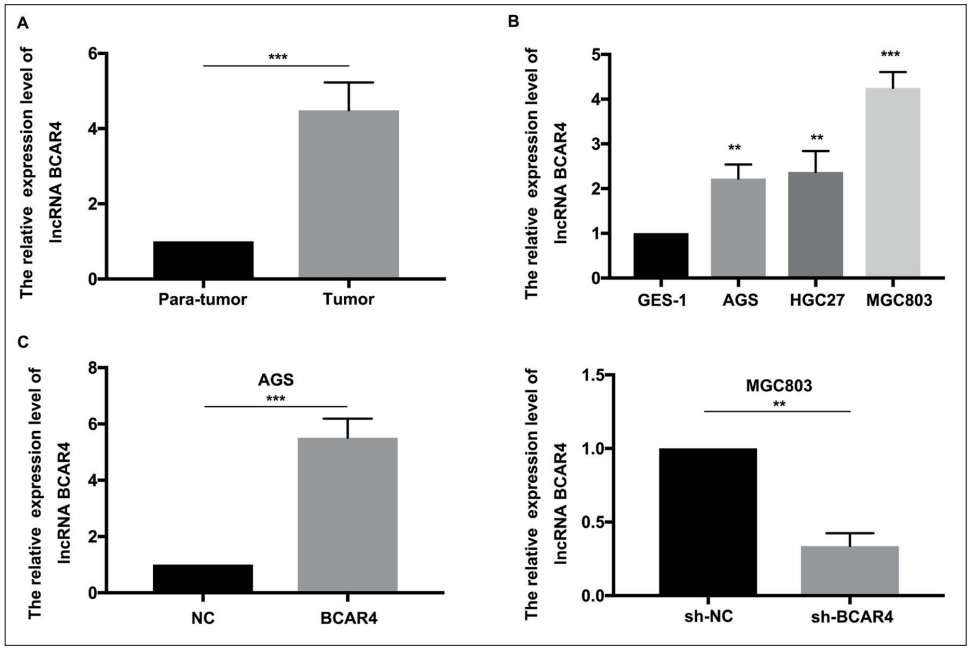

Figure 1. Long non-coding RNA BCAR4 was up-regulated in GC. **A,** Expression level of lncRNA BCAR4 in GC tissues and para-tumor tissues; **B,** Expression of lncRNA BCAR4 in GC cell lines. **C,** Transfection efficiency was evaluated by qRT-PCR. Data were presented as mean ± SD of three independent experiments. **p<0.01***p<0.001.

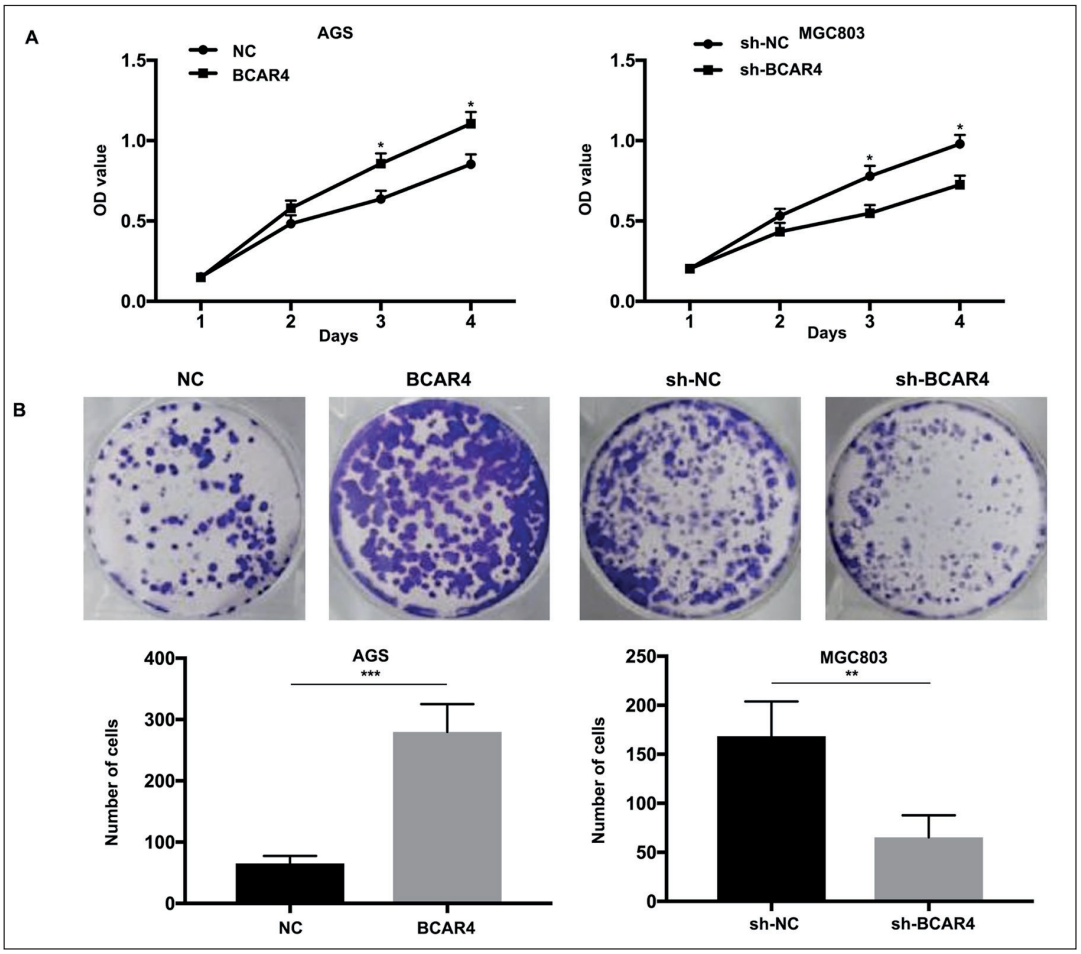

Figure 2. LncRNA BCAR4 over-expression promoted cell proliferation *in vitro*. **A,** Cell proliferation ability was determined by CCK-8 assay. **B,** Colony formation assay was used to detect cell proliferation (magnification × 40). Data were presented as mean ± SD of three independent experiments. *p<0.05, **p<0.01, ***p<0.001.

volumes in lncRNA BCAR4 up-regulated group were significantly larger than control group. Consistently, tumor weight was recorded in Figure 4B. Besides, we examined the expression level of lncRNA BCAR4 in generated tumors by qRT-PCR. The results showed that BCAR4 over-expression significantly increased the expression level of BCAR4 (Figure 4C). Therefore, results indicated that lncRNA BCAR4 promoted tumor formation *in vivo*.

The Underlying Mechanism of LncRNA BCAR4 in GC Progression

To reveal the underlying molecular mechanism of lncRNA BCAR4 in GC progression, we validated whether MAPK/ERK signaling participated in GC progression. Western blotting assay was used to detect the protein expression level of Mek, phosphorylation of Mek, Erk, phosphorylation of Mek, and GAPDH in selected cell lines. As shown in Figure 5, over-expression of lncRNA BCAR4 significantly up-regulated the protein level of phosphorylation of Mek and phosphorylation of Erk. However, down-regulated lncRNA BCAR4 markedly down-regulated the protein level of phosphorylation of Mek and Erk. Hence, all data indicated that lncRNA BCAR4 might exert its physiological function in GC progression *via* regulating MAPK/ERK signaling.

Discussion

GC is one of the most common malignancies that seriously affects the physical and mental health of human. The incidence of GC has greatly increased in recent years[7,8]. Although the diagnosis and treatment strategies for GC have been developed, the prognosis of most patients is still poor due to diagnosis at advanced stages[9].

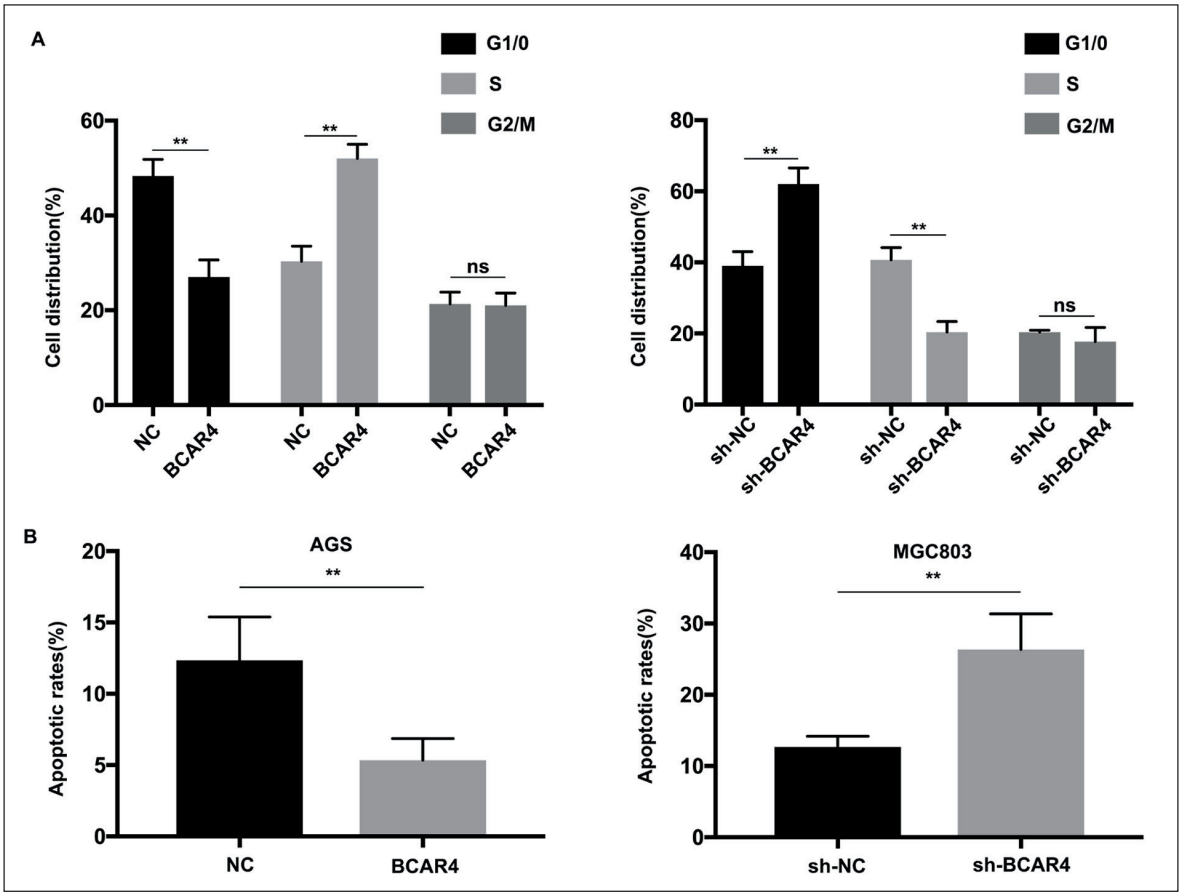

Figure 3. Up-regulated lncRNA BCAR4 accelerated cell cycle progression and suppressed cell apoptosis *in vitro*. **A,** Cell cycle progression was detected by flow cytometric analysis in transfected groups; **B,** Flow cytometric analysis was performed to detect the apoptotic rates in transfected cells. Data were presented as mean ± SD of three independent experiments. $^{**}p<0.01$.

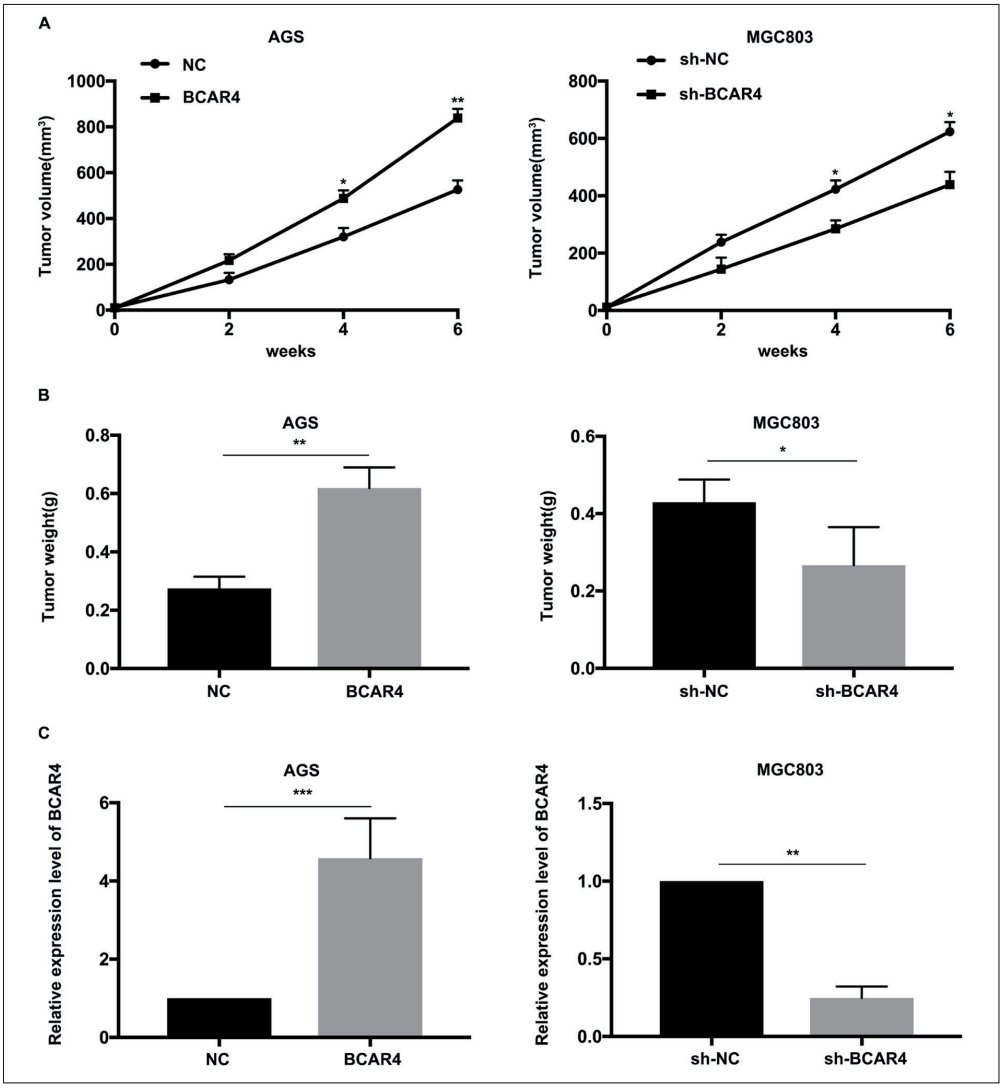

Figure 4. Over-expressed lncRNA BCAR4 promoted tumor formation *in vivo*. **A,** After tumor extraction, tumor volume was calculated and made into a graph; **B,** Tumor weight was recorded; ***C,*** Relative expression of lncRNA BCAR4 in tumors was examined by qRT-PCR. Data were presented as mean ± SD of three independent experiments. *p<0.05, **p<0.01, ***p<0.001.

Hence, searching for novel biomarkers and efficient therapies for GC is essential.

In the past few decades, multiple studies have focused on the abnormal expression of protein-encoding RNAs in cancers. However, increasing evidence from genomics and transcriptome studies have shown that lncRNAs play vital roles in cancer progression. LncRNAs have been considered as novel biomarkers and molecular therapeutic targets for diverse cancers[10]. Dysfunction of lncRNAs can also lead to a variety of human diseases[11]. However, the specific functions of most lncRNAs have not been fully elucidated. Concerning, lncRNA BCAR4 it has been proved to function as an oncogene in cervical cancer[12]. LncRNA BCAR4 has been reported to exert its effect on cell proliferation and metastasis in non-small cell lung cancer *via* regulating the epithelial-mesenchymal transition[13]. However, the role of lncRNA BCAR4 in GC progression still remains unknown. Herein, the aim of this work was to investigate the biological function of lncRNA BCAR4 in GC.

In the current research, we first examined the expression level of lncRNA BCAR4 in GC tissues and cells. We found that lncRNA BCAR4 was significantly up-regulated in both GC tissues and cell lines. CCK8 assay and colony formation assay revealed that lncRNA BCAR4 over-expression markedly promoted cell proliferation.

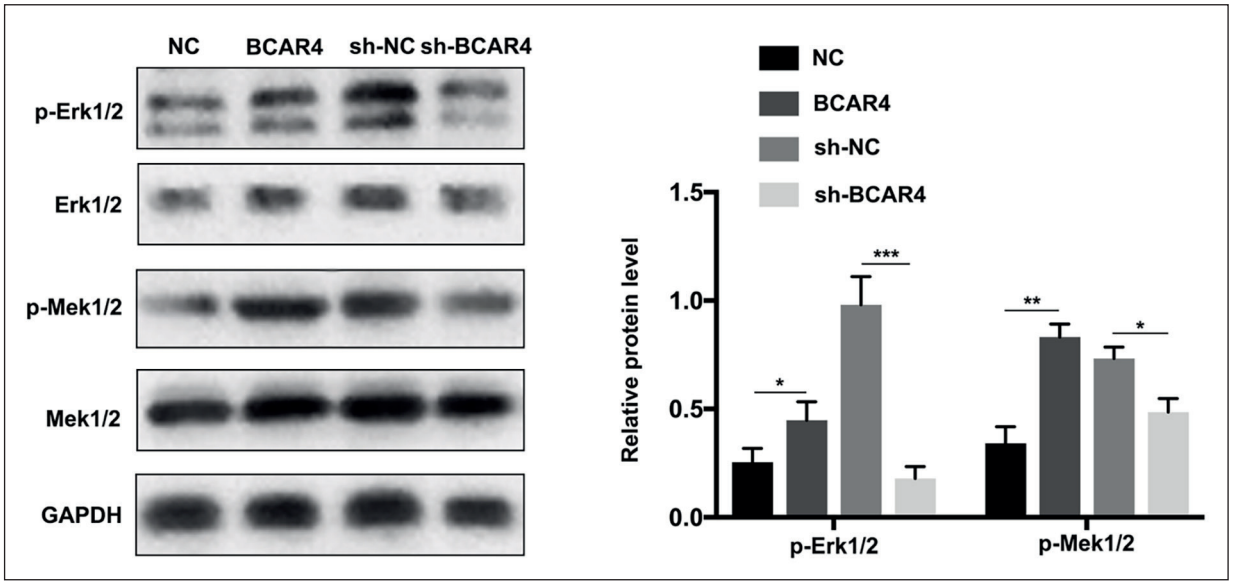

Figure 5. The underlying mechanism of lncRNA BCAR4 in GC progression. Mek1/2, p-Mek1/2, Erk1/2, p-Erk1/2 protein expression levels were examined by Western blotting in transfected cell lines. Data were presented as mean ± SD of three independent experiments. *p<0.05, **p<0.01, ***p<0.001.

Moreover, flow cytometry demonstrated that up-regulated lncRNA BCAR4 accelerated cell cycle and inhibited cell apoptosis *in vitro*. Tumor formation assay elucidated that over-expressed lncRNA BCAR4 promoted tumorigenicity *in vivo*. To investigate the underlying mechanism, we detected the protein level of some markers in MAPK/ERK signaling by Western Blotting. The MAPK/ERK signal transduction pathway is an important pro-proliferative and anti-apoptotic pathway in cells, which may affect the activity of downstream effector molecules, including cell cycle regulatory proteins and apoptosis-related proteins[14]. Meanwhile, it plays an essential role in the proliferation and apoptosis of malignancies[15]. Western blotting assay demonstrated that lncRNA BCAR4 may serve as an oncogene in GC by regulating MAPK/ERK signaling.

Conclusions

We revealed that lncRNA BCAR4 was significantly up-regulated in GC tissues and cell lines. Over-expression of lncRNA BCAR4 promoted cell proliferation and suppressed cell apoptosis *in vitro*, and promoted tumor formation *in vivo*. Besides, Western blotting assay showed that lncRNA BCAR4 played an oncogenic role in GC *via* regulating MAPK/ERK signaling.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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