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The long noncoding RNA BC0209135 inhibits the cell invasion through Wnt/β-catenin signaling in colorectal cancer

Q. ZHENG[1], Y. LIN[2], P. CHEN[1], Y.-P. FAN[2]

[1]Department of General Surgery, Ningbo No. 2 Hospital, Ningbo, China
[2]Department of Gastroenterology, Ningbo No. 2 Hospital, Ningbo, China

Abstract. – OBJECTIVE: Previous studies have shown that BC029135, a long noncoding RNA (lncRNA) with high conservation, was decreased with high fold change in colorectal cancer (CRC) by using microarray assay. In the present work, we investigated the functions of BC029135 in CRC, as well as the potential mechanisms underlying the malignant phenotype caused by loss of BC029135.

MATERIALS AND METHODS: Bioinformatics analysis of lncRNA BC029135 was performed to identify the potential function. The cell lines of HCT-116, SW480, SW620, LoVo, SW1116, and HT29 were used in this study. The mRNA expression of BC029135 and β-actinin was measured by Real-Time Polymerase Chain Reaction (RT-PCR). Western blot analysis was used to determine protein expression of β-actinin, MMP-2, cyclin D1, and c-Myc.

RESULTS: Our research showed that the expression of BC029135 in CRC tissues and cell lines was significantly lower than in adjacent normal tissues. In addition, overexpression of lncRNA BC029135 could inhibit the invasion of CRC cells. We also found that lncRNA BC029135 inhibits Wnt/β-catenin signaling in CRC cell lines.

CONCLUSIONS: We suggest that up-regulation of BC029135 suppresses CRC invasion and inactivates Wnt/β-catenin signaling. It will provide new insights for the treatment of colorectal cancer and new clues for clinical treatment.

Key Words:
LncRNA, BC029135, Colorectal cancer, Wnt/β-catenin signaling

Introduction

Colorectal cancer (CRC) is the third most common cancer and fourth leading cause of cancer-related death [1]. More than 10% of CRC patients are diagnosed with later stage, and about 30% of patients diagnosed with early-stage CRC will develop metastatic disease[2]. Colorectal cancer has become a life-threatening disease and seriously influences people's life quality. Therefore, a sophisticated understanding of its mechanism could shed new light on new diagnostic methods and active intervention.

The development of cancer consists of cell transformation, angiogenesis, cell migration, and invasion[3]. Invasion is an important process by which the normal cells or tissues were invaded by abnormal cells[4]. It has been known that tumor cells evade growth suppression by reducing the expression, activation or function of tumor suppressors such as P53, PTEN, and c-myc[5]. However, although many researches were performed to identify the potential mechanism of invasion, the specific mechanism by which tumor cells invade normal tissues remains unclear. In recent years, some researchers found that inhibit the invasion through small molecules will alleviate the progress of tumor[6].

Long noncoding RNA, a specific noncoding RNA, longer than 200 nt, has been widely studied in many diseases, including heart development[7], obesity[8], tumor development[9], diabetes mellitus[10]. LncRNA can be classified as intergenic lncRNA, intronic lncRNA, antisense lncRNA, promoter-associated lncRNA, UTR associated lncRNA[11]. LncRNA serves significant function, including structural or trafficking roles[12], cell differentiation and apoptosis[13]. At the same time, LncRNA functions by a broad range of mechanisms, including regulating the neighboring gene[14], miRNA-sponge action[15], and coding small peptide to suppress the colon cancer[16]. However, the relationship between long noncoding RNA and colorectal cancer remains unknown. There is no doubt that exploring the role and mechanism of lncRNA in the pathophysiology of colorectal

Corresponding Author: Yan Lin, MD; e-mail: nblinyan@aliyun.com

cancer will deepen the understanding of cancer development.

In previous studies, some researchers screened dysregulated lncRNA in colorectal cancer using microarray. In these dysregulated lncRNAs, a long coding RNA named BC029135 attracted our attention due to its high conservation and fold change. We performed bioinformatics analysis of lncRNA BC029135 to identify the potential functions, and verified them in cell lines. We found that overexpression of lncRNA BC029135 could inhibit the invasion of CRC cells. Moreover, we also found that lncRNA BC029135 inhibits Wnt/β-catenin signaling in CRC cell lines. Our research will provide new insights for the treatment of colorectal cancer and new clues for clinical treatment.

Materials and Methods

Cell Lines

The cell lines, including HCT-116, SW480, SW620, LoVo, SW1116, and HT29, used in this study were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). SW480 and SW620 were cultured in Leibovitz's L-15 medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS, Life Technologies, Gaithersburg, MD, USA). HCT-116 and LoVo were cultured in Ham's F12K medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% FBS (Life Technologies, Gaithersburg, MD, USA). SW116 and HT-29 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% FBS (Life Technologies, Gaithersburg, MD, USA). Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

Plasmid Construction and Transfection

To generate lncRNA BC029135 overexpression vector, the lncRNA BC029135 coding sequence was amplified from normal human complementary Deoxyribose Nucleic Acid (cDNA) and inserted into the KpnI and XhoI sites of the pCDNA construct. Primers are provided in Table I. All constructs were verified by direct sequencing. The pcDNA3.1 vector was used as negative control. Lipofectamine3000 (Invitrogen, Carlsbad, CA, USA) was used to transfect cells according to the manufacturer's protocol.

RNA Quantification and Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) Assay

Total RNA of CRC tissues, adjacent to normal tissues, and CRC cell lines were extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription reactions were performed by M-MLV reverse transcriptase (Promega, Madison, WI, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control for the expression normalization and quantification of BC029135 and β-catenin. Amplification of cDNA was used by Platinum SYBR Green Real-time qPCR Super-Mix UDG reagents (Invitrogen, Carlsbad, CA, USA) and the MX3000P system (Stratagene, La Jolla, CA, USA). Concrete steps were denatured at 95°C for 15 s, then 40 cycles of 95°C for 15 s, 50°C for 15 s, and 68°C for 20 s. BC029135 expression fold change was calculated by the $2^{-\Delta\Delta Ct}$ method following normalization to GAPDH. Primers are provided in Table I.

Transwell Invasion and Migration Assay

Resuspended cells with 100 μL serum-free medium were plated in 24-well transwell chamber of each insert (8-μm pore size, Corning Incorporated, Corning, NY, USA) with a Matrigel-coated membrane (BD Bioscience, Franklin Lakes, NJ, USA) for the transwell invasion assay and a non-Matrigel-coated membrane for the transwell migration assay. Lower chambers of the inserts were filled with Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS. Chambers were incubated at 37°C in a humidified incubator containing 5% CO_2. Twenty-four hours later, cells invaded/migrated to the lower surface of the insert were fixed, stained, and counted under a light microscope.

Table I. Primers used in this study.

Targets	Primers
Lnc BC029135	F5'-TGAGACTGGGCACTTGGAAA-3'
Lnc BC029135	R5'-TAGGTCCAGGGATTGCTTGG-3'
β-catenin	F5'-TCCATGCCTGCCCAATTCAT-3'
β-catenin	R5-GCAAGTCAACACAGCACAGG-3'
GAPDH	F5'-GGAGCGAGATCCCTCCAAAAT-3'
GAPDH	R5'-GGCTGTTGTCATACTTCTCATGG-3

Abbreviations: F, forward primer; R, reverse primer.

Western Blot Analysis

Proteins from cells were extracted by using the radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China). The extracted proteins were then subjected to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and transferred into the polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk and incubated with anti-β-catenin, anti-MMP2, anti-c-Myc, anti-Cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-β-actin antibodies (Sigma-Aldrich, St. Louis, MO, USA) overnight at 4°C. After washing three times (5 min each time), membranes were incubated with horse radish peroxidase (HRP) conjugated secondary antibodies (Abcam, Cambridge, MA, USA) for 2 h at room temperature. The bands were visualized by using the ECL kit (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis

All the graphs-plotting and data analysis were performed by using the Statistical Product and Service Solutions (SPSS) software package (version 17.0, SPSS Inc., Chicago, IL, USA). All the data were presented as mean ± standard deviation. The significant differences between different groups were analyzed by *t*-test (comparison for two groups). Comparison between groups was done using One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). $p < 0.05$ was considered to be statistically significant.

Results

Bioinformatics Analysis of lncRNA BC029135

To better understand the potential mechanism of lncRNA in colorectal cancer, lncRNA BC029135 was chosen due to its high conservation and fold change. Firstly, we performed bioinformatics analysis of lncRNA BC029135. The conservation levels were evaluated by using UCSC (http://genome.ucsc.edu/index.html). The analysis result showed that lncRNA BC029135 is highly conserved in rat, mouse and human (Figure 1A). Next, we calculated the coding potential of lncRNA BC029135 using Coding Potential Analyses (Peking University, Beijing, China). ln-

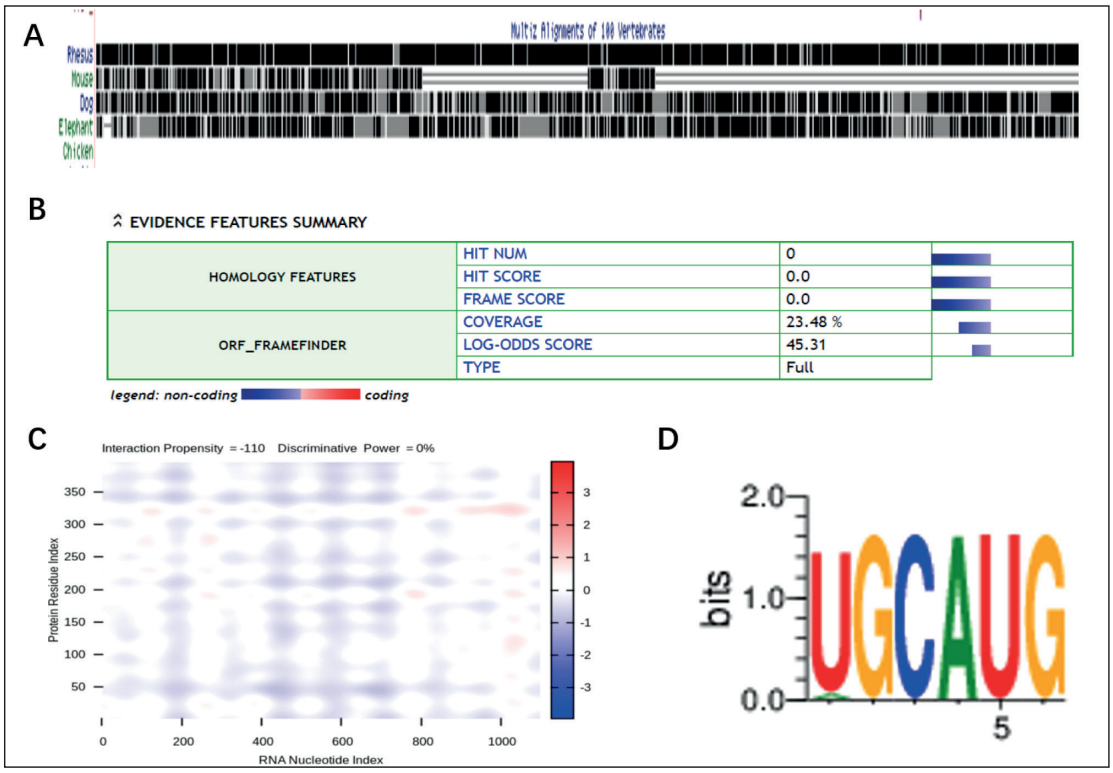

Figure 1. Bioinformatics analysis of lncRNA BC029135. ***A,*** LncRNA BC029135 is highly conserved in rat, mouse and human. ***B,*** LncRNA BC029135 had no credible protein coding open reading frame-with a coding potential score of 0.0. ***C,*** CAT-RAPID analysis was performed to calculate the interaction with potential protein RBFOX1.

cRNA BC029135 had no credible protein-coding open reading frame with a coding potential score of 0.0 (Figure 1B). To further identify the potential protein that may interact with lncRNA BC029135, CAT-RAPID analysis was performed to calculate the interaction with potential protein RBFOX1 (Figure 1C). Prediction of transcription factor binding sites indicated potential interacted sequence (Figure 1D).

LncRNA BC029135 is Down-Regulated in CRC Tissues and CRC Cell Lines

To investigate the expression level of lncRNA BC029135 in CRC, RT-PCR was performed to assess BC029135 expression in the 95 CRC and 95 adjacent normal tissues and normalized to GAPDH (Figure 2A). In addition, the mRNA expression of BC029135 was also detected in normal tissues and CRC cell lines (H29, SW116, SW480, SW620, and HCT-116 cells) (Figure 2B). We found that the expression of BC029135 in adjacent normal tissues was significantly higher than CRC tissues. The expression of BC029135 in CRC cell lines was significantly lower than that in normal tissues. Therefore, the results obtained from cell lines were consistent with those in tumor tissues. Compared with other CRC cell lines, the expression of BC029135 decreased remarkably in SW620 and HCT-116 cell lines. Therefore, we chose these two cell lines as model cells.

Differential Expression of LncRNA BC029135 in CRC Tissues and Its Association with CRC Patients' Clinical Characteristics and Survival

To further explore the clinical significance of BC029135 levels, CRC patients were divided into "high-level" (n=48) and "low-level" (n=47) BC029135 groups according to the median value of the BC029135 expression. As shown in Table II, low BC029135 level in CRS tissues was significantly associated with a more advanced TNM stage ($p = 0.031$) and a higher risk of lymph node ($p = 0.018$) and distant metastases ($p=0.034$). These clinical data indicate that BC029135 may affect metastasis of CRC, and it could be used as a new prognostic marker for CRC.

Effect of lncRNA BC029135 on CRC Cells Invasion

Cell invasion is an important reason for tumor progression and metastasis. To confirm whether lncRNA BC029135 has a direct functional role in promoting CRC cell invasion, we examined CRC cell invasion by using transwell invasion assay. The number of invaded SW620 and HCT-

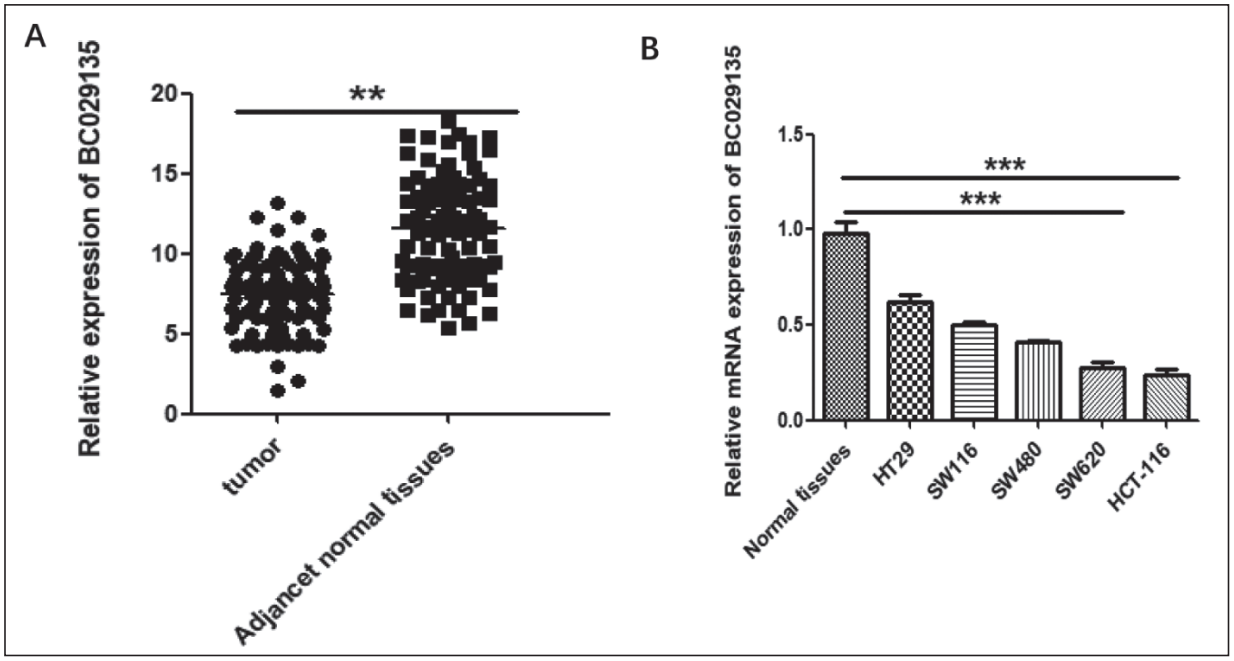

Figure 2. The mRNA expression level of lncRNA BC029135 in CRC tissues and cell lines. ***A,*** qRT-PCR was performed to assess BC029135 expression in the 95 CRC and 95 adjacent normal tissues. ***B,*** qRT-PCR was performed to examine the expression of BC029135 in cell lines. $^{**}p < 0.05$, $^{***}p < 0.001$.

Table II. Association between lncRNA BC029135 expression and clinicpathological characteristics in 95 CRC patients.

Variable	lncRNA BC029135 Expression		*p*-value
	Low expression (n = 47)	High expression (n = 48)	
Age (years)			0.920
< 59	24	25	
≥ 59	23	23	
Gender			0.085
Male	29	31	
Female	18	17	
TNM stage			0.031
I-II	20	31	
III-IV	27	17	
Tumor size (cm)			0.770
≥ 5	28	30	
< 5	19	18	
Tumor location			0.755
Rectum	25	24	
Colon	22	24	
CEA (ng/mL)			0.588
≥ 5	18	21	
< 5	29	27	
CA199 (U/mL)			0.938
≥ 35	17	17	
< 35	30	31	
Lymph node metastasis			0.018
Absent	18	30	
Present	19	18	
Distant metastasis			0.034
Absent	29	39	
Present	28	9	

116 cells decreased after the transfection with BC029135 overexpression vector (pcBC029135) as compared with the control (pcDNA3.1) (Figure 3A-3D). These results suggested that the up-regulation of lncRNA BC029135 suppresses the invasion of CRC cells.

Up-Regulation of lncRNA029135 Inhibits Wnt/β-Catenin Signaling in CRC Cell Lines

To explore the molecular mechanism by which lncRNABC029135 stimulates CRC cell lines growth and invasion, we examined the role of lncRNA BC029135 on Wnt/β-catenin signaling pathway downstream targets in CRC cell line, including MMP-2, cyclin D1, and c-Myc. The expression of BC029135 was elevated significantly by the pcBC029135 in SW620 and HCT-116 cell lines (Figure 4A). Upon the BC029135 up-regulation, β-catenin expression was down-regulated in SW620 and HCT-116 cells (Figure 4B). In consistent with RNA expression of β-catenin, the protein expression of β-catenin and its downstream targets, MMP-2, cyclin D1, and c-Myc were all reduced in BC029135 over-expressing SW620 and HCT-116 cells (Figure 4C). These results indicated that lncRNA BC029135 up-regulation suppresses CRC cell growth and invasion through inhibiting Wnt/β-catenin pathway.

Discussion

In this study, we identified the long noncoding RNA BC029135 and verified its function in CRC cells. Moreover, we found that lncRNA BC029135 could inhibit the cell invasion through Wnt/β-catenin signaling pathway. Taken together, we found an unknown lncRNA and studied the function and mechanism in colorectal cancer. Our research will provide new insights for the treatment of colorectal cancer and new clues for clinical treatment.

LncRNA serves significant function, including structural or trafficking roles[17], cell differentiation[15], and apoptosis[18]. LncRNA also has a broad range of mechanisms, including regulating the neighboring gene[13], miRNA-sponge action[19], and

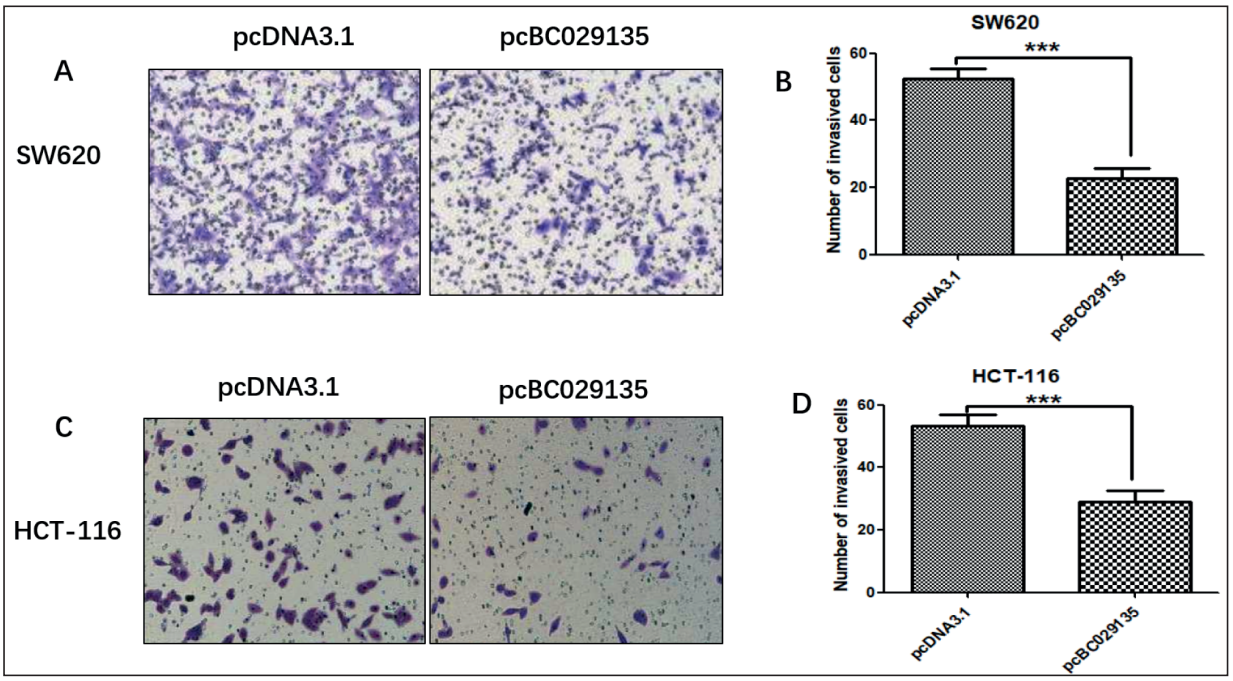

Figure 3. Effect of lncRNA BC029135 on CRC cells invasion. ***A-D,*** The number of invaded SW620 and HCT-116 cells was calculated after transfection with BC029135 overexpression vector (pcBC029135) as compared with the control (pcDNA3.1).

coding small peptide to suppress the colon cancer. Dysregulated lncRNA is relevant to tumor development and metastasis in various cancers[20]. lncRNA GAPLINC was reported to promote CRC cell invasion *via* binding to PSF/NONO and activating SNAI2 expression[21]. In the present study,

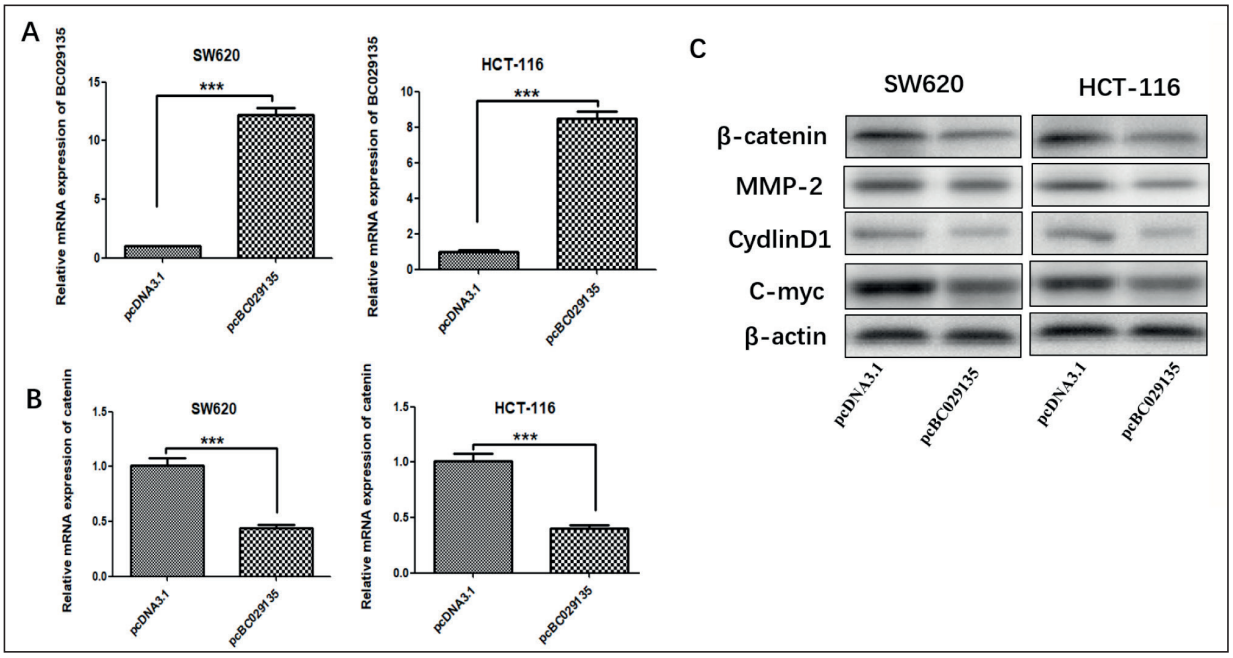

Figure 4. Up-regulation of lncRNA029135 inhibits Wnt/β-catenin signaling in CRC cell lines. ***A,*** qRT-PCR was performed to assess the expression of BC029135 in normal tissues and BC029135 overexpression cells. ***B,*** qRT-PCR was performed to assess the expression of β-actin in normal tissues and BC029135 overexpression cells. ***C,*** Western blot analysis was used to examine the protein expression of β-actin, MMP-2, cyclin D1, and c-Myc. ***$p < 0.001$.

we verified the loss of lncRNA BC029135 in CRC cell. Moreover, we found that lncRNA BC029135 could inhibit the invasion of CRC cells by transwell assays. Long non-coding RNAs have been shown to be associated with cancer development, demonstrating potential applications as novel diagnostic molecular markers in clinical treatment, as well as direct targets for therapeutic intervention.

Wnt/β-catenin signaling pathway is critical for accommodating cell proliferation, invasion, apoptosis, and differentiation by regulating downstream target genes, such as MMP-2, cyclin D1, and c-Myc[22,23]. Our results revealed that β-catenin, cyclin D1, c-Myc, and MMP-2 were downregulated in the BC029135 overexpression group. These data demonstrated that the overexpression of lncRNA BC029135 inhibits CRC cell growth and invasion through Wnt/β-catenin pathway. Taken together, our findings demonstrated that the loss of lncRNA BC029135 plays a role in the progression of colorectal cancer, and further experimentation regarding the mechanism by which lncRNA BC029135 contributes to development are clearly warranted.

Conclusions

We have elucidated the biological features and functions of lncRNA BC029135 which was downregulated in CRC tissues, and expounded its correlation with the invasion ability and prognosis of colorectal cancer. Furthermore, our data demonstrated that lncRNA BC029135 could function through Wnt/β-catenin signaling pathway, an important pathway in colorectal cancer. Understanding the precise role of lncRNA BC029135 might provide new insights for the therapeutics of colorectal cancer.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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