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Long non-coding RNA DLEU7-AS1 promotes the occurrence and development of colorectal cancer via Wnt/β-catenin pathway

X.-B. LIU[1], C. HAN[2], C.-Z. SUN[3]

[1]Department of General Surgery, Rizhao People's Hospital, Rizhao, Shandong, China
[2]Department of Thyroid Breast Surgery, Binzhou Central Hospital, Binzhou, Shandong, China
[3]Department of General Surgery, Jinxiang Hospital Affiliated to Jining Medical College, Jining, Shandong, China

Abstract. – OBJECTIVE: To investigate the expression features of long non-coding RNA (lncRNA) DLEU7-AS1 in colorectal cancer (CRC), so as to further study its role in the occurrence and development of CRC and its potential regulatory mechanism.

PATIENTS AND METHODS: The expression levels of lncRNA DLEU7-AS1 in 82 pairs of CRC tissues and para-carcinoma normal tissues were detected via quantitative Real-time polymerase chain reaction (qRT-PCR), and the correlation of DLEU7-AS1 expression with pathological indexes of CRC and patients' prognosis was analyzed. Besides, the expression of DLEU7-AS1 in CRC cells was further detected via qRT-PCR. The DLEU7-AS1 knockdown expression model was established using small interfering RNA in CRC cell lines HT-29 and HCT-116, and the effect of DLEU7-AS1 on biological functions of CRC cells was analyzed via Cell Counting Kit-8 (CCK-8) and transwell invasion/migration assay. Finally, its potential mechanism was investigated via Western blotting.

RESULTS: The results of qRT-PCR showed that the expression level of DLEU7-AS1 in CRC was significantly higher than that in normal tissues, and the difference was statistically significant. Compared with those in patients with low DLEU7-AS1 expression, the tumor stage in patients with high DLEU7-AS1 expression was higher, the prevalence rates of lymph node metastasis and distant metastasis were higher, and the overall survival rate was lower. Compared with those in the negative control group, the cell proliferation, invasion, and migration capacities were decreased significantly in DLEU7-AS1 knockdown expression group. Moreover, the results of Western blotting revealed that the expressions of key proteins in Wnt/β-catenin pathway, including β-catenin, c-myc, and cyclinD1, were decreased in si-DLEU7-AS1.

CONCLUSIONS: The expression of DLEU7-AS1 is significantly increased in CRC, which is markedly associated with CRC staging, lymph node metastasis, distant metastasis and poor prognosis. DLEU7-AS1 may promote the proliferation, invasion and migration capacities of CRC through regulating the Wnt/β-catenin pathway.

Key Words:
Long non-coding RNA, DLEU7-AS1, Colorectal cancer, Prognosis.

Introduction

Colorectal cancer (CRC) is a kind of common malignant tumor seriously threatening the human life and health. Like other tumors, its pathogenesis remains unclear so far[1]. Heredity, diet, unhealthy lifestyle and precancerous lesions are closely associated with its occurrence[2,3]. More than half of CRC patients have suffered from micro-metastasis in clinic before radical surgery, which is the direct cause of postoperative metastasis and recurrence of CRC[4]. Its pathogenesis has not been fully clarified yet, so the difficulty in diagnosis and treatment is one of the important reasons for its high morbidity and mortality rate[5]. Therefore, clarifying the molecular mechanism of CRC metastasis, predicting and diagnosing CRC metastasis and predicting the prognosis, are important contents in the CRC research.

The occurrence and development of CRC is a long-term, multi-gene and multi-stage complex process. However, the exact molecular pathogenesis of CRC is still not clear. With the rapid development of molecular biology and gene

Corresponding Author: Changzheng Sun, MD; e-mail: raoran90849@163.com

diagnosis technique, etc., it has been found that its possible mechanism is the vicious cell transformation and irreversible gene modification caused by the long-term interaction between genetic and environmental factors, which is mainly manifested as the activation of oncogenes and inactivation of tumor suppressor gene[6]. These changes eventually make the cell-related key physiological functions out of control, including proliferation, apoptosis, differentiation and other signal transduction[7]. Despite great achievements, whether there are other genes or epigenetic regulatory mechanisms involved in its occurrence and development still need further study.

With the continuous deepening of research, researchers have found that non-coding RNA is closely related to the tumor occurrence and development[8]. 98% RNAs in human genome are the non-coding RNAs, while only 2% transcripts are the coding RNAs. Non-coding RNA is the functional RNA molecule that cannot be translated into protein, including long non-coding RNA (lncRNA) and short non-coding RNA (piRNA, siRNA, and microRNA)[9]. LncRNA is a kind of RNA fragment with more than 200 bp in length, which does not encode protein. The abnormal expression of lncRNA molecules can be detected in almost all tumor tissues, and more and more studies have confirmed that the lncRNA molecule plays an extremely important role in the occurrence and development of tumor[10]. LncRNA can regulate the relevant protein-coding genes through many ways at different levels and play an extremely important role in the pathogenesis of tumor[11,12]. The role of lncRNA in CRC is poorly understood[13]; some lncRNAs, such as CRNDE and CCAT1, can promote CRC[14,15], while some, such as GAS5 and lncRNA-LET, can inhibit CRC[16]. In the pathogenesis of CRC, lncRNA is involved in the activation of Wnt pathway and epidermal growth factor receptor (EGFR) pathway, inhibition of transforming growth factor-β (TGF-β), P53 mutation and epithelial-mesenchymal transition, etc[17,18]., but the research in this area is still at the initial stage.

In this study, the expression levels of lncRNA DLEU7-AS1 in 82 pairs of CRC tissues and para-carcinoma normal tissues were analyzed, and the effect of DLEU7-AS1 on biological functions of CRC cells was investigated. Our results suggested that DLEU7-AS1 may serve as a new target in the treatment of CRC.

Patients and Methods

Patients and CRC Samples

A total of 82 pairs of surgically resected tumor tissue and para-carcinoma tissue samples of CRC patients were collected. According to the 7th version of Union for International Cancer Control/American Joint Committee on Cancer (UICC/AJCC) CRC tumor lymph node metastasis (TNM) staging criteria, all patients enrolled were diagnosed as CRC *via* the postoperative pathological analysis, and they did not receive the preoperative anti-tumor therapy, such as radiotherapy or chemotherapy. This study was approved by the Ethical Committee of Rizhao People's Hospital, and patients and their families had been fully informed that the samples would be used for scientific research and signed the informed consent.

Cell Lines and Reagents

Four CRC cell lines (HCT-8, HT-29, HCT-116 and SW-620) and one fetal human colonic epithelial cell line (FHC) were purchased from ATCC (Manassas, VA, USA); high-glucose Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Life Technologies (Rockville, MD, USA); cells were cultured in an incubator containing 5% CO_2 at 37°C, and the medium used was the high-glucose DMEM containing 10% fetal bovine serum (FBS).

Transfection

The negative control (siRNA) and siRNA containing DLEU7-AS1 interference sequence (si-DLEU7-AS1) were purchased from GenePharma (Shanghai, China). The cells were paved onto the 6-well plate and cultured until the cell density reached 70%. SiRNA transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the instructions of manufacturer. Cells were collected after 48 h for quantitative Real-time polymerase chain reaction (qRT-PCR) and cell function test.

Cell Proliferation Assay

Cells were collected at 48 h after transfection and paved onto the 96-well plate (2000 cells/well). Cells were incubated for 6 h, 24 h, 48 h and 72 h, respectively, and added with Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) reagent for incubation for another 2 h. Then the optical density (OD) of each well at the absorption wavelength of 490 nm was measured using the microplate reader and the data were analyzed.

Transwell Migration/Invasion Assay

At 48 h after transfection, the cells were digested with trypsin and resuspended using the serum-free medium. After cell counting, the cell density was adjusted to 2.0×10^5/mL; the transwell chambers containing and not containing matrix gel were placed in the 24-well plate; 200 μL cell suspension was added into the upper chamber, while 500 μL medium containing 10% FBS was added into the lower chamber. The plate was placed in an incubator at 37°C for incubation. After 48 h, the chamber was taken out, fixed with 4% paraformaldehyde for 30 min, stained with crystal violet for 15 min and washed with PBS. The inner face of basement membrane was carefully cleaned, and the inner-layer cells were removed. Cells passing through the membrane stained on the outer layer of basement membrane were observed under the microscope, and five visual fields were randomly selected for counting.

qRT-PCR

Total RNA was extracted from CRC cell lines and tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and then RNA was reversely transcribed into cDNA using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The qRT-PCR was performed using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) and StepOne Plus Real-time PCR system (Applied Biosystems, Foster City, CA, USA). The following primers were used for qRT-PCR: DLEU7-AS1: forward: 5'-GAGGGAGACACTTGGAAAAC-3', reverse: 5'-CACGTTGTTGGCCTTCGAGT-3'; β-actin: forward: 5'-CCTGGCACCCAGCACAAT-3', reverse: 5'-GCTGATCCACATCTGCTGGAA-3'. Data were analyzed using the ABI Step One software and the relative expression level of mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western Blotting

The transfected cells were lysed with cell lysis buffer, shaken on ice for 30 min and centrifuged at 14000 rpm and 4°C for 15 min. The total protein concentration was calculated using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). The extracted protein was separated via 10% SDS-PAGE and then transferred onto the polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Western blotting analysis was performed according to standard procedures. β-catenin, c-myc, cyclinD1, Tublin primary antibodies, anti-mouse and anti-rabbit secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS, Armonk, NY, USA) 22.0 was used for data processing, and data were presented as mean ± standard deviation ($\bar{x}\pm s$). The *t*-test was used for continuous variables, and x^2-test or Fisher's exact test was used for classified variables. Kaplan-Meier method was used to assess the prognosis and survival time of patients, and Log-rank test was used to compare the differences among different curves. $p<0.05$ suggested that the difference was statistically significant.

Results

DLEU7-AS1 was Highly Expressed in CRC Tissues and Cell Lines

The expressions of DLEU7-AS1 in 82 pairs of CRC tissues and the corresponding para-carcinoma tissues and CRC cell lines were detected *via* qRT-PCR. The results showed that compared with that in para-carcinoma tissues, the expression level of DLEU7-AS1 was significantly increased in CRC tissues, and the difference was statistically significant (Figure 1A). Compared with that in intestinal mucosal epithelial cells (FHC), the expression of DLEU7-AS1 was significantly high in CRC cells, and the difference was statistically significant (Figure 2A). Moreover, the expression levels of DLEU7-AS1 in HT-29 and HCT-116 were the highest, so we selected these two cell lines for subsequent experiments.

DLEU7-AS1 Expression was Correlated with Clinical Staging, Lymph Node Metastasis, Distant Metastasis and Overall Survival of CRC Patients

The expression of DLEU7-AS1 was divided into high expression group and low expression group according to the qRT-PCR results of DLEU7-AS1 expressions in 82 pairs of CRC tissues and para-carcinoma tissues. The correlation of DLEU7-AS1 expression with the patient's age, gender, tumor site, clinical staging, lymph node metastasis, and distant metastasis was analyzed *via* x^2-test. The high-expression DLEU7-AS1 was positively correlated with CRC clinical staging, lymph node metastasis and distant metastasis, but not correlated with age, gender and tumor site

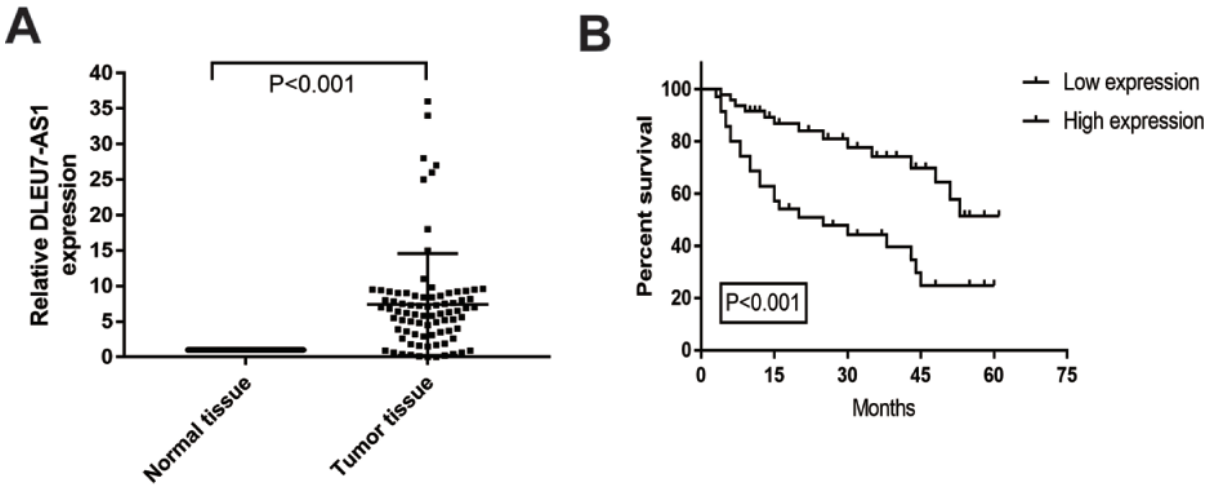

Figure 1. ***(A)*** The expression of DLEU7-AS1 in CRC tissue was significantly increased; ***(B)*** Kaplan-Meier survival curves of patients with CRC based on DLEU7-AS1 expression. Patients in the high expression group had a significantly more unfavorable prognosis than those in low expression group.

(Table I). In addition, in order to investigate the relationship between the expression of DLEU7-AS1 and the prognosis of CRC patients, relevant follow-up data were collected. Kaplan-Meier survival curve revealed that the high-expression DLEU7-AS1 was associated with poor prognosis of CRC, and the higher the expression level of DLEU7-AS1 was, the worse the prognosis would be (p<0.001; Figure 1B). This result suggested that HOTTIP may serve as a new biological index for predicting the prognosis of RCC.

Knockdown of DLEU7-AS1 Inhibited the Cell Proliferation

In order to explore the effect of DLEU7-AS1 on the proliferation capacity of CRC cells, the DLEU7-AS1 interfering expression model was successfully established (Figure 2B) and its proliferation was detected *via* CCK-8 in control group and DLEU7-AS1 interfering expression group. The cell proliferation rate in si-DLEU7-AS1 group was significantly decreased compared with that in si-NC group (Figure 2C-D).

Table I. Association of lncRNA DLEU7-AS1 expression with clinicopathologic characteristics of CRC.

Parameters	Number of cases	DLEU7-AS1 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.381
<60	35	22	13	
≥60	47	25	22	
Gender				0.170
Male	40	26	14	
Female	42	21	21	
Tumor location				0.598
Rectum	56	31	25	
Colon	26	16	10	
T stage				0.011
T1-T2	46	32	14	
T3-T4	36	15	21	
Lymph node metastasis				0.042
No	48	32	16	
Yes	34	15	19	
Distance metastasis				0.040
No	63	40	23	
Yes	19	7	12	

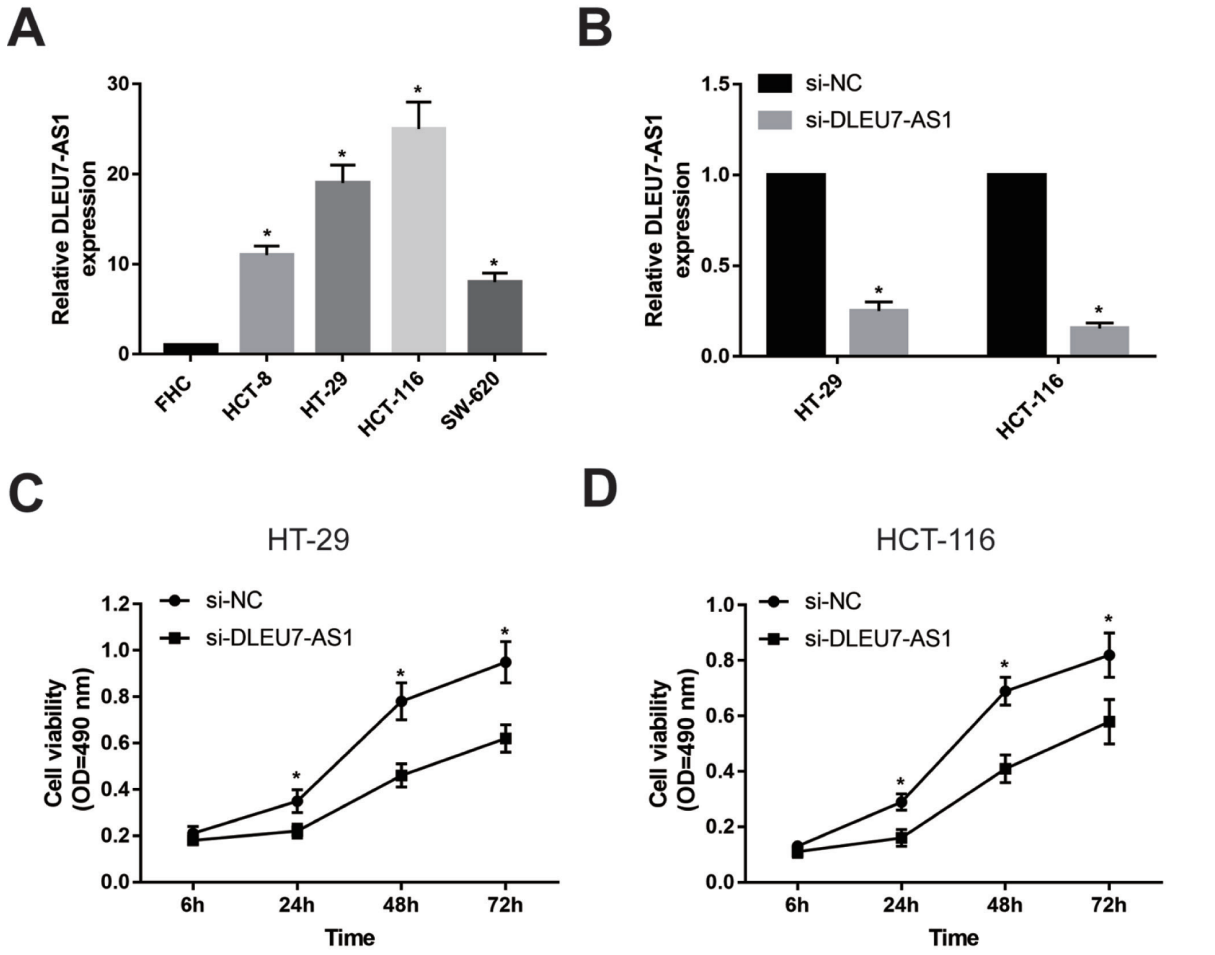

Figure 2. ***(A)*** qRT-PCR analysis of DLEU7-AS1 expression in CRC cell lines and intestinal mucosal epithelial cells FHC; ***(B)*** qRT-PCR were used to verify the efficiency of DLEU7-AS1 knockdown; ***(C-D)*** Growth curve analysis showing the cell growth of HT-29 and HCT-116 cells with DLEU7-AS1 knockdown.

Knockdown of DLEU7-AS1 Inhibited the cell Migration and Invasion

The effect of DLEU7-AS1 on the migration and invasion capacities of CRC cells was investigated via Transwell migration/invasion assay. The results of migration assay (Figure 3A-B) showed that the number of CRC cells passing through the membrane in Transwell chamber after knockdown of DLEU7-AS1 was significantly reduced compared with that in si-NC group, suggesting that the migration capacity is inhibited. The results of invasion assay were consistent with those above (Figure 3C-D).

Knockdown of DLEU7-AS1 Inhibited the Expression of Wnt/β-catenin Signaling Pathway

In order to analyze the potential mechanism of DLEU7-AS1 in promoting the cell proliferation, invasion and migration capacities, the changes in expressions of key proteins (β-catenin, c-myc, and cyclinD1) in Wnt/β-catenin pathway after knockdown of DLEU7-AS1 were detected *via* Western blotting. The results showed that the expression levels of the above-mentioned proteins were decreased significantly after knockdown of DLEU7-AS1 (Figure 4).

Discussion

CRC is one of the common malignant tumors in the world. In recent years, the morbidity and mortality rates of CRC have been gradually increasing; besides, the early diagnosis rate of CRC patients in China is very low, and most of them have been in the middle and late stages when treated,

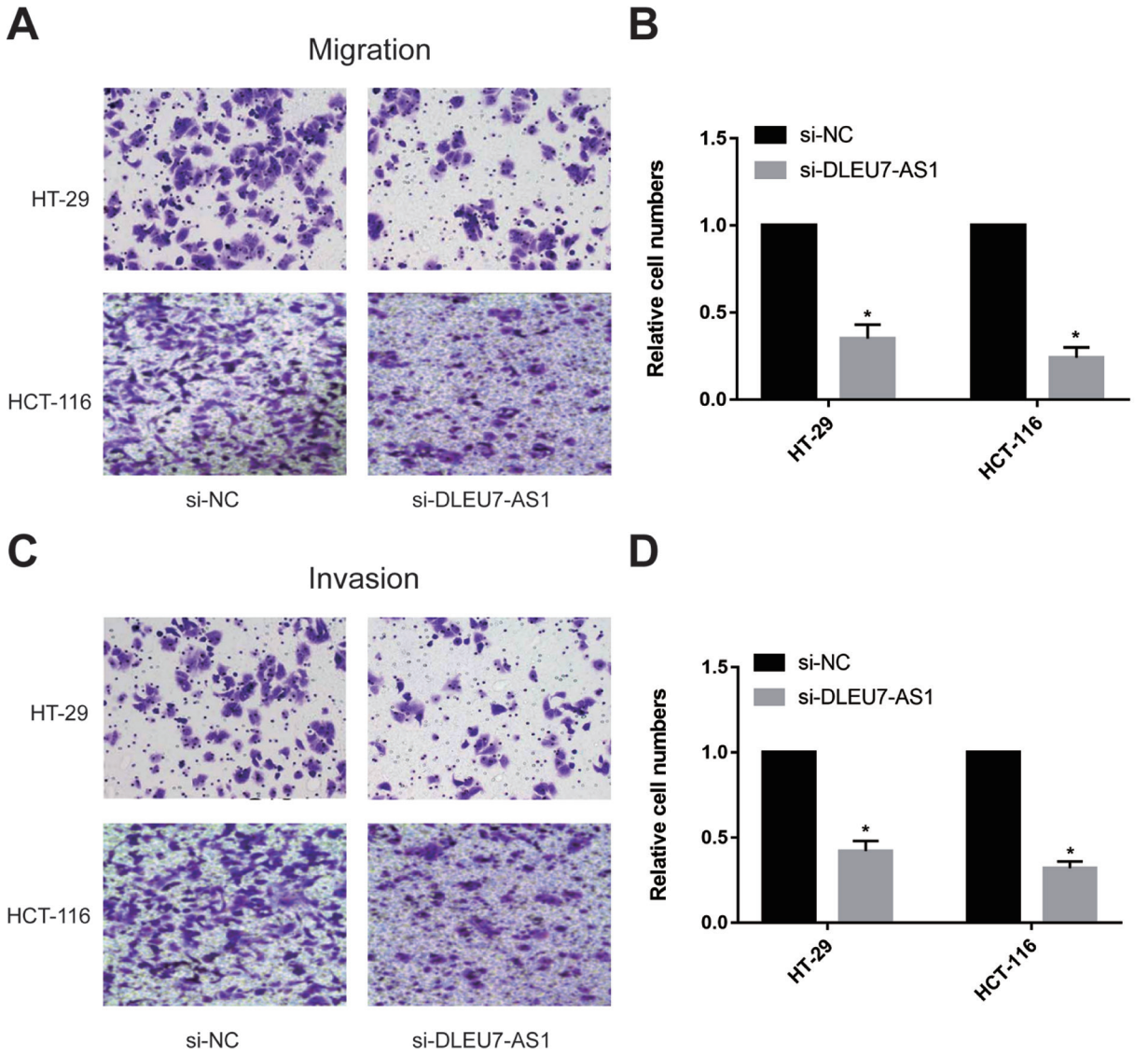

Figure 3. ***(A, B)*** HT-29 and HCT-116 cells transfected with si-DLEU7-AS1 displayed significantly lower migration capacity; ***(C,D)*** HT-29 and HCT-116 cells transfected with si-DLEU7-AS1 displayed significantly lower invasion capacity.

so the advanced tumor accounts for the majority[4]. The early diagnosis, metastasis, and recurrence of CRC and the adjuvant therapy after operation of advanced CRC have been the emphases in current research. Recent studies have shown that lncRNA plays an important role in a variety of diseases, including tumors. Many lncRNAs are abnormally expressed in CRC, which may play important roles in the diagnosis, treatment, and prognosis of CRC[19-21]. Therefore, searching for the abnormally-expressed lncRNA in CRC and analyzing its correlation with clinical prognosis will help improve the diagnosis and treatment levels of CRC, and improve the clinical prognosis of patients.

In this study, the expression of lncRNA DLEU7-AS1 in CRC and its role in the occurrence and development of CRC were investigated. First, the expressions of DLEU7-AS1 in 82 pairs of CRC tissues and para-carcinoma tissues were verified. The results showed that the DLEU7-AS1 expression was significantly up-regulated and positively correlated with CRC staging, lymph node metastasis, distant metastasis and poor prognosis. Thus, we believe that DLEU7-AS1 may play a tumor-promoting role in CRC. In order to further investigate the effect of DLEU7-AS1 on biological functions of CRC, the DLEU7-AS1 knockdown expression model was established using

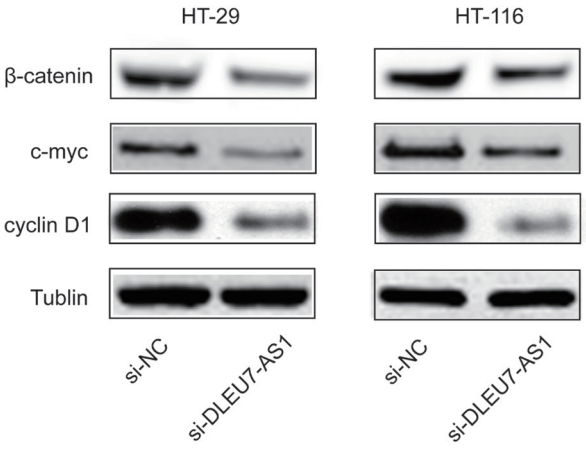

Figure 4. Knockdown of DLEU7-AS1 expression significantly decreased the expression of β-catenin, c-myc and cyclin D1.

small-interfering RNA. The results of CCK-8 and invasion/migration assay revealed that DLEU7-AS1 could promote the occurrence and development of CRC and exert important functions in CRC. However, its specific molecular mechanism remains unclear.

Wnt/β-catenin signaling pathway is an important signaling pathway related to tumorigenesis[22]. The abnormal activation of Wnt pathway in nasopharyngeal carcinoma and esophageal squamous carcinoma promotes the formation of tumor[23]. Wnt/β-catenin signaling pathway is activated in most gastric cancers and promotes the proliferation of gastric cancer[24]. The overexpression of Wnt-1 is associated with the proliferation, progression and poor prognosis of non-small cell lung cancer[25]. More than 90% CRC can occur upon the activation of typical Wnt/β-catenin signaling pathway[26]. When the Wnt signaling pathway is activated, β-catenin will gather in the nucleus, leading to the loss of epithelial structure, and such a phenomenon is significantly associated with tumor invasion and metastasis[27].

In order to explore whether DLEU7-AS1 promotes the occurrence and development of CRC through regulating Wnt/β-catenin, the changes in expressions of key proteins (β-catenin, c-myc, and cyclinD1) in Wnt/β-catenin pathway after knockdown of DLEU7-AS1 were detected *via* Western blotting. The results revealed that the expression levels of the above proteins were significantly decreased after knockdown of DLEU7-AS1, indicating that DLEU7-AS1 has a positive regulatory relation with Wnt/β-catenin pathway.

Conclusions

The expression of DLEU7-AS1 is significantly increased in CRC, which is markedly associated with CRC staging, lymph node metastasis, distant metastasis and poor prognosis. DLEU7-AS1 may promote the proliferation, invasion and migration capacities of CRC through regulating the Wnt/β-catenin pathway.

Conflict of interest

The authors declare no conflicts of interest.

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