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

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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 siRNA interference 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 more advanced, the positive rates of lymph node metastasis and distant metastasis were higher, and the overall survival rate was lower. Compared with 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

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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 microR[illegible][9]. LncRNA is a kind of RNA fragment wit[illegible] than 200 bp in length, which does not e[illegible]de protein. The abnormal expression of lncR[illegible] molecules can be detected in almost all tu[illegible] tissues, and more and more s[illegible] have co[illegible] firmed that the lncRNA mo[illegible]e p[illegible]s an extremely important role i[illegible]e occu[illegible]nce and development of tumor[10]. RNA [illegible]te the relevant prote[illegible] co[illegible]es thr[illegible]h many ways at diff[illegible]t level[illegible] play an extremely importa[illegible]le in the p[illegible]enesis of tumor[11,12]. The[illegible] RNA in C[illegible]s poorly understood[13]; [illegible]me ln[illegible]As, such as CRNDE and CCA[illegible]can promote[illegible][14,15], while some, such a[illegible]AS5 and lncRN[illegible]ET, can inhibit CRC[illegible] the p[illegible]ogenesis of CRC, lncRNA is inv[illegible]d in t[illegible]ctivation of Wnt pathway and epide[illegible]wth fa[illegible] receptor (EGFR) pa[illegible]ay, I[illegible]ion [illegible]ansforming growth fac[illegible]TGF[illegible]utation and epithelial-mesen[illegible]mal tra[illegible]ion, etc[17,18]., but the research in[illegible]e is still at the initial stage.

[illegible]y, the expression levels of lncRNA [illegible]LEU7-AS1 in 82 pairs of CRC tissues and pa[illegible]arcinoma normal tissues were analyzed, and th[illegible]ect 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 resect[illegible] mor tissue and para-carcinoma tiss[illegible] of CRC patients were collected. [illegible]rding t[illegible] the 7th version of Union for Inter[illegible]nal Cancer Control/American Joint Committ[illegible] Cancer (UICC/AJCC) CRC tumor ly[illegible]h node[illegible]tasis (TNM) staging criteria, al[illegible]ients enrol[illegible] diagnosed as CRC *via* t[illegible]ostope[illegible]tive pat[illegible] gical analysis, and th[illegible]d not [illegible]ve the pre-operative anti-tumo[illegible]the[illegible]s[illegible]as radi[illegible]rapy or chemotherapy[illegible]s stu[illegible]s appr[illegible]by the Ethical Comm[illegible]e of Rizha[illegible]ni[illegible] Hospital, and patient[illegible]ir families [illegible]en fully informed th[illegible]e sa[illegible] would be used for scientific research and signe[illegible] informed consent.

Cell Lines and Reagents

[illegible]our CRC cell l[illegible]nes (HCT-8, HT-29, HCT-116 [illegible]SW-620) an[illegible]e fetal human colonic epithel[illegible]ll line (FH[illegible] were purchased from ATCC (M[illegible] V[illegible]SA); high-glucose Dulbecco's modifie[illegible] medium (DMEM) and fetal bovine serum (FBS) were purchased from Life Technologies (Rockville, MD, USA); cells were cultured in [illegible]r 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 [illegible]sely transcribed into cDNA using Primesc[illegible] Reagent (TaKaRa, Otsu, Shiga, Japan). The [illegible]T-PCR was performed using SYBR®Premix [illegible] Taq™ (TaKaRa, Otsu, Shiga, Japan) and StepO[illegible] Plus Real-time PCR system (Ap[illegible]iosystem[illegible] Foster City, CA, USA). The [illegible]low[illegible] primers were used for qRT-PCR: [illegible]U7-AS[illegible]orward: 5'-GAGGGAGACACTT[illegible]AAAC[illegible]; 5'-CACGTTGTTGGC[illegible]TTC[illegible], p[illegible]: forward: 5'-CCTG[illegible]CCCA[illegible]'AAT-3', reverse: 5'-GCTG[illegible]'CACATC[illegible]GGAA-3'. Data were an[illegible]ng the AB[illegible]tep One software and [illegible]e rel[illegible] expression level of mRNA w[illegible]alculated usi[illegible] $2^{-\Delta\Delta Ct}$ method.

Western Blotting

[illegible]ansf[illegible]d cells were lysed with cell lysis buffe[illegible]on ice[illegible]0 min and centrifuged [illegible]4000[illegible]and 4[illegible]or 15 min. The total pro[illegible] concen[illegible]as calculated using the bicinchoninic ac[illegible]BCA) protein assay kit (Pierce, R[illegible], USA). The extracted protein was [illegible]10% SDS-PAGE and then transferred onto the polyvinylidene difluoride (PVDF) [illegible]brane (Millipore, Billerica, MA, USA). W[illegible]n 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 [illegible](SPSS, Armonk, NY, USA) 22.0 was [illegible]d for data processing, and data were prese[illegible]as mean ± standard deviation ($\bar{x}\pm s$). The *t*-tes[illegible]used for continuous variables, and x^2[illegible]t or Fi[illegible]exact test was used for classif[illegible]variables. [illegible] Meier method was use[illegible]assess[illegible]he prog[illegible] and survival time of [illegible]nts, a[illegible]Log-rank test was used to compa[illegible]th[illegible]es amo[illegible]iffe-rent curves. $p<0$[illegible]sugge[illegible]at the [illegible]erence was statistical[illegible]gnificant.

Results

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

[illegible]he expressi[illegible]of DLEU7-AS1 in 82 pairs o[illegible]C tissues [illegible]the corresponding para-carcinoma tissues [illegible]nd CRC cell lines were detected [illegible]-PCR. The results showed that compared with that in para-carcinoma tissues, [illegible]ression 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

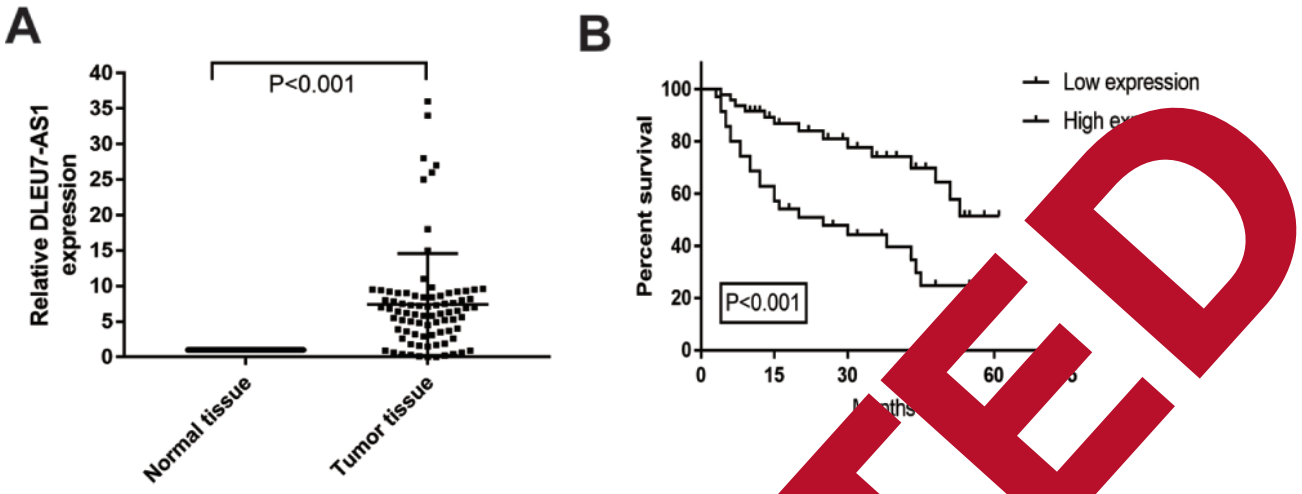

Figure 1. *(A)* The expression of DLEU7-AS1 in CRC tissue was significantly [illegible]eased, [illegible]aplan-Meier survival curves of patients with CRC based on DLEU7-AS1 expression. Patients in the high expression grou[illegible] significantly more unfavorable prognosis than those in low expression 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-M[illegible]r survival curve revealed that the high-exp[illegible] DLEU7-AS1 was associated with poor prog[illegible]is of CRC, and the higher the expression lev[illegible]f DLEU7-AS1 was, the worse the prognosis wo[illegible] be (p<0.001; Figure 1B). This [illegible]suggeste[illegible] that HOTTIP may serve as a [illegible]al index for predicting the prognosi[illegible]RCC.

[illegible]ckdown o[illegible]LEU7-AS1 Inhibited t[illegible]Cell Prolif[illegible]tion

I[illegible]r to [illegible]ore the effect of DLEU7-AS1 on the p[illegible]ation capacity of CRC cells, the DLEU7-AS1 interfering expression model was [illegible]fully established (Figure 2B) and its proli[illegible]ra[illegible]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 ln[illegible]A DL[illegible] expression with clinicopathologic characteristics of CRC.

Parameters	Number [illegible]f cases	DLEU7-AS1 expression		*p*-value
		Low (%)	High (%)	
Age (year[illegible]				0.381
<60	[illegible]	22	13	
≥60	47	25	22	
G[illegible]				0.170
Mal[illegible]	40	26	14	
Female	42	21	21	
[illegible]r loc[illegible]				0.598
[illegible]n	56	31	25	
Co[illegible]	26	16	10	
T[illegible]				0.011
[illegible]	46	32	14	
[illegible]T4	36	15	21	
[illegible]ymph node metastasis				0.042
[illegible]	48	32	16	
[illegible]	34	15	19	
Dis[illegible]nce metastasis				0.040
No	63	40	23	
Yes	19	7	12	

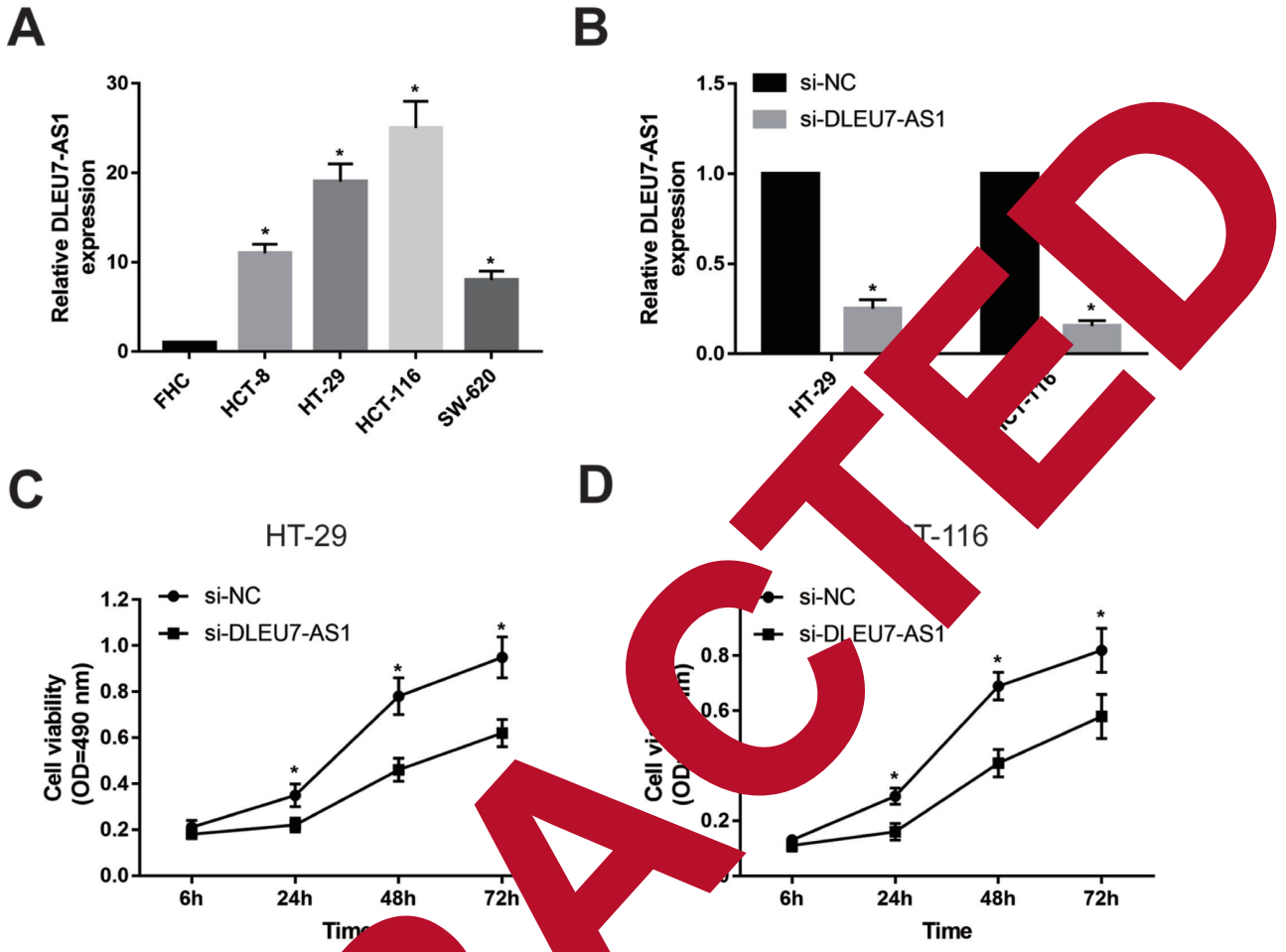

Figure 2. ***(A)*** qRT-PCR analysis LEU7-A expression RC cell lines and intestinal mucosal epithelial cells FHC; ***(B)*** qRT-PCR were used to verify t ciency o U7-AS1 kn ckdown; ***(C-D)*** Growth curve analysis showing the cell growth of HT-29 and HCT-116 cells wi U7-A

Knockdown 7-AS1 Inh ed the cell Mig tion a nvasion

The eff f DLEU7-A the migration and invasio pacities of CRC c was investigated via T well m tion/invasion assay. The results c ratio ay (Fig re 3A-B) showed that the numb cells p g through the membrane Trans amb er knockdown of DLEU7-A as sig reduced compared with that in s C group, gesting that the migration capaci ibited. The results of invasion assay were sist those above (Figure 3C-D).

ckdown of DLEU7-AS1 Inhibited the E ssion 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,

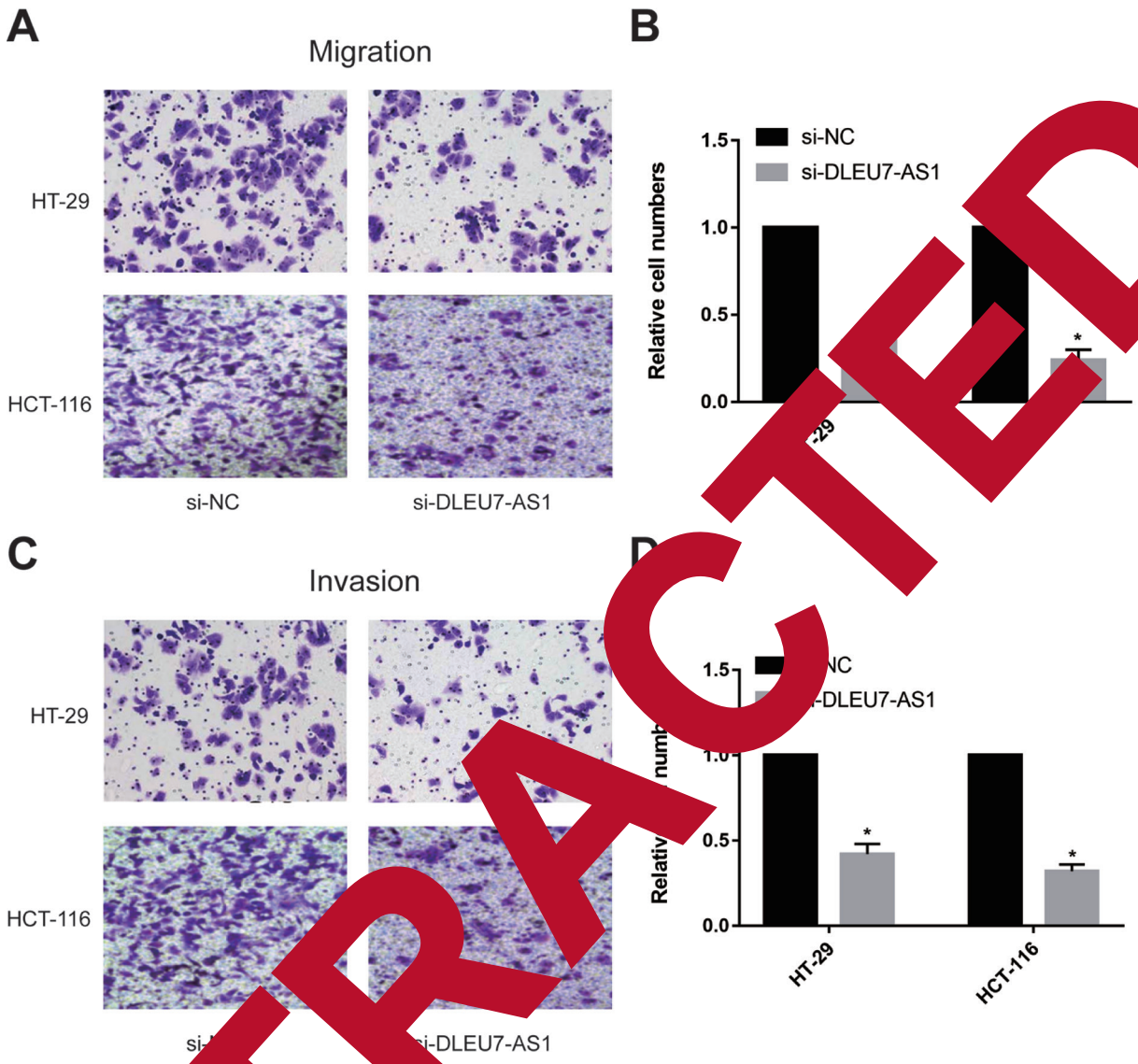

Figure 3. ***(A, B)*** ...ICT-116 cells ...ected with si-DLEU7-AS1 displayed significantly lower migration capacity; ***(C,D)*** HT-29 and ...T-116 ...ansfected with si-DLEU7-AS1 displayed significantly lower invasion capacity.

so th...vanced ...nor accounts for the majority[4]. Th... dia...is, metastasis, and recurrence of CRC ...djuvan...rapy after operation of ...ance... have ...n the emphases in current ...ch. Re...ies have shown that lncRNA pla...n impo...role in a variety of diseases, in...tumors. Many lncRNAs are abnormal-...xpr...n CRC, which may play important ...les in the diagnosis, treatment, and prognosis of ...[19-21]. Therefore, searching for the abnormal-ly ...essed 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

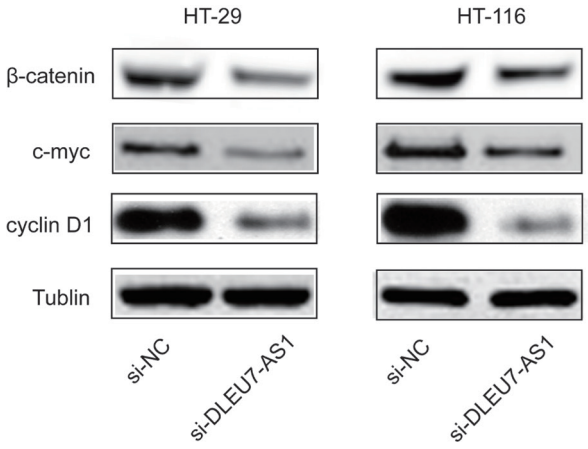

Figure 4. Knockdown of DLEU7-AS1 expression significantly decreased the expression of β-catenin, c-myc and cyclin D1.

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 mecha[illegible] remains unclear.

Wnt/β-catenin signaling pathway is an i[illegible]rtant signaling pathway related to tumorig[illegible]sis[22]. The abnormal activation of Wnt pathv[illegible] in nasopharyngeal carcinoma, [illegible]sophage[illegible] squamous carcinoma promot[illegible]ation o[illegible] tumor[23]. Wnt/β-catenin sig[illegible]g path[illegible] is activated in most gastric ca[illegible] and [illegible] proliferation of gastri[illegible] overexp[illegible]sion of Wnt-1 is ass[illegible]ted wit[illegible]proliferation, progression and [illegible]prognosis o[illegible]small cell lung cancer[25]. [illegible] 90% CR[illegible]n occur upon the acti[illegible]ion o[illegible]al Wnt/β-catenin signaling p[illegible]ay[26]. When [illegible]Wnt signaling pathway [illegible]tivated, β-caten[illegible]l gather in the nucl[illegible]eading [illegible]the loss of epithelial structure, an[illegible] a ph[illegible]henon is significantly associated with t[illegible]asion a[illegible]etastasis[27].

[illegible] ord[illegible]explo[illegible]whether DLEU7-AS1 pro[illegible] the [illegible]e and development of CRC thr[illegible]h regula[illegible] Wnt/β-catenin, the changes in ex[illegible]s of key proteins (β-catenin, c-myc, and [illegible]) Wnt/β-catenin pathway after knock[illegible]wn of DLEU7-AS1 were detected *via* Western [illegible]ting. The results revealed that the expression l[illegible]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 ass[illegible]ted with CRC staging, lymph node metasta[illegible] metastasis and poor prognosis. DLE[illegible]AS1 may promote the proliferation, invasio[illegible] migration capacities of CRC through regula[illegible]he Wnt/β-catenin pathway.

Conflict of interest

The authors declare no [illegible]st.

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