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LINC00657 promotes the development of colon cancer by activating PI3K/AKT pathway

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Abstract. – OBJECTIVE: The aim of this study was to explore whether LINC00657 can regulate cell proliferation and invasion by regulating the PI3K/AKT pathway and thus participate in the occurrence of colon cancer.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was applied to detect the expression levels of LINC00657 and E-cad in colon cancer tissues and corresponding adjacent tissues obtained from 80 patients, and the correlation was analyzed between LINC00657 expression and clinical information of patients such as prognosis, tumor size, tumor stage, and distant met- sis. The expression of LINC00657 and E- colon cancer cell lines was also examine- nd the effect of LINC00657 on tumor cell pro- tion was evaluated by cell counting kit-8 (CC- assay and colony formation experiments. Mea- while, transwell assay was pe- to eval- ate the influence of LINC00- -PN7 on cell invasive ability. In a- on, th- ffect of LINC00657 on CAPN7 a- PI3K/A- thway was detected by Western- s-

RESULTS: The exp- ion- LINC00- and E-cad in tum- ssues d- ed remarkably, especially i- ents who o- d distant metastasis. Co- with patient- h high-ly-expressed -C00- he patients with lower level of -C00657 ha- rse prognosis and an adva- tumor size a- M stage. Similarly, LIN- 57 and E-cad also- owed a decrease in c- cance- l lines. After overexpression of -00657- ll viability and invasive ability decr- arkabl- ile cell apoptosis rate increas- nifica- n addition, high expres- of LI- 65- an *in vitro* model signifi- ca- prome- PN7 expression and inhibited -vation o- K/AKT pathway.

-USIONS: LINC00657 had a low expres- cancer tissues, which could accel- ate cell proliferation and invasion by activat- PI3K/AKT pathway and inhibiting CAPN7 ex- ion.

Key Words:
LINC00657, Cell proliferation, Colon cancer, PI3K/AKT pathway.

-odu-

Colorect- is one of t- st common malignan- mors- digestive system. In the past two decades, t- cidence of colorectal can- e world has- on the rise, and its i- nce rate has been ra-ked third in the ove- malignant tu- r and fourth in the cause of -gnant tumor- th[1]. With the improvement of li- standards- the increase of meat intake, the- nce- olorectal cancer in China has increas- y year[2]. Most patients with colo-rectal cancer are non-hereditary sporadic cases, -a mainly affected by environment, diet, -bits, and chronic inflammation. The pathological progression is usually from adenoma to atypical hyperplasia, then to adenocarcinoma, and finally to metastatic adenocarcinoma[3,4]. In this process, the expression levels of a series of genes including APC, KRAS, and TP53 have been successively changed, eventually leading to somatic mutations which then induce the occurrence of tumors[5]. With the deepening of research on colorectal cancer, the researchers found that some epigenetic-related genes are also involved in the occurrence and evolution of colorectal cancer.

In the past, long-chain non-coding RNA had been regarded as a by-product in the process of gene transcription, which thus did not receive much attention. With the increasing application of various advanced experimental techniques such as gene chips, more and more lncRNAs have been discovered, and research on their functions and mechanisms of action has been gradually deepened.

LncRNA is a class of non-coding RNAs with a transcription length greater than 200 nucleotides[6]. Different lncRNAs often have different mechanisms of action, including splicing regulation, chromosomal remodeling, regulation of

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protein activity or protein localization, mRNA degradation, and so on[7-9]. Studies have demonstrated that a large number of lncRNAs may show an abnormal expression in tumor tissues. With the deepening of research, some specific lncRNA may become a new breakthrough in cancer prevention and treatment. For example, with high tissue specificity and sensitivity, CCAT1 expression in colorectal cancer tissues is 200 times higher than that in normal intestinal mucosa tissues[10]. Further researches have showed that CCAT1 is associated with tumor grade and TNM stage, suggesting it may serve as an independent biological indicator for the prediction of patient's prognosis[11].

The current work indicates that the correlation between lncRNA and colorectal tumor is mainly reflected in following several aspects. Firstly, lncRNA can be used as a molecular marker for early diagnosis and prognosis assessment of colorectal cancer; secondly, lncRNA is expected to become a new targeted drug; and thirdly, lncRNA can improve the chemoresistance[12]. In this study, we first explored the role of LINC00657 in colon cancer and elucidated its mechanisms regu[illegible] cell proliferation and migration.

Patients and Methods

Sample Collection

A total of 80 specimen[illegible]re obt[illegible]ed from patients with colon canc[illegible] Shaan[illegible] People's Hospital fro[illegible] No[illegible] 15 to [illegible]-tember 2018. Each [illegible]imen w[illegible]ken from tumor tissues and t[illegible]responding [illegible]al tissues located in the [illegible]ower tumor [illegible]gin. All collected spec[illegible]ens w[illegible]nfirmed by pathological dia[illegible]s. Fresh sp[illegible]ens were quickly placed [illegible]quid nitrogen afte[illegible]ng isolated. All the [illegible]nts ha[illegible]ever received any chemotherap[illegible]diothe[illegible]y or other treatments before the surge[illegible] the p[illegible]t's clinical data were [illegible]lecte[illegible] as gender, depth of tumor i[illegible]on, a[illegible] without regional lymph node [illegible]astasis. [illegible]s study has been approved by th[illegible] Committee of the Shaanxi Provincial [illegible]pl[illegible]ital. The written informed consent [illegible]f this study was obtained from all participants.

Cell Culture

Human colon cancer cell lines including HCT116, Caco2, Caco205, SW620, SW480 and normal intestinal mucosal epithelial cells NCM460 were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The culture medium was Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FB[illegible] penicillin-streptomycin (HyClone, S[illegible] Logan, UT, USA).

Cell Transfection

Transient transfection[illegible] performe[illegible] Lipofectamine 3000 r[illegible]nt (In[illegible]trogen, lsbad, CA, USA). C[illegible] were [illegible]ed one day before transfectio[illegible] a[illegible]ber w[illegible]djusted to 1.0-2.0[illegible]10^5/w[illegible]fter [illegible] were adherently gr[illegible]to 70% o[illegible], 900 µL of serum-f[illegible]um was ad[illegible] each well. 1.5 µL/w[illegible]f Lip[illegible] and LINC00657/CAPN7 overexpression plasm[illegible] 2.5 µg/well of empty control plasmid were r[illegible]tively diluted in 50 µL cell of serum-free OPTI-MEM medium and gently mixed and incubated for 5 min at room temperature. Afterward, the mixture was added to cells for 4[illegible]ours of incubation; then, the serum-[illegible]m was replaced with complete medium. [illegible] transfection for 36-48 hours, the cells were collected for the subsequent experi-

Cloning Formation Experiment

The cells in logarithmic growth phase were seeded into a six-well plate with 3000 cells per well and cultured at 37°C incubator with 5% CO_2 for 10-14 days until macroscopic clones appeared. After the cells were fixed with methanol for 30 min, the fixative was removed and PBS was used to wash cells twice. Then, Giemsa dye solution was used to stain the cells for more than 30 min, and then dried in air. The number of clones visible to the naked eye was counted using a transparent film with a grid, and the colony formation rate = (number of clones / number of cells seeded) × 100%.

Transwell Experiment

Cold serum-free DMEM medium was used to dilute Matrigel gel at a ratio of 1:5. 50 µL of glue was evenly spread on the bottom of the chamber. Cells were resuspended and diluted to a cell density of 5×10^5/ml, and 100 µL of cell suspension was added to each chamber. After 24-72 h, the cells were washed twice with phosphate-buffered saline (PBS), and the upper Matrigel and unmigrated cells were wiped off. The migrated cells were immobilized for 30 min

with methanol, and the Giemsa stain was used to stain them for 30 min in the dark. Subsequently, the chamber was placed on a glass slide and randomly photographed under a 20× microscope in 5 fields of view. Finally, cell counting and statistical analysis of cell invasive ability were performed.

Apoptosis

After transfection for 48 hours, the cells were digested with the appropriate amount of trypsin, and the suspension cells were gently pipetted and counted. 50,000-100,000 cells were collected for centrifugation, and after discarding the supernatant, the cells were gently resuspended with 195 μL Annexin V-FITC binding solution. 5 μL of Annexin V-FITC was added to incubate the cells for 15 min at 4°C in the dark, and 5 μL of propidium iodide staining solution was subsequently used to incubate for 5 min. At the same time, a tube without Annexin V-FITC and Propidium Iodide (PI) was used as a negative control. Flow cytometry was then performed immediately.

Total RNA Extraction and Quantita… Polymerase Chain Reaction (qPCR) A…y

The total RNA of the tissue sample… cells was extracted using TRIzol Reag… (Invitrogen, Carlsbad, CA, … and di… solved in RNase-free wate… …ne… …as then reverse transcribed to o… com… …hentary Deoxyribose Nucleic A… DNA)… quantitative PCR a… …li… …as pe… …med according to … SYBR … mix Ex Taq TMII (Perfect R… …ime) kit in… …tions (TaKaRa, Otsu, S… …a… …n), and the … …eviously obtained cD… …was … …titatively detected. The PC… …amplificati… …onditions were: pre-de… …ration at 94°C f… …min, followed by 4… …cles a… …4°C for 30 s, 55°C for 30 s, an… …C fo… …s. The primers used were as follow… …00657 …ATGATGAATGCGACAG… …C R: …GCAGTGTCTTGTTGTT… …E-ca… …ACACCTTTGCATACAGAAC… R: T… …CGATTACACCCAGACTGC; ZE… …CAGCTTGATACCTGTGAATGGG… …GGTCGTGTGGGACT; N-cad F: …GGACTTAGAGCGTGCTCATT R: CCTGTC…GCCAACTGTTTCA; ZO-1 F: TGGCTCGA…CACTAGA R: AGGTGGCTTTGGCTTAACACT; CAPN7 F: ATGGGGCAAGCTACCATTATCA R: TCATTGTAGGATTGTTGGTGAGG; Bax F: CCCGAGAGGTCTTTTTCCGAG R: CCAGCCCATGATGGTTCTGAT; Bcl-2 F: GAAGCGTCCCACGGAACTG R: GTGCAGGAGGGTGTCGTTG.

Plasmid Construction

The construction of LINC00657/… …N7 ove… rexpression plasmid was complet… … Shanghai Heyuan Biotechnology Co., Ltd (S… …ai, China). using adenoviral vecto… Adeno… …V-E-GFP-3FLAG. Overexpre… …l lentivi… … control lentivirus (pLe… …F1α-F… …FP-F2A… o-CMV-MCS) were … …hased … …m the same company.

Western Bl… …g Assay

The prot… …les were tak… … and placed on ice. A… thaw… …ey were bo…ed for 5 min, immediately placed … … and then centrifuged at 1… …/min at 4°C … …3 min. Afterward, 3… … of protein sample … as subjected to 10% … -PAGE (sod… …m dodecyl sulphate-polya… …mide gel e… …rophoresis), and transferred t… VDF (poly… …ylidene difluoride) membrane (M… … …a, MA, USA) by a conventional me… …hich was incubated with different …rimary antibodies at 4°C overnight. In the next … second antibody was used to incubate the …e…ne, which was then exposed using enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA).

Cell Counting Kit-8 (CCK-8) Assay

The cells were digested and collected 24 h after transfection, and seeded into 96-well plates at 2*103/well, with 6 replicate wells set in each group. After the cells were attached to the wall in the next day, cell viability was measured by the CCK-8 method (Dojindo, Kumamoto, Japan). 2 h before the test, 10 μL of CCK-8 solution was added to each well and incubated at 37°C for 2 h. The absorbance of each well at 450 nm was measured by a microplate reader.

Statistical Analysis

The statistical analysis software Statistical Product and Service Solutions (SPSS) 20 software (IBM, Armonk, NY, USA) was used to analyze the correlation of the data. Measurement data of each group were expressed by means ± SEM, and the mean difference between two groups was analyzed by *t*-test. The chi-square test was used for the analysis of classification data. $p < 0.05$ was considered statistically significant.

Results

LINC00657 Was Lowly Expressed in Colon Cancer Tissues

PCR was performed to detect the level of LINC00657 in colorectal carcinoma. The results showed that LINC00657 in colon cancer tissues was significantly lower than that in adjacent tissues (Figure 1A). Patients were divided into metastatic and non-metastatic groups based on tumor metastasis, and the level of LINC00657 was found markedly decreased in metastatic patients' tumor tissues (Figure 1B). Correlation analysis between LINC00657 expression and clinical information such as patient's age and gender revealed that low expression of LINC00657 was remarkably associated with tumor size, TNM stage, and distant metastasis, and that patients with low expression of LINC00657 had a worse prognosis than patients with high expression (Table I, Figure 1C), suggesting that abnormal expression of LINC00657 may be involved in the progression of colorectal carcinoma. To investigate the effect of LINC00657 on tumor metastasis, we examined the expression of E-cad in tumor tissues, which showed a decrease compared with normal tissues, especially in patients with distant metastases (Figure 1D, 1E).

High Expression of LINC00657 Could Inhibit Cell Invasion

Further, we found the expression [illegible]C00657 was also underexpressed in [illegible]veral [illegible] cancer cell lines, with SW62[illegible]pression b[illegible] lowest, so it was selec[illegible]as a s[illegible]sequen[illegible] model (Figure 2A). [illegible]nwhile [illegible]cad was also found decreased i[illegible] [illegible]l lines (Figure 2B). By plasmi[illegible]ansfe[illegible] we [illegible]xpressed LINC006[illegible] *vitro* (Fi[illegible]2[illegible]nd found that highly [illegible]d LINC00[illegible]gnificantly up-regula[illegible]the [illegible]nd protein level of E-cad (Figure 2D, 2E). Exp[illegible]n of other EMT-related gen[illegible]ed that ZE[illegible]d N-cad decreased w[illegible] LINC00657 was highly expressed, while [illegible] was signif[illegible]antly enhanced (Figure 2F). [illegible]swell exper[illegible]nts revealed that overexpress[illegible]f LINC00[illegible] could markedly weaken cell inva[illegible]bili[illegible]gure 2G).

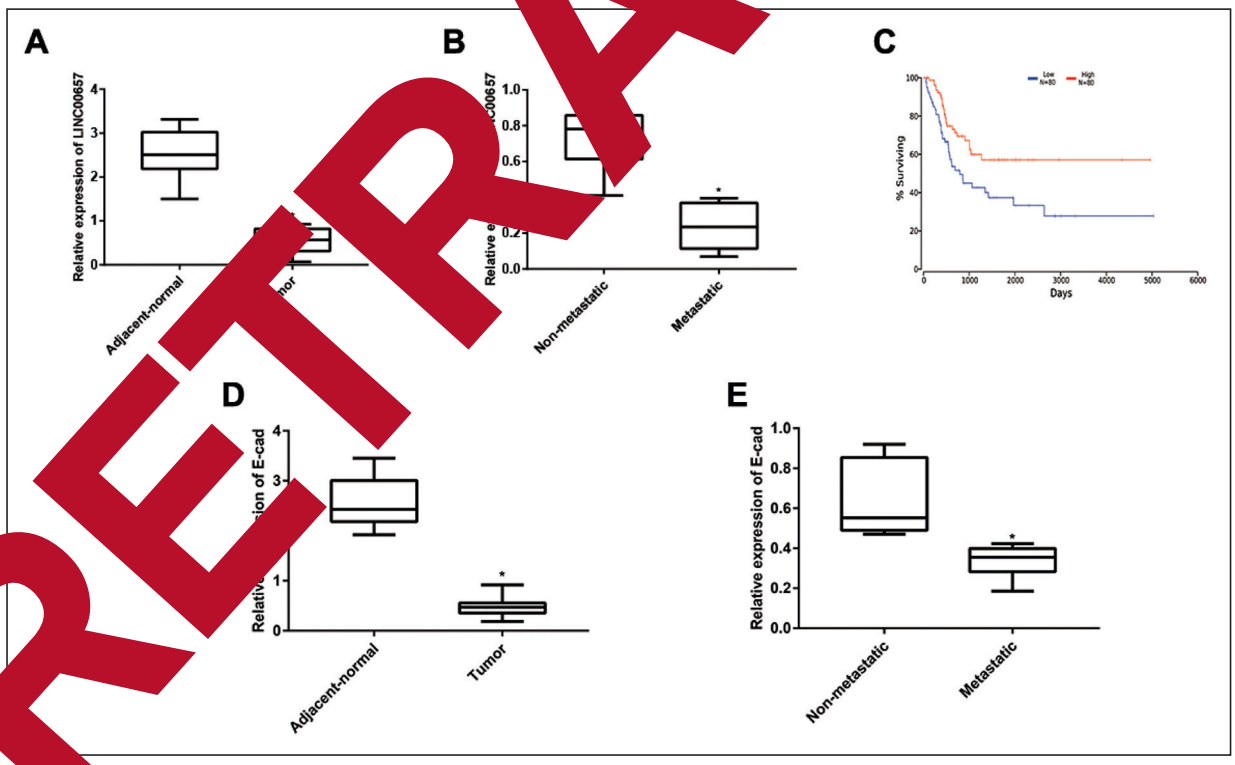

Figure 1. LINC00657 has a low expression in tumor tissues. ***A,*** LINC00657 is lowly expressed in colon cancer tissues. ***B,*** LINC00657 is lower in patients with distant metastasis than in patients without metastasis. ***C,*** Prognosis of patients with lowly expressed LINC00657 is poorer than those with highly expressed one. ***D,*** E-cad is lowly expressed in colon cancer tissues. ***E,*** E-cad is lower in patients with distant metastasis than in patients without metastasis.

Table I. Relation of LncRNA LINC00657 expression and clinicopathologic features in patients with colon cancer.

Clinicopathologic features	Number of cases	LncRNA LINC00657 expression		P
		Low (n = 40)	High (n = 40)	
Age (years)		40	40	0.1165
≤ 55	37	22	15	
> 55	43	18	25	
Gender				3556
Male	50	27	23	
Female	30	13	17	
Tumor size (cm)				0.0
≥ 5	45	29	16	
< 5	35	11	24	
TNM stages				82*
N0-2	38	18	20	
N3-N4	32	22	10	
Metastasis				0.0285*
Yes	37	25	12	
No	43	15	28	

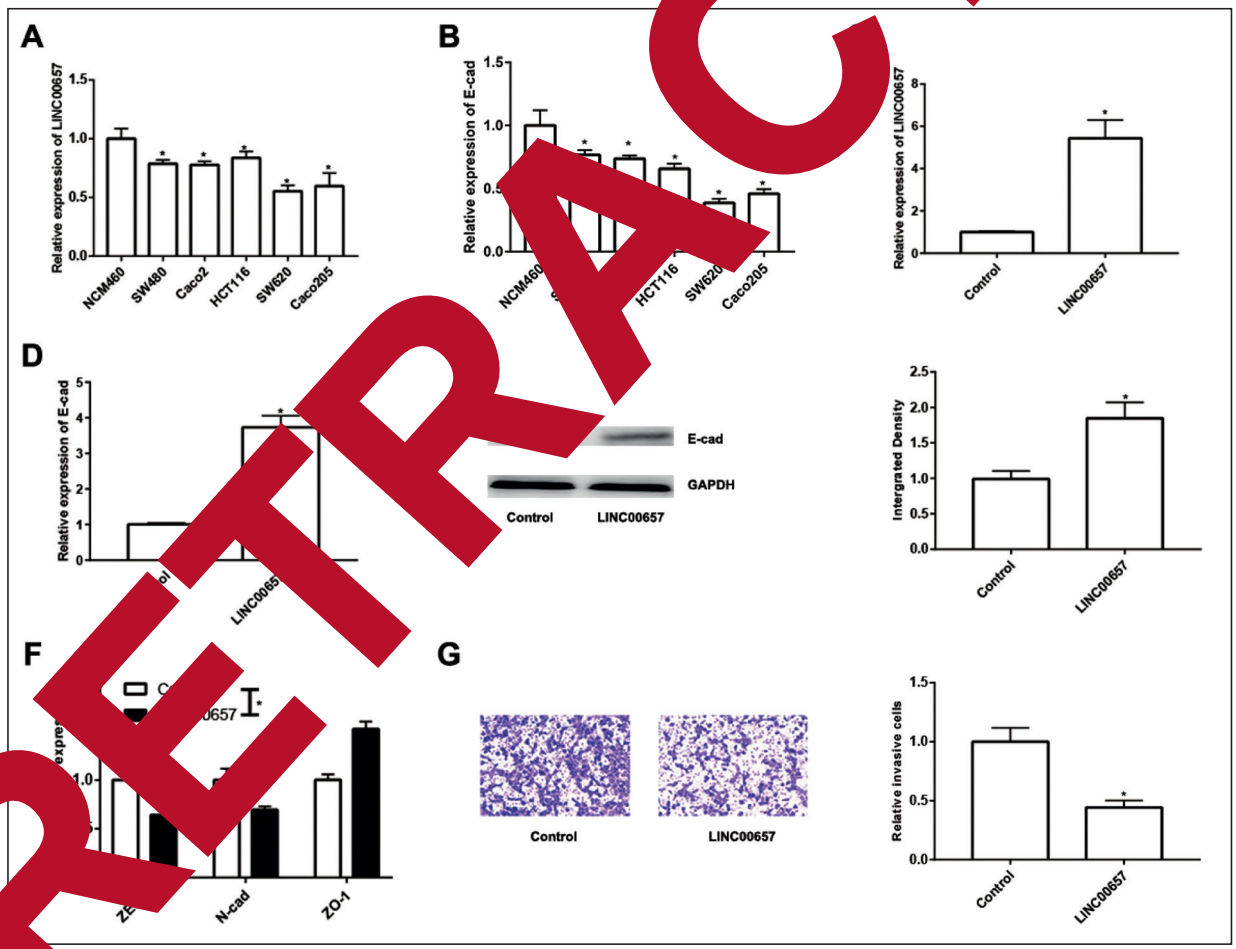

Figure 2. High expression of LINC00657 can inhibit cell invasion. ***A,*** LINC00657 is lowly expressed in colon cancer cells. ***B,*** E-cad is lowly expressed in colon cancer cells. ***C,*** Expression of LINC00657 is enhanced in SW620 after transfection of overexpression plasmid. ***D,*** High expression of LINC00657 promotes mRNA expression of E-cad. ***E,*** High expression of LINC00657 promotes E-cad protein expression. ***F,*** High expression of LINC00657 inhibits the expression of ZEB1 and N-cad but promotes ZO-1 expression. ***G,*** High expression of LINC00657 inhibits cell invasion.

High Expression of LINC00657 Was Able to Reduce Cell Proliferation Rate

The results of the CCK-8 assay revealed that the proliferation rate of colon cancer cells was significantly reduced after overexpression of LINC00657 (Figure 3A). At the same time, the clone formation experiments demonstrated LINC00657 was capable of inhibiting cell proliferative ability (Figure 3B). However, cell apoptosis showed a significant increase after high expression of LINC00657 (Figure 3C). In addition, the level of Bax-2, an apoptosis-inducing protein, was strikingly decreased (Figure 3D, 3E).

LINC00657 Could Regulate CAPN7 Expression

To explore the regulatory mechanism of LINC00657, we predicted its potential target gene through bioinformatics and CAPN7 was found. A significant increase in CAPN7 level occurred after up-regulation of LINC00657 (Figure 4A, 4B). Additionally, overexpression of CAPN7 could inhibit cell clonality, suggesting that LINC00657 may function through CA[illegible]7 (Figure 4C). PI3K/AKT pathway might [illegible] vital role in cell proliferation, and the W[illegible]n blotting results showed that overexpressio[illegible] LINC00657 could remarkably deactivate [illegible] PI3K/AKT pathway (Figure 4D).

Discussion

The concealed symptoms of colon cancer in the early stage create an undesirable situati[illegible] most patients have been in the middl[illegible] ced stage when diagnosed. Comprehe[illegible]ve use o[illegible] surgery, chemotherapy, radiother[illegible] still cannot enhance the five-year survival rate [illegible] olorectal carcinoma due to high rec[illegible]nce, [illegible]istant metastasis; so in-depth stu[illegible]g the pathe[illegible] of colon cancer, lookin[illegible] marke[illegible] of early gnosis as well as pr[illegible]is eve[illegible]ion and new targets for treatme[illegible] a[illegible]i[illegible]ly cru[illegible][13,14].

At present, re[illegible]ches [illegible]orectal[illegible]cer-related lncRNA[illegible]ve found [illegible]t[illegible]hange of lncRNA ex[illegible]is correlate[illegible] the occurrence of c[illegible]ectal[illegible]er, and some lncRNAs are involved in the prog[illegible]n and development of tum[illegible] example, M[illegible] can regulate tumor c[illegible]oliferation and be ass[illegible]ciated with TNM sta[illegible] of patients w[illegible]h colorectal cancer. The lower [illegible]legree of dif[illegible]ntiation, the deeper the depth o[illegible]sion and [illegible]ower expression of MEG3 in colo[illegible]can[illegible]ssues; therefore, MEG3 can be seen as [illegible] suppressor gene involved in the occurrence and development of tumors. Studies [illegible]oved that MEG3 can enhance p53 protein ve[illegible]ough inhibiting expression of MDM2, which promotes the binding of p53 and GDF15 and induces GDF15 expression, thereby redu-

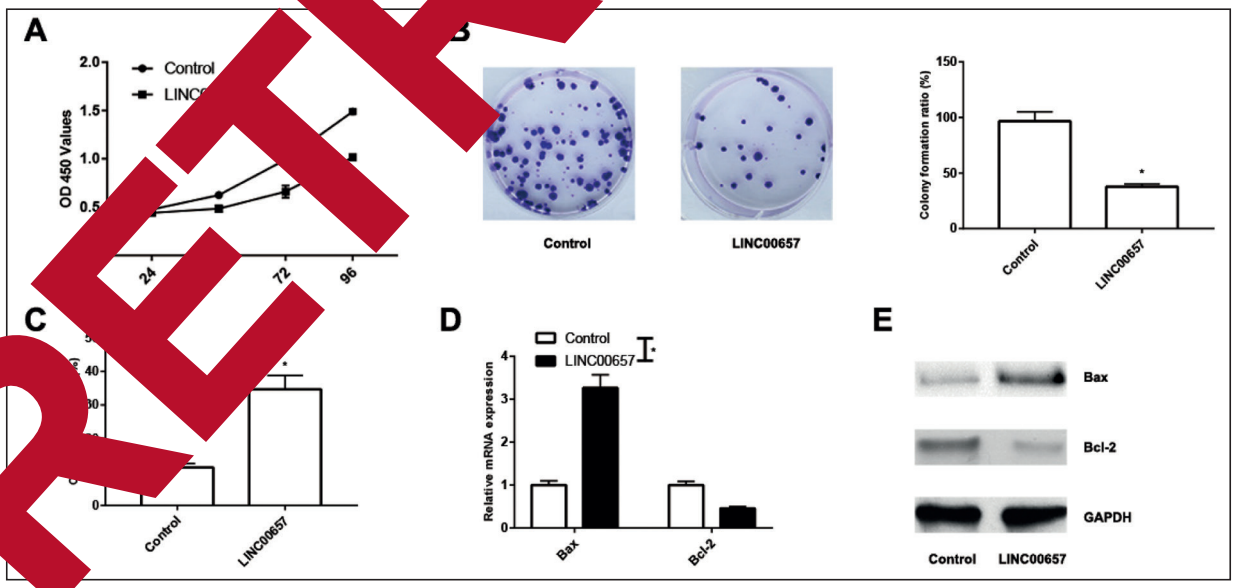

Figure 3. High expression of LINC00657 can inhibit cell proliferation. ***A,*** High expression of LINC00657 inhibits cell proliferation. ***B,*** High expression of LINC00657 inhibits cell colony formation. ***C,*** Increased cell apoptosis occurs after upregulation of LINC00657. ***D-E,*** High expression of LINC00657 promotes Bax expression and inhibits Bcl-2 expression.

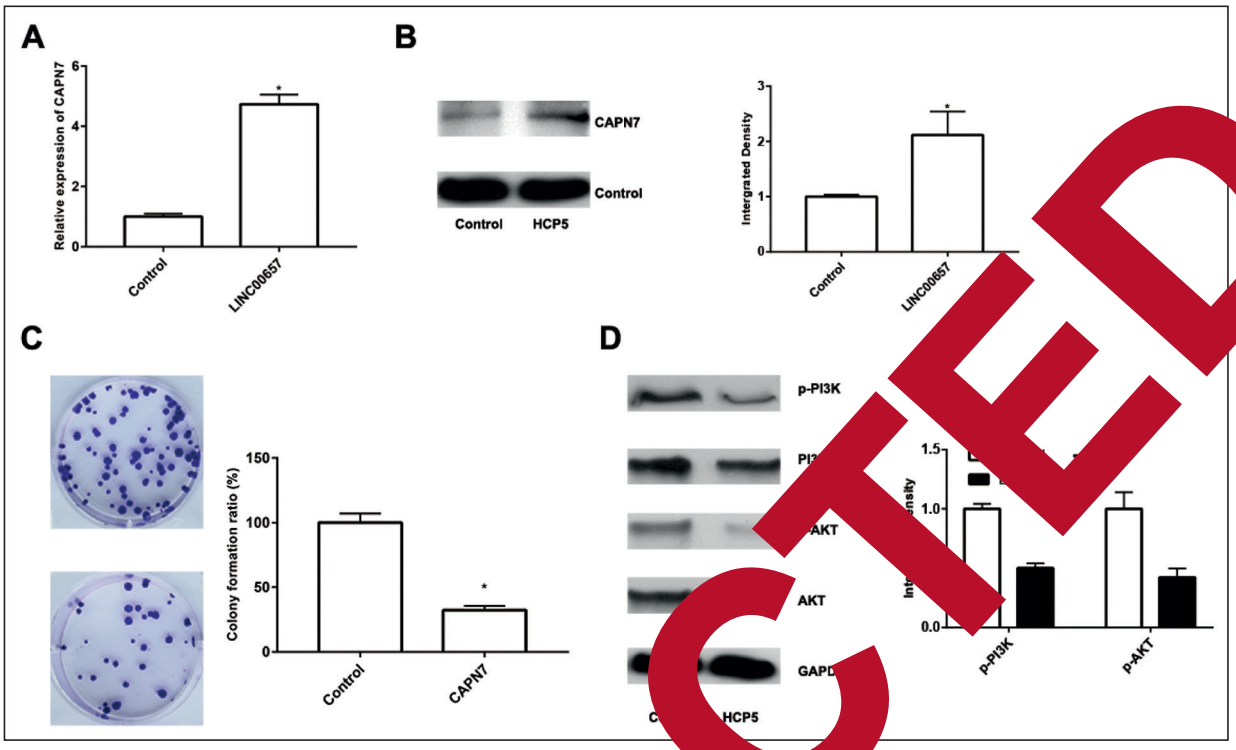

Figure 4. High expression of LINC00657 inhibits PI3K/AKT pathway. High expression of LINC00657 promotes CAPN7 expression. ***C,*** High expression of CAPN7 promotes colony formation. ***D,*** High expression of LINC00657 inhibits the activation of PI3K/AKT pathway.

cing cell proliferation rate[15]. Ln LET has low expression in colorectal es, and hypoxia-induced histone d tylase inhibit lncRNA-LET expressio hibiti lation process of his e d ncd by lncRNA-LET p ter reg LncRNA is also associated v nvasion an astasis of colorectal can pression o RNA in colorectal ade carcin issues and metastatic lymph no was analyz gene expression profilin nd it was found the expression of E 00043 was remarkably up-regulated in tatic ons[17]. HULC has been known to ha expre in liver cancer tissue; ever, s al und to express highly in metast s of colorectal cancer compar with pri y colorectal cancer lesion and no testinal mucosa[18].

APN), first discovered by Guroff[19], e a class of calcium-dependent cysteine pro- s in cells. Members of this family include m han 10 subtypes, which can be divided into typical and atypical types according to their structure. It is currently considered to be a family of Ca^{2+}-dependent hydrolyzed proteases that are widely distributed in most cells of microorganisms and humans[20]. Typical calpains include calpain1 and calpain2, both of which were the first calpains to be discovered and named for calcium ion concentration required for their enzymatic activity. Reports[21-23] have illustrated that calpains can affect the function of substrate proteins through proteolysis, and participate in the regulation of cell proliferation, apoptosis, differentiation, migration, and erosion. CAPN 7 is an atypical member of the calpains family and lacks the EF-chiral calcium-binding domain[23], rendering its proteolytic activity independent of calcium. Studies[24-27] have demonstrated that changes in the activity or abnormal expression of calpains family members are involved in the progression of many diseases. However, studies on CAPN 7 are rarely reported.

In this investigation, LINC00657 was found remarkably underexpressed in colon cancer tissues, and the survival rate of patients with low expression of LINC00657 was strikingly lower than those with highly expressed LINC00657. In an *in vitro* cell model, cell proliferation rate was found reduced after overexpression of

LINC00657, while the level of apoptosis was enhanced. Further researches on the mechanisms indicated that LINC00657 could promote the expression of CAPN7 and thus inhibit cell invasion. Additionally, the highly expressed LINC00657 was able to inhibit the PI3K/AKT pathway remarkably.

Conclusions

We showed that LINC00657 had a low expression in patients with colon cancer, and the underexpressed LINC00657 can enhance cell proliferative and invasive ability, the mechanism of which may be related to the inhibition of CAPN7 and activation of PI3K/AKT pathway.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of in[illegible]s.

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