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 metastasis. The expression of LINC00657 and E-cad in colon cancer cell lines was also examined, and the effect of LINC00657 on tumor cell proliferation was evaluated by cell counting kit-8 (CCK-8) assay and colony formation experiments. Meanwhile, transwell assay was performed to evaluate the influence of LINC00657 and CAPN7 on cell invasive ability. In addition, the effect of LINC00657 on CAPN7 and PI3K/AKT pathway was detected by Western blot assay.

RESULTS: The expression levels of LINC00657 and E-cad in tumor tissues decreased remarkably, especially in patients who occurred distant metastasis. Compared with patients with highly-expressed LINC00657, the patients with lower level of LINC00657 had a worse prognosis and an advanced tumor size and TNM stage. Similarly, LINC00657 and E-cad also showed a decrease in colon cancer cell lines. After overexpression of LINC00657, cell viability and invasive ability decreased remarkably while cell apoptosis rate increased significantly. In addition, high expression of LINC00657 in an in vitro model significantly promoted CAPN7 expression and inhibited activation of PI3K/AKT pathway.

CONCLUSIONS: LINC00657 had a low expression in colon cancer tissues, which could accelerate cell proliferation and invasion by activating PI3K/AKT pathway and inhibiting CAPN7 expression.

Key Words: LINC00657, Cell proliferation, Colon cancer, PI3K/AKT pathway.
protein activity or protein localization, mRNA degradation, and so on. 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. 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.

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. In this study, we first explored the role of LINC00657 in colon cancer and elucidated its mechanisms regulating cell proliferation and migration.

Patients and Methods

Sample Collection

A total of 80 specimens were obtained from patients with colon cancer in Shaanxi Provincial People’s Hospital from November 2015 to September 2018. Each specimen was taken from tumor tissues and the corresponding normal tissues located in the upper or lower tumor margin. All collected specimens were confirmed by pathological diagnosis. Fresh specimens were quickly placed in liquid nitrogen after being isolated. All the patients had never received any chemotherapy, radiotherapy or other treatments before the surgery. Also, the patient’s clinical data were collected such as age, gender, depth of tumor invasion, and with or without regional lymph node metastasis. This study has been approved by the Ethics Committee of the Shaanxi Provincial People’s Hospital. The written informed consent for 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 (FBS) and 1% penicillin-streptomycin (HyClone, South Logan, UT, USA).

Cell Transfection

Transient transfection was performed using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA). Cells were seeded one day before transfection, and the number was adjusted to 1.0-2.0 × 10^6/well. After cells were adherently grown to 70% of confluency, 900 μL of serum-free medium was added in each well. 1.5 μL/well of Lip3000 and LINC00657/CAPN7 overexpression plasmid or 2.5 μg/well of empty control plasmid were respectively diluted in 50 μL/well of serum-free OPTI-MEM medium and gently mixed and incubated for 5 min at room temperature. Afterward, the mixture was added to the cells for 4-6 hours of incubation; then, the serum-free medium was replaced with complete medium. After transfection for 36-48 hours, the cells were collected for the subsequent experiment.

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% CO₂ for 10-14 days until macroscopic clones appeared. 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 5×10⁶/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
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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 Quantitative Polymerase Chain Reaction (qPCR) Assay**

The total RNA of the tissue samples or cells was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and dissolved in RNase-free water, which was then reverse transcribed to obtain complementary Deoxyribose Nucleic Acid (cDNA). Real-Time quantitative PCR amplification was performed according to the SYBR® Premix Ex Taq TMII (Perfect Real Time) kit instructions (TakaRa, Otsu, Shiga, Japan), and the previously obtained cDNA was quantitatively detected. The PCR amplification conditions were: pre-denaturation at 94°C for 5 min, followed by 40 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s. The primers used were as follows: LINC00657 F: GATGATGAGTGCTAGATGC R: ACAGCAGTGTCTTGTTGT; E-cad F: TTACACCTTTGCATACAGACC R: TTTACGATTACACCCAGACTGC; ZEB1 F: CAGCTTGATACCTGTGAATGGG R: TATCTGTGGTCGTGTGGGACT; N-cad F: TGGACTTAGAGCGTGCTCATT R: CCTGTCAGGCACTAAGCT; ZO-1 F: TGGCTCGACGGCACTAGA R: AGGTGGCTTTGGCTAACACT; CAPN7 F: ATGGGGCAAGCTACCATTTATCA R: TCATTTGAGGATTGTGGTGAGG; Bax F: CCCGAGAGGGTCTTTTTC- CGAG R: CCAGCCCATGATGTTTCTGAT; Bel-2 F: GAAGCGTCCCACGGAACACTG R: GTGCAGAGGGTGTCCGTGTT.

**Plasmid Construction**

The construction of LINC00657/CAPN7 overexpression plasmid was completed by Shanghai Heyuan Biotechnology Co., Ltd (Shanghai, China), using adenoviral vector-pAdeno-mCMV-E-GFP-3FLAG. Overexpression lentivirus and control lentivirus (pLenti-EFla-EGFP-F2A-Puro-CMV-MCS) were purchased from the same company.

**Western Blotting Assay**

The protein samples were taken out and placed on ice. After thawed, they were boiled for 5 min, immediately placed on ice, and then centrifuged at 15 000 r/min at 4°C for 3 min. Afterward, 30 μg of protein sample was subjected to 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis), and transferred to a PVDF (polyvinylidene difluoride) membrane (Millipore, Billerica, MA, USA) by a conventional method, which was incubated with different primary antibodies at 4°C overnight. In the next day, the second antibody was used to incubate the membrane, 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*10^3/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 of LINC00657 was also underexpressed in several colon cancer cell lines, with SW620 expression being the lowest, so it was selected as a subsequent cell model (Figure 2A). Meanwhile, E-cad was also found decreased in tumor cell lines (Figure 2B). By plasmid transfection, we overexpressed LINC00657 in vitro (Figure 2C) and found that highly expressed LINC00657 significantly up-regulated the RNA and protein level of E-cad (Figure 2D, 2E). Expression of other EMT-related genes showed that ZEB1 and N-cad decreased when LINC00657 was highly expressed, while ZO-1 was significantly enhanced (Figure 2F). Transwell experiments revealed that overexpression of LINC00657 could markedly weaken cell invasive ability (Figure 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.
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Table 1. Relation of LncRNA LINC00657 expression and clinicopathologic features in patients with colon cancer.

<table>
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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 CAPN7 (Figure 4C). PI3K/AKT pathway might play a vital role in cell proliferation, and the Western blotting results showed that overexpression of LINC00657 could remarkably deactivate the PI3K/AKT pathway (Figure 4D).

**Discussion**

The concealed symptoms of colon cancer in the early stage create an undesirable situation that most patients have been in the middle or advanced stage when diagnosed. Comprehensive use of surgery, chemotherapy, radiotherapy still cannot enhance the five-year survival rate of colorectal carcinoma due to high recurrence, and distant metastasis; so in-depth studying the pathogenesis of colon cancer, looking for markers of early diagnosis as well as prognosis evaluation and new targets for treatment are particularly crucial\(^{13,14}\).

At present, researches on colorectal cancer-related IncRNAs have found that the change of IncRNA expression is correlated with the occurrence of colorectal cancer, and some IncRNAs are involved in the progression and development of tumors. For example, MEG3 can regulate tumor cell proliferation and be associated with TNM staging of patients with colorectal cancer. The lower the degree of differentiation, the deeper the depth of invasion and the lower expression of MEG3 in colorectal cancer tissues; therefore, MEG3 can be seen as a tumor suppressor gene involved in the occurrence and development of tumors. Studies have proved that MEG3 can enhance p53 protein level through inhibiting expression of MDM2, which promotes the binding of p53 and GDF15 and induces GDF15 expression, thereby redu-
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LncRNA-LET has a low expression in colorectal cancer tissues, and hypoxia-induced histone deacetylase can inhibit lncRNA-LET expression by inhibiting the regulation process of histone acetylation mediated by lncRNA-LET promoter region. LncRNA is also associated with invasion and metastasis of colorectal cancer. The expression of lncRNA in colorectal adenocarcinoma tissues and metastatic lymph nodes was analyzed by gene expression profiling, and it was found that the expression of ENST00043047 was remarkably up-regulated in metastatic lesions. HULC has been known to have a high expression in liver cancer tissue; however, it was also found to express highly in liver-metastatic lesions of colorectal cancer compared with primary colorectal cancer lesion and normal intestinal mucosa.

Calpains (CAPN), first discovered by Guroff, are a class of calcium-dependent cysteine proteases in cells. Members of this family include more than 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. 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 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, rendering its proteolytic activity independent of calcium. Studies 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...
LINCC00657, while the level of apoptosis was enhanced. Further researches on the mechanisms indicated that LINCC00657 could promote the expression of CAPN7 and thus inhibit cell invasion. Additionally, the highly expressed LINCC00657 was able to inhibit the PI3K/AKT pathway remarkably.

Conclusions

We showed that LINCC00657 had a low expression in patients with colon cancer, and the underexpressed LINCC00657 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.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

References

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