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Silencing of linc00337 inhibits proliferation, cell cycle progression, migration, and invasion of colorectal cancer cells through the MEK/ERK pathway

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Abstract. – OBJECTIVE: To explore the expression and biological functions of linc00337 in colorectal cancer (CRC), as well as its underlying mechanism.

PATIENTS AND METHODS: The relative expression of linc00337 in 47 cases of CRC tissues and cells was detected via quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). The si-linc00337 interference sequences were designed and transiently transfected into CRC cells. The interference efficiency was detected via qRT-PCR. Regulatory effect of linc00337 on proliferation of CRC cells was detected via colony formation assay. Cell cycle distribution and apoptosis rate after interference in linc00337 expression were determined using flow cytometry. Moreover, the effects of linc00337 knockdown on cell migration and invasion were detected using transwell assay. At last, the effect of si-linc00337 on the MEK/ERK signaling pathway was detected using Western blotting.

RESULTS: The results of qRT-PCR showed that among the 47 cases of CRC tissues, the expression of linc00337 was up-regulated in 40 cases. Similarly, it was highly expressed in CRC cell lines. The results of colony formation assay manifested that cell proliferation declined after interference in linc00337 expression. The results of flow cytometry and transwell assay showed that interference in linc00337 expression arrested the cell cycle in G1/G0 phase, increased the apoptosis rate, and inhibited the invasion and migration of CRC cells. According to the results of Western blotting, expressions of molecular markers in the MEK/ERK pathway after interference in linc00337 expression were significantly changed.

CONCLUSIONS: Linc00337 is up-regulated in CRC tissues and cells. Interference in linc00337 expression can inhibit cell proliferation, migration, and invasion and promote apoptosis through the MEK/ERK pathway.

Key Words:
Colorectal cancer, Linc00337, Biological function, MEK/ERK pathway.

Introduction

Colorectal cancer (CRC) is one of the common tumors in the world. In China, CRC is a common lethal tumor, and its morbidity rate shows a constantly increasing trend[1]. Malignant proliferation and metastasis of tumors are the most important cause of death in CRC patients[2]. In the past few decades, researchers have been committed exploring the molecular mechanism of the malignant phenotype of CRC, but its molecular mechanism remains unclear.

Long non-coding ribonucleic acids (lncRNAs) widely exist in the nucleus and cytoplasm. They are a kind of RNAs with more than 200 nucleotides in length, which cannot encode proteins but can regulate gene expressions[3-5]. LncRNAs play an important role in transcriptional regulation, post-transcriptional regulation, and regulation, such as chromosome modification, X chromosome silencing, DNA methylation, interference in intranuclear transport, transcriptional activation or silencing[6-8]. During the occurrence and devel-

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opment of malignant tumors, many abnormally expressed lncRNAs can be identified. Svoboda et al[9] reported that lncRNA HOTAIR is differentially expressed in CRC tissues, which is closely related to the tumor stage, lymph node metastasis, and prognosis. Alaiyan et al[10] found that the expression of lncRNA CCAT1 is gradually up-regulated during the malignant progression of CRC, and its expression changes are also related to tumor metastasis. Therefore, it is speculated that the abnormally expressed lncRNAs may be related to the occurrence and development of malignant tumors.

It is reported that linc00337 is up-regulated in gastric cancer, and it inhibits p21 expression by recruiting EZH2, thereby promoting proliferation of gastric cancer[11]. However, the expression and function of linc00337 in CRC have not been reported yet. In the present study, therefore, the relative expression of linc00337 in CRC was detected. It was found that the expression of linc00337 was up-regulated. According to further *in vitro* experiments, interference in linc00337 could inhibit proliferation and metastasis, and promote apoptosis of CRC cells. This investigation provides molecular targets and research directions for the clinical treatment of CRC patients.

Patients and Methods

Cell Culture and Transfection

Human CRC HCT116, LOVO, HT-29, RKO, and SW480 cell lines and human normal colorectal epithelial cell lines NCM460 were purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). All cells were cultured in the Roswell Park Memorial Institute-1640 (RPMI-1640) medium or Dulbecco's Modified Eagle's Medium high (DMEM-H; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/μL penicillin, and 100 μg/mL streptomycin according to the instructions in an incubator with 5% CO_2 at 37°C under saturated humidity. Transfection was conducted using Lipofectamine 2000. si-NC: CCUCAACUCCUGCUUCCAA, si-linc00337: CCAAUUGCAUAAUAUGGUU.

Tissue Specimens

The clinical specimens of 47 CRC patients treated in Jiangdu People's Hospital Affiliated to Medical College of Yangzhou University from January 2016 to December 2017 were collected. The tumor and para-carcinoma tissue specimens were cryopreserved in liquid nitrogen. None of the patients underwent preoperative anti-tumor therapy, such as radiotherapy or chemotherapy, and they were finally diagnosed *via* routine pathological examination. Specimens were obtained upon the consent of patients. This research was approved by the Ethics Committee of Jiangdu People's Hospital Affiliated to the Medical College of Yangzhou University.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

The total RNA was extracted from cells and tissues using TRIzol (Invitrogen, Carlsbad, CA, USA), and reversely transcribed into cDNA using PrimeScript™ reverse transcription kit (TaKaRa, Dalian, China). Subsequently, qRT-PCR was conducted according to the instructions of SYBR® Premix Ex Taq™ kit (TaKaRa, Dalian, China). QRT-PCR and data acquisition were performed using the ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA), with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference. Primers and interference sequences were as follows: Linc00337 Forward: GTTTGACTTGTTCAGGCACA, Reverse: GTCCTCCAAAGACGAGAACAG. GAPDH Forward: ACGGATTTGGTCGTATTGGGC, Reverse: TTGACGGTGCCATGGAATTTG.

Colony Formation Assay

The cells in experimental group and control group were inoculated into a 6-well plate (1000 cells/well) and cultured for about 15 d until visible colonies formed. After the cells were washed with phosphate-buffered saline (PBS) several times, they were fixed with 40 g/L paraformaldehyde for 30 min, stained with 10 g/L crystal violet dye for 10 min, dried and photographed. Three replicates were set in each group.

Cell Cycle Progression

The cells in experimental group and control group were collected, fixed with 700 mL/L cold ethanol at 4°C overnight, and stained with propidium iodide (PI; 0.05 mg/mL) and RNase (2 mg/mL) at room temperature for 30 min, followed by detection of cell cycle *via* flow cytometry. The percentage of cell distribution in G0/G1, S, and G2/M phase was evaluated using the Cell Lab Quanta SC software.

Apoptosis

After transfection, the cells in experimental group and control group were cultured for 48 h, and 1×10^5 cells were taken and centrifuged at 1000 rpm for 5 min. The supernatant was discarded, and the cells were resuspended with 500 μL of binding buffer. Then, 5 μL of Annexin V-FITC (fluorescein isothiocyanate) was added and mixed evenly, and 5 μL of propidium iodide was also added and mixed evenly, followed by incubation at room temperature for 15 min in a dark place. Finally, the apoptosis was detected using the flow cytometer (BD FACSCalibur, Detroit, MI, USA), and the apoptosis rate was calculated.

Transwell Assay

The cells were routinely cultured until the logarithmic growth phase and digested with trypsin. The cell suspension was rinsed, and added into the upper transwell chamber (5×10^4/well) with 200 μL of serum-free basal medium. 700 μL of medium with 10% FBS was added into the bottom chamber as chemical inducer. After routine culture in the incubator for 48 h, the cells on the upper surface of the filter membrane were wiped off, fixed with 4% formaldehyde solution, and stained with crystal violet dye.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (SPSS Inc., IBM, Armonk, NY, USA) was used for statistical analysis. Measurement data were expressed as ($\bar{x} \pm s$), and enumeration data were expressed as percentage (%). Measurement data were compared using the Student's *t*-test. $p<0.05$ suggested the statistically significant difference.

Results

Linc00337 Expression Was Up-Regulated

First, qRT-PCR was performed to detect the relative expression of linc00337 in CRC tissues. The results showed that linc00337 expression was up-regulated in 40 cases compared with para-carcinoma tissues (Figure 1A and 1B). Then, the relative expression of linc00337 in CRC cells

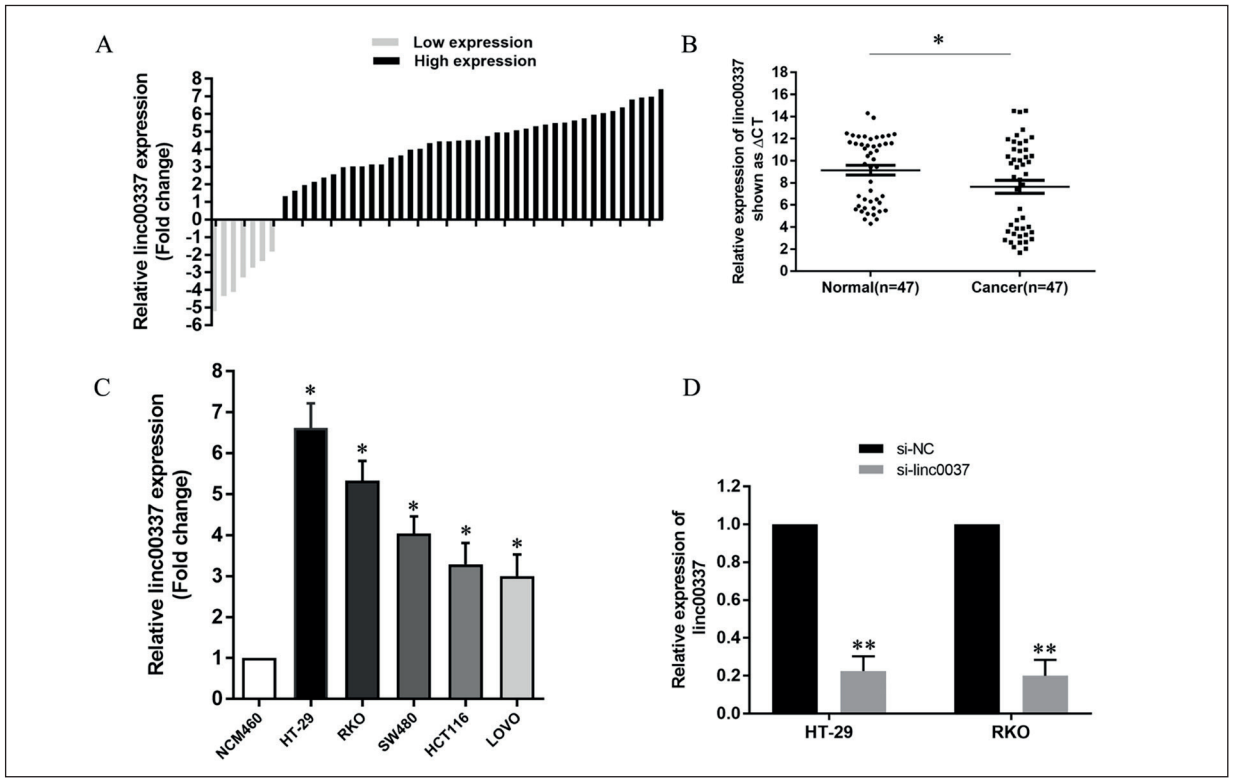

Figure 1. Linc00337 is up-regulated in CRC tissues and cells. **A**, Relative expression of linc00337 in 47 cases of CRC tissues and para-carcinoma tissues is detected via qRT-PCR. The results show that the linc00337 expression is up-regulated in 40 cases of CRC tissues compared with para-carcinoma tissues, with GAPDH as an internal reference. **B**, Linc00337 is up-regulated in CRC tissues. **C**, Relative expression of linc00337 in CRC cells is detected *via* qRT-PCR, and it is also up-regulated. **D**, Interference efficiency of si-linc00337 detected *via* qRT-PCR.

was detected using qRT-PCR. The results showed that linc00337 expression was also up-regulated (Figure 1C). To study the biological functions of linc00337, specific interference sequences of si-linc00337 were designed and transiently transfected into cells. After 48 h, the transfection efficiency was determined (Figure 1D).

Effects of linc00337 on CRC Cell Proliferation and Cycle

The effect of linc00337 on the proliferation of CRC cells was determined using colony formation assay. It was found that cell proliferation declined after interference in linc00337 expression (Figure 2A and 2B). Then, the effect of linc00337 expression on cell cycle distribution was studied *via* flow cytometry, and it was found that the cell cycle progression was arrested in G1/G0 phase after knockdown of linc00337 (Figure 2C and 2D).

Linc00337 Regulated MEK/ERK Pathway

The results of flow cytometry showed that the apoptosis rate increased after interference in linc00337 expression (Figure 3A and 3B). To explore the effect of linc00337 on CRC cell migration, the changes in cell migration were detected using transwell assay. It was found that after interference in linc00337 expression, the cell migration declined (Figure 3C and 3D). Then, the potential molecular mechanism of biological functions of linc00337 was explored.

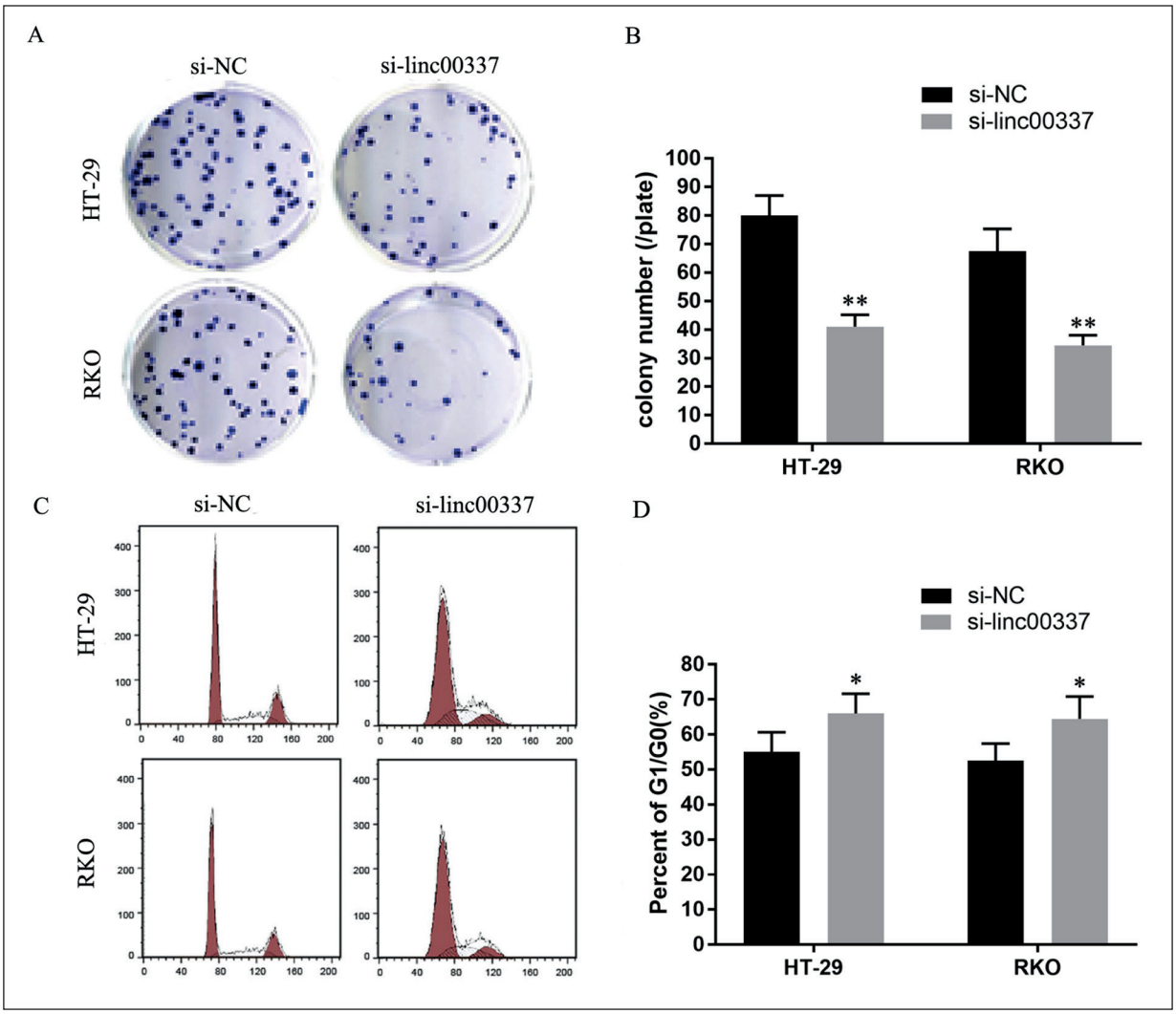

Figure 2. Effects of linc00337 on CRC cell proliferation and cell cycle. **A, B,** Results of colony formation assay reveal that cell proliferation declines after interference in linc00337 expression (40×). **C, D,** Results of flow cytometry reveal that the cell cycle is arrested in G1/G0 phase after interference in linc00337 expression.

The results of Western blotting manifested that there were changes in the expressions of molecular markers in the MEK/ERK pathway after interference in linc00337 expression (Figure 3E and 3F).

Discussion

Great progress has been made in the research on the incidence and development of CRC in the past few decades. Conventional treatments

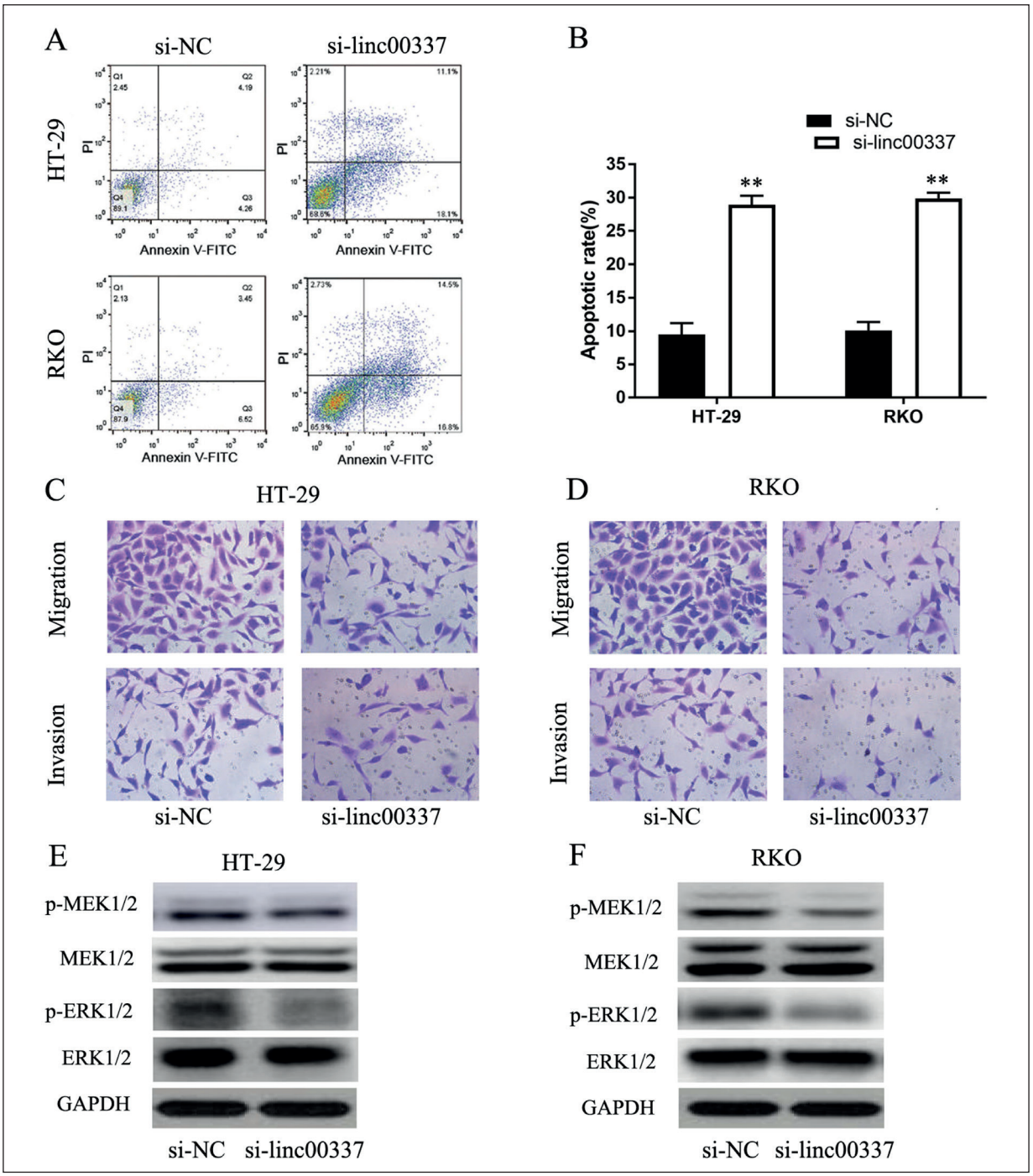

Figure 3. Linc00337 regulates the MEK/ERK signaling pathway. **A, B,** Results of flow cytometry manifest that interference in linc00337 expression increases the apoptosis rate. **C, D,** Results of Transwell assay manifest that both cell migration and invasion decline in si-linc00337 group compared with control group (40×). **E, F,** Results of Western blotting manifest that there are changes in the expressions of molecular markers in the MEK/ERK pathway after interference in linc00337 expression.

for CRC include surgery, chemotherapy, and molecular targeted therapy. However, effective and sensitive screening methods for CRC are lacking and difficult to be applied. Therefore, many CRC patients are diagnosed as CRC accompanied lymph node metastasis or distant metastasis, seriously affecting the prognosis[12,13]. In addition, CRC is characterized by a high degree of heterogeneity. Therapeutic regimens are different for patients in different stages of tumor progression, and the prevalence of drug resistance in molecular targeted therapy is high[14,15]. Therefore, it is of great significance for improving the prognosis of patients to further clarify the molecular mechanism of the incidence and development of CRC, and search for early diagnostic indexes and therapeutic targets. Individualized treatment is encouraged in the future according to different gene expression profiles and pathological features of patients.

LncRNAs are discovered non-coding RNAs in recent years, attracting extensive attention due to their diverse functions. LncRNAs can affect the biological behaviors of tumors by regulating genes and miRNA expressions, and also participate in chromosomal remodeling, transcriptional regulation, and RNA degradation[16]. In addition, due to tissue specificity, lncRNAs are significantly more sensitive in disease diagnosis than DNAs, protein-encoding RNAs, and protein markers[17]. The roles of lncRNA H19, MALAT1, and CCAT1 have been widely recognized in the incidence and development of CRC[18]. Therefore, further exploring the function of lncRNAs in CRC not only has important significance for revealing the mechanism of CRC occurrence and development, but also may provide evidence for searching for new diagnostic indexes and therapeutic targets for CRC.

There have been no reports about the expression and function of linc00337 in CRC. In this study, it was found for the first time that linc00337 was up-regulated in CRC tissues and cells, serving a potential oncogenic role. The abnormal activation of the MEK/ERK pathway can lead to inhibited cell apoptosis and differentiation, malignant transformation, abnormal proliferation and tumorigenesis, and further facilitate the tumor cell proliferation[19,20]. Moreover, lncRNAs, as important regulators, promote the incidence and development of tumors through the MEK/ERK signaling pathway[21]. In this study, it was shown through *in vitro* experiments that there were changes in the expressions of molecular markers in the MEK/ERK pathway after interference in linc00337 expression.

Conclusions

To sum up, linc00337 is upregulated in CRC, which promotes proliferation, invasion, and metastasis and inhibits apoptosis of CRC cells by regulating the MEK/ERK pathway. Our findings provide a theoretical basis for reversing the malignant phenotype of CRC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ, He J. Cancer statistics in China, 2015. CA Cancer J Clin 2016; 66: 115-132.
2) Fisichella R, Cappellani A, Berretta S. MiR-431 inhibits colorectal cancer cell invasion via repressing CUL4B. Eur Rev Med Pharmacol Sci 2018; 22: 5051-5052.
3) Mastrokostas A, Gavana M, Gkrizioti M, Smyrnakis E, Cholongitas E, Benos A, Haidich AB. Discrepancies and misconceptions of perceived colorectal cancer screening barriers between primary health professionals and unscreened population. A comparative study in Greece. J BUON 2018; 23: 67-76.
4) Flippot R, Beinse G, Boileve A, Vibert J, Malouf GG. Long non-coding RNAs in genitourinary malignancies: a whole new world. Nat Rev Urol 2019; 16: 484-504.
5) Tam C, Wong JH, Tsui S, Zuo T, Chan TF, Ng TB. LncRNAs with miRNAs in regulation of gastric, liver, and colorectal cancers: updates in recent years. Appl Microbiol Biotechnol 2019; 103: 4649-4677.
6) He RZ, Luo DX, Mo YY. Emerging roles of lncRNAs in the post-transcriptional regulation in cancer. Genes Dis 2019; 6: 6-15.
7) Tang X, Qiao X, Chen C, Liu Y, Zhu J, Liu J. Regulation mechanism of long noncoding RNAs in colon cancer development and progression. Yonsei Med J 2019; 60: 319-325.
8) Mishra S, Verma SS, Rai V, Awasthee N, Chava S, Hui KM, Kumar AP, Challagundla KB, Sethi G, Gupta SC. Long non-coding RNAs are emerging targets of phytochemicals for cancer and other chronic diseases. Cell Mol Life Sci 2019; 76: 1947-1966.
9) Svoboda M, Slyskova J, Schneiderova M, Makovicky P, Bielik L, Levy M, Lipska L, Hemmelova B, Kala Z, Protivankova M, Vycital O, Liska V, Schwarzova L, Vod-

ickova L, Vodicka P. HOTAIR long non-coding RNA is a negative prognostic factor not only in primary tumors, but also in the blood of colorectal cancer patients. Carcinogenesis 2014; 35: 1510-1515.

10) Alaiyan B, Ilyayev N, Stojadinovic A, Izadjoo M, Roistacher M, Pavlov V, Tzivin V, Halle D, Pan H, Trink B, Gure AO, Nissan A. Differential expression of colon cancer associated transcript1 (CCAT1) along the colonic adenoma-carcinoma sequence. BMC Cancer 2013; 13: 196.

11) Hu B, Wang X, Li L. Long noncoding RNA LINC00337 promote gastric cancer proliferation through repressing p21 mediated by EZH2. Am J Transl Res 2019; 11: 3238-3245.

12) Kuwai T, Yamada T, Toyokawa T, Iwase H, Kudo T, Esaka N, Ohta H, Yamashita H, Hosoda Y, Watanabe N, Harada N. Local recurrence of diminutive colorectal polyps after cold forceps polypectomy with jumbo forceps followed by magnified narrow-band imaging: a multicenter prospective study. Endoscopy 2019; 51: 253-260.

13) Chang J, Lin G, Ye M, Tong D, Zhao J, Zhu D, Yu Q, Zhang W, Li W. Decreased mean platelet volume predicts poor prognosis in metastatic colorectal cancer patients treated with first-line chemotherapy: results from mCRC biomarker study. BMC Cancer 2019; 19: 15.

14) Hu H, Huang J, Lan P, Wang L, Huang M, Wang J, Deng Y. CEA clearance pattern as a predictor of tumor response to neoadjuvant treatment in rectal cancer: a post-hoc analysis of FOWARC trial. BMC Cancer 2018; 18: 1145.

15) Ramphal W, Boeding J, Gobardhan PD, Rutten H, de Winter L, Crolla R, Schreinemakers J. Oncologic outcome and recurrence rate following anastomotic leakage after curative resection for colorectal cancer. Surg Oncol 2018; 27: 730-736.

16) Horita K, Kurosaki H, Nakatake M, Ito M, Kono H, Nakamura T. Long noncoding RNA UCA1 enhances sensitivity to oncolytic vaccinia virus by sponging miR-18a/miR-182 and modulating the Cdc42/filopodia axis in colorectal cancer. Biochem Biophys Res Commun 2019; 516: 831-838.

17) Slaby O. Non-coding RNAs as biomarkers for colorectal cancer screening and early detection. Adv Exp Med Biol 2016; 937: 153-170.

18) Tam C, Wong JH, Tsui S, Zuo T, Chan TF, Ng TB. LncRNAs with miRNAs in regulation of gastric, liver, and colorectal cancers: updates in recent years. Appl Microbiol Biotechnol 2019; 103: 4649-4677.

19) Li J, Ye T, Liu Y, Kong L, Sun Z, Liu D, Wang J, Xing HR. Transcriptional activation of Gstp1 by MEK/ERK signaling confers chemo-resistance to cisplatin in lung cancer stem cells. Front Oncol 2019; 9: 476.

20) Bejarano L, Bosso G, Louzame J, Serrano R, Gomez-Casero E, Martinez-Torrecuadrada J, Martinez S, Blanco-Aparicio C, Pastor J, Blasco MA. Multiple cancer pathways regulate telomere protection. EMBO Mol Med 2019; 11: e10292.

21) Sun J, Guo Y, Bie B, Zhu M, Tian H, Tian J, Li J, Yang Y, Ji F, Kong G, Li Z. Silencing of long noncoding RNA HOXD-AS1 inhibits proliferation, cell cycle progression, migration and invasion of hepatocellular carcinoma cells through MEK/ERK pathway. J Cell Biochem 2019 Jun 23. doi: 10.1002/jcb.29206. [Epub ahead of print].