LINC00538 promotes the progression of colon cancer through inhibiting NKD2 expression

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Summary

Purpose: The purpose of this study was to explore the possible role and mechanism of LINC00538 in the pathogenesis of colon cancer.

Methods: The expression levels of LINC00538 in 70 pairs of colon cancer tissue samples and adjacent ones were examined by qRT-PCR, and survival analysis of patients was performed according to the result. Meanwhile, colon cancer cell lines were screened. In addition, LINC00538 siRNA was transfected into colon cancer cells using liposome method, and then cell proliferation and cell cycle were examined by CCK8 and EDU assays, while cell apoptosis was detected by flow cytometry. Finally, the mechanism of LINC00538 in colon cancer was further explored by RNA-binding protein immunoprecipitation and chromatin immunoprecipitation.

Results: The expression of LINC00538 in colon cancer tissues was remarkably higher than that in normal ones, and the overall survival of patients with colon cancer was negatively correlated with the expression of LINC00538. After transfection of LINC00538 siRNA, the proliferation rate of colon cancer cell lines including HCT116 and RKO cells was weakened, the S phase of the cell cycle was shortened, while the cell apoptosis was elevated. In addition, further mechanism studies demonstrated that LINC00538 can bind to EZH2 and inhibit the expression of NKD2, thereby regulating the proliferation and apoptosis of colon cancer cells.

Conclusions: This study demonstrated for the first time that LINC00538 was highly expressed in colon cancer and was associated with poor prognosis of patients. Knockdown of LINC00538 in colon cancer cell lines was able to inhibit the cell proliferation and cell cycle, while it promoted the apoptosis. Its mechanism of participating in the development of colon cancer may be through the down-regulation of NKD2 and the regulation of EZH2.

Key words: colon cancer, LINC00538, EZH2, NKD2

Introduction

Currently, colorectal cancer ranks third in the incidence rate of malignant tumors worldwide, and second in mortality, which is considered as a major threat to public health [1,2]. Colorectal cancer is a multi-step biological process involving the dysregulation of multiple oncogenes and tumor suppressor genes [3]. The overall survival rate of patients with colorectal cancer is still relatively low, although we have gained a relatively deep understanding of this malignancy the diagnostic technology is progressing and the treatment means are becoming more and more effective [4]. With its increasing incidence, there is an urgent need for a deeper understanding of the specific molecular mechanism in the pathogenesis of colorectal cancer, so as to promote the discovery of more effective treatment strategies and ultimately improve substantially the prognosis of patients [5].

So far, with the improvement of genome-wide and transcriptome sequencing technology,
it has been found that the whole genome of most mammals can be transcribed, but most of the transcripts are limited or do not have the ability to encode proteins at all, and these genes are called long-non coding (nc)RNA [6]. A large number of studies have shown that IncRNA plays an irreplaceable role in various biological processes such as embryonic development, epigenetics, transcription and translation, various biological behaviors of cells, and tumorigenesis [7]. In addition, extensive studies have provided strong evidence that IncRNA plays a pivotal role in a series of human diseases, including various types of cancer, such as breast cancer [8], pancreatic cancer, lung cancer [9], gastric cancer [10] and so on. Recent studies have shown that IncRNAs also play a modulator role in the occurrence and development of colorectal cancer, indicating their potential as new therapeutic targets. However, studies on the molecular mechanism and biological functions of IncRNA in the formation and progression of colorectal cancer are still in an initial stage. So far, researchers have only made some limited achievements in elucidating specific molecular mechanisms, but these limited achievements are enough to indicate that IncRNA is a potential biological target for the diagnosis and treatment of this disease [11,12].

In this study, we reported for the first time that a new IncRNA, LINC00538, was remarkably up-regulated in colon cancer tissues and cell lines. In addition, after downregulation of LINC00538, we detected the proliferation and migration ability as well as cell cycle and apoptosis of colon cancer cells to further explore the mechanism of LINC00538’s involvement in colon cancer, so as to provide new insights and new targets for the pathogenesis and treatment of this malignancy.

Methods

Data acquisition and patient data

All the selected specimens were colon cancer tissues collected from colon cancer patients from January 2017 to January 2019 in the hospital. No patients were treated with adjuvant therapy such as radiotherapy and chemotherapy. The postoperative specimens were confirmed by pathology as colon cancer. The specimens from the control group were from adjacent tissues of the same patient, and no cancer cells were found after pathological examination. Tumor staging was performed according to the current TNM staging system. All specimens were frozen in liquid nitrogen and stored in a -80°C refrigerator. The study was approved by the ethics committee of Youjiang Medical University and all patients signed informed consent. This study was conducted in accordance with the Declaration of Helsinki.

Cell culture and transfection

Normal colon cells (NCM460) and colon cancer cell lines HCT116, SW480, SW620 and RKO were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA) and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) and high glucose complete medium containing 10% fetal bovine serum (FBS) in a 37°C, 5% CO₂, incubator. Cell transfection was performed according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Detailed cell transfection was performed according to the instructions. siRNA at the concentration of 50nM was added to each well. After 6h of culture, fresh DMEM containing 10% FBS was added. When the cell confluence reached 75-85%, subsequent experiments were carried out. The transfection sequence was as follows: si-LINC00538-1: 5’-CAG CAA AGC ACU AAU GAA A-3’; si-LINC00538-2: 5’-CCA ACA AGC UCU AGA UUA U-3’; si-EZH2: 5’-CAG AAC AAG UAA AGG AAA A-3’; si-NKD2: 5’-GUU GCA GGA UGG AGG GUG A-3’ (Shanghai Jima, China).

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

After homogenization of the tissue or washing the cells three times with PBS, appropriate amount of TRIzol (Invitrogen, Carlsbad, CA, USA) was used to lyse the samples, and total RNA was extracted and then stored at -80°C for later use. After being measured by a microcuclei quantitation meter, the extracted RNA was reverse-transcribed to obtain complementary deoxyribose nucleic acid (cDNA), and the SYBR Green method was used for subsequent RT-qPCR detection. 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 1 min and 30 s. The primer sequences were: LINC00538 (F: 5’-CTGGCTTGAGGGCATCTCTT-3’; R: 5’-TTGGCTTGACGAGC-3’); KL2 (F: 5’-TGTCGCTTCACACGAGC-3’; R: 5’-TGCGGACTCTTGTTG-3’); EZH2 (F: 5’-AATCAGAGTACATGCGACTGAGA-3’; R: 5’-TCGTCGAGGATCTTGGTAACTC-3’); P21 (F: 5’-TGTCCGTCAGAACGCTTCGCTAACACTTG-3’; R: 5’-GGTGGGTTATGGTCTTCAAAAGG-3’); RND1 (F: 5’-GGGAGGACAGTGTTCCCTACAG-3’; R: 5’-CTCCGTACATCTGGCTGAGC-3’); PTEN (F: 5’-TGAGTTGATCTAGCTTTACAGCT-3’; R: 5’-GGTGGTTATGCTTTCTCATTACAGG-3’); RND1 (F: 5’-GATGCGATGCATCTTGTTGCG-3’; R: 5’-CTGGCTTGAGGGCATCTCTT-3’); LATS2 (F: 5’-ACTTTTCCTGCCACGACTTATTC-3’; R: 5’-GATGAGGCGGAGGATCCGAC-3’); KLF2 (F: 5’-TTCGCTCTTTACTGTCGACG-3’; R: 5’-GGTGGGTTATGGTCTTCAAAAGG-3’); NMD2 (F: 5’-GAGGACAGTGTTCCCTACAG-3’; R: 5’-CTCCGTACATCTGGCTGAGC-3’).

Cell Counting Kit (CCK-8) assay for cell viability

Twenty-four h after transfection, cells were digested, collected and plated into 96-well plates at 2×10³ per well, and 6 replicate wells were set in each group. After the cells were attached, the viability of the cells was determined by CCK-8 method (Dojindo, Kumamoto, Japan). Two h before the test, 10 μL of CCK-8 solution was added to each well and incubated with cells at 37°C for 2 h. The absorbance of each well was measured at 450 nm by a microplate reader.
**Edu assay for cell proliferation**

Cells in logarithmic growth phase were seeded in 96-well plates, and each well was incubated with 100 μL of 50 μM EdU medium for 2 h, and then the medium was discarded. The cells were washed with PBS, and 50 μL of cell fixative solution was added to incubate at room temperature. Fifty μL of 2 mg/mL glycine was added and incubated with cells for 5 min on a bleaching shaker. Subsequently, 100 μL of PBS and 100 μL of permeabilization buffer were added; after washed once with PBS, 100 μL of Apollo® staining reaction solution was added and incubated in the dark for 50 min at room temperature. After washed with 100 μL of permeabilization buffer 2~3 times and methanol for 1~2 times, 100 μL of 1× Hoechst 33342 reaction solution was added and incubated with cells in the dark on decolorization shaker for 30 min. After 1× Hoechst 33342 reaction solution was discarded, and fluorescence microscope was used to observe and take photos of the cells.

**Flow cytometry for cell cycle analysis**

Cells in logarithmic growth phase were prepared by trypsinization to prepare a single cell suspension. After centrifuged twice in a centrifuge tube, cells were washed with PBS for 2 times, and the supernatant was discarded; after fixed by pre-cooled 75% ethanol, cells were centrifuged and treated with 0.5 propidium iodide (PI) at 4°C for 20~30 min. Then, flow cytometry was performed as follows: determination of DNA content in each cycle at 488 nm excitation wavelength, repeated experiments 3 times, and the average was taken.

**Flow cytometry for cell apoptosis**

Cells in the logarithmic growth phase were washed twice with PBS and then digested with trypsin without ethylene diamine tetraacetic acid (EDTA) to remove the supernatant. The cells were resuspended with PBS, and the supernatant was removed. Subsequently, 1 μL of propidium iodide (PI) and 5 μL of Annexin V-FITC were added in 100 μL of cell suspension, and incubated at room temperature in the dark for 15 min. Afterwards, the cells were put on flow cytometer after 15 min and tested within 1 h.

**Nuclear separation**

The cell suspension was placed to Eppendorf (EP) tube, partly for lysis of whole cells, and partly for separation of nuclei and cytoplasm. The supernatant was transferred to a RNA free EP tube and RNAiso plus reagent was added for total RNA extraction. The remaining cells were added to buffer A (1.0mM MgCl₂) thoroughly mixed, and placed on ice for 5 min. After centrifugation for 5 min, the supernatant was transferred to a new EP tube. After standing for 5 min, the supernatant was centrifuged, and the pellet was resuspended in buffer S1 (0.25mM sucrose in Buffer A). After mixing well, buffer S2 (1.1mM sucrose in Buffer A) was slowly added from the top of the liquid surface. The supernatant was transferred to an RNA free EP tube, and RNAiso plus reagent was added for total RNA extraction; centrifugation was continued. RIPA buffer was used to lyse cells for 30 min, and the supernatant was the nucleus.

**Figure 1.** LINC00538 is highly expressed in colon cancer tissues. **A:** LINC00538 expression is significantly elevated in tumor tissues compared with adjacent tissues. **B:** LINC00538 is increased in tumor tissues of stage III-IV patients compared with patients with stage I-II colon cancer. **C:** Kaplan–Meier analysis suggests that the higher the expression level of LINC00538, the worse the prognosis of patients. **D:** The expression levels of LINC00538 in colon cancer cell lines HCT116, SW480, SW620 and RKO are higher than that of the normal cell line NCM460. (*p<0.05).
RNA binding protein immunoprecipitation (RIP) test

After the cell lysate was obtained, magnetic beads were prepared and placed on ice. RNA-binding protein immunoprecipitation was then performed. RNA purification was carried out by phenol, chloroform, Salt Solution I, Salt Solution II, precipitate enhancer, absolute ethanol (no RNase), dissolved in 10-20 μL of diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China), and stored at -80°C. Finally, qRT-PCR was used to detect the expression of each gene in EZH2 protein and IgG protein precipitate.

Chromatin immunoprecipitation (CHIP)

The cells were taken for formaldehyde cross-linking and ultrasonication. After the ultrasonication was completed, the insoluble matter was removed by centrifugation. The cells were treated at 65°C for 5 h, and electrophoresis was performed to detect the effect of ultrasonic disruption. In the sonicated product, ChIP Dilution Buffer and Protein A Agarose/Salmon Sperm DNA were added. The supernatant was centrifuged and 20 μL were used as input. One μL of antibody was added to each ET. After overnight incubation at 4°C, 60 μL of Protein A Agarose/Salmon Sperm DNA was added to each tube. After the completion of the cross-linking overnight at 65°C, the DNA fragments were recovered and finally analyzed by PCR.

Statistics

Analyses were performed using SPSS 19.0 (SPSS IBM, Armonk, NY USA) statistical software. The measurement data were expressed as mean ± standard deviation, and the data between groups were compared by t-test. Survival analysis was performed by GraphPad Prism (Version X; La Jolla, CA, USA), and the difference was statistically significant at p<0.05.

Figure 2. Low expression of LINC00538 inhibits cell proliferation. **A**: The expression level of LINC00538 is detected after transfection of si-LINC00538-1, si-LINC00538-2 and the corresponding control. **B**: CCK-8 detects the proliferation of HCT116 cells after expression of LINC00538 was down-regulated. **C**: CCK-8 detects the proliferation of RKO cells after expression of LINC00538 was down-regulated. **D**: EDU assay detects the proliferation of HCT116 and RKO cells after expression of LINC00538 was down-regulated. **E**: Flow cytometry detects HCT116 cell cycle after decreasing downregulation of LINC00538 expression. **F**: Flow cytometry detects RKO cell cycle after decreasing downregulation of LINC00538. **G**: Flow cytometry detects apoptosis of HCT116 and RKO cells after downregulation of LINC00538. (**p<0.05**).
Results

LINC00538 is highly expressed in colon cancer tissues

We detected the expression of LINC00538 in 70 colon cancer tissue samples and adjacent ones by RT-p PCR. The results showed that the expression of LINC00538 in colon cancer tissues was remarkably higher than that in adjacent samples (p<0.05) (Figure 1A). The expression of LINC00538 in the colon cancer patients who were in stage III and IV was remarkably higher than those in stage I and II (Figure 1B). The tissue samples were further divided into high expression group and low expression group according to the median value of LINC00538 expression in colon cancer tissues. Patients with high expression of LINC00538 had a reduced total survival time compared with those with low expression (p=0.0014) (Figure 1C). In addition, qRT-PCR was performed to examine the LINC00538 expression in colon cancer cell lines (HCT116, SW480, SW620 and RKO) and normal colon cells (NCM460), and it was found to be remarkably higher in the former than in the latter (Figure 1D), suggesting that LINC00538 might be involved in the development of colon cancer.

LINC00538 promotes colon cancer cell proliferation

Because of the high expression of LINC00538 in HCT116 and RKO, these two cell lines were selected for subsequent cell experiments. Subsequently, LINC00538 siRNA was transfected into HCT116 and RKO, and then the transfection efficiency was confirmed by qRT-PCR. As shown in Figure 2A, LINC00538 expression was remarkably reduced, with si-LINC00538-1 more effective. In addition, cell proliferation was detected by CCK8 assay at 24, 48, and 72 h, and the results

Figure 3. LINC00538 inhibits NKD2 expression by binding to EZH2. A: Detection of the expression of LINC00538 in cytoplasm and nucleus of HCT116 by qRT-PCR. B: Detection of the expression of LINC00538 in cytoplasm and nucleus of RKO by qRT-PCR. C: Increased expression of NKD2 in HCT116 cells after decreasing the expression level of LINC00538. D: Increased expression of NKD2 in RKO cells after decreasing the expression level of LINC00538. E: NKD2 levels are increased in colon cancer cell lines HCT116, SW480, SW620 and RKO compared to the normal cell line NCM460. F: RIP experiments show that LINC00538 can be combined with EZH2. G: ChIP-qPCR experiments show that EZH2 can bind to the NKD2 promoter region in HCT116 and RKO cells. H: Detection of expression level of LINC00538 in HCT116 and RKO cells after transfection of si-EZH2 and corresponding controls. I: Increased expression of NKD2 in HCT116 and RKO cells after decreasing EZH2 expression levels. J: Decreased binding capacity of EZH2 and NKD2 promoter regions in HCT116 cells after decreasing the expression level of LINC00538. K: Decreased binding capacity of EZH2 and NKD2 promoter regions in RKO cells after decreasing the expression level of LINC00538 (*p<0.05).
showed that the cell viability of colon cancer cells was remarkably decreased after down-regulation of LINC00538 (Figures 2B, 2C); meanwhile, the result of EDU assay was consistent with that of CCK8 (Figure 2D). Subsequently, the function of LINC00538 in colon cancer cell lines was further explored and analyzed. The cell cycle was detected after the cells were treated with LINC00538 small interfering RNA, and the results revealed that the ratio of S phase decreased while that of G0/G1 increased (Figures 2E, 2F). At the same time, flow cytometry was performed to detect cell apoptosis; and it was found that knockdown of LINC00538 resulted in increased apoptosis percentage, indicating that low expression of LINC00538 could inhibit colon cancer cell proliferation and promote cell apoptosis.

**LINC00538 inhibits NKD2 expression by combining EZH2**

To further investigate the molecular mechanism of LINC00538 in colon cancer cells, we first examined the subcellular localization of LINC00538 in HCT116 and RKO cells. The distribution of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U1 indicated that the cytoplasmic nuclei were successfully isolated, and most of LINC00538 was

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**Figure 4.** LINC00538 regulates cell proliferation by NKD2. **A:** Detection of NKD2 expression levels in HCT116 and RKO cells after transfection of si-NKD2 and corresponding controls. **B:** CCK-8 assay detects the proliferation of RKO cells that is reduced after simultaneous downregulation of LINC00538 and NKD2. **C:** CCK-8 assay detects the proliferation of HCT116 cells that is reduced after simultaneous downregulation of LINC00538 and NKD2. **D:** EDU assay detects the proliferation of HCT116 cells that is reduced after simultaneous downregulation of LINC00538 and NKD2 (40×). **E:** EDU assay detects the proliferation of RKO cells that is reduced after simultaneous downregulation of LINC00538 and NKD2 (40×). **F:** Flow cytometry detects the cell cycle distribution of HCT116 cells that is reduced after simultaneous downregulation of LINC00538 and NKD2 expression. **G:** Flow cytometry detects the cell cycle distribution of RKO cells that is reduced after simultaneous downregulation of LINC00538 and NKD2 expression. **H:** Flow cytometry detects cell apoptosis of HCT116 cells that is reduced after simultaneous downregulation of LINC00538 and NKD2 expression. **I:** Flow cytometry detects cell apoptosis of RKO cells that is reduced after simultaneous downregulation of LINC00538 and NKD2 expression. (*p<0.05).
LINC00538 promotes the occurrence of colon cancer

LINC00538 regulates cell proliferation via NKD2

To determine whether NKD2 is involved in the effect of low LINC00538 expression on cell functions, we down-regulated NKD2 via transfection with si-NKD2 (Figure 4A). Subsequently, CCK8 assay was performed to detect cell proliferation, and the results showed that knockdown of NKD2 could obviously enhance cell proliferation ability, while simultaneous down-regulation of LINC00538 and si-NKD2 could partially reduce the enhancement of cell proliferation ability induced by si-NKD2 (Figures 4B,4C). In addition, the result of EDU proliferation assay showed that low expression of LINC00538 could directly bind to EZH2 (Figure 3F). To further determine whether LINC00538 regulates NKD2 expression by interacting with EZH2, we performed chromatin immunoprecipitation analysis, and the results indicated that EZH2 could directly bind to the NKD2 promoter and mediate H3K27me3 methylation modification (Figure 3G). Furthermore, we knocked out EZH2 (Figure 3H) to detect the expression of NKD2, which was found conspicuously increased (Figure 3I). However, low expression of LINC00538 reduced the binding capacity of EZH2 to the NKD2 promoter and inhibited H3K27me3 methylation modification (Figures 3J,3K). The above results demonstrated that LINC00538 might be involved in the development of colon cancer by reducing the expression of NKD2 through acting on EZH2.

Discussion

In recent years, with the rapid development of diagnosis and treatment technology, the mortality rate of patients with colorectal cancer has been remarkably reduced, and their quality of life has been remarkably improved. However, metastasis and recurrence of colorectal cancer are still the key factors leading to high mortality and poor prognosis of colorectal cancer patients, so it is urgent to further study the pathogenesis of this cancer and finally determine more effective methods to treat this fatal disease [15].

In recent years, non-coding RNA has been a hot topic in the research field related to malignancies. Many studies have verified and analyzed the key role of non-coding RNA in the occurrence and development of tumors from different perspectives [14]. LINC00538, as a member of many long non-coding RNAs, is believed to be involved in regulating the cellular behavior of some breast cancers in previous studies [15], which is likely to have a subtle impact on the occurrence and progression of colorectal cancer. In this study, the expression of LINC00538 in colon cancer tissues and cells was found remarkably increased, and knockdown of LINC00538 could inhibit the proliferation of colon cancer cells while promote apoptosis.

Drosophila zeste gene Enhancer of zeste homolog 2 (EZH2) belongs to a group of multi-comb protein complexes, which promotes epigenetic gene silencing [16,17]. Maintaining an appropriate level of EZH2 at the molecular level is important for the normal function of cells, and its abnormal expression will lead to abnormal proliferation of cells and the occurrence of tumors [18]. Currently, EZH2 has been evaluated as a new biomarker for cancer treatment targets. In this study, we confirmed that LINC00538 can directly bind to EZH2, which can further combine with the NKD2 promoter region.

The human NKD (Naked Cuticle) gene family includes two homologues, NKDI and NKD2. N-section of NKD2 contains most of the functional areas, including tyrosine, EF-hand loci, Dishevelled area, processing area, vesicle recognition area, and membrane targeting binding sites. Promoter methylation of NKD2 occurs in glioblastoma cells [19]. Furthermore, overexpression of NKD2 inhibited proliferation, invasion and migration of tumor cells in metastatic human and mouse colon cancer cells; meanwhile, in vivo experi-
ments in colon cancer cell lines have confirmed the same result [20]. In addition, it was found that NKD2 can inhibit the classical Wnt signaling pathway to achieve the purpose of inhibiting the growth and migration of tumor cells [21]. In this study, NKD2 expression was found remarkably increased in colon cancer cells and was regulated by EZH2 and LINC00538, which could regulate cell proliferation and apoptosis through modulating NKD2.

However, our experiments still have some shortcomings since most of our results were obtained by using colon cancer-related cell lines, and the role and significance of LINC00538 in the pathogenesis of colon cancer should be further explored by establishing an in vivo tumor model.

Conclusions

This study demonstrated for the first time that LINC00538 was highly expressed in colon cancer and was associated with poor prognosis of colon cancer patients. In addition, downregulation of LINC00538 was found to be able to inhibit the proliferation and promote apoptosis of colon cancer cells through binding to and modulating EZH2, so as to participate in the development of colon cancer.

Conflict of interests

The authors declare no conflict of interests.

References