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ORIGINAL ARTICLE

LncRNA CCAT2 promotes proliferation and suppresses apoptosis of colorectal cancer cells

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Summary

Purpose: *To investigate the effects of long non-coding ribonucleic acid (lncRNA) colorectal cancer (CRC)-associated transcript 2 (CCAT2) expression on proliferation and apoptosis of colorectal cancer (CRC) cells.*

Methods: *Data of lncRNA expression in CRC were downloaded from the cancer genome atlas (TCGA) database for differential expression and survival analyses, and real-time fluorescence quantitative polymerase chain reaction (qRT-PCR) was employed to analyze the expression level of lncRNA CCAT2 in 80 cases of CRC and adjacent tissues collected as well as normal colorectal cells and CRC cell lines selected. The cells successfully transfected were collected for the detection of the effects on apoptosis and proliferation. Then, immunofluorescence assay was performed to measure the protein expression levels of apoptotic protein markers B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax).*

Results: *It was found through differential expression analysis that the expression of lncRNA CCAT2 showed a significant difference in CRC tissues, and CRC patients with a high expression level of lncRNA CCAT2 had poor prognosis. Based on the results of qRT-PCR assay, lncRNA CCAT2 was significantly highly expressed in CRC tissues. After transfection with mimic and NC, its expression was obviously higher in mimic group than that in NC group, and cell lines with over-expressed lncRNA CCAT2 were successfully constructed. The flow cytometry results showed that the proportion of apoptotic cells was 5% in mimic group and about 13% in NC group. According to the results of immunofluorescence assay, Bax was mainly located in the cytoplasm, and the fluorescence intensity was decreased significantly in mimic group, indicating that Bax expression was inhibited.*

Conclusions: *LncRNA CCAT2 is differentially expressed in CRC, and its expression is significantly upregulated in CRC. LncRNA CCAT2 can promote the growth and proliferation and suppress the apoptosis of CRC cells. The changes in lncRNA CCAT2 expression are associated with poor prognosis.*

Key words: *lncRNA CCAT2, CRC cells, cell proliferation, apoptosis*

Introduction

Colorectal cancer (CRC) is currently the third most common malignant tumor in the world and also the fourth leading cause of cancer-related death, with a yearly increasing incidence rate [1-3]. Significant achievements have been made in the treatment of early CRC, but the long-term survival rate of advanced CRC is still low. In the current medical environment, the effective survival rate of CRC is increasing, which is mainly related to chemotherapy with 5-fluorouracil (5-FU) and oxaliplatin, and chemotherapy before surgery has been used as standard therapy [4,5]. However, recurrence is detected in 20-40% of patients with advanced CRC after radical operation [6,7], which is mainly caused by lack of biomarkers for early clinical diagnosis and postoperative prognosis as well as spe-

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cific molecules for targeted therapy, and decreased treatment effectiveness at present [5]. Therefore, finding out biomarkers to improve the sensitivity of diagnosis of CRC remains the focus of intense research.

According to results of whole genome sequencing, about 2% of genes in mammalian genomes can encode proteins, and most of the remaining genes regulate the proteins encoded by genes and are called as non-coding ribonucleic acids (ncRNAs). Detailed studies on the functional mechanisms of ncRNAs have revealed that long ncRNAs (lncRNAs) containing over 200 nucleotides are regarded as diagnostic biomarkers and prognostic factors [8-10]. Previous studies have identified a range of lncRNAs abnormally expressed in cancers [11,12]. Elevated relative expression of lncRNA HOTAIR in primary tumors and blood of CRC patients is correlated with poor prognosis [13]. LncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) has been proven to be upregulated in clinical CRC tissue samples and highly expressed in CRC, and the prognosis of CRC patients with high expression of MALAT1 is particulary poor, so it is an independent risk factor for CRC [13,14].

As a lncRNA, colon cancer-associated transcript 2 (CCAT2), mainly located in the nucleus, is tissue- and cell type-specific, which is a RNA of 1,752 bases transcribed from the 8q24 region of the human genome and contains a single nucleotide polymorphism (SNP) rs6983267 [15] that is related to an increased risk for CRC and other cancer types, including prostate, ovarian and breast cancer [16-18]. The molecular and cellular mechanisms such a SNP mutation in increasing the risk for cancers are unclear, but it is discovered that there are deoxyribonucleic acid (DNA) enhancer elements in the genomic regions spanning rs6983267, such as transcripts binding to transcription factor 7-like 2 (TCF7L2) [19]. It has been reported that CCAT2 is remarkably overexpressed in many tumors including CRC, breast and lung cancer. Currently, CCAT2 has been reported to be able to reduce the sensitivity to chemical drugs, and promote the growth and metastasis of tumors, which is an attribute related to cancer stem cells (CSCs) [20,21]. This study mainly aimed to explore the expression of CCAT2 in CRC, its effects on the proliferation and apoptosis of CRC cells, and its potential as a prognostic marker of CRC.

Methods

Materials

Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA), a fully automatic microplate reader (Thermo, Waltham, MA, USA), a flow cytometer (Miltenyi, Bergisch Gladbach, Germany), a RNA isolation purification kit (Roche, Basel, Switzerland), MTS reagent (Promega, Madison, WI, USA), B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax) antibodies (CST, Danvers, MA, USA), and a centrifuge (Hitachi, Tokyo, Japan).

Subjects and grouping

CRC and adjacent tissues were collected from patients with surgery alone operated in our hospital from July 2014 to January 2016. Colorectal cancer cell line, FHC cell line, HT29 cell line, Lovo cell line and HCT-116 cell line were used in this study. With CRC as the research object, colon cancer HCT-116 cells were selected as the objects of the study, transfected with lncRNA CCAT2 negative control (NC) and lncRNA CCAT2 mimic, and divided into NC group and mimic group according to different genes transfected. This study was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University. Signed written informed consents were obtained from all participants before the study entry.

Downloading and processing of CRC lncRNA data from public database

To find colorectal-associated lncRNAs, all CRC-related lncRNA data were downloaded from the cancer genome atlas (TCGA) public database and then screened based on the significance in expression and prognosis. Analysis methods: An expression matrix was established, with expression level of each gene as the row name and each sample as the column name. Then differential expression analysis was carried out using the Limma package in R language to screen out differentially expressed lncRNAs. The clinical progress of CRC patients was downloaded for survival analysis in combination with the expression level of lncRNAs, so as to find out differential lncRNAs related to the CRC prognosis.

Real-time fluorescence quantitative polymerase chain reaction (qRT-PCR)

Cell pellet was collected to extract the total RNAs using the Pure Link kits, and then the quality of extracted RNAs was evaluated based on A_{260}/A_{280}. Next, the RNAs were reversely transcribed into complementary DNAs (cDNAs), followed by amplification. SYBR Green PCR Kit and Mastercycler® Ep Realplex2 were used for reverse transcription (RT)-qPCR under 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min for 40 cycles, with cDNAs (2 μL) as a template. With glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference, the relative expression level of lncRNA CCAT2 was calculated by $2^{-\Delta\Delta Ct}$ method. The primer pairs used: LncRNA CCAT2-F: 5'-AGACAGTGCCAGCCAACC-3'; R: 5'-TGCCAAACCCTTCCCTTA-3'. GAPDH-F: 5'-AATGGACAACTGGTCGTGGAC-3'; R: 5'-CCCTCCAGGGGATCTGTTTG-3'.

Methyl thiazolyl tetrazolium (MTT) assay

Cells were seeded in a 96-well plates at 3000 cells/well, with three replicates set in each group. During the assay, the absorbance of cells was monitored and record-

ed for 6 days. At certain time points after cell growth, the supernatant was gently discarded, and the cells were added with an equal volume of MTT reagent and incubated for 2 h. The relative absorbance in each group was calculated by the following formula:

$$\text{Relative absorbance} = \text{optical density (OD)}_{\text{mimic}} - \text{OD}_{\text{blank control group}} / \text{OD}_{\text{NC}} - \text{OD}_{\text{blank control group}}$$

Apoptosis determined through flow cytometry

The cells were trypsinized after the fusion rate was over 80%. After the digestion was terminated, they were centrifuged at 1200 g for 5 min, and the cell pellet was collected, re-suspended and washed twice with pre-cooled phosphate-buffered saline (PBS). Subsequently, appropriate volumes of FITC and propidium iodide (PI) were added, based on the volume of the cell pellet for incubation for 3 min and 10 min, respectively. Finally, the cells were detected by flow cytometry (Beckman Coulter, Brea, USA).

Transfection with lncRNA CCAT2 NC and lncRNA CCAT2 mimic

Serum-free medium was prepared, and the transfection reagents were taken out and thawed. Then, cells were inoculated into a 6-well plate at 200,000 cells/well and cultured for 24 h. After the cells were stably attached, transfection was started using Lipofectamine® 2000 Transfection Reagent (10 μL/250 μL mimic and NC) (Invitrogen, Carlsbad, CA, USA). About another 24 h later, the medium containing the mixture and the fresh medium containing 10% fetal bovine serum (FBS) were placed in advance. RT-qPCR was utilized to evaluate the transfection efficiency.

Immunofluorescence assay

The assay was performed over three days. On the first day, cells were seeded into 6-well plates at a density of 20,000 cells/well, and sterile slides were placed. On the next day, the cells were collected, fixed with 4% paraformaldehyde, blocked with 5% bovine serum albumin (BSA), and added with Bax and Bcl-2 antibodies. On the third day, the cells were added with corresponding secondary antibodies, washed with PBS at least for three times to ensure that they were thoroughly cleaned, and incubated with fluorescein-labeled rabbit secondary antibodies. Thereafter, 4',6-diamidino-2-phenylindole (DAPI) staining solution was added dropwise for 5 min for staining, and then images were acquired.

Statistics

SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used to process and analyse the research data obtained from assays. Data are shown as mean±SD. The t-test was used for analysis of the measurement data. Differences between two groups were analysed using Student's t-test. Comparisons between multiple groups was done using one-way ANOVA test, followed by *post-hoc* test. $P<0.05$ indicated that there were significant differences in the experimental results.

Results

LncRNA CCAT2 served as a biomarker for colorectal cancer prediction

According to CRC analysis lncRNAs via TCGA database, CCAT2 was markedly overexpressed in CRC tissues. The correlation between lncRNA CCAT2 expression level and survival of CRC patients was analyzed, and it was revealed that the survival of CRC patients with a high expression level of lncRNA CCAT2 was shortened, with severely poor prognosis ($p<0.05$) (Figure 1).

LncRNA CCAT2 expression was elevated in colorectal cancer tissues and cells

qRT-PCR was adopted to quantify the expression of lncRNA CCAT2 in 80 cases of CRC tissues, corresponding adjacent tissues and CRC cell lines.

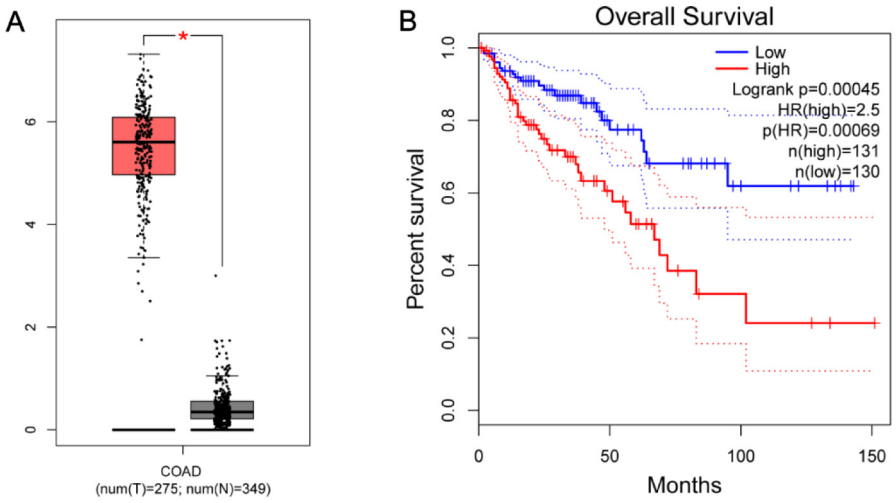

Figure 1. A: Expression of lncRNA CCAT2 in CRC based on TCGA database, and **B:** Association between lncRNA CCAT2 and CRC prognosis.

The results demonstrated that the expression level of lncRNA CCAT2 was clearly higher in CRC tissues than in adjacent tissues ($p<0.01$), and it was higher in CRC cells than in FHC cells. Compared with HT29 and LOVO cell lines, HCT-116 cell line had a relatively low expression level of lncRNA CCAT2 ($p<0.05$) (Figure 2).

LncRNA CCAT2 promoted proliferation of colorectal cancer cells

HCT-116 cells were selected for exogenous overexpression of lncRNA CCAT2, and qRT-PCR assay was carried out. The results revealed that the expression level of CCAT2 was significantly higher in the mimic group than in the NC group

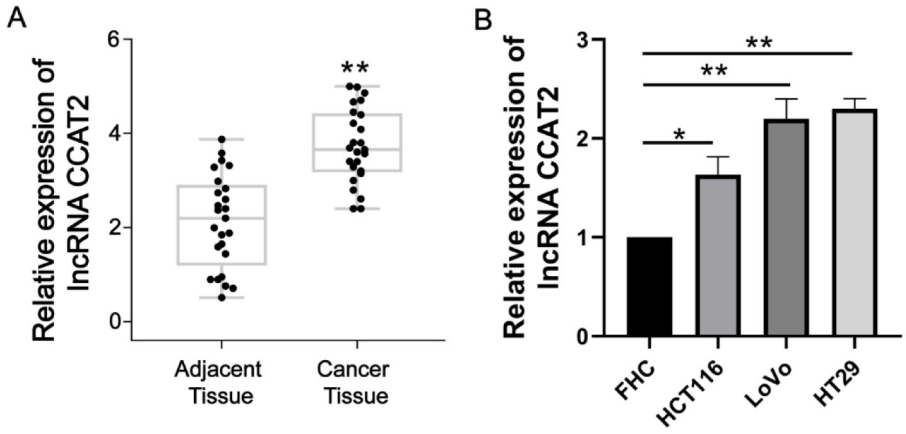

Figure 2. Expression level of lncRNA CCAT2 in CRC tissues and cells. **A:** The expression level of lncRNA CCAT2 is evidently higher in CRC tissues than in adjacent tissues ($p<0.01$), and **B:** Such a level is elevated in CRC cell lines ($p<0.001$), and HCT116 cell line has a lower expression level of lncRNA CCAT2 than HT29 and LOVO cell lines ($p<0.05$) (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

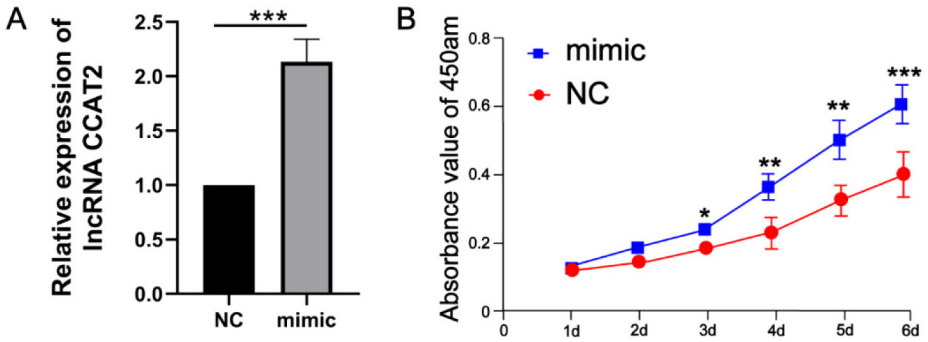

Figure 3. Effects of lncRNA CCAT2 on proliferation of CRC cells. **A:** Changes in lncRNA CCAT2 expression in NC group and mimic group. The expression of lncRNA CCAT2 is raised in mimic group compared with NC group ($p<0.01$). **B:** Effects of lncRNA CCAT2 on the proliferation of CRC cells. Mimic group exhibits a greater proliferation rate in comparison with NC group ($p<0.05$) (*$p<0.05$, **$p<0.01$).

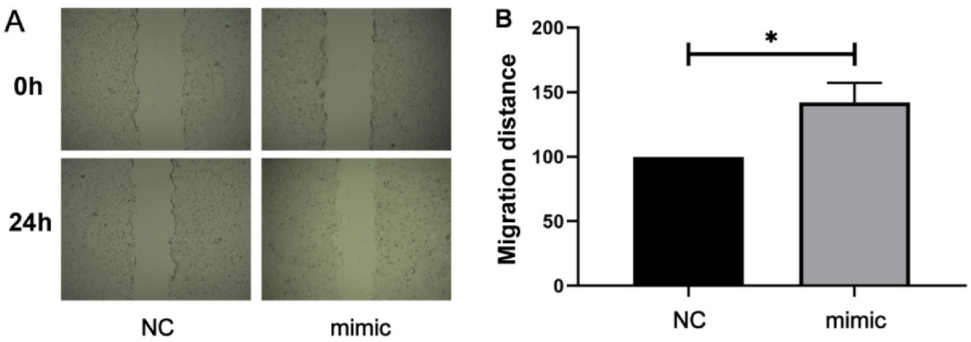

Figure 4. Effects of lncRNA CCAT2 overexpression on the migration of cells. **A** and **B**: The intercellular distance is shorter in the mimic group than in the NC group (magnification: 10×) (*$p<0.05$).

(p<0.001). The changes in cell growth were detected for 6 consecutive days, and it was discovered that a difference was detected in cell proliferation between mimic group and NC group from the third day (p<0.05), which was most evident on the sixth day (p<0.001) (Figure 3).

LncRNA CCAT2 overexpression facilitated migration of colorectal cancer cells

The changes in cell migration distance were detected after 24 h. The results revealed that the intercellular distance in the mimic group was shortened, and that in the NC group was basically unchanged, suggesting that lncRNA CCAT2 promotes the migration of CRC cells (Figure 4).

LncRNA CCAT2 overexpression suppressed the apoptosis of colorectal cancer cells

The results of flow cytometry demonstrated that the percentage of apoptotic cells was 5% in the mimic group and about 13% in the NC group. Immunofluorescence assay results showed that in the mimic group Bax was mainly located in the cytoplasm, with overtly reduced fluorescence intensity, indicating that Bax expression is inhibited. Besides, red fluorescent cells were observed in the cytoplasm, and the fluorescence intensity was stronger than in the NC group, suggesting that the protein expression level of Bcl-2 in the mimic group was significantly increased (p<0.05), and that lncRNA CCAT2 was capable of repressing the apoptosis of CRC cells (Figure 5).

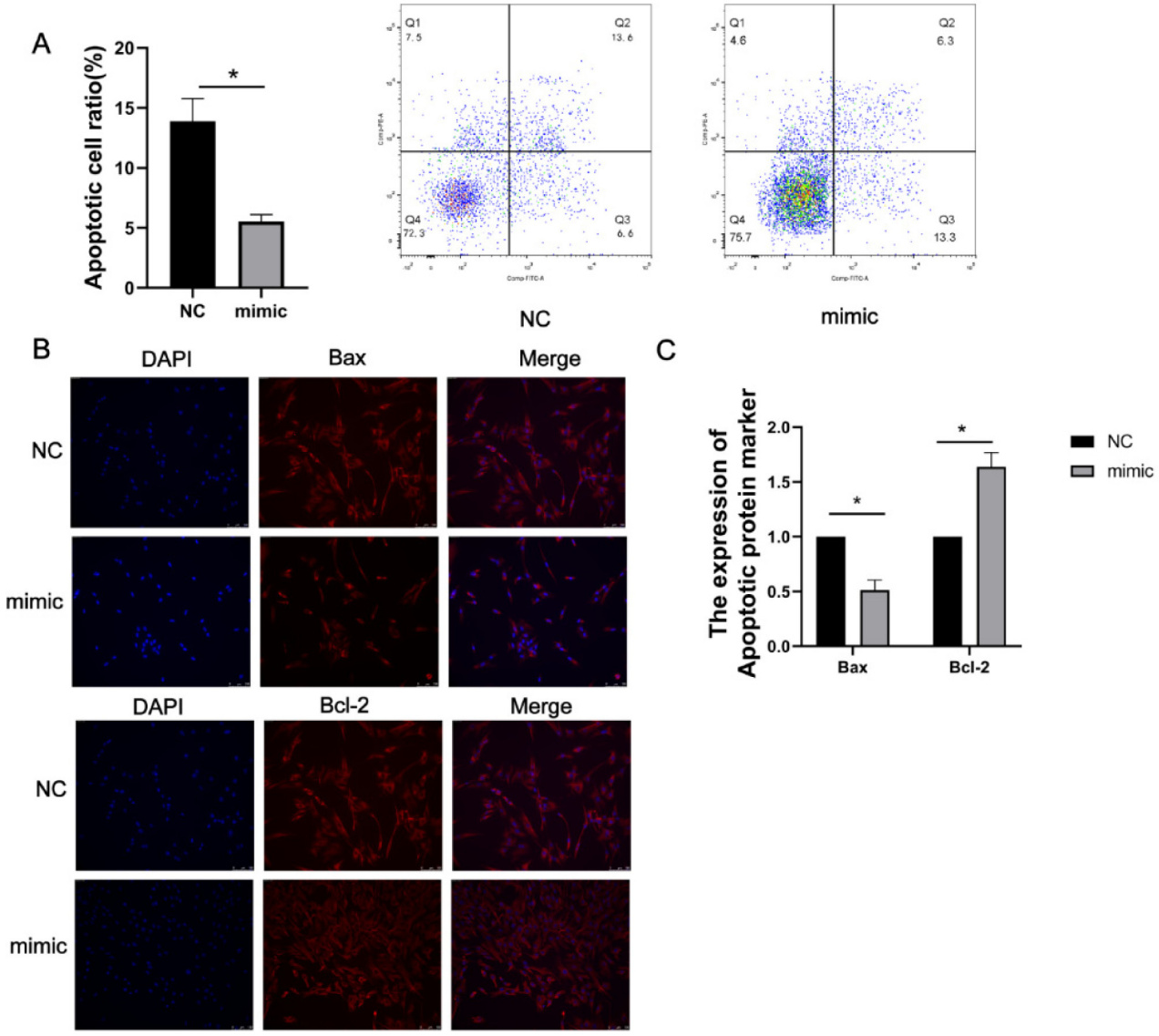

Figure 5. Effects of lncRNA CCAT2 overexpression on CRC cells. **A** and **B**: The apoptosis ratio of CRC cells is obviously smaller in the mimic group than in the NC group (p<0.05) (magnification: 400×). **C** and **D**: mimic group has a dramatically decreased expression level of Bax and a remarkably raised expression level of Bcl-2 based on immunofluorescence assay detection (p<0.05) (*p<0.05 *vs.* mimic group).

Discussion

Recent studies have manifested that lncRNAs, as new regulatory mechanisms, have attracted increasing attention and play crucial roles in various diseases and even cancers. LncRNA CCAT2 has been first identified as a region in the 8q24 chromosome position, which contains a nucleotide polymorphism and is expressed in CRC with stable microsatellite. It is overexpressed in CRC, promotes the growth and metastasis of tumors, reduces the sensitivity to chemotherapy of CSC and is co-regulated by miR-145 and miR-21 [22]. The role of lncRNAs has been understood by more and more people, and its coexpression with other genes and assembly with cell structures have also been extensively studied. However, their specific mechanisms still need to be further investigated [22-24].

In this study, lncRNAs were first downloaded from TCGA database for data analysis and processing, and it was uncovered that lncRNA CCAT2 was differentially expressed in CRC tissues, and its expression was elevated in CRC. Then, the relationship between lncRNA CCAT2 expression level and CRC survival was analyzed, and the results showed that the survival of patients with a high level of lncRNA CCAT2 expression was overtly shortened, implying that lncRNA CCAT2 has the potential to be a biomarker for poor prognosis of CRC. Next, three types of CRC cells and 80 cases of CRC tissues were selected, which revealed that lncRNA CCAT2 was markedly overexpressed in CRC tissues and cells, while it was relatively lowly expressed in HCT-116 cell line. To explore the functions of lncRNA CCAT2, HCT-116 cells were transfected with lncRNA CCAT2 NC and lncRNA CCAT2 mimic, the transfection efficiency was confirmed, and the cell line with overexpressed lncRNA CCAT2 were constructed. It has been proven that lncRNA CCAT2 is obviously highly expressed in other tumors and acts as an oncogene. However, its role in CRC is rarely reported. In this study, the changes in absorbance at 490 nm of the cell supernatant, which reflected the cell growth rate, were continuously monitored, and it was observed that the absorbance was significantly higher in the mimic group than in the NC group since the third day, and the difference was statistically significant from the fourth day to the sixth day, and reached the maximum on the sixth day. These results indicate that lncRNA CCAT2 is able to promote cell growth and proliferation. The changes in migration distance of cells were examined at 24 h after scratching, and the experimental data showed that in the mimic group the intercellular distance was shortened, and the cell migration became faster, supporting the conclusion that lncRNA CCAT2 promotes the migration of CRC cells. Finally, to investigate the effects on apoptosis, cell suspension was collected and detected using a flow cytometer. The proportion of apoptotic cells in the mimic group was 5%, significantly lower than in the NC group. In addition, the protein expression levels of Bax and Bcl-2, apoptotic protein marker, were also detected via immunofluorescence assay to reflect changes in apoptosis. Compared with those in the NC group, Bax protein expression level was distinctly reduced, while Bcl-2 protein expression level was substantially raised in the mimic group. The results of the above two experiments showed that overexpressing lncRNA CCAT2 can inhibit the apoptosis of CRC cells.

Conclusions

In conclusion, this study found that lncRNA CCAT2 can facilitate the growth and proliferation and repress the apoptosis of CRC cells. However, its mechanisms will be further investigated in subsequent studies, so as to explore genes co-expressed with lncRNA CCAT2 and functional molecular mechanisms of lncRNA CCAT2.

Conflict of interests

The authors declare no conflict of interests.

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