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Research Paper

Long noncoding RNA CCAT1 functions as a ceRNA to antagonize the effect of miR-410 on the down-regulation of ITPKB in human HCT-116 and HCT-8 cells

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ABSTRACT

Colorectal cancer is one of the most common malignancies, which has seriously affected people's health. Abnormal expression of long non-coding RNAs and microRNAs are closely related to the process of occurrence, development, invasion and metastasis of colorectal cancer. However, the effect of lnc *CCAT1* on human HCT-116/HCT-8 cells and its potential mechanism were investigated. In present study, differential expression of *CCAT1*, miR-410 and *ITPKB* were detected in colon cancer tissues and adjacent parts. Then the prediction programs were applied to predict the target genes of miR-410. The complementary bindings of miR-410 with lnc *CCAT1* and *ITPKB* were assessed by luciferase assays. The interaction between LncRNA *CCAT1* and miR-410 was analyzed. In addition, the mRNA and protein of *ITPKB* and apoptosis factors were examined in cells after miR-410 overexpression or silencing. Meanwhile, MTT and flow cytometer were used to detect the cells proliferation and apoptosis level. Results showed that *CCAT1* and miR-410 were up-regulated in colon cancer tissues, but *ITPKB* was down-regulated. Lnc *CCAT1* and *ITPKB* were predicted to be the targets of miR-410 and the prediction were verified by luciferase assays. The expression of lnc *CCAT1* and *ITPKB* were inhibited by miR-410 in human HCT-116/HCT-8 cells. Meanwhile, lnc *CCAT1* could lead to a decrease of miR-410. Furthermore, miR-410 overexpression could promote cell proliferation and reduce apoptosis. In summary, these data demonstrated that miR-410 could promote cell proliferation and reduce apoptosis by inhibiting *ITPKB* expression and the expression of lnc *CCAT1* antagonized the effect of miR-410.

INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignant tumors of the digestive tract caused serious harm to human life and health [1]. The morbidity and mortality of colorectal cancer are higher due to a variety of factors including colon polyps, ulcerative colitis, lifestyle, diet, age, obesity, environment and genes interaction [2, 3]. Thus, the effective methods are urgently needed for the CRC patients' treatment. However, its pathogenesis has not been fully elucidated.

Long non-coding RNAs (lnc RNAs) are formed by RNApol II transcription with a length of more than 200nt non-coding RNA molecules and have no protein coding function due to the lack of effective open reading frame (ORF) [4, 5]. *CCAT1* was first found to be

up-regulated in colorectal cancer [6]. Previous studies have found that *CCAT1* could be activated by *c-Myc* and played a regulatory role in the development, progression, metastasis and invasion of colorectal cancer [7–10]. Furthermore, hepatocellular carcinoma [11, 12], gastric cancer [9], gallbladder cancer [13], breast cancer [14] cells proliferation and migration levels were up-regulated which were confirmed to be related to the abnormal expression of *CCAT1*. However, the regulation mechanism of *CCAT1* in colon cancer is still not clear.

MicroRNAs (miRNAs) are highly conserved RNAs participate in a series of life process by regulating the expression of genes resulting in mRNAs degradation or post-transcriptional inhibition [15]. Studies have showed that miRNAs are widely involved in the occurrence, development, prognosis and recurrence of colorectal cancer [16–20]. Among the reported miRNAs, miR-410 has be proved to function as a tumor suppressor in human glioma though regulating *MET* [21]. ITPKB, a member of 3-kinases family, is associated with calcium signaling pathway. It may play a vital role in immune disorders, Alzheimer's disease, multiple sclerosis and malignant melanoma [22, 23]. Meanwhile, research has shown that *ITPKB* could be down-regulated by miR-375 in SCLC and promote cell growth in SCLC cell line [24]. However, little work has been reported on miR-410 and *ITPKB* function in colon cancer.

In the present study, we aimed to investigate the expression, function and interaction of miR-410, *CCAT1* and *ITPKB* in human HCT-116 cells to reveal the underlying mechanisms. Our findings demonstrated that miR-410 could inhibit cell proliferation and promote apoptosis by inhibiting *ITPKB* expression and the expression of *CCAT1* antagonized the effect of miR-410 on the down-regulation of its target *ITPKB* in human HCT-116 cells. Which laid the foundation for the deeply study of colon cancer.

RESULTS

Patient statistics

A total of 30 colon cancer patients were included in this study. The surgeries were performed from January 2010 to December 2011. The colon cancer tissues and adjacent parts were collected and stored at -80°C. The proportion of male was 63.3% and the average age was 60.9 ± 12.1. All the patients were Han population in north of China.

The genes expression in different colon cancer tissues

Differences in expression of miR-410, *CCAT1* and *ITPKB* were detected in 30 different colon cancer tissues and adjacent parts using qPCR. As shown in Figure 1A, miR-410 and *CCAT1* expression levels were gradually up-regulated in colon cancer tissues ($p<0.01$), but the expression of *ITPKB* mRNA was significantly higher in adjacent parts ($p<0.01$) (Figure 1A).

MiR-410 directly target *ITPKB* and *CCAT1*

The prediction programs (Target Scan, starBase v2.0 and miRGen) were used to identify potential binding sites with miR-410 in the 3'UTR and the target relationship was higher between the miR-410 and *ITPKB*/lnc *CCAT1* 3'UTR among the results. Besides, *ITPKB* and *CCAT1* had been generally proved to participate in cancer cell proliferation. In our previous research, miR-410 and *CCAT1* was markedly up-regulated and *ITPKB* expression level was gradually reduced in colon cancer tissues and adjacent parts. As shown in Figure 1B-1C, miR-410 could closely bind to the target sites in the 3'UTR of *ITPKB*/lnc *CCAT1*. Compared with the control group, luciferase activity was significantly lower in group (miR-410 mimics + ITPKB-WT) and group (miR-410 mimics + CCAT1-WT). Luciferase results showed that miR-410 had a high binding ability with *ITPKB*/lnc *CCAT1* 3'UTR (Figure 1B-1C) ($p<0.05$).

MiR-410 down-regulated *ITPKB* and the interaction between miR-410 and lnc *CCAT1*

As shown in Figure 2A-2B, lnc *CCAT1* was significant down-regulated in miR-410 mimics transfecting cells, and a higher expression was found in cells transfected with miR-410 inhibitor. On the other hand, lnc *CCAT1* was widely expressed in HCT-116 cells and HCT-8 cells transfecting with pCDNA-CCAT1 (Figure 2C). Compared with the control group, lnc *CCAT1* overexpression could down-regulate miR-410 (Figure 2D). Therefore, there was an interaction between miR-410 and lnc *CCAT1*. Besides, *ITPKB* mRNA and protein expressed in miR-410 inhibitor group was significantly increased, indicating that miR-410 could reduce the expression of *ITPKB* ($p<0.01$) (Figure 3).

MiR-410 promote the human HCT-116/HCT-8 cells proliferation and inhibit cells apoptosis

The human HCT-116/HCT-8 cells proliferation and apoptosis were detected by MTT and flow cytometry after miR-410 transfection. Results showed that miR-410 mimics group induced a significant increase on the growth rate of human HCT-116/HCT-8 cells ($p<0.01$) (Figure 4). After 48 h transfection, the apoptosis results showed that the apoptosis rates were 4.31% (miR-410 mimics group), 19.41% (miR-410 inhibitor group) and 11.98% (miR-shNC) in HCT-116 cells. Meanwhile, the apoptosis rates were 4.8% (miR-410 mimics), 21.47% (miR-410 inhibitor) and 10.48% (miR-shNC) in HCT-8 cells. Suggesting that miR-410 could inhibit both HCT-116 and HCT-8 cells apoptosis (Figure 5).

The caspase-3 and Bcl-2 expression levels detection

We further detected the apoptosis related protein caspase-3 and Bcl-2 after miR-410 overexpression in HCT-116 and HCT-8 cells. The results showed that caspase-3 level was higher in miR-410 inhibitor group compared with miR-410 shNC group. However, Bcl-2 protein level showed an opposite trend. In other words, *ITPKB* up-regulation caused an increase of caspase-3 and a decrease of Bcl-2 (Figure 3B).

DISCUSSION

Nowadays, tumor resection and concurrent chemo radiotherapy are the main means for the treatment of CRC, however, it has a poor prognosis. Lnc RNAs and miRNAs have been verified to participate in cancer development and progression by regulating the cell proliferation, differentiation and apoptosis [25–32]. Besides, the potential role played by these molecules in the pathogenesis of CRC attracts more and more attention, however, the current

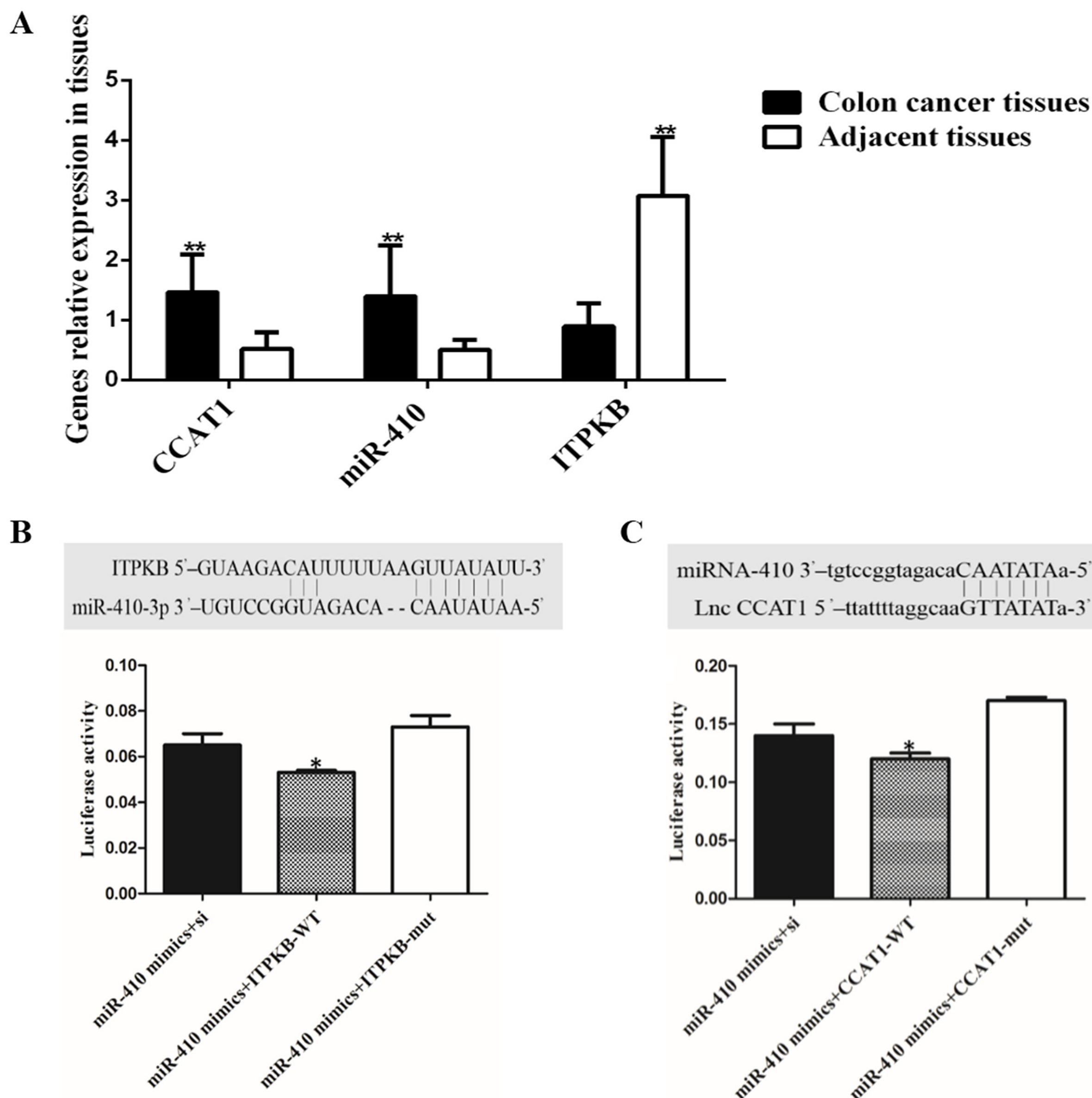


Figure 1: Relative expression of genes mRNA in colon cancer tissue and target genes of miR-410 validation. **(A)** miR-410, *CCAT1* and *ITPKB* mRNA in colon cancer tissues and adjacent part; **(B)** Binding site and binding capacity between miR-410 and *ITPKB*; **(C)** Target site and luciferase activity were analyzed for relationship between miR-410 and *CCAT1*. *p<0.05 was a significantly different; **p<0.01, the difference was extremely significant.

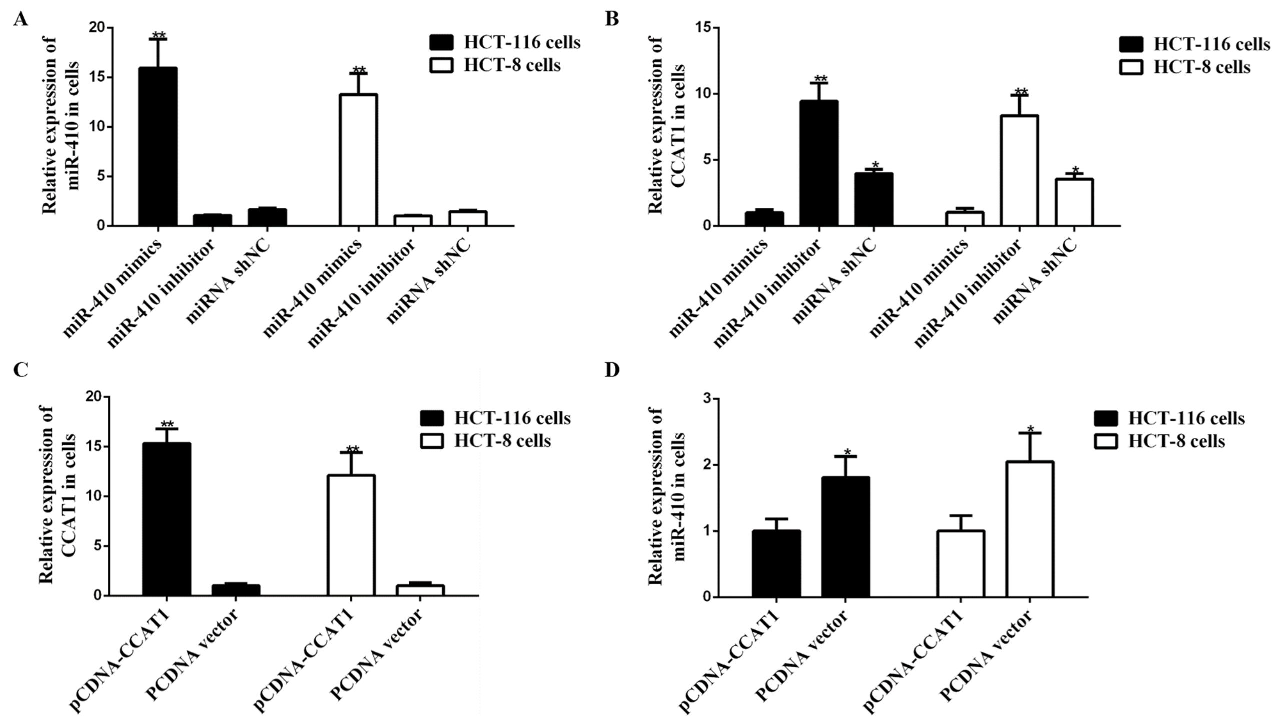


Figure 2: Interaction between miR-410 and lnc *CCAT1*. **(A-B)** miR-410 mimics, inhibitor and miR-shNC were transfected into HCT-116/HCT-8 cells, then *CCAT1* were analyzed by qPCR. **(C-D)** Effect of *CCAT1* on miR-410 were analyzed in HCT-116/HCT-8 cells. *p<0.05 was a significantly different; **p <0.01, the difference was extremely significant.

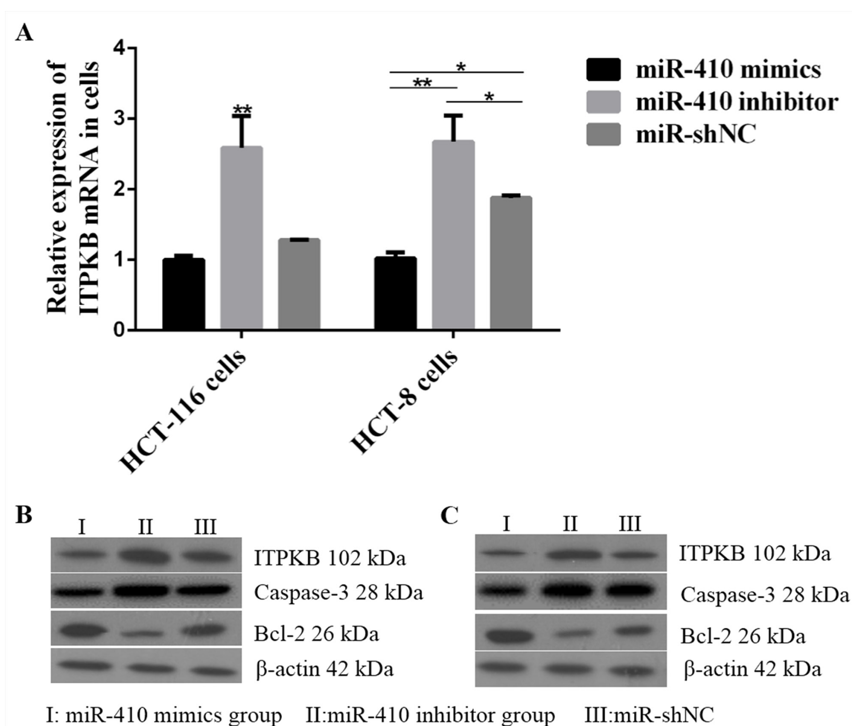


Figure 3: Effects of miR-410 on *ITPKB* and apoptosis factors in HCT-116/HCT-8 cells. **(A)** *ITPKB* mRNA expression levels detected by qPCR after miR-410 mimics or miR-410 inhibitor or miR-shNC transfecting into HCT-116/HCT-8 cells. **(B)** ITPKB and apoptosis factors proteins were detected in HCT-116 cells. **(C)** miR-410 mimics or miR-410 inhibitor or miR-shNC transfected into HCT-8 cells, then ITPKB and apoptosis factors proteins were detected by western blot. *p<0.05 was a significantly different; **p<0.01, the difference was extremely significant.

mechanism research is still not comprehensive. Our study aims to investigate the interaction among lnc *CCAT1*, miR-410 and its target gene *ITPKB* in human HCT-116/HCT-8 cells proliferation and apoptosis.

So far, a variety of miRNAs have been shown to be involved in the pathogenesis of colon cancer, such as miR-34a [33], miR-506 [34], miR-675 [35] and miR-200 [36]. In previous study, miR-410 has be proved to function as

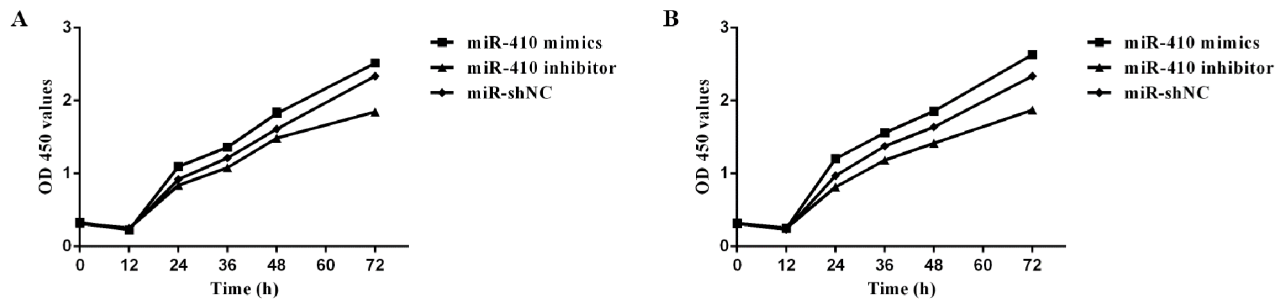
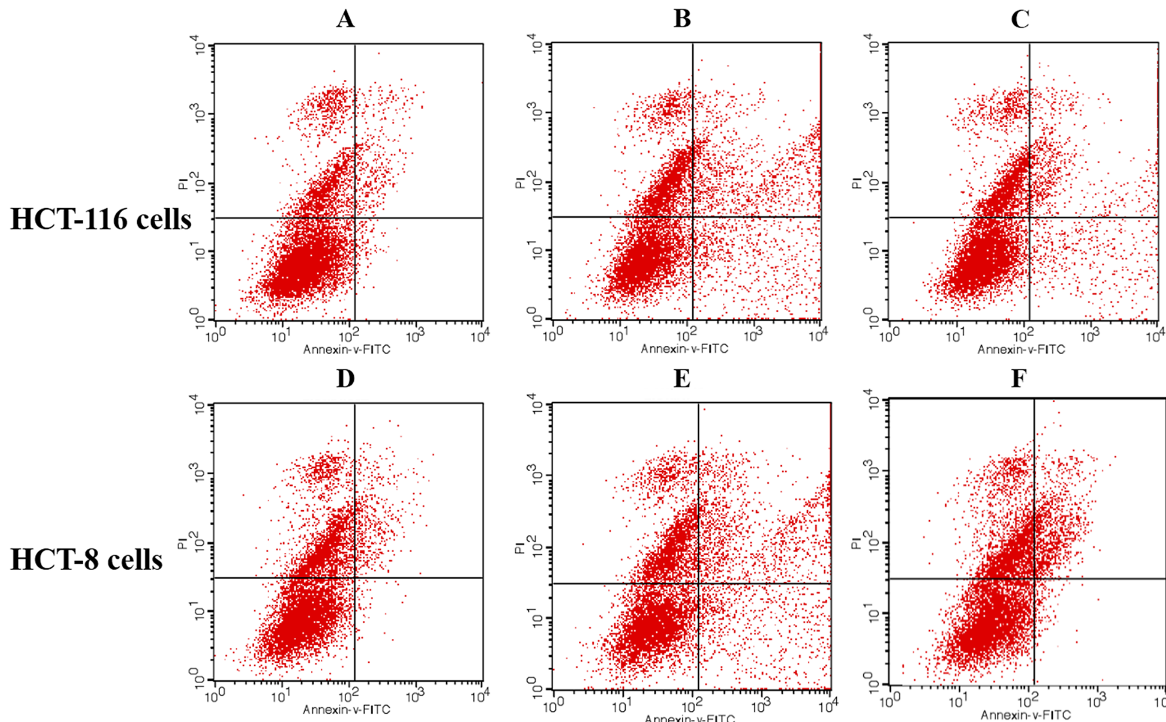


Figure 4: HCT-116/HCT-8 cell proliferation ability. (A) HCT-116 cell growth rate counted after transfection. **(B)** Growth rate were detected in HCT-8 cells transfected miR-410 mimics, inhibitor and miR-shNC.



Cells group/data	HCT-116-UR(%Total)	HCT-116-LR(%Total)	HCT-8-UR(%Total)	HCT-8-LR(%Total)
miR-410 mimics	3.48	0.83	4.34	0.46
miR-410 inhibitor	13.17	6.24	15.27	6.20
miR-410 shNC	7.88	4.10	9.26	1.22

Figure 5: HCT-116/HCT-8 cell apoptosis rate. (A-C) HCT-116 cells transfected miR-410 mimics, inhibitor and miR-shNC, then apoptosis rate was detected by flow cytometer. **(D-F)** HCT-8 cells apoptosis rate was detected after transfection. $^{**}p<0.01$, the difference was extremely significant.

Table 1: Primer sequences of qPCR

Symbol	Primer	Primer Sequence (5′–3′)
hsa-miR-410-3p	RT-Primer	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTG
	F-Primer	AGACAGGCCA
	R-Primer	ACACTCCAGCTGGGAATATAACACAGATG
		CAGTGCAGGGTCCGAGGT
U6	RT-Primer	AACGCTTCACGAATTTGCGT
	F-Primer	CTCGCTTCGGCAGCACA
	R-Primer	AACGCTTCACGAATTTGCGT
ITPKB	F-Primer	TTAAAGCCATCTCGTCCCTAC
	R-Primer	GCCCAAAGCTCCATAAACAAC
CCAT1	F-Primer	CATTGGGAAAGGTGCCGAGA
	R-Primer	ACGCTTAGCCATACAGAGCC
β-actin	F-Primer	CTCCATCCTGGCCTCGCTGT
	R-Primer	GCTGTCACCTTCACCGTTCC

a tumor suppressor in human glioma by regulating *MET*. Thus, miR-410 was chose to conduct further research. The prediction programs (Target Scan, starBase v2.0 and miRGen) were used to identify potential binding sites and the target relationship between miR-410 and *ITPKB*/lnc *CCAT1* was verified using luciferase assays. Luciferase assays showed that miR-410 could target *ITPKB*/lnc *CCAT1* 3'UTR. The qPCR and western blot results on the tissues confirmed this relationship. Meanwhile, miR-410 mimics could promote the human HCT-116/HCT-8 cells proliferation and attenuate apoptosis. In addition, the caspase-3 expression level was reduced and Bcl-2 was significant increase in miR-410 mimics group.

Lnc *CCAT1* has been involving in the development, progression, metastasis and invasion of colorectal cancer [8, 10]. Emerging evidence have confirmed that lnc RNAs might function as a competing endogenous RNA (ceRNA) or a molecular sponge in modulating miRNAs. Meanwhile, studies indicated that lnc *CCAT1* could negatively regulate the expression of miRNA-218-5p and let-7 in gallbladder cancer [13] and hepatocellular carcinoma [11]. In addition, there were less research on lnc *CCAT1* in colon cancer. In this study, we predicted that the miR-410 target sites were in the 3'UTR of lnc *CCAT1*. Dual-luciferase reporter assay confirmed the interaction between miR-410 and lnc *CCAT1* which was similar to previous studies [37]. Lnc *CCAT1* overexpression could improve the ITPKB level. This relationship was detected on the tissue using qPCR and western blot.

In conclusion, the interaction among miR-410, *CCAT1* and *ITPKB* was detected in human HCT-116/HCT-8 cells. MiR-410 could directly target lnc *CCAT1* and *ITPKB*. Meanwhile, miR-410 mimics could promote the human HCT-116/HCT-8 cells proliferation and attenuate apoptosis. In addition, the caspase-3 expression level was reduced and Bcl-2 was significant increase in miR-410 mimics group. Simultaneously, lnc *CCAT1* functioned as a ceRNA to antagonize the effect of miR-410 on the down-regulation of *ITPKB*. These findings might provide a basis for the treatment of colon cancer.

MATERIALS AND METHODS

Tissue samples

The 30 CRC tissues and adjacent tissues were collected from the Department of Colorectal and Anal Surgery in Third Affiliated Hospital of Jilin University. The study was approved by the Ethics Committee of Third Affiliated Hospital of Jilin University. Tissue samples were stored in liquid nitrogen.

Cell culture and transfection

The prediction programs (Target Scan, starBase v2.0 and miRGen) were applied to predict the target genes of miR-410. The human HCT-116/HCT-8 cell lines were purchased from Shanghai Institute for Biological Sciences (Shanghai, China). All cells were cultivated in DMEM medium (GIBCO, USA) with 10 % FBS (HyClone, USA), 100 units/ml penicillin and 100 mg/ml streptomycin at 37 °C in incubator containing 5% CO_2. The miR-410 mimics, miR-410 inhibitor, shNC, WT-vectors (*CCAT1-WT*, *ITPKB-WT*), mut-vectors (*CCAT1-mut*, *ITPKB-mut*), pCDNA-*CCAT1* and PCDNA3.1 vector were synthesized and

purchased from RiboBio Company (Guangzhou, China). The HCT-116/HCT-8 cells transfection and co-transfection were performed using FuGENE HD Transfection Reagent (Promage, USA) according to the manufacturer's instructions.

Real-time PCR and western blot detection

Total RNA was isolated using Trizol reagent and cDNAs were synthesized by a RT-PCR Kit (Takara, Japan). Specific primers were designed for RT-PCR and qPCR reaction (Table 1). The conditions and procedures were referred to the instructions. The proteins were extracted with RIPA buffer (Boster, China) and the BCA Protein Assay Kit (Boster, China) was used to detect protein concentration referring to the instructions. Proteins were isolated by SDS-PAGE and transferred to PVDF membrane. The membrane was blocked with 5% skim milk powder and then incubated with rabbit anti-ITPKB antibody, 1:200 ((Bioss, China), rabbit anti-Caspase-3 antibody, 1:300 (Bioss, China), rabbit anti-Bcl-2 antibody, 1:300 (Bioss, China) and mouse anti-β-actin antibody, 1:6000 (Bioss, China). The proteins bandings were detected with ECL Western Blotting Substrate (Invitrogen, USA).

Luciferase assays

The *ITPKB/CCAT1* wild type (WT), *ITPKB/CCAT1* mutant type (mut) and si were respectively co-transfected with mi-410 mimics into HCT-116/HCT-8 cells by FuGENE HD Transfection Reagent. Firefly and Renilla luciferase activities were measured using a dual-luciferase reporter gene assay system at 48 h after transfection.

Cell proliferation and apoptosis assay

The HCT-116/HCT-8 cells were transfected with miR-410 mimic, miR-410 inhibitor and shNC. Cell cycle progression and levels of apoptosis were analyzed using Cell Cycle and Apoptosis Detection Kit (Beyotime Biotechnology, China) according to manufacturer's protocol.

Statistical analysis

Data were reported as mean ± standard deviation (SD). The differences among groups were analyzed by one-way Analysis of Variance followed by Fisher's LSD test. Survival curves were plotted after the test. Statistical analysis results were performed using SPSS19.0 software for windows and a significant difference was considered with $p<0.05$.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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