Int J Clin Exp Pathol 2016;9(11):11169-11178
www.ijcep.com /ISSN:1936-2625/IJCEP0021946

Original Article

Down-regulation of lncRNA CCAT1 inhibits colon cancer cell proliferation and promotes apoptosis by targeting apoptosis-related proteins

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Received December 15, 2015; Accepted February 25, 2016; Epub November 1, 2016; Published November 15, 2016

Abstract: Colon carcinoma is one of the leading causes of death from cancer and is characterized by a heterogenic pool of cells with distinct differentiation patterns. In this study, to screen the key lncRNA which is related with cell proliferation and apoptosis, qRT-PCR assay was performed and lncRNA CCAT1 was selected. Quantitative RT-PCR was used to observe the expression level of lncRNA CCAT1 in colon cancer tissues and the adjacent non-tumor tissues. siRNA intervention on colon cancer cells SW620 and HT29 was then performed. After intervention, protein expression of Caspase-3, Caspase-9, Bax and Bcl-2 in control, si-scramble and si-lncRNA CCAT1 group was compared. Cell proliferation, apoptosis and cell cycle were also performed to analyze the relationship between the silence of lncRNA CCAT1 and disease progression. Results showed the mRNA expression of lncRNA CCAT1 in colon cancer tissue was significantly higher than that in adjacent non-tumor tissue. After lncRNA CCAT1 in SW620 and HT29 cells intervened with siRNA, the apoptosis rate of SW620 and HT29 cells increased, the cell population of total in si-LncRNA CCAT1 group increased at G0/G1 stage, while decreased at S and G2/M stages compared with control and si-Scramble group. Western blot analysis showed the silence of lncRNA CCAT1 promoted the protein expression of apoptotic protein Bax, caspase 3 and caspase 9, and reduced the level of Bcl-2. Therefore, lncRNA CCAT1 may be a target for colon cancer treatment.

Keywords: Colon cancer, lncRNA CCAT1, apoptosis, bax, caspase-3

Introduction

Colon cancer is one of the best-understood neoplasms from a genetic perspective 1, 2, 3, yet it remains the second most common cause of cancer-related death, indicating that some of its cancer cells are not eradicated by current therapies 4, 5 [1]. With the change of diet and population structure and the aging of the population, the morbidity and mortality of colon cancer are on the rise [2]. Due to the particularity, once colon cancer is diagnosed, the treatment is too late which results in the overall 5-year survival rate is about 50% [3]. Although colon cancer has been treated with chemotherapy, radiation therapy, targeted therapy, and other comprehensive treatment therapy, the prognosis for most patients is poor [4]. The present studies showed the occurrence of development of colon cancer may be related to the activation of proto-oncogenes and inactivation of tumor suppressor genes, epigenetic regulation of individual genome and environmental factors, but the exact molecular mechanism is poorly understood [5]. Therefore, deeply study on the pathogenesis of colon cancer early diagnosis and treatment is of great significance to improve the quality of life in patients with colon cancer.

Long noncoding RNAs (lncRNAs) are a recently discovered class of non-protein coding RNAs, which have now increasingly been shown to be involved in a wide variety of biological processes as regulatory molecules [6]. It plays key roles in the progression and metastasis of some carcinomas [7]. They are key regulators of diverse biological processes such as transcriptional regulation, cell growth and differentiation [8]. Dysregulation of some lncRNAs has been shown in various types of cancers, such as

breast cancer and gastric carcinoma. LncRNAs play important roles in regulating gene expression at epigenetic, transcriptional and post-transcriptional levels [9]. Therefore, study on LncRNA is becoming a hot spot of the medical profession.

In order to further study the mechanism of lncRNA in colon cancer, we screened the differentially expressed LncRNA molecules in colon cancer tissues and adjacent tissues by LncRNA PCR array. Then, the effect of screened lncRNA on the proliferation and apoptosis of colon cancer was studied.

Material and methods

Tissues and cell line

Colon cancer tissue and matched adjacent non-tumor tissues (>2 cm from the tumor) were obtained from 71 colon cancer patients who underwent surgery in Zhongshan hospital affiliated to Fudan university from January, 2014 to June, 2015. The samples were snap-frozen in liquid nitrogen, and appropriate informed consent and approval from the Human Research Ethics Committee were obtained. The tissues were carefully dissected by the pathologist.

The colon cancer cell lines (SW620, HCT116 and HT29) and colon cell line (293-T) were cultured in DMEM (Dulbecco's modified eagle medium) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C and the mixed medium of DMEM + Ham's F12 (v:v=1:1) added with 2.5 mmol/L L-glutamine, 0.3 mg/mL G418, 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin at 34°C, respectively, under the atmosphere of 5% CO_2. The medium was altered every 24 h and the cells at exponential phase were used for current experiments. All the samples were stored at -80°C for further use.

RNA extraction and qRT-PCR assay

TRIzol solubilization and extraction is a relatively recently developed general method for deproteinizing RNA [10]. In this study, the total RNA was extracted and isolated from BMSCs using the Trizol reagent (Invitrogen, Carlsbad, CA). RNA quality was assessed with the Thermo Scientific NanoDrop1000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). RT-PCR was performed using QuantiTect Primer Assay (Qiagen GmbH, Hilden, Germany) and QuantiTect SYBR Green RT-PCR Kit (Qiagen GmbH, Hilden, Germany) on a LightCycler 480 Instrument (Roche Diagnostics, Mannheim, Germany). The detection and quantification contained the following steps: first, reverse transcription was performed at 55°C for 30 min, initial activation for 15 min at 95°C, next 40 cycles of denaturation were conducted at 94°C for 15 s, then annealing for 30 s at 55°C, extension for 30 s at 72°C. Fluorescence data was collected at the extension step. The relative expression of the target gene was determined using the $2^{-\Delta Ct}$ method [11].

LncRNA PCR array

A PCR-array is a high throughput RT-PCR system. A 96-well PCR plate was prepared with a different primer-probe in each well as the PCR array. RNA was respectively converted into cDNA, digested by a restriction enzyme, and then ligated to an adaptor by using the cohesive ends created by the enzyme. After mixing the ligated samples into a single tube, PCR amplification was performed using an adaptor primer and a gene-specific primer. The experimental procedure was performed as previously described [12].

Knockdown of 1ncRNA-CCAT1

According to SIRT1 gene sequence, we designed and synthesized siRNAs using Silencer trade mark siRNA construction kit (Huntingdon, UK) and the sequences were shown as follows: si-CCAT1-1: 5'-CAUACCAAUUGAACCGAGCCUUGUA-3'; si-CCAT1-2: 5'-CAUUAACCUGCUAUCCUCUUUACAA-3'; si-CCAT1-3: 5'-CCCUGUUAAGUAAACGAACACGAAA-3' and si-Scramble: 5'-CATCAATTGAA CCGAGCCTTACGTA-3'. Tubes with si-CCAT1 sequences were centrifuged at 12000 g for 2 min and then sterile DEPC was added into si-CCAT1 solution to 20 μM. For the negative control, 62.5 μL DEPC was added into samples. Colon cancer cells were seeded in 6-well plates and cultured to the cell confluence was 30~50%. Si-CCAT1 was transfected into colon cancer cells by lipofectimine kit according to the manufacturer's instructions. One tube was added 8 μL siRNA and 250 μL Opti-MEM. The other tube was added 250 μL Opti-MEM and 10 μL Lipofectimine 2000. The samples of the two tubes were mixed and place for 15 min. The mixed solution was added

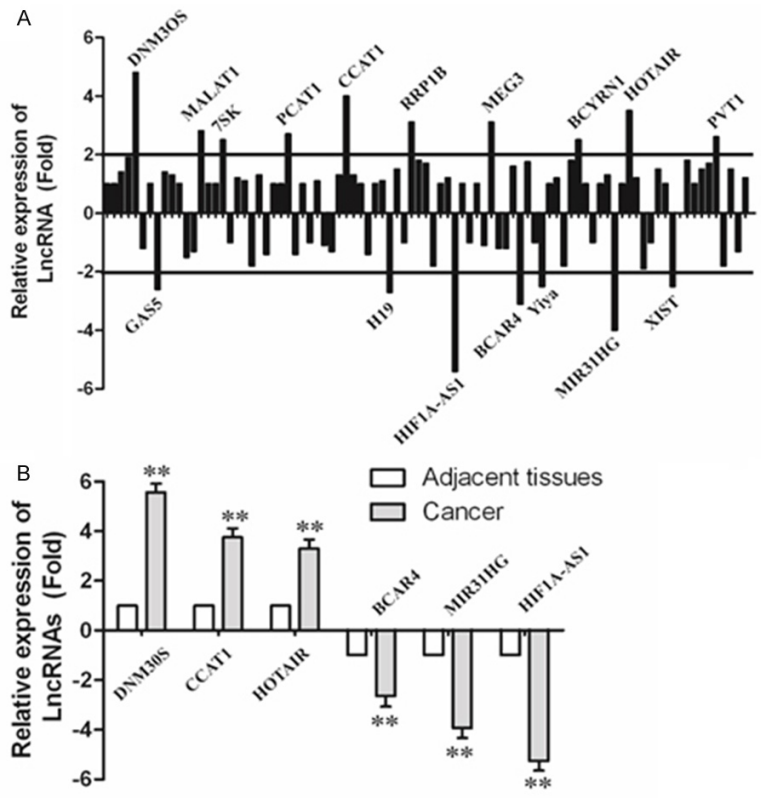

Figure 1. A: The differentially expressed LncRNAs in colon cancer tissue and matched adjacent non-tumor tissues were screened by LncRNA PCR array. B: The 6 LncRNAs with the most significant fold change were selected for validation in tissues. **P<0.01, compared with the matched adjacent non-tumor tissues, LncRNA level in colon cancer tissues had statistical differences.

into colon cancer cells after washed with PBS for twice.

Western blot analysis

Total proteins were prepared from transfected Ishikawa or HEC-1-B cells using radioimmunoprecipitation assay (RIPA) lysis buffer (Sigma, USA) supplemented with protease inhibitors. An equal amount (50 μg) of cellular lysates was separated on 10% SDS-polyacrylamide gel electrophoresis (PAGE) minigels and transferred to nitrocellulose filter membranes (Hybond, Escondido, CA, USA). The membrane was blocked with Tris-buffered saline Tween-20 (TBST) containing 5% skimmed milk powder for 1 h at room temperature, followed by incubation in TBST containing 5% BSA (Sigma, St. Louis, MO, USA) and primary antibodies overnight at 4°C. Primary antibodies were detected using a peroxidase-coupled goat anti-rabbit secondary antibody (1:8000, ZSBio, Beijing, China) and EZ-ECL chemiluminescence Detection kit for HRP (Biological Industries, Beit-Haemek, Israel). The following primary antibodies were used: rabbit mAb miR-381 (1:1000, Cell Signaling Technology, Danvers, MA, USA) and rabbit pAb β-actin (1:1000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

Cell counting kit-8 (CCK-8)

100 mL suspension of siRNA-CCAT1 and scramble siRNA-CCAT1 were plated in the 96-well plate supplemented with DMEM added 10% FBS. After cultured for 1 d, 2 d, 3 d, 4 d and 5 d at 37°C with 5% CO_2, CCK-8 (10 mL) was added into each well. The cells were incubated for another 2 h and the OD (optical density) values were measured using enzyme-labeled instrument (Varian, Palo Alto, CA, USA) at 450 nm. The cell viability was than calculated as follows:

Cell Viability% = (ODt - ODb)/(ODc - ODb) × 100%

Where ODt, ODb and ODc were the OD values of BX647187 siRNA-MG63 group, scramble siRNA- MG63 group and MG63 group, respectively.

Flow cytometry

To study the influence of expression changes of CCAT1 on the apoptosis of colon cancer cells, flow cytometry was used. The colon cancer cell SW620 and HT29 at logarithmic phase were selected and plated in a 96-well plate at a density of 2 × 103 cells/well in supplemented RPMI 1640 and incubated for 16 h before the cells were subjected to treatment in triplicate wells. After treatment, the cells were washed twice in phosphate-buffered saline (PBS) (2.68 mM KCl, 1.47 M KH_2PO_4, 8 mM Na_2KPO_4, 136.75 mM NaCl) and counted. Fifty to one hundred thousand cells were selected and centrifuged 5 min at 1000 r/min. Annexin V-FITC mixed liquor of 195 μL was added to resuspend cytotrophoblast cells and 5 μL was added to mix.

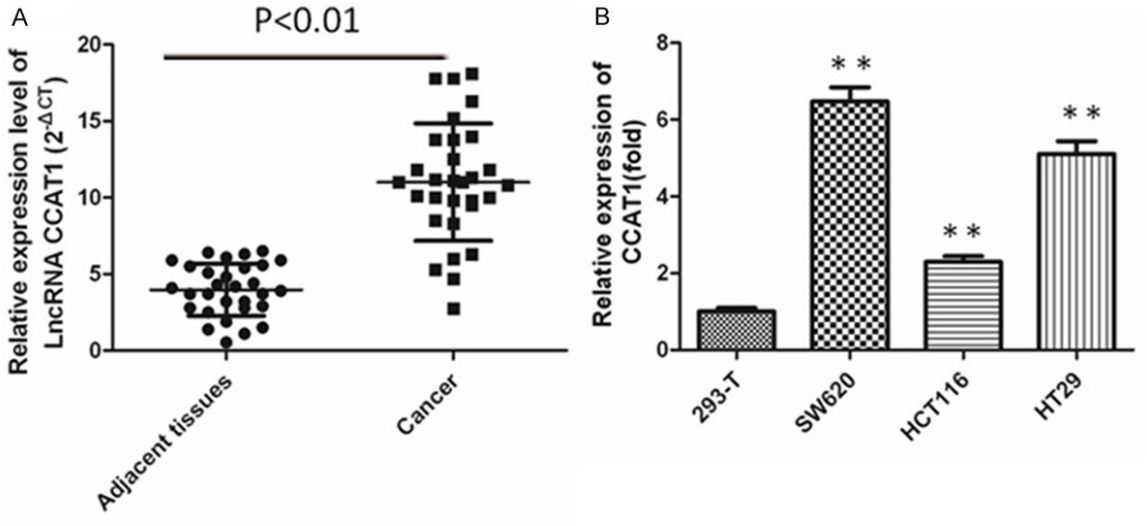

Figure 2. A: The mRNA level of LncRNA CCAT1 in colon cancer tissue and matched adjacent non-tumor tissues from 30 patients with colon cancer were determined by qRT-PCR. B: The mRNA level of LncRNA CCAT1 in colon cancer cell line SW620, HCT116 and HT29, and the normal colon cell 293-T was determined by qRT-PCR. **P<0.01, compared with 293-T cell, the level of LncRNA CCAT1 in colon cancer cell line SW620, HCT116 and HT29 had statistical differences.

Centrifugation at 1000 r/min for 5 min was performed after cultivation 10 min. Sample was obtained after discarding supernatant and 10 μL propidium iodide (PI) was added. Afterwards, the sample was stilled in dark for 30 min. Finally, the apoptosis was detected using flow cytometry (FCM) on the Moflo (Dako Cytomation, Glostrup, Denmark).

Statistical analysis

Statistical analysis was performed using SPSS 18.0. Data are expressed as the mean ± standard error of the mean (SEM) of three independent experiments. Pearson's product-moment correlation coefficient was used to assess the correlation between MDC1 mRNA and protein levels and miR-381 levels in EC. A paired Student's t-test was used to evaluate differences between two groups. Multiple group comparisons were analyzed using ANOVA with a post hoc test. P<0.05 was considered to indicate a statistically significant result.

Results

Expression of LncRNA

Total RNA was extracted from the colon cancer tissues of 71 patients, and the concentration and purity of RNA were determined by nucleic acid analyzer. Results showed the optical density A260/A280 were between 1.8~2.0 which indicated there had no degradation in RNA degradation and the quality of RNA is well.

LncRNA differentially expressed spectrum analysis showed 88 LncRNAs were differentially expressed in colon cancer tissues and adjacent non-tumor tissues and differences are more than 2 times which had statistical significance (**Figure 1A**). Among them, the expression of 10 LncRNAs was upregulated in colon cancer tissue and 7 were downregulated. Then, 6 LncRNAs with the most significant fold change were selected for further verify in tissues and results showed the results of 6 LncRNAs were the same with that of PCR array (**Figure 1B**). The expression of LncRNA CCAT1 in colon cancer tissues was upregulated 3.49 times the value of LncRNA CCAT1 in the adjacent non-tumor tissues. Considering the previous studies, LncRNA CCAT1 was selected for further study.

LncRNA CCAT1 was up-regulated in cancer tissues

The expression values of LncRNA CCAT1 in colon cancer tissues and adjacent non-tumor tissues were determined by real-time quantitative reverse transcription PCR (qRT-PCR). Results showed the expression level of LncRNA CCAT1 in colon cancer tissues was 3.53 times the expression of LncRNA CCAT1 in the adja-

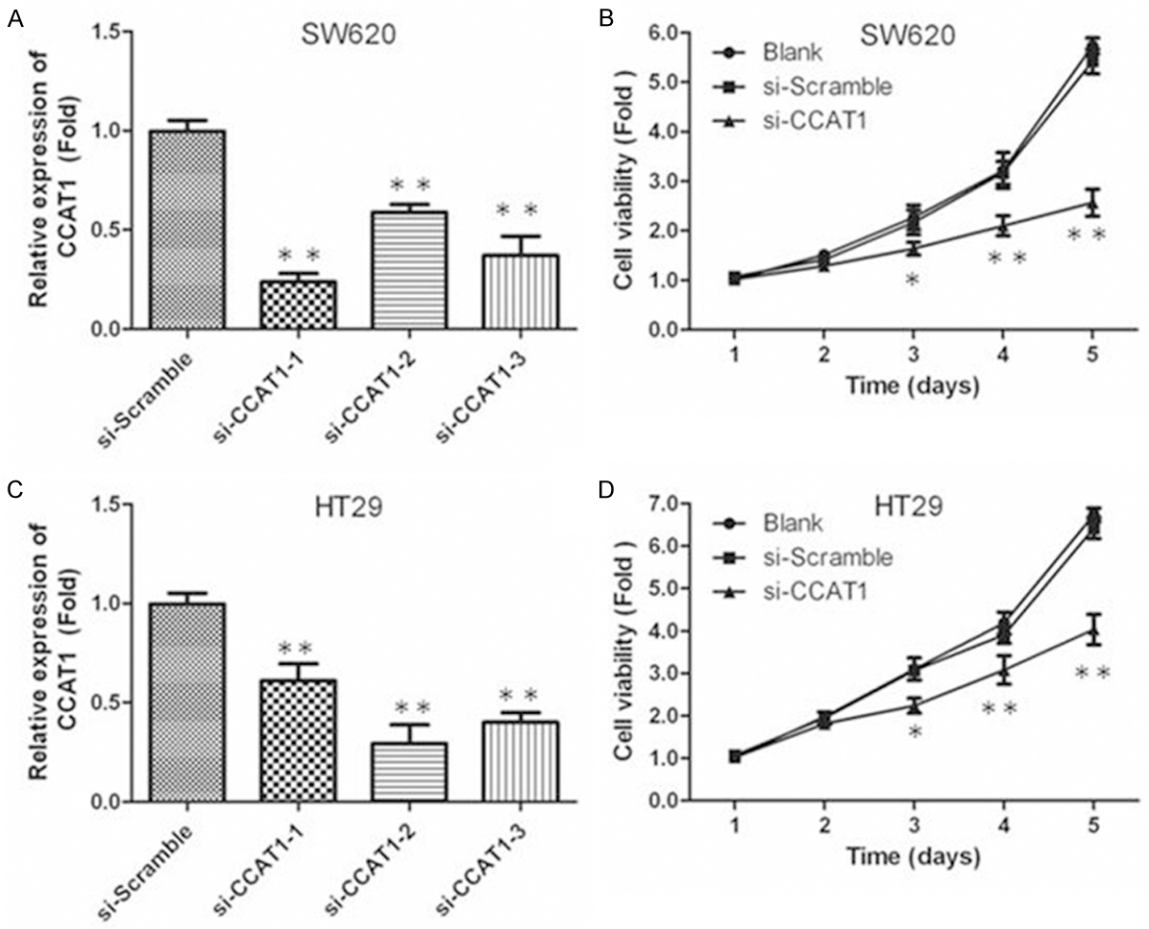

Figure 3. A, C: The relative expression of LncRNA CCAT1 after interference with siRNA. **p<0.01, compared with si-Scramble group, the level of LncRNA CCAT1 in si-CCAT1-1, si-CCAT1-2 and si-CCAT1-3 had statistical differences. B, D: The cell viability of SW620 and HT29 in the control, si-Scramble and si-CCAT1 group determined by CCK-8 assay. *p<0.05 and **p<0.01, compared with the blank group, the viability of SW620 and HT29 cells in si-CCAT1 had statistical differences.

cent non-tumor tissues (P<*0.01*) (**Figure 2A**). The mRNA levels of LncRNA CCAT1 in colon cancer cell line SW620, HCT116 and HT29 and the normal colon cell line 293-T were also determined by qRT-PCR. Results showed CCAT1 was highly expressed in SW620 and HT29 (**Figure 2B**). Therefore, we speculated LncRNA CCAT1 have certain relevance with the development and progression of colon cancer.

Knockdown of LncRNA CCAT1 suppressed the proliferation of colon cancer cells

Cell transfection efficiency decides whether subsequent biology research goes well. According to the results of preliminary experiments, we found that the expression of CCAT1 in colon cancer cell line SW620 and HT29 was high and we also calculated the transfection efficiency of siRNA by RT-PCR. Results showed 50 μmol/ml siRNA had the best transfection efficiency. Therefore, cancer cell line SW620 and HT29 at this concentration were taken as models for functional deficiency study. After colon cancer cells were transfected with siRNA for 48 h, the expression level of CCAT1 was significantly downregulated compared with the control (P< *0.05*, **Figure 3A** and **3B**).

The effect of CCAT1 on the proliferation of colon cancer cell was determined by CCK-8 assay. Results showed compared with the control, the proliferation capacity of SW620 and HT29 decreased significantly after transfection with 1 d, 2 d, 3 d, 4 d and 5 d (P<0.05, **Figure 3C** and **3D**). Thus it can be seen that CCAT1 expression had an obvious promoting role on colon cancer cell proliferation.

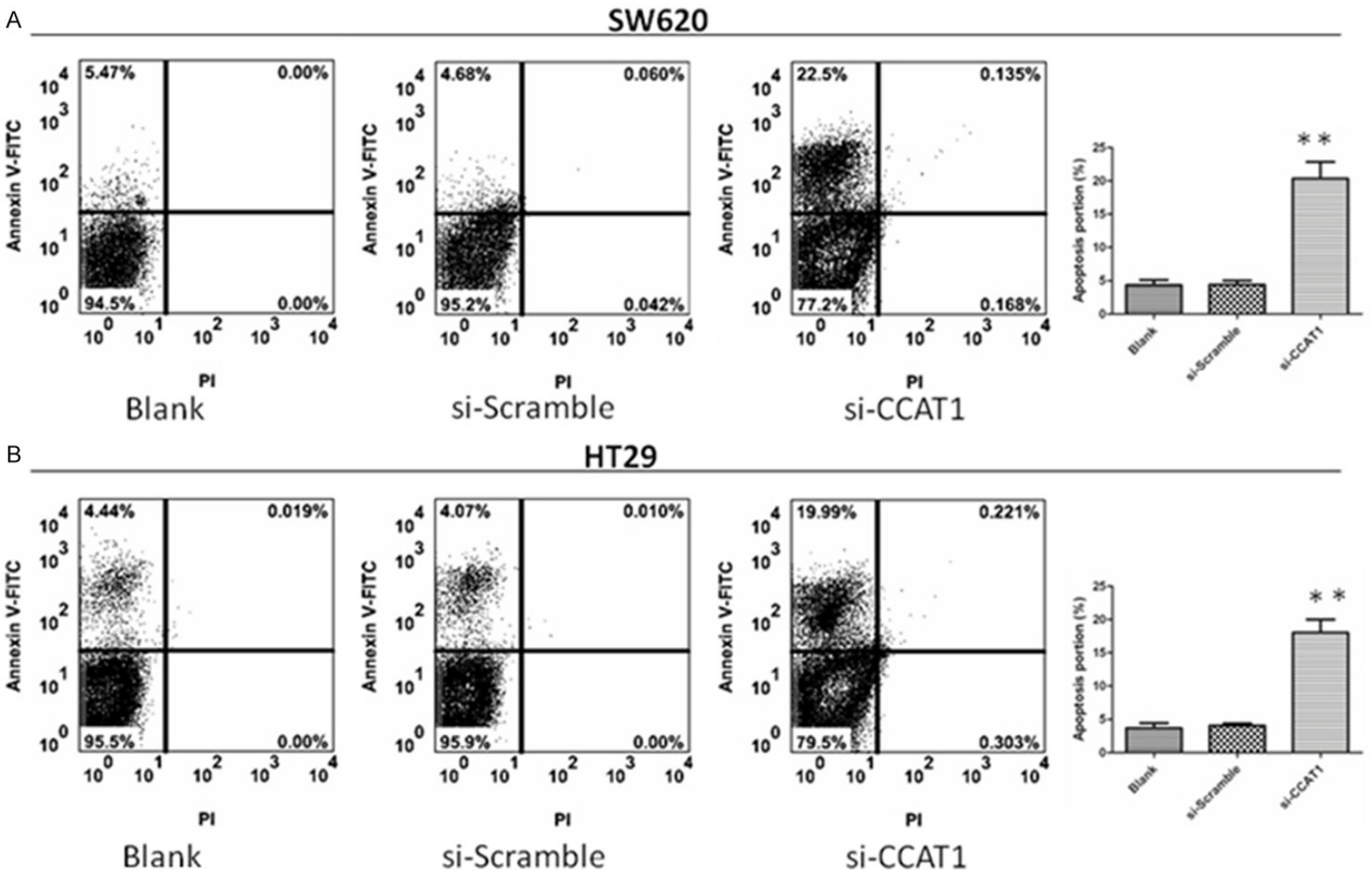

Figure 4. The percent of apoptotic SW620 and HT29 cell in blank, si-Scramble and si-CCAT1 group before and after transfection with siRNA. **P<0.01, compared with the blank group. The percentages of apoptotic SW620 and HT29 cell in si-CCAT1 group had statistical differences.

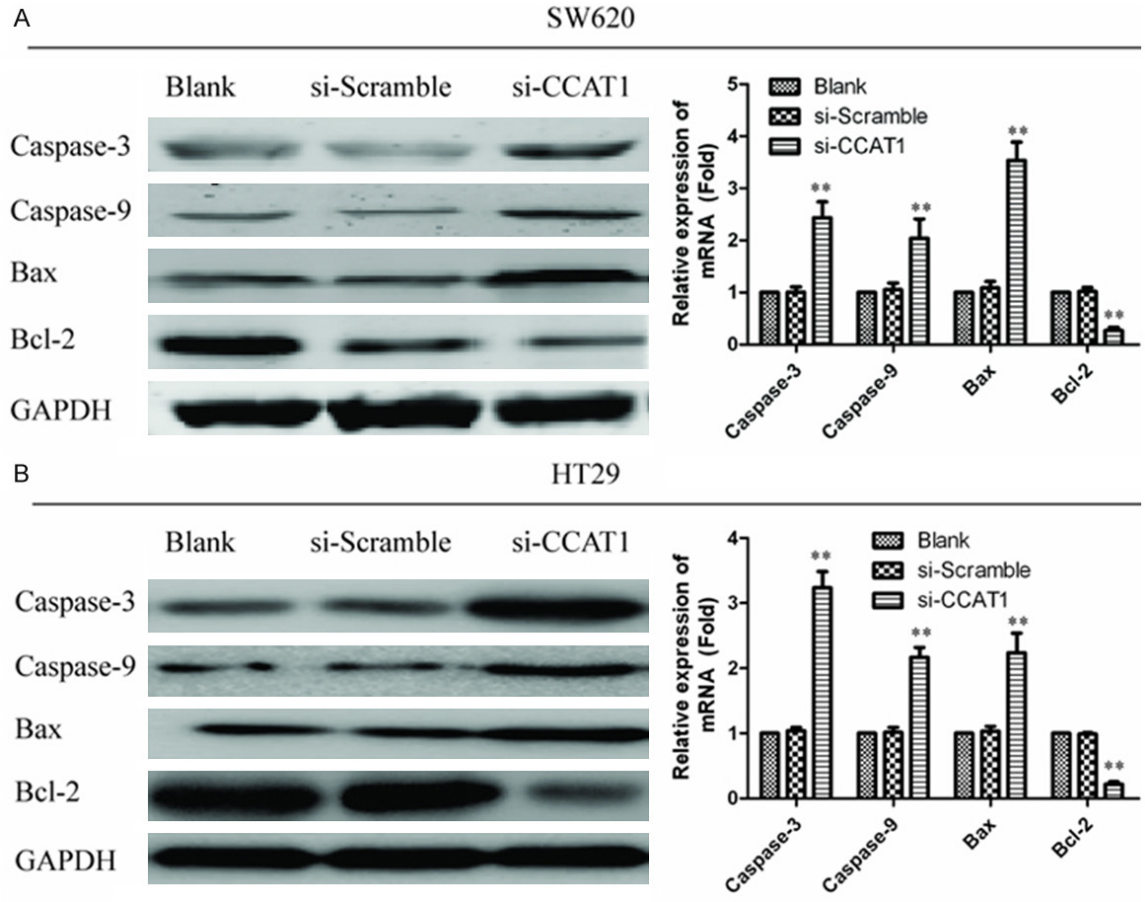

Figure 5. A and B: Inhibition on lncRNA CCAT1 promoted the protein expression of apoptotic protein Bax, caspase 3 and caspase 9, and reduced the level of Bcl-2 in colon cancer cell SW620 and HT29, respectively.

Knockdown of LncRNA CCAT1 promoted the apoptosis of colon cancer cells

To further study whether colon cancer cell proliferation was mediated by inhibiting apoptosis, we determined the cell apoptosis situation of SW620 and HT29 by flow cytometry and the expression of apoptosis related proteins (Bax, Bcl-2, caspase 3 and caspase 9) by western blotting after the expression of CCAT1 was downregulated by transfection with siRNA. Results showed the apoptosis rate was evidently higher blank control and negative control group ($P<0.05$, **Figure 4A** and **4B**). It indicated downregulation of CCAT1 can promote cell apoptosis.

Knockdown of LncRNA CCAT1 influenced the expression of related proteins

To further clarify the effect of low expression of CCAT1 on colon cancer cell apoptosis, we determined the changes of expression levels of several apoptosis related proteins. Results showed the expression of pro-apoptotic protein Bax increased significantly after the knockdown of CCAT1 and the ratio of Bax/Bc1-2 increased about 3 times. In addition, the expression levels of caspase 3 and caspase 9 increased markedly after the knockdown of CCAT1 compared with the control ($P<0.05$, **Figure 5A** and **5B**). Those indicated the downregulation of CCAT1 can promote the apoptosis of colon cancer cells.

Discussion

Colon carcinoma is one of the leading causes of death from cancer and is characterized by a heterogenic pool of cells with distinct differentiation patterns [13]. It has been viewed as the result of progressive accumulation of genetic and epigenetic abnormalities [14]. So far, the most effective treatment for colon carcinoma is

surgery. However, the treatment is not satisfactory and more than one-third of the patients recurred after surgery [15]. Long noncoding RNAs (lncRNAs) are an important class of pervasive genes involved in a variety of biological functions. They are aberrantly expressed in many types of cancers [16]. They play an important role in many important areas of epigenetics, stem cell biology, cancer, signaling and brain function [17]. They are increasingly implicated in cancer biology, contributing to essential cancer cell functions such as proliferation, invasion, and metastasis [18]. Emerging evidences implicate lncRNAs are deregulated in cancer development [19]. are involved in human tumorigenesis and misregulated in many cancers, including colon cancer [20]. Colon Cancer Associated Transcript-1 (CCAT1) is a lncRNA, previously shown to be significantly up-regulated in colon cancer [21]. It was reported can promote the progression of gastric carcinoma and activated by c-Myc [22]. However, the regulatory effect of CCAT1 in colon cancer remains unclear.

In this study, we used LncRNAs PCR array to screen the differential expressed LncRNAs in colon carcinoma tissues compared with those in the matched adjacent non-tumor tissues. Results showed there were 10 different LncRNAs were upregulated while 7 were downregulated in colon carcinoma tissues. According to the previous researches, we screened 6 different lncRNAs for qRT-PCR validation. The results of qRT-PCR validation for 1ncRNA DNM30S, HOTAIR, CCAT1, BCAR4, MIR31HG and HIF1A-AS10 were consistent with that of PCR array. Moreover, previous study showed CCAT1 was upregulated in gastric carcinoma tissue and the upregulated CCAT1 can promote the proliferation and transfer of gastric carcinoma cells. Therefore, CCAT1 was selected for further study. Our study on the mRNA level of CCAT1 in colon carcinoma tissues and the matched adjacent non-tumor tissues also showed CCAT1 was significantly raised in colon carcinoma tissues. To study the effect of CCAT1 on the proliferation and apoptosis of colon carcinoma cells, siRNA was transfected to colon carcinoma cells, and the proliferation and apoptosis of colon carcinoma cells was determined by CCK-8 and flow cytometry, respectively. Results showed the level of CCAT1 was reduced after transfection with siRNA and the knockdown of CCAT1 significantly reduced the proliferation of SW620 and HT29 cells and enhanced the apoptosis of them. The abnormally expressed CCAT1 was reported promote cell proliferation and invasion of hepatocellular carcinoma cells [23]. Zhang et al reported the overexpression of long non-coding RNA CCAT1 is a novel biomarker of poor prognosis in patients with breast cancer [24]. Moreover, C-Myc-activated long noncoding RNA CCAT1 can promote colon cancer cell proliferation and invasion [20]. Then, we determined the expression levels of apoptosis-related proteins. Results showed the inhibition on CCAT1 promoted the protein expression of apoptotic protein Bax, caspase 3 and caspase 9, and reduced the level of Bcl-2 which improved the ratio of Bax/Bcl-2. Pro-apoptotic Bax is a soluble and monomeric protein under normal physiological conditions [25]. The pro-apoptotic protein, Bax, has been reported to translocate from cytosol to mitochondria following exposure of cells to apoptotic stresses [26]. The association of Bax with mitochondria is an essential step in the implementation of apoptosis [27]. Bcl-2 is the prototypical member of a large family of apoptosis-regulating proteins, consisting of blockers and promoters of cell death [28]. Bcl-2 protein together with the pro-apoptotic protein bax, are thought to function by forming homo- and heterotypic dimers which control the progression to apoptosis [29]. Caspase-3 is a frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins [30]. Activation of pro-caspase-3 is a central event in the execution phase of apoptosis and appears to serve as the convergence point of different apoptotic signaling pathways [31]. Caspase-9 is a member of caspase family of cysteine proteases that have been implicated in apoptosis and cytokine processing [32].

All those indicated CCAT1 play an important role in colon cancer. This study help us to understand the molecular mechanism about how CCAT1 influence the proliferation and apoptosis of colon cancer cells which would provide key data on inhibiting the progression of colon cancer.

Disclosure of conflict of interest

None.

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