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Downregulating long non-coding RNA CCAT5 inhibits tumor growth, invasion and metastasis in colorectal cancer through suppressing STAT3

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Abstract. – OBJECTIVE: Recent researches have proved that long noncoding RNAs (lncRNAs) play an important role in tumorigenesis. In this research, lncRNA CCAT5 was explored to identify its role in the development of colorectal cancer (CRC).

PATIENTS AND METHODS: Real time-quantitative polymerase chain reaction (RT-qPCR) was utilized to measure CCAT5 expression of CRC tissues. Besides, function assays including healing assay and transwell assay were conducted. Furthermore, RT-qPCR and Western blot assay were used to explore the underlying mechanism.

RESULTS: By comparison with CCAT5 expression in adjacent tissues, the CCAT5 expression level was significantly higher in CRC samples. Moreover, after CCAT5 was downregulated, cell migration and cell invasion abilities were suppressed. Besides, knockdown of CCAT5, the mRNA and protein expression of STAT3 was repressed. Furthermore, it was found that STAT3 expression was positively correlated to CCAT5 expression in CRC tissues.

CONCLUSIONS: Results suggest that CCAT5 could promote cell migration and invasion of CRC by upregulating STAT3, which may offer a potential therapeutic target in CRC.

Key Words:
Long non-coding RNA, CCAT5, Colorectal cancer, STAT3.

Introduction

Colorectal cancer (CRC) is the third most prevalent malignant tumor and is also the fourth leading cause of cancer-related deaths in the world[1]. Almost 1.36 million cases were newly diagnosed of CRC annually all over the world and nearly 0.6 million cases died because of CRC in 2012[2]. Despite of the technological advances in early detection and intervention for the past decades, the overall survival rate remains disappointing for those patients with advanced stage who eventually develop recurrence and metastasis[3]. Therefore, it's urgent to investigate the underlying molecular mechanisms of tumorigenesis and progression in CRC and figure out new strategies for the early diagnosis and therapy.

Genome sequencing technology has revealed that only 2% of the transcripts encode proteins while most of genome sequence transcripts are non-coding RNAs (ncRNAs). Long noncoding RNAs (lncRNAs) are a subtype of ncRNAs which are longer than 200 nucleotides. Some researches have reported that lncRNAs function as key regulators in numerous biological processes, including the development of diverse cancers. For example, through regulation of miR-34c expression and targeting MUC2, lncRNA AF147447 represses cell proliferation and cell invasion in gastric cancer infected with *Helicobacter pylori*[4]. Downregulated linc-ITGB1 inhibits cell invasion, cell migration, and epithelial-mesenchymal transition in non-small cell lung cancer by decreasing Snail expression[5]. Through regulating the stability of DNMT1 and depressing the expression of tumor suppressors, lncRNA LUCAT1 promotes esophageal squamous cell carcinoma formation and cell metastasis[6]. LncRNA SChLAP1 contributes to the development of aggressive prostate cancer by antagonizing the function of the SWI/SNF complex[7]. However, the role of lncRNA CCAT5 in CRC and its underlying molecular mechanism have not been studied so far.

In this study, we found out that the expression of CCAT5 was remarkably higher in CRC tissues. Moreover, CCAT5 promoted the migra-

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tion and invasion of CRC cells *in vitro*. Moreover, our further experiment explored the underlying mechanism of how CCAT5 functioned in CRC development.

Patients and Methods

Cell Lines and Clinical Samples

A total of 55 CRC patients were enrolled for human tissues who received surgery at The First Affiliated Hospital of Chongqing Medical University. No radiotherapy or chemotherapy was performed before the surgery. The Ethics Committee of The First Affiliated Hospital of Chongqing Medical University approved this study protocol, and all the participants provided the written informed consents.

Cell Culture

Human CRC cell lines (HCT116, HT29, SW620, and SW480) and normal human colonic epithelial cell line (NCM460) were got from the Chinese Academy of Science (Shan[illegible] China). Culture medium consisted of 10[illegible] bovine serum (FBS; Life Technologies, G[illegible]ersburg, MD, USA), Roswell Park Memo[illegible] Institute-1640 (RPMI-1640, HyClone, So[illegible] Logan, UT, USA) as well as p[illegible] Beside[illegible] cells were cultured in an i[illegible]bator[illegible]taining 5% CO_2 at 37°C.

Cell Transfection

Lentivirus e[illegible]ing sho[illegible]pin RNA (shRNA) direc[illegible]g[illegible]t PCAT-1 [illegible]provided by GenePha[illegible] and t[illegible]loned into the pLenti-EF1a-EG[illegible]2A-Puro ve[illegible] (Biosettia Inc., San Diego[illegible], USA). Then, 29[illegible]lls were used for the [illegible]kaging [illegible]e viruses, the CCAT5 lentivirus[illegible]CC[illegible]5) and the empty vector.

Wound Healing Assay

[illegible]er tra[illegible], cells were cultured in RPM[illegible]40 medium overnight. Then, cells were [illegible]h a plastic tip and cultured in serum-free RPMI-1640. Each assay was repeated in triplicate independently. Wound closure was [illegible]ed at 24 h.

Transwell Assay

After transfection, 5×10^4 cells in 200 µL serum-free RPMI-1640 were added to the top chamber of an 8 µm pore size insert (Corning, Corning, NY, USA) with 50 µg Matrigel (BD, Bedford, MA, USA). And RPMI-1640 and FBS were added to the lower chamber. 48 h later, the top surface of chambers was treated by methanol for 30 min after wiped by cotton swab. Then, they were stained in crystal violet for 20 min. The data for i[illegible] membrane was counted in three fields.

RNA Extraction and Real Time-Quantitative Polymerase Chain Reaction (RT-qPC[illegible]

TRIzol reagent (Invi[illegible]en, Carlsbad, USA) was utilized for [illegible]rating [illegible]total R[illegible] By reverse Transcri[illegible]tio[illegible] (T[illegible]a Biot[illegible]chnology Co., Ltd., Da[illegible], Chi[illegible]e total [illegible]A was reverse-transcr[illegible] to cDNA[illegible] 2[illegible] method was utilize[illegible]ulating re[illegible]expression. Following [illegible] the [illegible]ers using for RT-qPCR: CCAT5 [illegible]imers forw[illegible] 5′-GTGACTTCGCCT-GT[illegible]A-3′, reverse[illegible]GGCCTCTATCTG-T[illegible]CTTTATTCC-3″; GAPDH primers forward 5[illegible]CAAAATC[illegible]ATG GGGCAATGCTGG-3′ a[illegible]everse 5′-[illegible]ATGG CATGGACTGTGGT-CA[illegible]A-3′. T[illegible]nocycling conditions were as follow[illegible]95°C, 5 sec for 40 cycles at 95°C, 35 sec at 60°C.

Western Blot Analysis

Protein was extracted from cells by Reagent radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China). Bicinchoninic acid (BCA) protein assay kit was used for quantifying protein concentrations (TaKaRa Biotechnology Co., Ltd., Dalian, China). Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was utilized to separate the target proteins. After replaced to the polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland), they were incubated with antibodies. Rabbit anti-GAPDH and rabbit anti-STAT3 (Cell Signaling Technology, CST, Danvers, MA, USA) were used in this study, as well as goat anti-rabbit secondary antibody (Cell Signaling Technology, CST, Danvers, MA, USA). Image J software (NIH, Bethesda, MD, USA) was applied for assessment of protein expression.

Statistical Analysis

All statistical analyses were carried out with Statistical Product and Service Solutions (SPSS) 20.0 (SPSS, Chicago, IL, USA). The Student's *t*-test was performed. The statistical significance was defined as $p<0.05$.

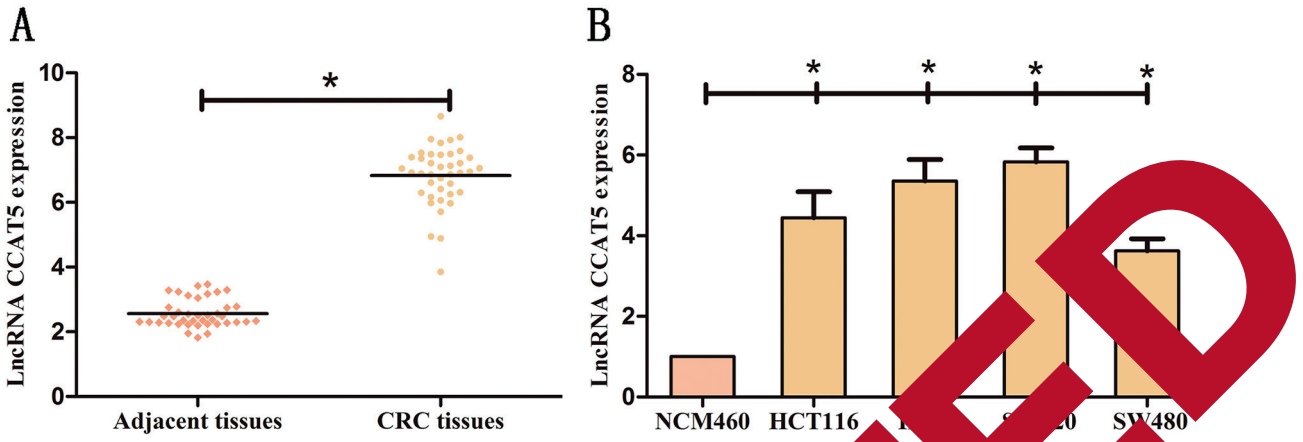

Figure 1. CCAT5 expression in CRC tissues and cells. **A,** CCAT5 expression was sig[illegible]ntly in[illegible]d in the [illegible]tissues compared with adjacent tissues. **B,** Expression levels of CCAT5 were determined [illegible] human CR[illegible] li[illegible]d normal human colonic epithelial cells (NCM460) by RT-qPCR. GAPDH was used as a[illegible]control. Data[illegible]ented as the mean ± standard error of the mean. *p<0.05.

Results

CCAT5 Expression Level in CRC Tissues and Cells

Firstly, CCAT5 expression was detected [illegible] RT-qPCR in 55 patients' tissues and fou[illegible] cell lines. As a result, CCAT5 was signific[illegible]y upregulated in tumor tissue samples (Figure [illegible] The CCAT5 expression level of CRC cells w[illegible] higher than that of normal hu[illegible]c epith[illegible] lial cells (NCM460) (Figure[illegible]

D[illegible]nregulation of CCAT5 Inhibited Cell M[illegible]ration an[illegible]vasion CRC Cells

In this study, [illegible]chose HT29 and SW620 CRC cel[illegible]s for t[illegible]knockdown of CCAT5. Then, CCAT[illegible]on was detected by RT-qPCR (Figure 2A and 2B). Moreover, results of wound [illegible]g assay showed that knockdown of CCAT5 [illegible]ntly repressed the ability of migration in CRC cells (Figure 3A). The outcome of the transwell assay also revealed that the number of invaded cells was remarkably decreased after CCAT5 was knocked down in CRC cells (Figure 3B).

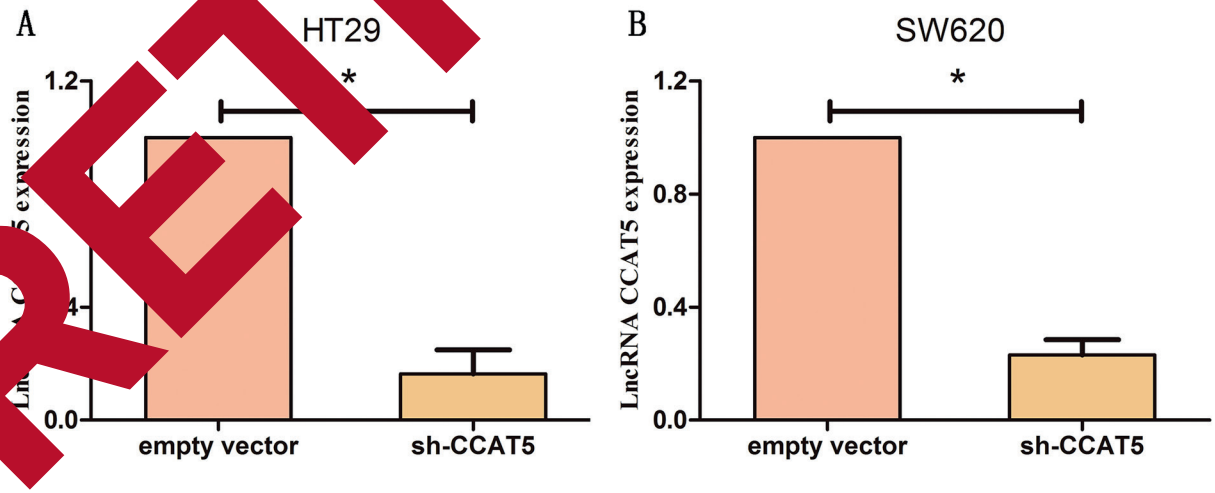

Figure 2. RT-qPCR was used to detect the transfection efficiency in CRC cells. **A,** CCAT5 expression in HT29 CRC cells transfected with CCAT5 lentiviruses (sh-CCAT5) and the empty vector was detected by RT-qPCR. **B,** CCAT5 expression in SW620 CRC cells transfected with CCAT5 lentiviruses (sh-CCAT5) and the empty vector was detected by RT-qPCR. GAPDH was used as an internal control. The results represent the average of three independent experiments (mean ± standard error of the mean). *p<0.05.

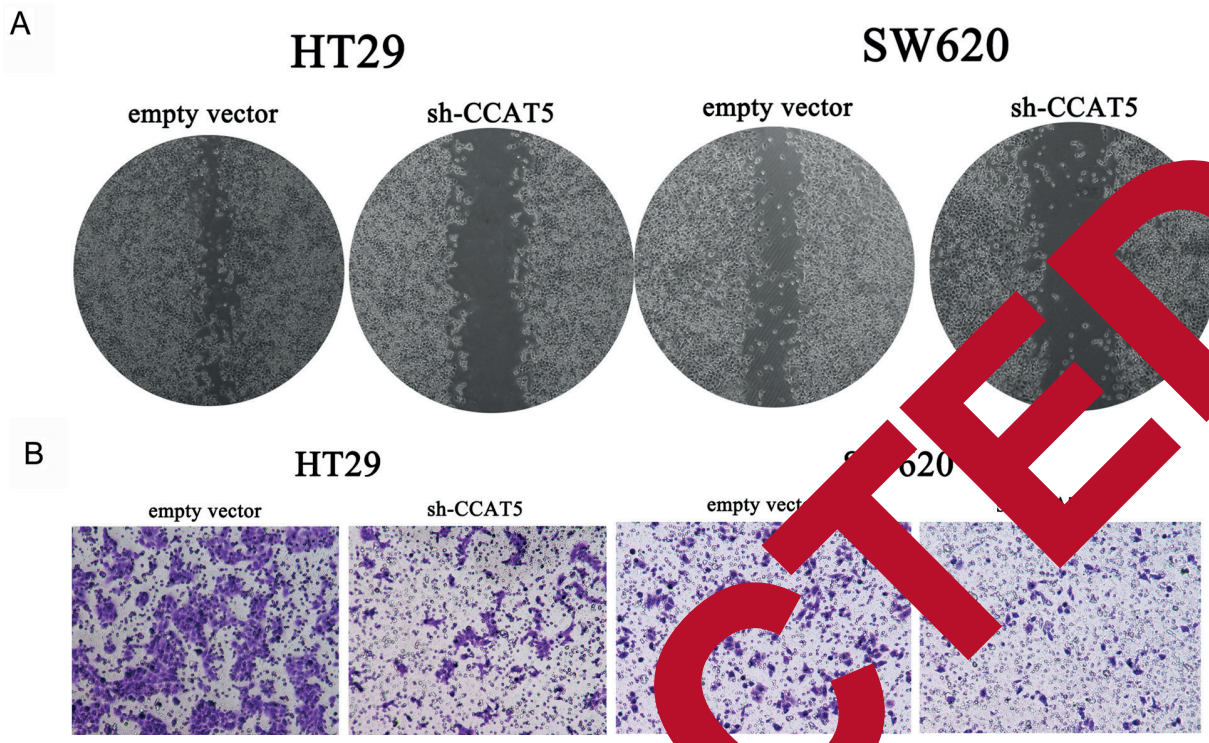

Figure 3. Downregulation of CCAT5 repressed CRC cell migration [illegible], Wound healing assay showed that knockdown of CCAT5 significantly reduced cell mig[illegible] in CRC cells ([illegible]n: 10×). **B,** Transwell assay showed that number of invaded cells was significantly decreased [illegible]n of CCAT5 in CRC cells (magnification: 40×). The results represent the average of three independent experimen[illegible]ean [illegible]d error of the mean). *$p<0.05$.

The Interaction Betwee[illegible]AT3[illegible]d CCAT5 in CRC

The RT-qPCR resul[illegible] sh[illegible]d th[illegible] sion level of STAT[illegible] CRC[illegible] was signifi-cantly lower in C[illegible]5 lentivir[illegible](sh-CCAT5) group when c[illegible]a[illegible]with the S[illegible] level in empty vecto[illegible]oup (F[illegible]4A). The result of the Western l[illegible]assay showe[illegible]t after CCAT5 was knock[illegible]own, STAT3 co[illegible]e downregulated at t[illegible]protein[illegible]el (Figure 4B). Furthermore, we [illegible]d th[illegible]TAT3 expression in CRC tissues was re[illegible]ly hig[illegible]hen compared with that [illegible]jace[illegible]sue[illegible]igure 4C). The correlation an[illegible]is de[illegible]ated that STAT3 expression le[illegible]was posit[illegible]ely correlated to CCAT5 expres-[illegible]issues (Figure 4D).

Discussion

Compelling evidence has suggested that lncRNAs play a crucial role in the carcinogenesis of CRC by the regulation of various cell biological behaviors. For instance, by the modulation of the cell proliferation activity and ERK/COX-2 pathway, lncRNA CCHE1 functions as an important oncogene in the development and progression of CRC[8]. LncRNA TP73AS1 markedly promotes cell apoptosis and depresses cell proliferation in CRC by functioning as a competing endogenous RNA for miR103 and further modulate the expression of PTEN[9]. LncRNA RUNX1-IT1 acts as a tumor suppressor in CRC by the inhibition of cell migration and cell proliferation and could function as a novel diagnostic biomarker[10]. Also, through SIRT1 mediated autophagy, lncRNA H19 promotes 5-Fu resistance in CRC by sponging to miR-194-5p[11].

CCAT5, also known as MNX1-AS1, is a newly discovered lncRNA which has been reported to promote malignancy in cancers. For instance, CCAT5 facilitates the development of cervical cancer by activating MAPK pathway[12]. Through altering expressions of CDK4, cyclin D, Bax, and Bcl-2, CCAT5 functions as an oncogene in ovarian cancer[13]. CCAT5 promotes also cell proliferation, cell invasion, and cell migration in glioblastoma progression by the inhibition of miR-4443[14].

We found that CCAT5 was upregulated both in CRC samples and CRC cells. Besides, knockdown of CCAT5 repressed cell migration and invasion in CRC cells. The above results indicated that CCAT5 promoted tumorigenesis of CRC and might act as an oncogene.

As a mutation of the signal transducer and activator of transcription (STAT) factors, STAT3 has been demonstrated to be expressed in various cell types and plays a crucial role in tumorigenesis[15,16]. For example, STAT3 is activated in more than 40% of breast cancers and promotes the development of breast tumor by regulating downstream target genes[17]. The upregulation of STAT3 promotes tumor progression and cell metastasis in ovarian cancer and exhibits a potential therapeutic target for ovarian cancer[18]. By inhibition of WP1066, the STAT3 signaling pathway depresses the growth and invasiveness of bladder cancer cells[19]. Moreover, IL-6/JAK/STAT3 pathway plays a crucial role in the progression of CRC, which may help to offer potential therapeutic approaches[20]. In the present work, we first discovered the interaction between STAT3 and CCAT5. The results showed that the expression level of STAT3 could be downregulated after knockdown of CCAT5. Furthermore, S... expression in CRC tissues was positively ... CCAT5 expression. All the results ab... suggested that CCAT5 might promote tumorig... of CRC through upregulating STAT3.

Con... ons

Above data ide... ed th... AT5 w... mark-ably upregulat... CRC tissu... esi... CCAT5 could facili... migration ... nvasion in CRC throu... upre... ing STAT... These find-ings su... that CCA... ay contribute to thera-py f... as a candida... get.

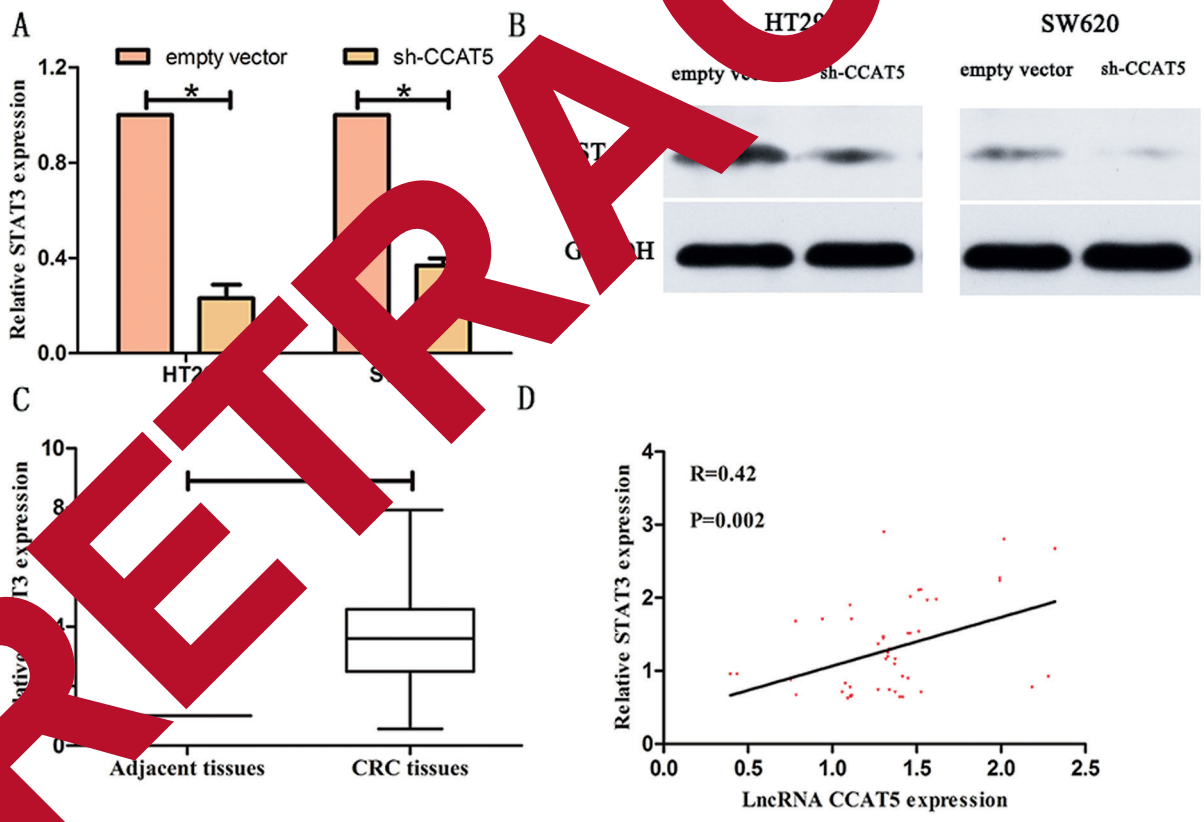

Figure 4. Interaction between CCAT5 and STAT3. **A,** RT-qPCR results showed that STAT3 expression was decreased in CCAT5 lentiviruses (sh-CCAT5) compared with the empty vector. **B,** Western blot assay revealed that STAT3 protein expression was decreased in CCAT5 lentiviruses (sh-CCAT5) compared with the empty vector. **C,** STAT3 was significantly upregulated in CRC tissues compared with adjacent tissues. **D,** The linear correlation between the expression level of STAT3 and CCAT5 in CRC tissues. The results represent the average of three independent experiments. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

Conflict of interest

The authors declare no conflicts of interest.

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