Long noncoding RNA colon cancer associated transcript-1 promotes the proliferation, migration and invasion of cervical cancer

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Abstract. Previous studies have revealed significant roles for long noncoding RNA (lncRNA) in the tumorigenesis, metastasis and invasion of various tumors, including cervical cancer. The present study aimed to investigate the potential roles of lncRNA colon cancer associated transcript 1 (CCAT1) in the metastasis and invasion of cervical cancer, and to reveal the potential underlying mechanism. The mRNA expression of lncRNA CCAT1 in cervical cancer tissue was measured using the reverse transcription-quantitative polymerase chain reaction, and the association between lncRNA CCAT1 and the metastasis of cervical cancer was analyzed. The effects of lncRNA CCAT1 expression on HeLa cell viability, and migration and invasion were also analyzed by MTT and Transwell assays. The results demonstrated that lncRNA CCAT1 was highly expressed in the cervical cancer tissue compared with the adjacent normal tissue. High expression of lncRNA CCAT1 was positively associated with tumor size, and there was correlation between high lncRNA CCAT1 expression and a poor survival rate of cervical cancer. The cell viability, and migratory and invasive abilities were suppressed by silencing CCAT1. The results of the present study indicate that lncRNA CCAT1 was highly expressed in cervical cancer, and may serve important roles in promoting the progression and metastasis of cervical cancer.

Introduction

Cervical cancer remains one of the most common female malignancies worldwide (1), and is characterized by

migration and invasion (2), resulting in a poor 5-year survival rate (3). Therefore, the development of novel methods for the improvement of cervical cancer treatment and prognosis is vital for improving the diagnosis and treatment of cervical cancer.

Long noncoding RNAs (lncRNAs; >200 nucleotides) have been demonstrated to exhibit crucial roles in tumorigenesis, migration and invasion in recent years (4,5). For example, the upregulated lncRNA HOX transcript antisense RNA (HOTAIR) promotes the progression of hepatocellular cancer (6), and lncRNA H19 promotes the tumorigenesis and metastasis of gastric cancer (7). Generally, the underlying mechanism of lncRNA gene expression regulation may be at the transcriptional or post-transcriptional level (8). To date, several lncRNAs, including focally amplified lncRNA in epithelial cancer (FAL1), HOTAIR, metastasis-associated lung adenocarcinoma transcript-1 and cervical carcinoma expressed proliferating cell nuclear antigen regulatory lncRNA, have been reported to serve pivotal roles in the progression or metastasis of cervical cancer (9-12). lncRNA colon cancer associated transcript 1 (CCAT1) is highly expressed in colon cancer tissue, and has been reported to be useful in the detection of colon cancer and tumor-associated tissues (13). The roles of lncRNA CCAT1 in the biology of tumors has only been reported in a number of tumor types (14,15). The proliferation and invasion of colon cancer cells was promoted by the c-myc-mediated activation of lncRNA CCAT1 (16). Ma *et al* (17) demonstrated that lncRNA CCAT1 promotes the development of gallbladder cancer by negatively modulating the expression of miR-218-5p. These previous studies indicate that lncRNA CCAT1 may function as an oncogene in tumors. However, few studies have reported the association between lncRNA CCAT1 expression, and the progression and development of cervical cancer.

The present study investigated the expression of lncRNA CCAT1 in cervical cancer tissue, and analyzed the association between lncRNA CCAT1 expression and HeLa cell viability, metastasis and invasion. The present study aimed to investigate the potential roles of lncRNA CCAT1 in the metastasis of cervical cancer and to identify the potential underlying mechanism.

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Materials and methods

Patients and samples. A total of 30 patients aged 23–62 years who were diagnosed with cervical cancer at Hebei Provincial People's Hospital (Shijiazhuang, China) between June 2014 and December 2015 were enrolled in the present study. Informed consent was obtained from all patients and all procedures were approved by the Protection of Human Ethics Committee of the Hebei Provincial People's Hospital. The cervical cancer diagnosis was pathologically confirmed, and cancer tissues and adjacent normal tissue were obtained from clinically ongoing surgical specimens with informed consent. Tissues size was measured and all of the samples were snap-frozen with liquid nitrogen and stored at -80°C until RNA extraction.

Cell culture and transfection. HeLa human cervical cancer cells (European Collection of Authenticated Cell Cultures; Public Health England; Salisbury, UK) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) at 37°C in an atmosphere containing 5% CO_2 . The full-length of CCAT1 encoding sequences were amplified by using the PfuUltra II fusion HS DNA Polymerase (Agilent Technologies, Inc., Santa Clara, CA, USA). The thermocycling conditions were: 95°C 45 sec, 60°C 15 sec, 95°C 30 sec, 30 cycles. Then CCAT1 sequences were cloned into pcDNA3.1 vector (Invitrogen; Thermo Fisher Scientific, Inc.) as pcDNA3.1-CCAT1. The experimental procedures were performed as previously described (18). Primers used for CCAT1 amplification were 5'-CTAGCTAGCACAACATCGACTTTGAAGTT-3' (forward) and 5'-CCCAAGCTTAAGACTTAATATACTTATATTTA-3' (reverse). The small interfering (si)RNA for CCAT1 was designed and synthesized by RiboBio (Guangzhou RiboBio Co., Ltd., Guangzhou, China), and the sequence was as follows: CCAT1 siRNA (si-CCAT1: 5'-CAUUAACCUGCUAUCCUCUUUACAA-3'). Si-CCAT1 was transfected into HeLa cells for 48 h by using the Lipofectamine 2000[®] (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Cells without transfections were used as a negative control.

MTT assay. Effects of lncRNA CCAT1 expression on cell proliferation were assessed using MTT assay as previously described (19). Cells cultured in DMEM medium containing 10% FBS at logarithmic stage (5×10^3) were transfected in 96-well plates. After 24 h cultivation, supernatant was removed and MTT (20 μl) was added every 24 h and incubated for 4 h. Subsequently, 150 μl dimethyl sulfoxide was added to the cells for 10 min. Absorbance of cells in each well was observed at 570 nm using an absorption spectrophotometer (Olympus Corporation, Tokyo, Japan).

Colony assay. HeLa cells were seeded onto 10 cm plates at a density of 1×10^3 cells/well. Cells were treated and allowed to grow for 14 days until visible clones appeared. Cell colony formation was detected by staining with 0.1% crystal violet solution, air dried and counted.

Cell migration and invasion assays. Cell migration and invasion assays were performed using Transwell migration chambers

(8 μm pore size). The membranes for the invasion assay were coated with a diluted extracellular matrix (Matrigel, consisting of the laminin and type IV collagen) solution (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Cells were seeded in the upper chamber at a density of 5×10^4 cells/well with serum-free medium following transfection. Medium containing 10% FBS, served as a chemoattractant in the lower chamber. After 24 h incubation at 37°C , non-invasive/migratory cells on the top of the membrane were scraped and removed by cotton swabs, and the invasive/migratory cells were fixed in 4% methanol (NIST, USA) for 30 min at room temperature. Then 0.1% crystal violet (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used to stain for 20 min at room temperature and subsequently five randomly selected fields of view were counted using light microscopy at 400x magnification.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from cervical cancer tissue was extracted using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.), followed by treatment with RQ1 RNase-free DNase I (Promega Corporation, Madison, WI, USA). Purified RNA mixed with nuclease-free water was used for cDNA synthesis using the reverse transcriptase PrimerScript 1st Strand cDNA Synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.). Expression was detected in an Eppendorf Mastercycler using the SYBR ExScript RT-PCR kit (Takara Biotechnology, Co., Ltd., Dalian, China) according to the manufacturer's protocol. The reaction procedure was as follows: 95°C 45 sec, 95°C 15 sec, 60°C 15 sec, 45 cycles. All the tests were repeated 3 times, and the relative quantitative analysis by $2^{-\Delta\Delta\text{Ct}}$ method for the determination of lncRNA CCAT1 changes (20). Primers used were: lncRNA CCAT1, 5'-TTTATGCTTGAGCCTTGA-3' (forward) and 5'-CTTGCCTGAAATACTTGC-3' (reverse); internal GAPDH primer: 5'-TGTTGCCATCAATGACCCCTT-3' (forward) and 5'-CTCCACGACGTACTCAGCG-3' (reverse). GAPDH was selected as the internal control.

Statistical analysis. All experiments were repeated three times independently. Data are presented as the mean $\pm$ standard deviation. Statistical analysis was performed using SPSS 19.0 software (IBM SPSS, Armonk, NY, USA). Significant differences were calculated using one-way analysis of variance followed by Dunnett's test. The correlation analysis was performed by using log rank to compare survival curves. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of lncRNA CCAT1 in cervical cancer tissue. The present study analyzed the relative mRNA expression of lncRNA CCAT1 in cervical cancer tissue by RT-qPCR analysis. The results demonstrated that lncRNA CCAT1 exhibited increased expression in the cervical cancer tissue compared with the adjacent tissue ($P < 0.05$; Fig. 1A). Consequently, the association between relative mRNA expression of lncRNA CCAT1 in tumor tissue and tumor size was also analyzed, and the results demonstrated that as the expression of lncRNA CCAT1 increased the tumor size increased (Fig. 1B). In

Figure 1. Expression of lncRNA CCAT1 in cervical cancer tissue. (A) lncRNA CCAT1 was highly expressed in cervical cancer tissue compared with adjacent normal tissue. * $P < 0.05$ vs. adjacent normal tissue. (B) lncRNA CCAT1 expression was positively associated with tumor size in cervical cancer. (C) High lncRNA CCAT1 expression was associated with a poor survival rate in cervical cancer. lncRNA, long noncoding RNA; CCAT1, colon cancer associated transcript 1.

Figure 2. lncRNA CCAT1 promotes the proliferation of HeLa cells. (A) lncRNA CCAT1 expression was increased in cells transfected with the overexpression plasmid and reduced in cells transfected with si-CCAT1, compared with control cells. (B) lncRNA CCAT1 overexpression increased cell viability and silencing of lncRNA CCAT1 reduced cell viability compared with control cells. (C) HeLa cell colonies were stained with 0.1% crystal violet. (D) Number of HeLa cell colonies counted by crystal violet staining. The number of colonies was significantly increased by overexpression of lncRNA CCAT1 and significantly reduced by silencing lncRNA CCAT1. * $P < 0.05$ vs. control. lncRNA, long noncoding RNA; CCAT1, colon cancer associated transcript 1; si-CCAT1, small interfering RNA against CCAT1; OD, optical density.

addition, the correlation between survival and lncRNA CCAT1 expression was also investigated, and the results demonstrated that high lncRNA CCAT1 expression was associated with a decreased survival rate compared with low CCAT1 expression (Fig. 1C). The results may indicate that the expression of lncRNA CCAT1 is positively associated with the tumor size, and associated with a poor survival rate in patients with cervical cancer.

Effects of lncRNA CCAT1 expression on HeLa cell viability in vitro. lncRNA CCAT1 was overexpressed and silenced in

HeLa cells (Fig. 2). Expression of lncRNA CCAT1 was significantly increased following pcDNA3.1-CCAT1 transfection compared with the control, while it was significantly reduced by transfection with si-CCAT1 transfection compared with the control ($P < 0.05$; Fig. 2A). Subsequently, the viability of HeLa cells was analyzed, and results demonstrated that cell viability was increased by lncRNA CCAT1 overexpression, while it was reduced by silencing CCAT1 expression compared with control cells (Fig. 2B), indicating a positive association between lncRNA CCAT1 expression and HeLa cell viability. In addition, the colony assay also revealed that lncRNA

Figure 3. Effects of lncRNA CCAT1 expression on the migration and invasion of HeLa cells. (A) The number of migratory cells was significantly increased by lncRNA CCAT1 overexpression and significantly reduced by silencing CCAT1 compared with control cells. (B) The number of invasive cells was significantly increased by lncRNA CCAT1 overexpression and significantly reduced by silencing CCAT1. * $P < 0.05$ vs. control. lncRNA, long noncoding RNA; CCAT1, colon cancer associated transcript 1; si-CCAT1, small interfering RNA against CCAT1.

CCAT1 overexpression increased the number of HeLa cell colonies compared with the control (Fig. 2C and D).

lncRNA CCAT1 overexpression promotes migration and invasion in HeLa cells. The influence of lncRNA CCAT1 expression on HeLa cell migration (Fig. 3A) and invasion (Fig. 3B) was investigated. The number of migratory or invasive cells was significantly increased by CCAT1 overexpression compared with the control group ($P < 0.05$), while the number of migratory or invasive cells was decreased by silencing CCAT1, indicating that lncRNA CCAT1 expression was positively associated with cell migration and invasion in HeLa cells.

Discussion

Increasing evidence has demonstrated that lncRNAs serve pivotal roles in the biology of various tumors, including cervical cancer (21). Previous studies have demonstrated that lncRNA CCAT1 is abundantly expressed in several types of cancer, including colon and gastric cancer (14,15). The present study analyzed the expression of lncRNA CCAT1 in cervical cancer tissue, and investigated the potential roles of lncRNA CCAT1 in the development and metastasis of cervical cancer. The results of the present study revealed that lncRNA CCAT1 was highly expressed in cervical cancer tissue and that its expression was positively associated with tumor size, indicating that the abnormal expression of lncRNA CCAT1 may be associated with cervical cancer.

In addition, the present study investigated the influence of lncRNA CCAT1 expression on the proliferation of HeLa cells and the results demonstrated that silencing lncRNA CCAT1 reduced the viability of HeLa cells compared with control HeLa cells. The roles of lncRNA CCAT1 in cervical cancer have not, to the best of our knowledge, previously

been completely investigated. However, previous studies have demonstrated that colon cancer cell proliferation is promoted by the overexpression of lncRNA CCAT1 (22,23). Similarly, Wang *et al* (24) demonstrated that upregulation of lncRNA CCAT1 promoted cell proliferation in esophageal squamous carcinoma and Liang *et al* (25) reported that lncRNA CCAT1 promoted the progression of hepatocellular carcinoma by a complex mechanism. Based on the results of the current study, we hypothesize that lncRNA CCAT1 upregulation may enhance cell proliferation in cervical cancer.

The present study also examined the effects of abnormal lncRNA CCAT1 expression on the migration and invasion of HeLa cells. Cell migration and invasion are involved in the metastasis of tumors (26). lncRNA CCAT1 was previously demonstrated to have crucial roles in the proliferation and invasion in various types of tumor, including colon cancer and hepatocellular cancer (22,27). Additionally, previous studies have revealed that several lncRNAs serve roles in the invasion and migration of cervical cancer cells, including FAL1, MALAT1 and LET lncRNAs (10,28,29). The results of the present study revealed that HeLa cell migration and invasion was suppressed by silencing lncRNA CCAT1, indicating that abnormal expression of lncRNA CCAT1 may be associated with cervical cancer migration and invasion.

In conclusion, the results of the present study revealed that lncRNA CCAT1 was upregulated in cervical cancer tissues, and that the abnormal expression of lncRNA CCAT1 may increase the progression and metastasis of cervical cancer by affecting cell proliferation, migration and invasion. The present study may provide a theoretical basis for the potential role of lncRNA CCAT1 as an oncogene in cervical cancer. Further studies are required to investigate the signaling pathways underlying the effects of abnormal lncRNA CCAT1 expression on cervical cancer in depth.

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