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Long non-coding RNA CCAT2 promotes cholangiocarcinoma cells migration and invasion by induction of epithelial-to-mesenchymal transition

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ABSTRACT

Cholangiocarcinoma (CCA) is one of the most aggressive malignancies in humans. Emerging evidence has indicated that abnormally expressed long non-coding RNAs (lncRNAs) could conduce to tumorigenesis and progression. Specifically, colon cancer-associated transcript 2 (CCAT2) has been reported to be overexpressed in several carcinomas. However, its clinical significance and functional roles in CCA is still unknown. qRT-PCR experiments were conducted to assess the CCAT2 expression in CCA tissue samples and cell lines. In addition, the link between CCAT2 expression and clinicopathological characteristics was analyzed. The potential effects of CCAT2 in CCA cells was evaluated *in vitro* including cell proliferation, colony-forming ability, apoptosis, migration, invasion and epithelial-to-mesenchymal transition (EMT). As a result, CCAT2 was aberrantly overexpressed in CCA tissue samples and cells, and this upregulation was correlated with tumor size, lymph node invasion, TNM stage and postoperative recurrence in CCA patients. Overexpression of CCAT2 could serve as an independent prognostic indicator for CCA. Additionally, overexpression of CCAT2 was a dismal prognostic indicator for patients with CCA. Furthermore, CCAT2 silencing caused tumor suppressive effects via reducing cell proliferation, migration and invasion, inducing cell apoptosis and reversing the EMT process in HuCCT1 and CCLP1 cells. Collectively, our data illustrated that lncRNA CCAT2 played an oncogenic role in CCA and may offer a potential therapeutic target for treating this fatal disease.

1. Introduction

Cholangiocarcinoma (CCA) is arisen from biliary epithelial cells lining the biliary tract and the second most common primary hepatobiliary carcinoma [1,2]. Approximately 80–90% of CCA are derived from extrahepatic bile duct. Extrahepatic CCA (ECC) can be further divided into perihilar and distal tumors according to their location [3]. This fatal disease was frequently progressed to advanced stages at the time of clinical presentation and the recurrence rate is high [4,5]. Surgical procedure as well as chemoradiotherapy has become the main approaches for treating CCA. Although these therapeutic methods may prolong lifespan and alleviate patient suffering, the prognostic outcome for CCA remains unfavorable [6,7]. Thus, it is urgent to find novel diagnostic and therapeutic targets for patients with CCA.

Long noncoding RNAs (lncRNAs) are defined as transcripts more than 200 nt in length and function as pivotal regulators of cellular transcription [8]. LncRNAs can regulate gene expression by acting as a 'sponge' to absorb miRNAs to participate in post-transcriptional processing [9,10] or regulating downstream effectors by EZH2-driven H3K27 methylation [11]. Recently, several lncRNAs are known as imperative factors involving in tumorigenesis and progression [12,13]. Colon cancer-associated transcript 2 (CCAT2), a novel lncRNA mapping to 8q24 genomic region, regulates metastatic progression and chromosomal instability in colon cancer [14]. Some investigations demonstrated that CCAT2 can also facilitate the metastasis and invasion of hepatocellular carcinoma [15], gastric cancer [16] and prostate cancer [17]. However, the expression levels, clinical features and biological functions of CCAT2 in CCA have not been characterized. This lack of data prompted us to investigate the role of CCAT2 in CCA.

In the study, the expression levels of CCAT2 in CCA tissues and matched non-tumorous tissues were determined by qRT-PCR experiment. The association of CCAT2 expression with clinicopathological features was explored. In addition, cell proliferation, apoptosis, migration, invasion and epithelial-to-mesenchymal transition (EMT) after silencing of CCAT2 were assessed. Collectively, our findings illuminate the pivotal role of CCAT2 in tumorigenesis and progression of CCA and shed light on the lncRNA-directed diagnostics and therapeutics in CCA.

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2. Materials and methods

2.1. Patients and sample collection

All of the tissue specimens were obtained from patients who underwent surgery at the Second Affiliated Hospital of Harbin Medical University between 2010 and 2012. This study was carried out with the approval of the Ethics Review Committees of Harbin Medical University (No.HMUIRB20170015). All the patients recruited in the study provided informed consent before this study. CCA tissue samples and paired adjacent non-tumorous bile duct tissue samples were snap-frozen in liquid nitrogen immediately after resection. Clinic-pathological follow-up data for the patients were collected.

2.2. Cell lines and culture

Human CCA cell lines including RBE and HCCC-9810 cells were acquired from the Cell Bank of Type Culture of the Chinese Academy of Sciences (Shanghai, China). QBC939, CCLP-1, Huh-28, HuCCT1 and KMBC and Human intrahepatic biliary epithelial cell (HIBEC) were preserved in our laboratory. Cells were maintained in RPMI-1640 (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Invitrogen Life Technologies, Carlsbad, CA, USA) in a humidified atmosphere at 37 °C and 5% CO_2.

2.3. qRT-PCR analysis and cell transfection

Total RNA was extracted from CCA tissue specimens and cells by using Trizol (Thermo Fisher Scientific, Waltham, MA, USA). The Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany) was used to reverse transcribe RNA into cDNA. FastStart Universal SYBR Green Master Kit (Roche, Germany) and a BIO-RAD C1000 Thermal Cycler were used for qRT-PCR experiments. GAPDH was used as an internal control. All primer sequences are summarized below: CCAT2 primers forward: 5′- AGACAGTGCCAGCCAACC-3′, reverse: 5′-TGCCA AACCCTTCCCTTA-3′; GAPDH primers forward: 5′-GGGAGCCAAAAGG GTCAT-3′, reverse: 5′-GAGTCCTTCCACGATACCAA-3′. Small interfering RNAs (siRNAs) which targeted CCAT2 and a si-NC (scrambled nonspecific control) were purchased from GenePharma (Shanghai, China). Lipofectamine 3000 (Thermo Fisher Scientific, USA) was used for transfection. The sequences of the siRNAs are listed below: si-CCAT2-1, 5′-GUGCAACUCUGCAAUUUAAUU-3′; si-CCAT2-2, 5′-CCAG GCAAUAAC UGUGCAAUU-3′; si-CCAT2-3, 5′-UUAACCUCUUCCUAUC UCAUU-3′ (Fig. S1A). Cells were harvested for further analyses at 48 h after transfection. Fluorescently labeled siRNAs were examined by flow cytometry to detect the transfection efficiency.

2.4. Cell counting kit-8 (CCK-8) assays

To examine the proliferation of HuCCT1 and CCLP1 cells after transfected with corresponding siRNA, CCK-8 assay was performed. Transfected cells were placed in 96-well plates at a density of 1500 cells per well. CCK-8 solution (Dojindo, Japan) was added to each well at 0, 24, 48, 72 and 96 h and incubated for 2 h at 37 °C atmosphere. The absorbance of each well at 450 nm was quantified and recorded by a microplate reader (Tecan, Männedorf, Switzerland).

2.5. Colony formation assays

Colony formation assays were used to assess clonogenic ability of transfected cells. A particular number of transfected cells were trypsinized in a single-cell suspension and planted in six-well dishes. Afterwards, the cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum for about 2 weeks. The visible colonies were fixed in paraformaldehyde and stained with crystal violet (Beyotime, Beijing, China) before manually counted under a light microscope (Olympus Corp., Tokyo, Japan).

2.6. Flow cytometry analyses of cell apoptosis

For the analysis of apoptosis, transfected cells in the exponential growth phase were trypsinized and washed thrice with phosphate-buffered saline (PBS) before adding Annexin V-FITC binding buffer. Then, Annexin V and propidium iodide (Beyotime, Haimen, China) were added to the cell suspension and incubated for 15 min at room temperature in the dark. At last, the stained cells were analyzed by using flow cytometry (FACScan, BD Biosciences).

2.7. Relative caspase activity determination

Caspase-3 and -9 were analyzed using the Caspase-3 Activity Kit and Caspase-9 Activity Kit (Solarbio, Beijing, China) at 48 h after transfection. In Brief, cell proteins were extracted and added to 96-well plates with 90 μL of the provided reaction buffer and caspase substrate. Reaction mixtures were measured by a microplate reader (Tecan, Switzerland) at 405 nm wavelengths after incubated at 37 °C for 4 h.

2.8. Scratch wound assays

Wound healing assays were carried out to measure cell motility after transfection. A scraped, acellular area was created by a 200 μL pipette tube. Photographs were taken at 0 h and 36 h to measure the motility of each group. Migration was quantified by counting the average distance that cells migrated towards the original wound field.

2.9. Transwell assays

BD 24-well transwell unit (Costar, Washington, DC, USA) was used to examine cell migration or invasion capacities followed by the manufacturer's directions. For the migration assay, cells (5×10^4 cells per well) suspended in 0.2 ml fetal bovine serum-free medium were placed in the upper compartment of inserts. While, complete medium was filled in the lower compartment of 24-well plates. After incubating 24 h at 37 °C with 5% CO_2, the cells attached to the lower membrane surface were fixed in 4% paraformaldehyde and stained with crystal violet solution (Beyotime, Beijing, China). For the invasion assay, Matrigel (BD Biosciences, San Jose, CA, USA) was coated in the top filter of the transwell unit and placed in an incubator at 37 °C for 4 h to form a reconstructed basement membrane. The methods used were identical to those applied to the migration assay.

2.10. Western blot assays

Western blot assays were performed as previously described [18]. E-cadherin, vimentin and GAPDH antibodies were obtained from Abcam (Cambridge, MA, USA). The HRP-conjugated secondary antibody was purchased from Cell Signaling Technology (Danvers, MA, USA).

2.11. Statistical analysis

Statistical analyses were analyzed by using GraphPad Prism 5.01 software (GraphPad software, La Jolla, CA, USA) and SPSS 19.0 statistical software package (IBM, Armonk, NY, USA). Significant differences between groups were evaluated by using Student's *t*-tests. The results are reported as the means ± standard deviation based on at least three replicates. The link between CCAT2 expression and clinicopathologic characteristics was calculated by Fisher's exact tests. Survival curves were evaluated by Kaplan-Meier analyses and log-rank tests. Statistical significance was set at $P < 0.05$.