

Original Article

CCAT1 promotes laryngeal squamous cell carcinoma cell proliferation and invasion

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Abstract: Long noncoding RNAs (lncRNAs) are proved to be deregulated in a number of cancers and play important roles in tumor biology. Colon cancer-associated transcript-1 (CCAT1) was initially found in colon cancer and promoted cancer cell development. However, the role of CCAT1 in laryngeal squamous cell carcinoma (LSCC) is still unknown. In this study, we demonstrated that CCAT1 expression was upregulated in the LSCC tissues compared with the normal adjacent tissues. Moreover, CCAT1 expression was higher in LSCC tissues than in adjacent normal tissues in 70% of patients. In addition, higher expression of CCAT1 was correlated with T3-4 grade and advanced clinical stage. Overexpression of CCAT1 promoted Hep-2 cell proliferation, migration and invasion. Ectopic expression of CCAT1 suppressed E-cadherin expression while enhanced the expression of N-cadherin and Vimentin. Furthermore, CCAT1 overexpression increased Hep-2 cell colony formation and cell cycle. We also demonstrated that CCAT1 overexpression suppressed the expression of let-7 and enhanced the expression of HMGA2 and Myc, the direct target genes of let-7. To conclude, our data demonstrated that CCAT1 played an oncogenic role in the development of LSCC.

Keywords: Long noncoding RNAs, lncRNAs, CCAT1, laryngeal squamous cell carcinoma

Introduction

Laryngeal squamous cell carcinoma (LSCC) is the second most common malignancy of the head and neck worldwide [1-5]. Patients with LSCC demonstrate low cure rate and high morbidity, thereby requiring novel diagnostic and therapeutic procedures [6-9]. Despite great advances achieved in the diagnosis of LSCC, there is no ideal biomarker for diagnosis and treatment of LSCC patients [10-12]. Therefore, it is crucial to identify novel useful molecular biomarkers for diagnosis and therapy of LSCC.

Long noncoding RNAs (lncRNAs) are a group of RNA molecules with length longer than 200 nucleotides [13-16]. Recent studies have demonstrated that lncRNAs play crucial roles in many biological and pathological processes such as development, proliferation, apoptosis, migration and invasion [17-21]. Moreover, lncRNAs can act as tumor suppressor genes or

oncogenes in different tumors including lung cancer, breast cancer, bladder cancer, colorectal cancer and osteosarcoma [22-26].

In our study, we demonstrated that colon cancer associated transcript-1 (CCAT1) expression was upregulated in the LSCC tissues. Overexpression of CCAT1 promoted Hep-2 cell proliferation, migration and invasion. Ectopic expression of CCAT1 suppressed the expression of E-cadherin and enhanced the expression of N-cadherin and Vimentin.

Materials and methods

Tissue samples

Our study was approved by the clinical Ethics Committee of The First Affiliated Hospital of Harbin Medical University. Written consents were obtained from all patients. Paired tissues of LSCC and their matched noncancerous sam-

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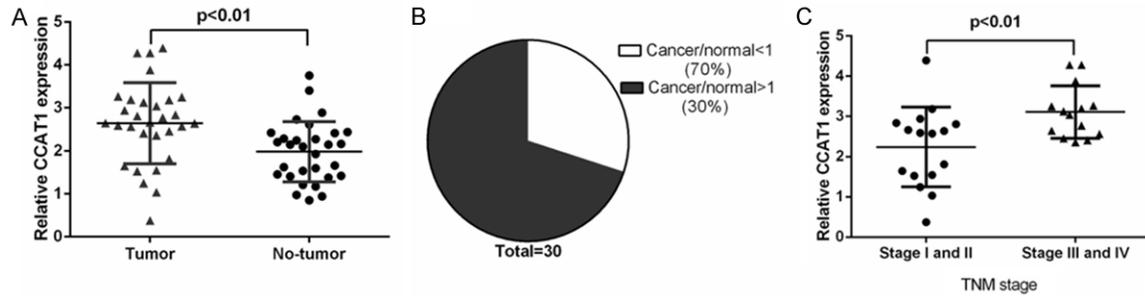


Figure 1. The expression of CCAT1 was upregulated in LSCC tissues. A. The expression of CCAT1 in the LSCC tissues and normal adjacent tissues was measured using qRT-PCR. B. CCAT1 expression was increased in LSCC tissues compared with adjacent normal tissues in 21 patients. C. Higher expression of CCAT1 was related with T3-4 clinical stage.

ples were obtained between 2012 and 2014 at our department. These patients did not receive chemotherapy or radiotherapy before admission. All samples were immediately frozen and stored in the liquid nitrogen following surgery.

Cell culture and transfected

Hep-2 cell (LSCC cell line) was collected from Cell Bank of the Shanghai of the Chinese Academy of Science. Cells were seeded in the DMEM medium (Dulbecco's modified Eagle's medium) (GIBCO, USA) supplemented with fetal bovine serum (FBS). pCDNA-CCAT1 and control vector was obtained from the GenePharma (Shanghai, China) and cell transfection was done using Lipofectamine 2000 (Invitrogen, USA) following to the instructions.

RNA extraction and real-time PCR

Total RNA was extracted from cell or tissues by TRIzol reagent (Invitrogen, USA) according to the manufacturer's information. Real-time PCR (qRT-PCR) was done to measure the miRNA and mRNA expression on the 7500 Real Time PCR System (Applied Biosystems, CA). The relative expression of CCAT1 and mRNA was normalized to GAPDH. The primers were used as following: CCAT1, forward 5'-TTTATGCTTGAGCCTTGA-3' and reverse 5'-CTTGCCTGAAATACTTGC-3'; GAPDH, forward 5'-GTCAACGGATTTGGTCTGTATT-3' and reverse 5'-AGTCTTCTGGGTGGCAGTGAT-3'.

Cell proliferation, cell cycle and colony formation analysis

CCK-8 (Cell counting kit-8; Dojindo, Japan) was used to determine the cell proliferation. Hep-2 cell was cultured in the 96-well plates and the

cell proliferation was measured for 0 hour, 24 hours, 48 hours and 72 hours. Cell proliferation was measured through absorbance detection at the 450 nm. For cell cycle analysis, Hep-2 cells were harvested, washed and fixed using cold 70% ethanol. Then, cells were stained by PI (propidium iodide) and RNase A for about 30 minutes and measured using flow cytometry (BD, Becton Dickinson). For colony formation, cell was trypsinized into the single-cell and seeded in the 6-well plates. After cultured for 2 weeks, the colony was fixed and counted with crystal violet (Sigma, Germany).

Cell invasion and migration

Wound healing analysis was performed to measure the cell migration. Cells were plated in the 6 well plates and wound was done with the pipette tip. Then, cells were cultured for additional 48 hours and the wound was detected by photomicrographs. For cell invasion analysis, transwell with Matrigel was used. Cells were cultured in the upper chamber with serum-free media and 10% FBS was added into the lower chamber. After 48 hours, the invaded cells in the lower membrane were stained with crystalviolet and counted using a microscope (Olympus, Japan).

Western blot assay

Total protein was isolated from cell or tissues using lysis buffer. Protein was separated by 12% SDS-PAGE and then transferred to PVDF (polyvinylidene fluoride) membranes (Millipore, MA). The PVDF membrane was blocked with milk and incubated with primary antibodies against (HMGA2, Myc, GAPDH, abcam) overnight. Protein was measured using ECL (en-

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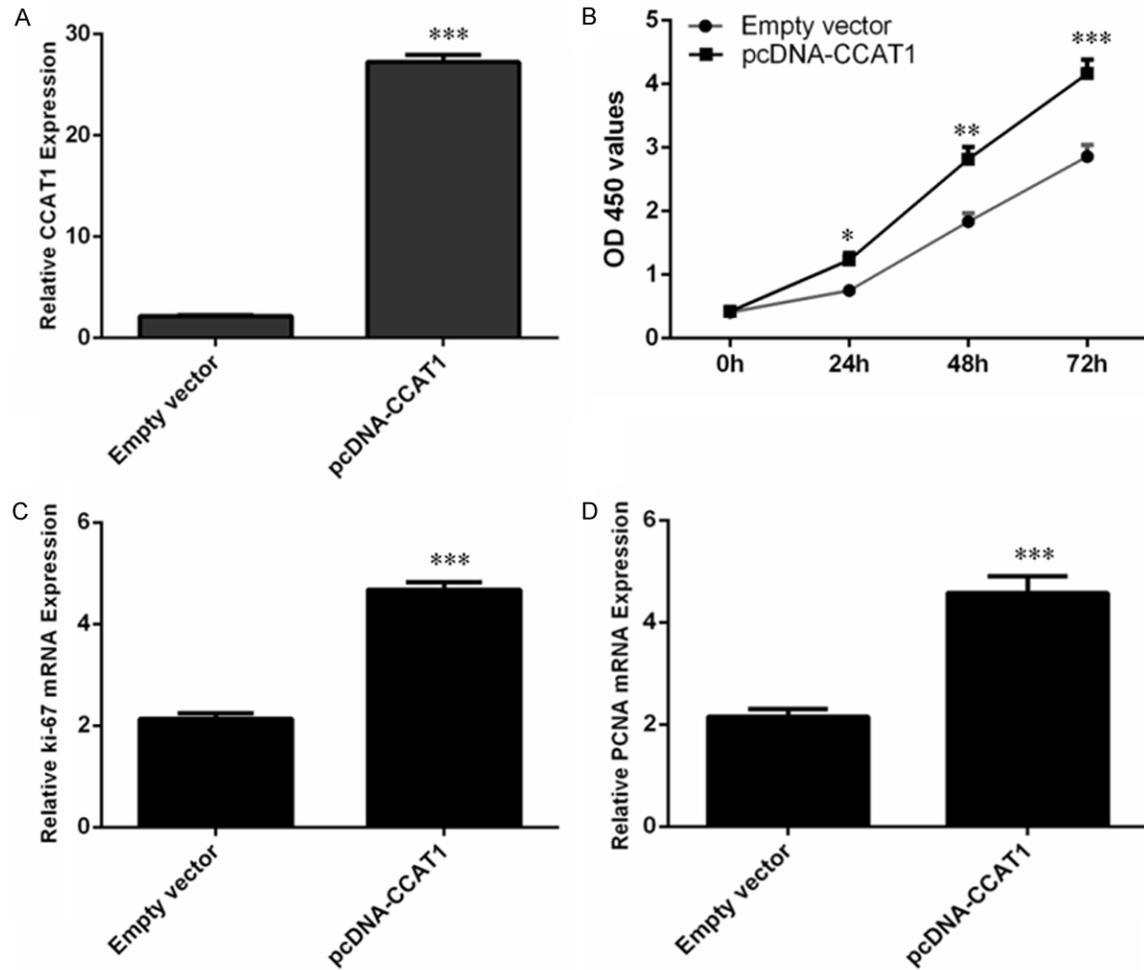


Figure 2. CCAT1 increased the LSCC cell proliferation. A. The expression of CCAT1 was detected in the Hep-2 cell using qRT-PCR. B. Ectopic expression of CCAT1 increased the Hep-2 cell proliferation. C. The expression of ki-67 was measured using qRT-PCR. D. The expression of PCNA was measured using qRT-PCR.

hanced chemiluminescence reagent) (Pierce) following to manufacturer's instructions.

Statistical analysis

Data were shown as mean \pm SD (standard deviation). The significance differences between more than two groups were determined one-way ANOVA and the difference between two groups was analyzed using the Student t-test. *P* value with less than 0.05 was considered statistically significant.

Result

CCAT1 expression was upregulated in LSCC tissues

We measured the CCAT1 expression in the 30 pairs of LSCC tissues. CCAT1 expression was

upregulated in LSCC tissues compared with the normal adjacent tissues (**Figure 1A**). Moreover, CCAT1 expression was increased in LSCC tissues compared with the adjacent normal tissues in 21 patients (70%, 21/30) (**Figure 1B**). In addition, higher expression of CCAT1 was correlated with T3-4 grade and advanced clinical stage (**Figure 1C**).

CCAT1 increased LSCC cell proliferation and promoted LSCC cell colony formation and cell cycle

We used the pcDNA-CCAT1 to measure the function of CCAT1 in Hep-2 cell. We found that pcDNA-CCAT1 could promote the CCAT1 expression in Hep-2 cell (**Figure 2A**). Ectopic expression of CCAT1 promoted Hep-2 cell proliferation (**Figure 2B**). Moreover, overexpression of CCAT1 increased the expression of ki-67 (**Figure 2C**)

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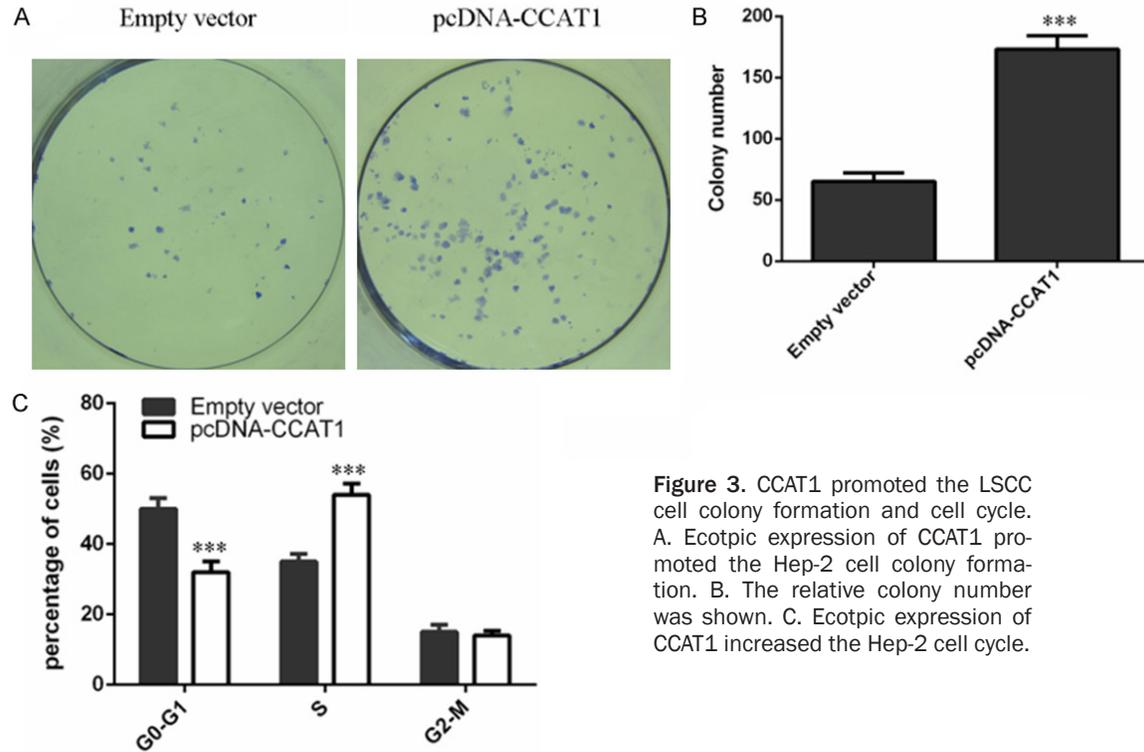


Figure 3. CCAT1 promoted the LSCC cell colony formation and cell cycle. A. Ectopic expression of CCAT1 promoted the Hep-2 cell colony formation. B. The relative colony number was shown. C. Ectopic expression of CCAT1 increased the Hep-2 cell cycle.

and PCNA (Figure 2D). CCAT1 overexpression increased Hep-2 cell colony formation (Figure 3A, 3B). Moreover, ectopic expression of CCAT1 also promoted Hep-2 cell cycle (Figure 3C).

CCAT1 enhanced LSCC cell migration and invasion

Wound healing analysis showed that CCAT1 overexpression enhanced Hep-2 cell migration (Figure 4A and 4C). Moreover, cell invasion analysis demonstrated that CCAT1 promoted Hep-2 cell invasion (Figure 4B, 4D). In addition, overexpression of CCAT1 could suppress the expression of E-cadherin and enhance the expression of N-cadherin and Vimentin (Figure 4E).

CCAT1 suppressed let-7 expression and enhanced HMGA2 and Myc expression

Overexpression of CCAT1 could promote the expression of let-7 expression (Figure 5A). Moreover, ectopic expression of CCAT1 enhanced the expression of HMGA2 (Figure 5B, 5C). In addition, ectopic expression of CCAT1 also promoted the expression of Myc (Figure 5D, 5E).

Discussion

In our study, CCAT1 expression was upregulated in the LSCC tissues compared with the normal adjacent tissues. Moreover, CCAT1 expression was higher in LSCC tissues compared with adjacent normal tissues in 70% patients. In addition, higher expression of CCAT1 correlated with T3-4 grade and advanced clinical stage. Overexpression of CCAT1 promoted Hep-2 cell proliferation, migration and invasion. Ectopic expression of CCAT1 could suppress the expression of E-cadherin and enhance the expression of N-cadherin and Vimentin. Furthermore, CCAT1 overexpression increased Hep-2 cell colony formation and cell cycle. We also demonstrated that CCAT1 overexpression suppressed let-7 expression and enhanced the expression of HMGA2 and Myc, the direct target genes of let-7. These data demonstrated that CCAT1 played an oncogenic role in the development of LSCC.

Previous studies had showed that CCAT1 played an important role in several tumors such as colorectal cancer, breast cancer, hepatocellular carcinoma, gallbladder cancer and gastric cancer [27-31]. For example, Ye et al [27] dem-

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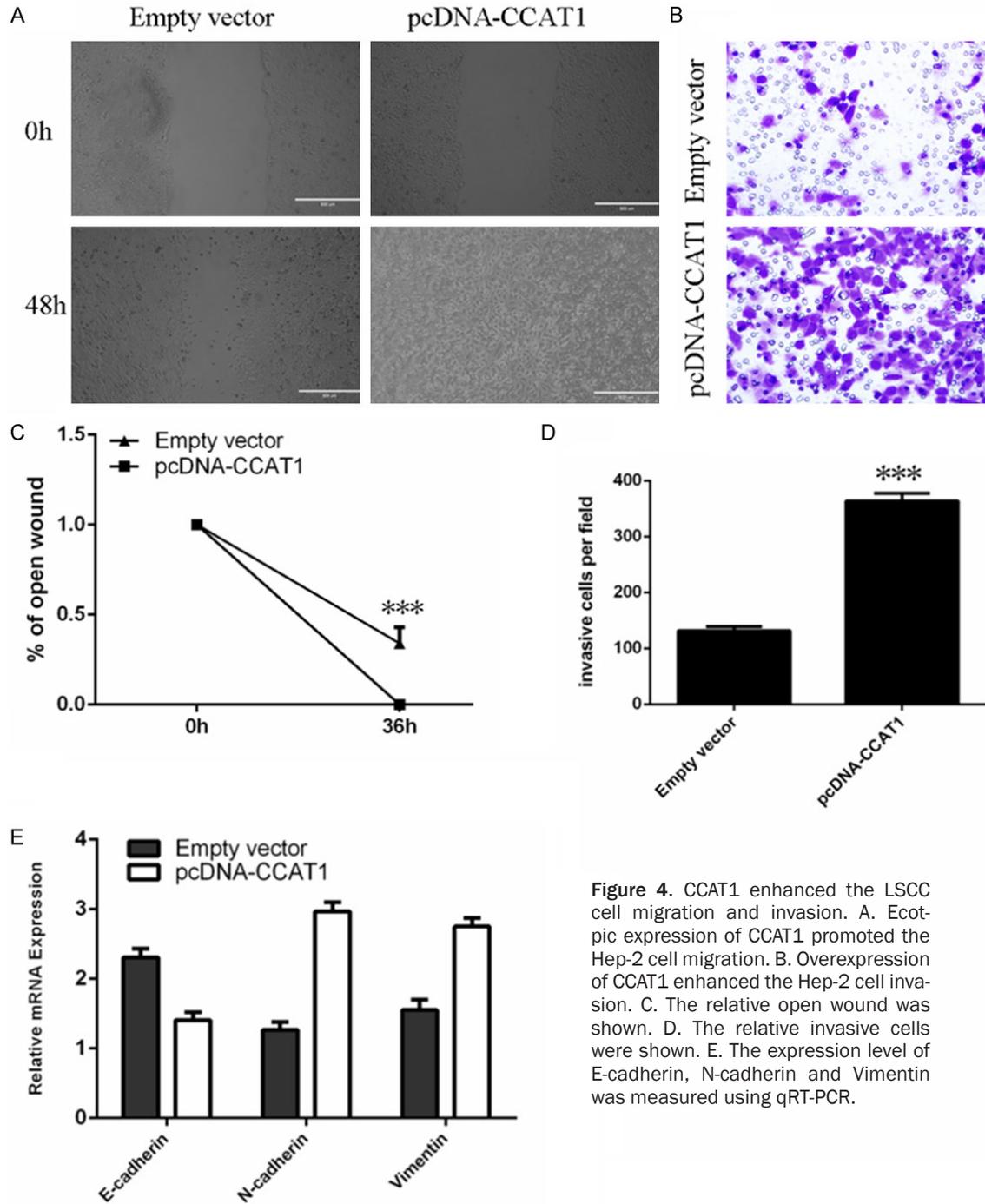


Figure 4. CCAT1 enhanced the LSCC cell migration and invasion. A. Ecotopic expression of CCAT1 promoted the Hep-2 cell migration. B. Overexpression of CCAT1 enhanced the Hep-2 cell invasion. C. The relative open wound was shown. D. The relative invasive cells were shown. E. The expression level of E-cadherin, N-cadherin and Vimentin was measured using qRT-PCR.

onstrated that CCAT1 expression was higher in the colorectal cancer tissues than in normal para-carcinoma tissues. The expression level of CCAT1 was correlated with tumor staging, local infiltration depth, vascular invasion and CA19-9 level. Zhang et al [28] showed that CCAT1 expression in breast tissues were higher than those in non-tumor tissues. High CCAT1

expression was correlated with advanced TNM stage, low differentiation grade and more lymph node metastases. Patients with high CCAT1 expression had a lower overall survival (OS) and progression-free survival. Yang et al [32] demonstrated that CCAT1 expression was upregulated in gastric cancer tissues compared with normal adjacent tissues. Deng et al

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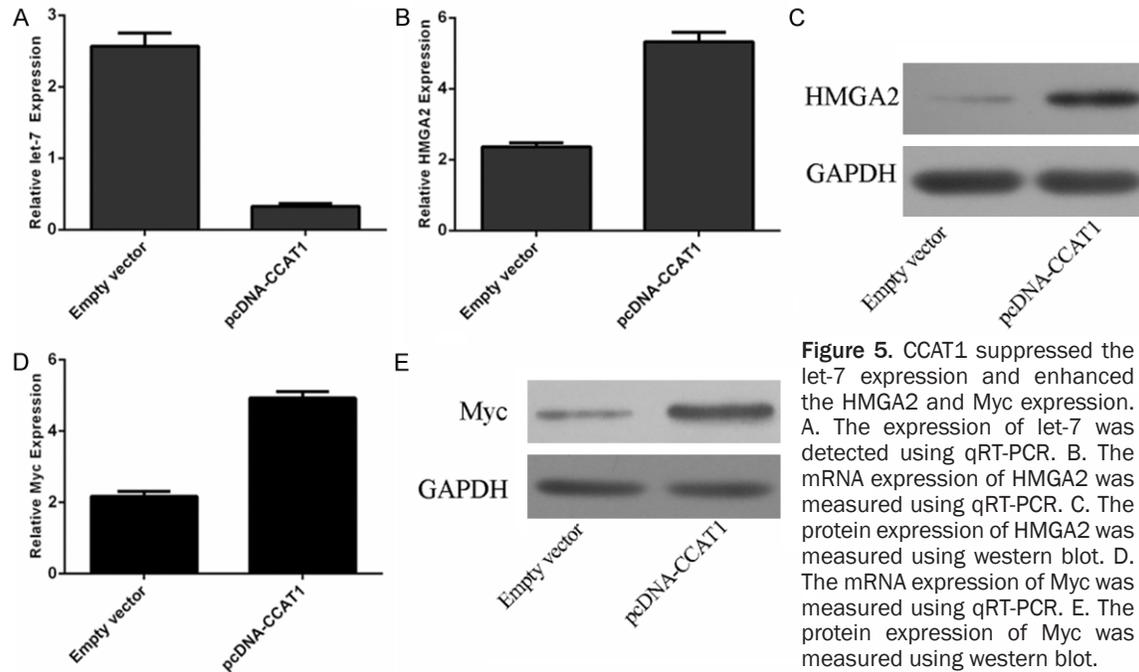


Figure 5. CCAT1 suppressed the let-7 expression and enhanced the HMGGA2 and Myc expression. A. The expression of let-7 was detected using qRT-PCR. B. The mRNA expression of HMGGA2 was measured using qRT-PCR. C. The protein expression of HMGGA2 was measured using western blot. D. The mRNA expression of Myc was measured using qRT-PCR. E. The protein expression of Myc was measured using western blot.

[33] found that CCAT1 expression was upregulated in hepatocellular carcinoma tissues compared with non-cancerous hepatic tissues. Higher expression of CCAT1 was associated with microvascular invasion, tumor size, poor prognosis and AFP. CCAT1 overexpression increased the hepatocellular carcinoma cell migration and proliferation. However, the role of CCAT1 in LSCC is still unknown. In our study, we found that CCAT1 expression was increased in LSCC tissues compared with the normal adjacent tissues. In addition, higher expression of CCAT1 was correlated with advanced clinical stage. Overexpression of CCAT1 promoted Hep-2 cell proliferation, migration and invasion. Ectopic expression of CCAT1 could suppress the E-cadherin expression and enhance the expression of N-cadherin and Vimentin. Furthermore, CCAT1 overexpression increased the Hep-2 cell colony formation and cell cycle. These results suggested that CCAT1 acted as an oncogene in the development of LSCC.

Increasing evidences have showed that lncRNA exerts functions by targeting miRNAs. For example, Ma et al [30] found that CCAT1 expression was increased in gallbladder cancer tissues. Overexpression of CCAT1 suppressed the expression of miR-218-5p and promoted the expression of miR-218-5p target gene Bmi1. Moreover, another study also showed

that CCAT1 inhibited the let-7 expression and enhanced c-Myc and HMGGA2 expression, the target genes of let-7 [33]. In line with these data, we also found that CCAT1 overexpression suppressed the let-7 expression and enhanced the expression of HMGGA2 and Myc, which were the direct target genes of let-7. Our data suggested that CCAT1 played an important role in LSCC development partly through targeting let-7 expression.

In conclusion, we demonstrated that CCAT1 expression was upregulated in LSCC tissues and CCAT1 overexpression promoted LSCC cell proliferation, colony formation, cell cycle, migration and invasion. In addition, overexpression of CCAT1 suppressed let-7 expression and enhanced HMGGA2 and Myc expression, which were the target genes of let-7. These data demonstrated that CCAT1 served as an oncogene in the development of LSCC.

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