Original Article
Overexpression of long non-coding RNA CCAT2 predicts a poor prognosis in patients with oral squamous cell carcinoma

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Abstract: Introduction: Long non-coding RNA CCAT2 (lncRNA CCAT2) have been demonstrated to be a critical role in cancer progression and prognosis. However, its effects in oral squamous cell carcinoma (OSCC) have not been explored. The aim of this study was to investigate the expression and clinical significance of lncRNA CCAT2 in human OSCC. Methods: Expression levels of lncRNA CCAT2 in 102 OSCC tissues and cell lines were examined by quantitative real-time PCR (qRT-PCR). The correlation of lncRNA CCAT2 with clinicopathological features and prognosis was analyzed. Moreover, in vitro assays were performed to explore its role in OSCC progression. Results: The expression level of lncRNA CCAT2 was significantly higher in tumor tissues and oral cancer cell lines than in adjacent non-tumor tissues and normal human oral keratinocyte cells. High expression of lncRNA CCAT2 was found to significantly correlate with tumor grade, TNM stage and distant metastasis. Moreover, OSCC patients with lncRNA CCAT2 higher expression have shown significantly poorer overall survival than those with lower lncRNA CCAT2 expression. Univariate and multivariate analyses suggested that high expression of lncRNA CCAT2 was an independent poor prognostic indicator for OSCC patients. In vitro assays, our data indicated that downregulation of CCAT2 decrease cell proliferation, migration, and invasion ability. Conclusions: High lncRNA CCAT2 expression is associated with poor prognosis in OSCC and may serve as a novel prognostic marker in OSCC.

Keywords: Oral squamous cell carcinoma, LncRNA CCAT2, overall survival, prognosis

Introduction

Oral squamous cell carcinoma (OSCC) is one of the most lethal malignancies worldwide and is also the most common oral cancer [1]. OSCC is accompanied by frequent metastasis, high recurrence and poor prognosis. It has been estimated that worldwide, more than 100,000 deaths result from OSCC annually [2]. Despite much progress in early detection and treatment, the 5-year survival rate worldwide for OSCC remains below 60% [2]. Therefore, it is necessary for us to discover the underlying molecular mechanisms and screen useful biomarkers and novel therapeutic targets of OSCC.

Long non-coding RNAs (lncRNAs) are more than 200 nucleotides in length with limited or no protein-coding capacity and serve as the primary regulatory non-coding RNA [3]. LncRNAs have been implicated in a large number of cellular processes, such as cell proliferation, cell cycle progression, cell growth and cell apoptosis [4, 5]. Emerging evidences indicate that lncRNAs may play complex and extensive roles in promoting the development and progression of cancer. For example, Chen et al. found that lncRNA HOTAIR was up-regulated in esophageal squamous cell carcinoma tissues and associated with tumor size, TNM stage and poor prognosis of esophageal squamous cell carcinoma [6]. Further experiments suggested that HOTAIR exerts its oncogenic functions via binding the PRC2 (polycomb repressive complex 2), which methylates histone H3 on K27 to promote gene repression [7]. Hua et al. indicated that lncRNA ANRIL up-regulated in human hepatocellular carcinoma tissues and correlated with a higher histologic grade and TNM stage [8]. They suggested that ANRIL expression served as an

Table 1. Clinicopathological features and the expression of lncRNA CCAT2 in OSCC patients

Parameters	Group	Total	lncRNA CCAT2		P value
			Low	High	
Age (years)	< 60	48	20	28	0.638
	≥ 60	54	25	29	
Gender	Male	62	28	34	0.792
	Female	40	17	23	
Tumor location	Tongue	40	16	24	0.385
	Floor of mouth	4	3	1	
	Buccal mucosa	25	12	13	
	Hard palate	7	4	3	
	Upper or lower gingival	26	10	16	
Tumor size	T1-T2	45	18	27	0.457
	T3-T4	57	27	30	
Tumor grade	G1	40	25	15	0.003
	G2/G3	62	20	42	
Distant metastasis	Yes	43	5	38	0.000
	No	59	40	19	
TNM stage	I-II	38	23	15	0.010
	III-IV	64	22	42	

independent predictor for overall survival of hepatocellular carcinoma patients. Sun et al. reported that GAS5 down-regulation was involved in gastric cancer tumorigenesis and progression, ectopic expression of GAS5 could decrease gastric cancer cell proliferation and induce apoptosis in via regulating E2F1 and P21 [9]. Dysregulation of certain lncRNAs are related to prognosis, metastasis, and recurrence in different cancer types. However, the role of lncRNAs in OSCC development has only recently been investigated and remains largely unknown.

LncRNA CCAT2 (colon cancer-associated transcript 2) was firstly identified and found highly expressed in microsatellite-stable colorectal cancer [10]. CCAT2 is located within the 8q24 gene desert region and includes a cancer-related single nucleotide polymorphism rs69832-76. Recently, various studies have shown that lncRNA CCAT2 plays a crucial role in regulation of cancer cell biological functions. In addition, overexpression of lncRNA CCAT2 has been found in several types of cancers such as esophageal squamous cell carcinoma [11], gastric cancer [12], breast cancer [13], and lung cancer [14]. However, the lncRNA CCAT2 expression in OSCC and underlying mechanism is still unknown.

In the present study, we investigated the expression of lncRNA CCAT2 in OSCC tissues and oral cancer cell lines. We then examined the relationships between CCAT2 levels in tumor tissues and the clinicopathological features of OSCC. Finally, we conducted in vitro assays to demonstrate the biological functions of CCAT2 in OSCC development and progression.

Material and methods

Patients and samples

The 102 OSCC patients were from the Department of Stomatology, 421 Hospital of PLA (Guangzhou, China), diagnosed with OSCC between January 2007 and June 2009. OSCC was confirmed in all cases by histological examination of tissue from biopsy specimens. Tumor stage was classified according to the 7th edition of the classification of malignant tumors of the American Joint Committee on Cancer [15]. Tumor grade was classified following the WHO criteria [16]. All patients did not receive chemotherapy or radiotherapy prior to surgery. Clinicopathological characteristics in our study are presented in **Table 1**. After surgical resection, tumor specimens and adjacent non-tumor tissues were collected and stored in liquid nitrogen until use. This study was approved by the Research Ethics Committee of the 421 Hospital of PLA. Written informed consent was obtained from all patients.

Cell culture and transfection

Three oral cancer cell lines (Tca8113, SCC25 and CAL27), and a human immortalized oral keratinocyte cell line (HOK16E6E7) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Oral cancer cells were cultured in RPMI 1640 or Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin, and 100 mg/ml streptomycin. HOK16E6E7 were cultured in keratinocyte growth medium containing 0.15 mM calcium and supplemented with epidermal

growth factor (Gibco). All cells were cultured at 37°C in a humidified atmosphere with 5% CO_2.

Tca8113 cells were transfected with either 50 nM siRNAs targeting CCAT2 (si-CCAT2) or scrambled negative controls (si-NC) (Gene-Pharma, China) using the Lipofectamine 2000 transfection reagent (Invitrogen) according to the instructions provided by the manufacturer. The target sequence for CCAT2 siRNAs was 5'-UUAACCUCUUCCUAUCUCAUU-3' [11]. After 48 hours, cells transfected with siRNA were harvested for qRT-PCR to determine the transfection efficiency.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNAs were extracted from tumorous and adjacent normal tissues or cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. For qRT-PCR, RNA was reverse transcribed to cDNA by using a Reverse Transcription Kit (Takara, Dalian, China). Real-time PCR analyses were performed with Power SYBR Green (Takara, Dalian China). Results were normalized to the expression of GAPDH. The PCR primers for CCAT2 or GAPDH were as follows: CCAT2 sense, 5'-CCCTGGTCA-AATTGCTTAACCT-3' and reverse, 5'-TTATTCGT-CCCTCTGTTTTATGGAT-3'; GAPDH sense, 5'-GC-ACCGTCAAG GCTGAGAAC-3' and reverse, 5'-TG-GTGAAGACGCCAGTGGA-3'. qRT-PCR and data collection were performed on ABI 7500. The fold change between tumor tissues and non-cancerous samples for lncRNA CCAT2 was calculated with the $2^{-\Delta\Delta CT}$ method. The expression levels of lncRNA CCAT2 in HOK16E6E7, Tca8113, SCC25, and CAL27 were detected using the same method. qRT-PCR was repeated in triplicate for each sample.

Cell proliferation assay

The proliferation of Tca8113 cells was evaluated using the MTT (Sigma) assay according to the manufacturer's instructions. Tca8113 cells that had been transfected with either si-CCAT2 or si-NC for 48 hours were reseeded into 96-well plates. Cell density was adjusted to 5×10^3/well, and the final volume was 150 μl/well. MTT solution (20 μl) was added to the plates 24, 48, 72, and 96 hours later. The cells were cultured for 4 hours at 37°C. Then, the medium was discarded and 150 μl DMSO was added and oscil-

lated for 15 min. The absorbance was measured at 490 nm using an enzyme-labeled analyzer. Three independent experiments were performed.

Cell migration assays

Migration ability was measured using a wound healing assay. Tca8113 cells transfected with either si-CCAT2 or si-NC were seeded into 6-well plates, incubated in their respective complete culture medium and grown to confluence over-night. The monolayer was scratched and then incubated in fresh medium for 24 hours. The width of the wound was measured after 24 hours. Three different locations were visualized and photographed with a phase-contrast inverted microscope (Leica, Solms, Germany).

Transwell invasion assay

Cell invasion assays was performed using 24-well Transwells (8-μm pore size, Corning Life Sciences) coated with 1 mg/ml Matrigel (BD Sciences). Tca8113 cells transfected with either si-CCAT2 or si-NC were collected and resuspended in serum-free medium at a concentration of 1×10^5 cells/ml, respectively. The cells were seeded in the upper chamber of the wells in 200 μl FBS-free medium, and the lower chambers were filled with 500 μl 20% FBS medium. Following incubation for 48 hours at 37°C, 5% CO_2, the cells on the filter surface were fixed with methanol, stained with 0.1% crystal violet, and photographed with a phase-contrast inverted microscope (Leica). The cells from at least five random microscopic fields (×100) were counted.

Statistical analysis

All statistical analyses were performed using SPSS 17.0 software (IBM, SPSS, Chicago, IL, USA). Comparison of continuous data was analyzed using an independent *t*-test between the two groups, whereas categorical data was analyzed by the chi-square test. Overall survival curves were plotted according to the Kaplan-Meier method, with the log-rank test applied for comparison. A Cox proportional hazards regression analysis was used for univariate and multivariate analyses of prognostic values. The data are shown as the mean ± SD from at least three independent experiments. Two-sided *P* values
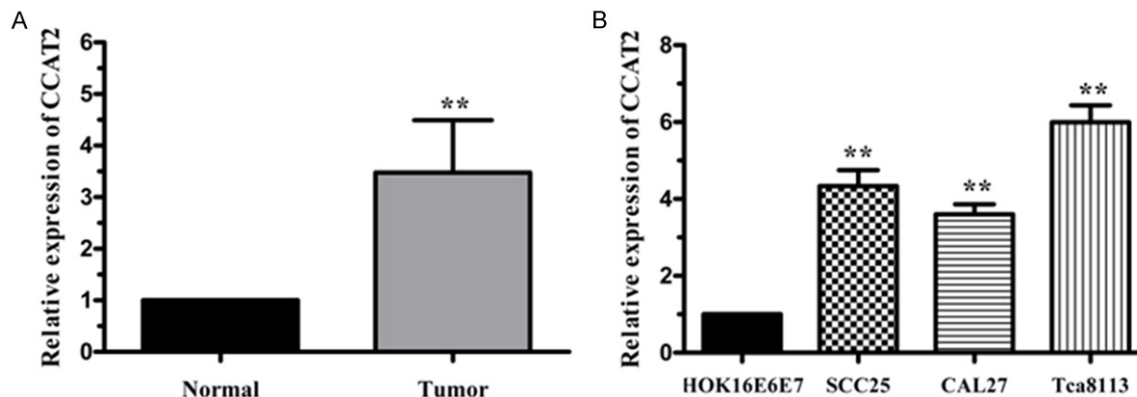
Figure 1. LncRNA CCAT2 expression levels in OSCC tissues and oral cancer cells. The levels of CCAT2 were assessed by qRT-PCR, using GAPDH as a normalisation control. A. The CCAT2 expression levels in OSCC samples were significantly higher than those in adjacent non-tumor tissues. B. Higher expression levels of CCAT2 were detected in 3 oral cancer cell lines than in immortalized oral keratinocyte cell line HOK16E6E7. Results were expressed as mean ± SD for three replicate determination. **$P < 0.01$.
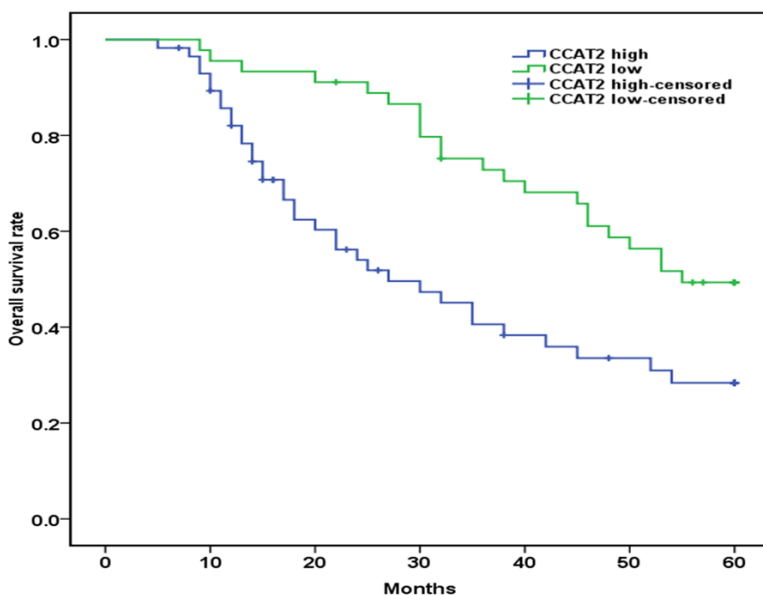

Figure 2. Kaplan-Meier overall survival curves according to the CCAT2 expression level. OSCC patients with high CCAT2 expression showed a significantly poorer prognosis than those with low CCAT2 expression (log-rank test, $P = 0.003$).

were calculated, and a probability level of 0.05 was chosen for statistical significance.

Result

LncRNA CCAT2 is upregulated in OSCC tissues and oral cancer cell lines

LncRNA CCAT2 expression levels were investigated in 102 paired OSCC tissues and adjacent histologically normal tissues by qRT-PCR. CCAT2 expression was significantly higher in tu-

mor tissues compared with adjacent normal tissues ($P < 0.01$, **Figure 1A**). Expression was also examined by qRT-PCR in three oral cancer cell lines (Tca8113, SCC25 and CAL27) and an immortalized oral keratinocyte cell line HOK16E6E7. This experiment showed that CCAT2 expression was higher in oral cancer cell lines than in HOK16E6E7 ($P < 0.01$, **Figure 1B**).

Relationship between lncRNA CCAT2 expression levels and clinicopathological features

To assess the correlation of lncRNA CCAT2 expression with clinicopathological data, CCAT2 expression levels in tumor tissues were categorized as low or high in relation to the median value of relative CCAT2 expression (3.46-fold). Clinicopathological factors were analyzed in the high and low CCAT2 expression groups. As shown in **Table 1**, there was no correlation between the relative CCAT2 expression levels and age, gender, tumor location, or tumor size ($P > 0.05$), but the relative CCAT2 expression levels were significantly positively correlated with TNM stage ($P = 0.010$), tumor grade ($P = 0.003$), and distant metastasis ($P < 0.001$). Taken together, these observations indicated that increased CCAT2 expres-

Table 2. Prognostic factors in Cox proportional hazards model

Variable	Univariate analysis			Multivariate analysis		
	Risk ratio	95% CI	*P* value	Risk ratio	95% CI	*P* value
Age	1.335	0.598-2.373	0.612			
≥ 60 vs. < 60						
Gender	0.612	0.654-2.370	0.708			
Male vs. Female						
Tumor location	1.516	0.824-2.910	0.564			
Tongue vs. (Floor of mouth + Buccal mucosa Hard palate Upper or lower gingival)						
Tumor size	1.805	0.712-3.335	0.489			
T1 + T2 vs. T3 + T4						
Tumor grade	2.845	1.552-5.417	0.008	2.455	1.341-5.152	< 0.001
G1 vs. G2 + G3						
Distant metastasis	3.581	1.652-7.526	< 0.001	3.064	1.580-6.473	< 0.001
Yes vs. No						
TNM Stage	3.154	1.306-6.058	< 0.001	2.847	1.522-5.937	< 0.001
I + II vs. III + IV						
CCAT2	2.945	1.506-5.974	0.002	2.718	1.429-5.682	< 0.001
High vs. Low						

sion is associated with the progression and development of OSCC.

High-level expression of lncRNA CCAT2 predicts poor prognosis in OSCC patients

Kaplan-Meier analysis and log-rank test were used to evaluate the effects of lncRNA CCAT2 expression and the clinicopathological characteristics on overall survival of OSCC patients. The results showed that patients with high CCAT2 expression had a significantly poorer prognosis than those with low expression (P < 0.01, **Figure 2**). Univariate analysis showed that the relative level of CCAT2 expression, TNM stage, tumor grade, and distant metastasis were correlated with overall survival rate of patients with OSCC (P < 0.05, **Table 2**). Multivariate analysis indicated that CCAT2 expression level, TNM stage, tumor grade, and distant metastasis were independent prognostic indicators for the overall survival of patients with OSCC (P < 0.05, **Table 2**). The results suggested that expression of CCAT2 in OSCC patients can be developed as an independent prognostic factor.

Suppressing CCAT2 expression decreases cell proliferation, migration and invasion in vitro

To further investigate the role of CCAT2 in human oral cancer cells, CCAT2 specific siRNA

(si-CCAT2) was transfected into Tca8113 cells, respectively. Nonspecific siRNA was used as a negative control (si-NC). As shown in **Figure 3A**, cells transfection with si-CCAT2 showed a significant decreased mRNA expression of CCAT2 compared to the si-NC group (P < 0.05).

MTT assays showed that the cell growth rate was downregulated in the si-CCAT2 transfected Tca8113 cells compared with the si-NC group (P < 0.05, **Figure 3B**). Furthermore, to analyze the role of lncRNA CCAT2 in cell migration and invasion, wound healing assays and transwell invasion assays were performed with Tca8113 cells. Wound healing assays showed that the migration ability of Tca8113 cells transfected with si-CCAT2 was significantly decreased compared with the si-NC group (P < 0.05, **Figure 3C**). Transwell invasion assay revealed that the invasion capacity of Tca8113 cells transfected with si-CCAT2 was notably down-regulated compared to si-NC group (P < 0.05, **Figure 3D**). Taken together, these data demonstrated that downregulation of lncRNA CCAT2 expression can inhibit cell proliferation, migration and invasion of oral cancer cells in vitro.

Discussion

The sequencing of the human genome revealed that the coding portion of the genome represents less than 2% of the genome. The remain-
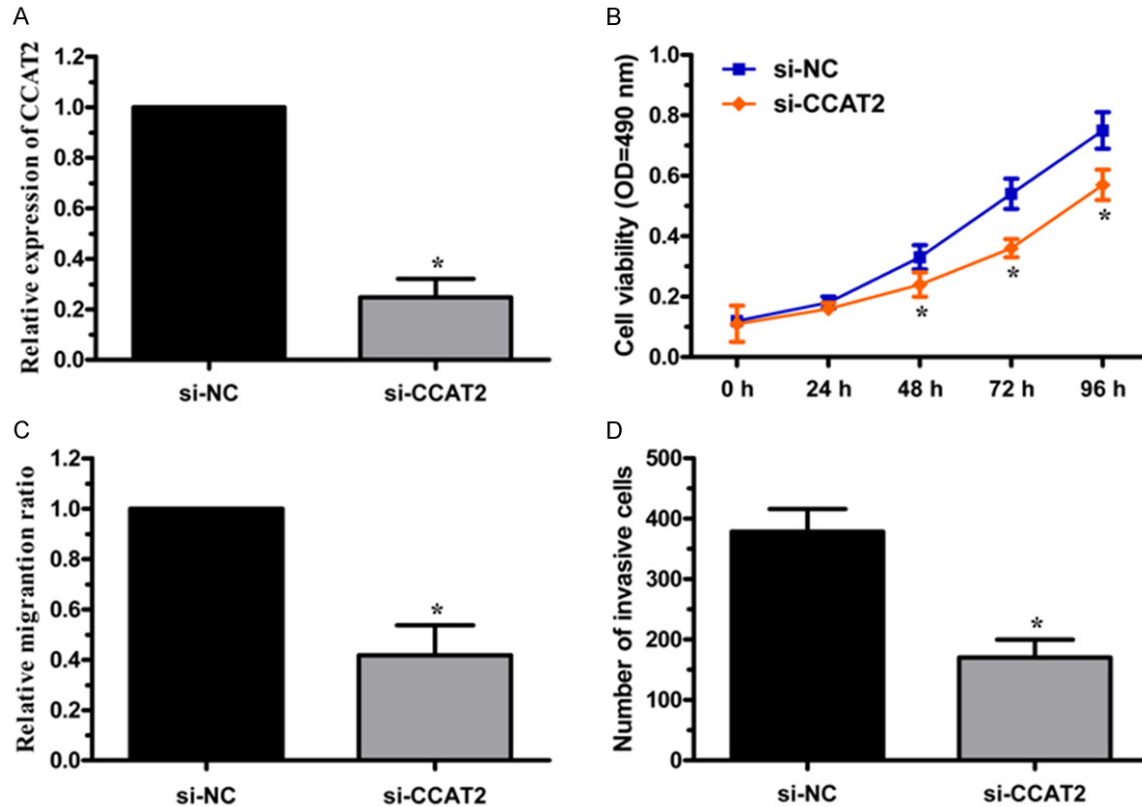
Figure 3. The knockdown of CCAT2 inhibits proliferation, migration and invasion in Tca8113 cells. A. qRT-PCR revealed that CCAT2 was efficiently knocked down by treatment with si-CCAT2 in Tca8113 cells. B. Tca8113 cells trasfected with si-CCAT2 displayed significantly lower proliferation ability compared with those transfected with si-NC. C. Tca8113 cells trasfected with si-CCAT2 displayed significantly lower migration ability compared with those transfected with si-NC. D. Tca8113 cells trasfected with si-CCAT2 displayed significantly lower invasion ability compared with those transfected with si-NC. Results are expressed as means ± SD for three replicate determinations. *P < 0.05.

ing 98% of transcription products of the genome consists of non-coding RNA sequences, including microRNAs and lncRNAs [17]. The aberrant expressions of specific lncRNAs in cancer could mark the spectrum of disease progression and these lncRNAs may serve as independent biomarkers for diagnosis and prognosis [18].

Several cancer-related lncRNAs have been identified, but only a few OSCC-related lncRNAs have been characterized. HOTAIR was found to be upregulated in the majority of OSCC and was associated with metastasis, the stage and histological differentiation [19]. HOTAIR overexpression can contribute to the regulation of E-cadherin through binding to EZH2 and H3-K27me3 with the E-cadherin promoter, which promote the metastasis and invasion of OSCC cells [20]. Furthermore, overexpression of lnc-RNA UCA1 also could promote metastatic abili-

ty of tongue squamous cell carcinoma cells [21]. However, the role and function of lncRNA CCAT2 in OSCC remains unknown.

In the present study, we explored the clinical significance of lncRNA CCAT2 in OSCC patients for the first time. By using qRT-PCR, we found that lncRNA CCAT2 was increased in OSCC tissues and oral cancer cell lines to a greater extent than in adjacent non-tumor tissues and normal human oral keratinocyte cell line. We also revealed that the relative expression level of lncRNA CCAT2 was associated with TNM stage and distant metastasis of OSCC patients. Furthermore, we analyzed a correlation between lncRNA CCAT2 expression level and prognosis of OSCC. Our results showed patients with high lncRNA CCAT2 expression had a shorter overall survival rate than those with low lncRNA CCAT2 group. These findings were further supported by the univariate and multivari-

ate analyses of Cox proportional hazards regression model, indicating that the expression of lncRNA CCAT2 could be an independent factor for predicting the prognosis of OSCC patients. Therefore, our data suggested that increased expression of lncRNA CCAT2 was associated with a high risk of death from OSCC.

Recent studies suggest that the expression levels of lncRNA CCAT2 were significantly elevated in esophageal squamous cell carcinoma tissues and were correlated with lymph node metastasis [11]. Additionally, silencing CCAT2 by siRNA led to inhibition of proliferation and invasion in non-small cell lung cancer cell lines [14]. To further understand the underlying mechanism of CCAT2 in OSCC progression, in vitro experiments were conducted. We found that down-regulated expression of lncRNA CCAT2 significantly decreased proliferation, migration and invasion capability of lung cancer cells in vitro. These data further support the importance of CCAT2 in cellular biology and oncogenesis of oral cancer cells and indicate that CCAT2 is involved in the development and progression of OSCC.

In summary, our studies demonstrated that lncRNA CCAT2 expression was up-regulated in OSCC and was associated with the biological aggressiveness and progression of OSCC. Moreover, down-regulated expression of CCAT2 could inhibit the proliferation, migration and invasion of oral cells in vitro. These findings demonstrated that lncRNA CCAT2 could serve as a potential biomarker and therapeutic target for OSCC.

Disclosure of conflict of interest

None.

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