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MISCELLANEOUS

Downregulation of lncRNA CASC2 promotes the postoperative local recurrence of early oral squamous cell carcinoma

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Abstract

Purpose LncRNA CASC2 plays a role as tumor suppressor gene in different types of human malignancies, while its involvement in oral squamous cell carcinoma (OSCC) is unknown. The present study aimed to investigate the involvement of lncRNA CASC2 in OSCC.

Methods In this study, the expression of lncRNA CASC2 in tumor tissues, adjacent healthy tissues, and plasma of 122 OSCC patients as well as in plasma of 52 healthy controls was detected by RT-qPCR. Diagnostic value of lncRNA CASC2 for OSCC was evaluated by ROC curve analysis. Patients were followed up for 5 years to record recurrence. LncRNA CASC2 expression vectors were transfected into cells of human OSCC cell lines, and the effects on cancer cell proliferation and miRNA-21 expression were analyzed by CCK-8 assay and RT-qPCR, respectively.

Results We found that CASC2 was significantly downregulated in OSCC patients than in healthy controls. Downregulation of CASC2 distinguished OSCC patients from healthy controls. Local recurrence was observed in 26 out of 122 patients and no distant recurrence was observed during follow-up. Compared with pretreatment levels, plasma levels of CASC2 were significantly increased in patients with local recurrence than in patients without recurrence. Transfection of CASC2 expression vectors led to significantly inhibited tumor cell proliferation and reduced miRNA-21 expression levels.

Conclusions We, therefore, conclude that downregulation of lncRNA CASC2 may participate in the postoperative local recurrence of early OSCC through miRNA-21.

Keywords Oral squamous cell carcinoma · LncRNA CASC2 · Local recurrence, miRNA

Introduction

Surgical resection is considered to be a promising treatment strategy for cancer patients at the early stages [1]. However, recurrence after surgical resection is still a major cause of cancer-related deaths [2]. Therefore, how to prevent recurrence after surgical resection is still a major challenge in the treatment of cancers. Oral cancer or mouth cancer is a type of head and neck cancer caused by the growth of cancerous tissues in oral cavity [3]. As the major type of oral cancer, oral squamous cell carcinoma (OSCC) accounts for more than 95% of all cases and causes more than 145,000 deaths worldwide every year [4, 5]. It has been proved that the recurrence rate of OSCC after proper surgical resection is still as high as 20%, leading to poor survival of those patients.

Long non-coding RNAs, or lncRNAs, are a group of non-coding RNAs composed of more than 200 nucleotides that have critical roles in both physiological and pathological processes [7]. Recent studies have also shown that lncRNAs may also participate in postoperative recurrence of cancers [8, 9]. LncRNA CASC2 is a well-characterized tumor suppressor lncRNA, and downregulation of lncRNA CASC2 promotes the progression of malignancies, such as bladder cancer [10] and renal cell carcinoma [11]. However, based on our knowledge, no studies on the involvement of lncRNA CASC2 in pathogenesis of OSCC have been reported. In the present study, we found that downregulation of lncRNA CASC2 may induce postoperative recurrence of OSCC through miRNA-21.

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Materials and methods

Subjects

A total of 266 patients with OSCC were diagnosed and treated in the hospital authors worked in from January 2009 to January 2013. Our study included 122 out of those 266 patients according to strict inclusion and exclusion criteria. Inclusion criteria: (1) patients diagnosed as stage I or II OSCC by pathological examinations; (2) patients received surgical resection of the primary tumor; (3) patients completed follow-up study. Exclusion criteria: (1) patients with metastatic-OSCC; (2) patients complicated with other severe malignancies; (3) patients died during follow-up before the diagnosis of recurrence. The 122 patients included 70 males and 52 females, and age ranged from 28 years to 69 years, with a mean age of 49.1 ± 6.1 years. Blood was extracted from those patients on the day of admission and on the day of the diagnosis of recurrence to prepare plasma. Plasma, tumor tissues, and adjacent healthy tissues were stored in liquid nitrogen before use. At the same time, 52 healthy controls who received normal physiological examinations were also subjected to blood extraction to serve as control group. Control group included 30 males and 26 females, and age ranged from 27 years to 68 years, with a mean age of 48.3 ± 65.6 years. No significant differences in age and gender were found between two groups. The ethics committee in which all authors worked approved this study. All participants signed informed consent.

Follow-up

All patients were followed up for 5 years to record local recurrence. Local recurrence was observed in 26 out of 122 patients and no distant recurrence was observed.

Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted using Trizol reagent (Invitrogen, USA). Reverse transcription was performed to synthesize cDNA using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) through the following thermal conditions: 50 °C for 30 min and 75 °C for 15 min. PCR reaction systems were prepared using SYBR® Green Real-Time PCR Master Mixes (Thermo Fisher Scientific, USA). PCR reactions were performed through the following thermal conditions: 95 °C for 50 s, followed by 40 cycles of 95 °C for 15 s and 60 °C for 40 s. Primers used in PCR reactions were: 5′-GCACATTGGACGGTGTTTCC-3′ (forward) and 5′-CCCAGTCCTTCACAGGTCAC-3′ (reverse) for lncRNA CASC2; 5′-GACCTCTATGCCAACACAGT-3′ (forward) and 5′-AGTACTTGCGCTCAGGAGGA-3′ (reverse) for β-actin. Amplification capabilities of two pairs of primers were similar. For the assay of miRNA-21 expression, miRNA samples were prepared using miRNA Isolation Kit (QIAGEN, Hidden, Germany). mirVana qRT-PCR miRNA Detection Kit (Thermo Fisher Scientific) was used to prepare PCR reaction systems and PCR reactions were performed with U6 as a endogenous control. Ct values were processed and normalized through $2^{-\Delta\Delta CT}$ method. Expression of lncRNA CASC2 was normalized to β-actin.

Cell lines, cell culture, and cell transfection

Cells of SCC090 and SCC25 two human oral squamous cell carcinoma cell lines were bought from ATCC. Cells of SCC090 cell line were cultured with ATCC-formulated Eagle's Minimum Essential Medium (Catalog No. 30-2003) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-Glutamine. Cells of SCC25 cell line were cultured in 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium supplemented with 1.2 g/L sodium bicarbonate, 2.5 mM L-glutamine, 0.5 mM sodium pyruvate, 15 mM HEPES, 400 ng/mL hydrocortisone, and 10% fetal bovine serum. Cells were cultured at 37 °C in a 5% CO_2 incubator. Full-length lncRNA CASC2 cDNA was amplified through PCR and was inserted into pEGFPC3 vector (Clontech, Palo Alto, CA, USA). Vectors were transfected into cancer cells using lipofectamine 2000 reagent (11668-019, Invitrogen, Carlsbad, USA). Cells without transfection were used as control cells and cells transfected with empty vectors were used as negative control cells. Expression of CASC2 was detected by RT-qPCR at 12 h after transfection. Subsequent studies were performed only in cases of overexpression rate of CASC2 compared with control cells and negative control cells reached 200%.

Cell proliferation assay

After transfection and confirmation of CASC2 overexpression, single-cell suspensions with a final density of 4×10^4 cells per well were prepared. Each well of a 96-well plate was filled with 4×10^3 cells in 0.1 mL cell suspension. Cells were cultured at 37 °C in a 5% CO_2 incubator, followed by addition of 10 μL CCK-8 solution 24, 48, 72, and 96 h later. Cells were cultured for additional 4 h and absorbance was measured using Fisherbrand™ accuSkan™ GO UV/Vis Microplate Spectrophotometer (Fisher Scientific) at 450 nm.

Statistical analysis

All data were processed using Graphpad Prism 6 software. Diagnostic values of plasma CASC2 for OSCC were analyzed by ROC curve analysis. Correlation between plasma

levels of CASC2 and clinicopathological data of patients were analyzed by Chi-square analysis. Gene expression and cell proliferation data were expressed as mean ± standard deviation. Comparisons between two groups and among multiple groups were analyzed by *t* test and one way analysis of variance and LSD test, respectively. $p < 0.05$ indicated a difference with statistically significant.

Results

CASC2 expression was downregulated in tumor tissues than in healthy control tissues in 122 patients with OSCC

Expression of CASC2 in tumor tissues and adjacent healthy tissues was detected by RT-qPCR. As shown in Fig. 1, expression levels of CASC2 were significantly lower in tumor tissues than those in adjacent healthy tissues in 112 out of 122 patients with OSCC ($p < 0.05$), indicating the involvement of CASC2 in OSCC.

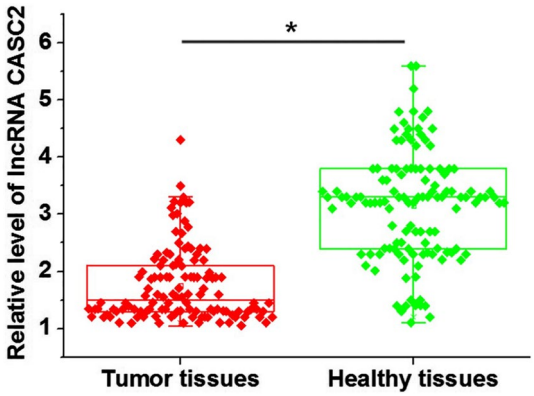

Fig. 1 CASC2 expression was downregulated in tumor tissues than that in healthy control tissues in 122 patients with OSCC (*$p < 0.05$)

Downregulated plasma levels of CASC2 distinguished OSCC patients from healthy controls

Plasma CASC2 in both OSCC patients and healthy controls was also detected by RT-qPCR. As shown in Fig. 2a, compared with healthy controls, significantly downregulated plasma levels of CASC2 were found in patients with OSCC ($p < 0.05$). ROC curve analysis was performed to evaluate the diagnostic value of plasma CASC2 for OSCC. As shown in Fig. 2b, the area under the curve was 0.8445, with standard error of 0.03655 and 95% confident interval of 0.7728–0.9162.

Plasma levels of CASC2 were correlated with tumor size

Correlation between plasma levels of CASC2 and clinicopathological data of patients with OSCC were analyzed by Chi-square analysis. As shown in Table 1, plasma levels of CASC2 were significantly correlated with tumor size ($p < 0.05$), but not age, gender, as well as smoking and drinking habits.

Plasma levels of CASC2 decreased in patients with local recurrence but increased in patients without recurrence

During follow-up, local recurrence was observed in 26 out of 122 patients and no distant recurrence was observed. Compared with pretreatment levels, plasma levels of CASC2 significantly decreased in patients with local recurrence (LR) (Fig. 3a, $p < 0.05$) but significantly increased in patients without recurrence (non-recurrence, NR) (Fig. 3b, $p < 0.05$) (Fig. 4).

LncRNA CASC2 overexpression led to promoted cancer cell proliferation

The above-mentioned data revealed that lncRNA CASC2 is very likely involved in the growth and local recurrence of

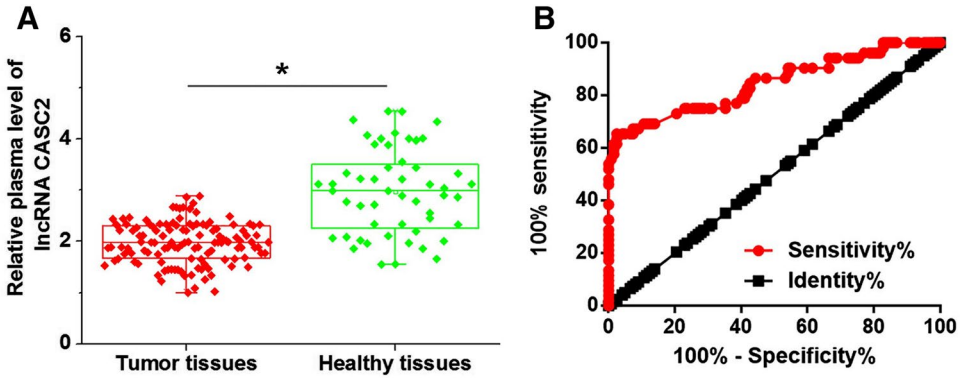

Fig. 2 Downregulated plasma levels of CASC2 distinguished OSCC patients from healthy controls. Data here show the comparison of plasma levels of CASC2 between OSCC patients and healthy controls (**a**) and ROC curve analysis of the diagnostic value of CASC2 for OSCC (**b**) (*$p < 0.05$)

Table 1 Correlation between plasma levels of CASC2 and clinicopathological data of patients with OSCC

Variables	Groups	Cases	High expression	Low expression	χ^2	*p* value
Age	> 50 (years)	58	31	27	0.53	0.47
	< 50 (years)	64	30	34		
Gender	Male	70	32	38	1.21	0.27
	Female	52	29	23		
Smoking	Yes	56	30	26	0.53	0.47
	No	66	31	35		
Drinking	Yes	68	31	37	1.20	0.27
	No	54	30	24		
Tumor size	> 2 cm	74	28	46	11.13	$p < 0.001$
	≤ 2 cm	48	33	15		

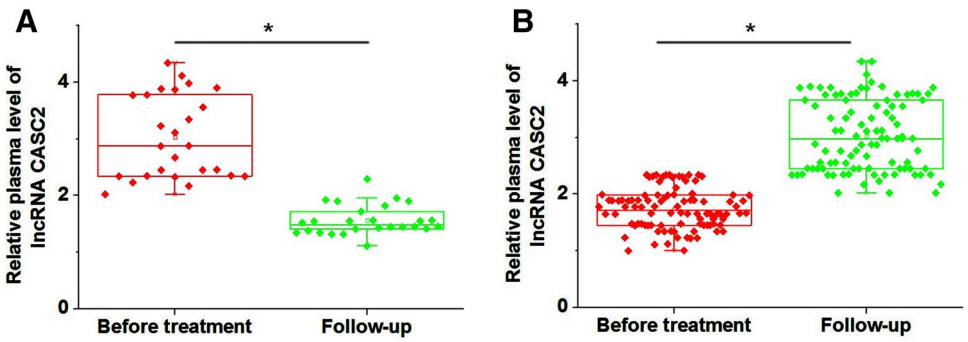

Fig. 3 Plasma levels of CASC2 decreased in patients with local recurrence but increased in patients without recurrence. Compared with pretreatment levels, plasma levels of CASC2 significantly decreased in patients with local recurrence (LR) (**a**) but significantly increased in patients without recurrence (non-recurrence, NR) (**b**) (*$p < 0.05$)

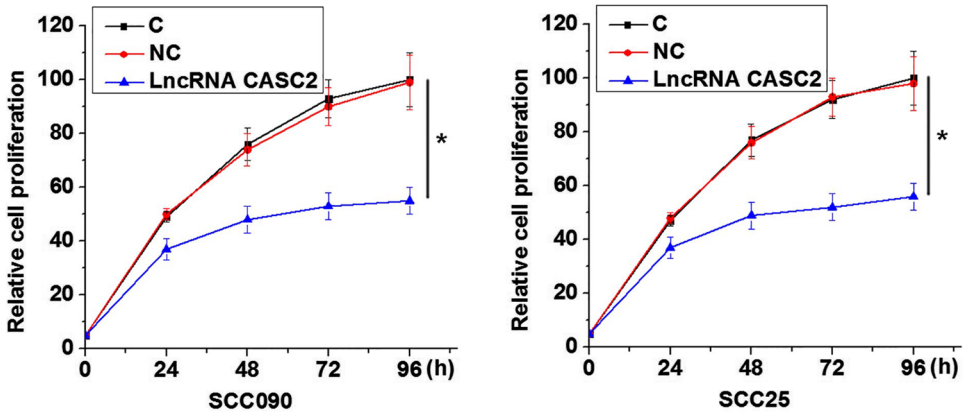

Fig. 4 LncRNA CASC2 overexpression led to promoted cancer cell proliferation (*$p < 0.05$)

OSCC. To further test this hypothesis, CASC2 expression vectors were transfected into cells of two human OSCC cell lines SCC090 and SCC25, and cell proliferation was detected by CCK-8 assay. Compared with control cells (C) and negative control cells (NC), lncRNA CASC2 overexpression led to significantly promoted proliferation of cells of both cell lines.

LncRNA CASC2 overexpression led to inhibited miRNA-21 expression

Compared with control group (C) and negative control group (NC), CASC2 expression vector transfection led to significantly reduced expression levels of miRNA-21 in cells of both human OSCC cell lines SCC090 and SCC25 (Fig. 5, $p < 0.05$).

Discussion

Although the functionality of lncRNA CASC2 has been characterized in several types of human malignancies, its involvement in OSCC is still unknown. The key finding of our study is that lncRNA CASC2 may participate in the recurrence of OSCC after surgical resection.

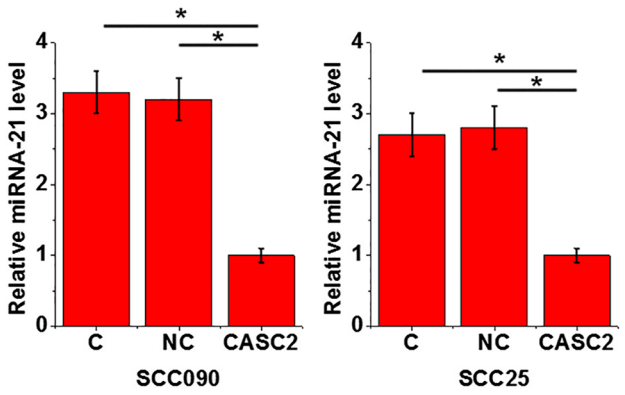

Fig. 5 LncRNA CASC2 overexpression led to inhibited miRNA-21 expression (*$p < 0.05$)

Identification of tumor suppression genes may provide novel therapeutic targets for cancer treatment.

LncRNAs, which have critical functions in almost all critical aspects of cancer development, are considered to be potential treatment targets for cancers [12, 13]. It has been shown that the development of OSCC globally affects the expression pattern of lncRNAs [14], indicating the involvement of lncRNAs in this disease. As a tumor suppressor lncRNA, downregulation of lncRNA CASC2 expression has been observed in several types of human cancers, such as bladder cancer [10] and renal cell carcinoma [11]. In our study, we observed significantly downregulated expression of lncRNA CASC2 in tumor tissues than in adjacent healthy tissues in 122 patients with OSCC. In addition, plasma levels of lncRNA CASC2 were also found to be lower in OSCC patients than those in healthy controls. Those data indicate the potential role of lncRNA CASC2 as a tumor suppressor lncRNA in OSCC.

Tumor metastasis is responsible for most cancer-related deaths [15]. Therefore, the early diagnosis and treatment is still critical for survival of cancer patients. In our study, we only included OSCC patients at stages I and II, which are the early stages of cancer development. ROC curve analysis showed that downregulation of plasma level of lncRNA CASC2 effectively distinguished patients with OSCC from healthy controls. In addition, plasma levels of lncRNA CASC2 showed no significant correlation with patients, age, gender, as well as smoking and drinking habits, which may potentially affect gene expression [16–18]. Those data suggest that plasma lncRNA CASC2 may serve as a potential diagnostic marker for OSCC. However, lncRNA CASC2 shows altered expression in several types of human diseases. Therefore, other diseases should be excluded and multiple markers should be combined to improve the diagnostic accuracy.

Interestingly, our follow-up data revealed that patients with local recurrence after surgical resection showed further downregulated plasma levels of lncRNA CASC2 during follow-up. However, patients without recurrence showed increased plasma levels of lncRNA CASC2. Therefore, plasma level of CASC2 may serve as an indicator of postoperative recurrence of patients with OSCC. We also provided evidence that the actions of lncRNA CASC2 in the postoperative recurrence of OSCC may be mediated by its regulatory role in cancer cell proliferation. Therefore, upregulation of lncRNA CASC2 may serve as a potential target for the prevention of the recurrence of OSCC.

However, the molecular mechanism of the actions of lncRNA CASC2 in the recurrence of OSCC is still unclear. It has been reported that lncRNA CASC2 may achieve its functions through the interaction with miRNAs, such as miRNA-21 [11], which has critical functions in epithelial-to-mesenchymal transition [19]. Our study also showed that lncRNA CASC2 overexpression mediated the downregulation of miRNA-21 in OSCC cells. Therefore, the actions of lncRNA CASC2 in OSCC are likely achieved through the interactions with miRNA-21.

In conclusion, lncRNA CASC2 is downregulated in patients with OSCC and downregulation of lncRNA CASC2 may lead to tumor growth and postoperative tumor recurrence possibly through miRNA-21.

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Compliance with ethical standards

Conflict of interest Yao Dong has no conflict of interest. Wei Wu has no conflict of interest.

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