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Original Article

Long non-coding RNA CASC2 serves as an onco-suppressor in human esophageal squamous cell carcinoma through inhibition of NF-κB pathway

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Abstract: Background: Recent evidence has proven that dysregulation of long non-coding RNAs (lncRNAs) plays critical roles in tumor progression. LncRNA CASC2 was documented to be aberrantly expressed in several kinds of human malignancies and involved in tumor progression. In this research, we aimed to determine the expression profiles and biological functions of CASC2 in esophageal squamous cell carcinoma (ESCC). Methods: qRT-PCR assay was used to detect the expression of CASC2 in both human ESCC cells and ESCC tissue samples. The association of CASC2 expression with clinicopathologic features of ESCC patients was also analyzed. The effects of CASC2 overexpression on cell proliferation, migration and invasion were explored in pcDNA3.1-CASC2-transfected ESCC cells through CCK-8 and transwell assays *in vitro*. ESCC cells with endogenously over expressed CASC2 were transplanted into null mice to examine *in vivo* tumor growth. Key components of the NF-κB signaling pathway were detected using western blotting assay. Results: Our data showed that the relative level of CASC2 in ESCC cells was aberrantly decreased compared to that in normal esophageal epithelial cells. Also, the expression of CASC2 in ESCC tissues was significantly down-regulated than that in adjacent non-tumor tissues. By statistical analyses, CASC2 expression was observed to be significantly correlated with lymph node status and TNM Stage. In ESCC cells, Forced overexpression of CASC2 exerted significant tumor suppressing functions through inhibiting ESCC cell proliferation, migration and invasion *in vitro*, as well as inhibiting tumor growth *in vivo*, including reductions of tumor size and tumor weight. More importantly, increased CASC2 level induced the down-regulation of p65 and p-IκBα proteins and elevation of IκBα protein of NF-κB pathway. Conclusions: Our data provided the first evidence that CASC2 played a critical onco-suppressive role in ESCC development through blocking NF-κB signaling pathway, and a therapeutic strategy through enhancing the CASC2 expression might benefit ESCC patients.

Keywords: Long non-coding RNA, CASC2, esophageal squamous cell carcinoma, proliferation, invasion, NF-κB signaling

Introduction

Esophageal squamous cell carcinoma (ESCC) remains the most prevailing histological subtype of esophageal cancer in developing countries, such as China and Iran [1, 2]. Although advances have been made in early diagnosis and personalized therapy in recent years, the overall 5-year survival rate of patients with ESCC is still less than 10-15% [3]. The etiology of ESCC is complicated and heterogeneous. Although multiple genetic and epigenetic alternations are found to be closely associated with ESCC, the underlying mechanisms of this malignancy remain poorly understood. Accordingly, identification of effective molecular carcinogenesis-associated markers and novel therapeutic approaches for ESCC remains an important aim of ESCC investigation.

Long noncoding RNAs (LncRNAs) comprise a very heterogeneous subclass of endogenous RNA molecules, which are more than 200 nucleotides in length and unable to be translated into proteins because of a lack of an intact open reading frame [4, 5]. Nowadays, accumulating studies documented that lncRNAs play important roles in a wide variety of biological processes, such as regulation of gene expression, posttranslational processing and tumori-

genesis [6, 7]. Increasing number of evidence indicated that some lncRNAs have oncogenic or onco-suppressive functions in the development and progression of ESCC. For example, several studies suggested that MALAT1 exhibits oncogenic activity in ESCC [8-10]. Zhang et al. [11] found that CCAT2 was up-regulated in ESCC tissues and associated with unfavorable overall survival in ESCC patients. Jiang et al. [12] reported that the relative level of TUG1, a pivotal oncogenic lncRNA, was remarkably higher in ESCC tissues compared with adjacent non-tumor tissues, and might help predict a poor prognostic outcome of ESCC patients. Moreover, Xu et al. [13] also reported that *in vitro* silencing of TUG1 repressed the proliferation and migration of ESCC cells and blocked the progression of cell cycle. However, for all we know, the involvement of lncRNAs in ESCC development and progression is just starting to be explored.

Among >3000 human lncRNAs, only <1% have been functionally featured [5]. LncRNA CASC2 (cancer susceptibility candidate 2), located at the 10q26 locus, was originally identified in human endometrial cancer [14]. Following studies have documented that CASC2 serves as a potential onco-suppressor in glioma [15], non-small cell lung cancer [16], colorectal cancer [17] and renal cell carcinoma [18]. To date, however, the regulatory functions of CASC2 in ESCC have not been reported so far.

In the current study, we for the first time validated the down-regulation of CASC2 in ESCC tissues and cell lines, and the onco-suppressive role of CASC2 in ESCC cell proliferation, migration and invasion was also determined *in vitro* and *in vivo*. Our research would undoubtedly provide a potential molecule target involved in the progression of ESCC.

Materials and methods

Patient and tissue specimens

133 pairs of ESCC tissue and the corresponding non-tumor esophageal epithelial tissue samples (located over 5 cm away from the tumor border) were obtained from patients with ESCC who underwent surgery resection between January 2013 and November 2015 at Ningbo Medical Center Lihuili Eastern Hospital (Ningbo, China). All tissues were histopathologically confirmed by two experienced pathologists, and no patient recruited in the study had undergone preoperative chemotherapy or radiotherapy. Clinical data was obtained from medical records. All specimens were immediately kept in RNA keeper tissue stabilizer (Vazyme, Nanjing, China) and stored at -80°C until further use. The study was approved by the Research Ethics Committee of Ningbo Medical Center Lihuili Eastern Hospital. All participants provided written informed consent for the use of their tissue samples in the present study.

Cell culture

Three human ESCC cell lines, EC-109, EC-1 and KYSE-410, were purchased from the Institute of Cell Biology, Shanghai, China. Primary cultures of normal esophageal epithelial cells (NEEC) were established from fresh samples of the corresponding normal esophageal epithelial tissue, as previously described [19]. These cells were grown in RPMI-1640 medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Gibco, Grand Island, NY), 100 μg/ml streptomycin and 100 units/ml penicillin (Sigma-Aldrich, USA) at 37°C and 5% CO_2 in a humidified chamber. The medium was changed every 2-3 d, and the cells were trypsinized at 80-90% confluence.

RNA extraction and qRT-PCR

Total RNA was isolated from tissues and cells using TRIzol reagent (Life Technologies, Scotland, UK). The yield and quality of total RNA was evaluated by measuring the absorbance at 260 and 280 nm using a spectrophotometer (Beckman Coulter, Inc., Brea, CA, USA). The cDNA was converted from total RNA by reverse transcribing the total RNA with a TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The expression of CACS2 mRNA was quantified using SYBR Premix EX Taq™ II kit (TaKaRa, RR820A) on the ABI 7500 Real-Time PCR System (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as the internal reference. Fold changes were calculated using a relative quantification ($2^{-\Delta\Delta Ct}$), according to a previous publication [20].

The primers of CASC2 and GAPDH used for qRT-PCR were synthesized by Invitrogen with the sequences as follows: CASC2 Forward 5'-

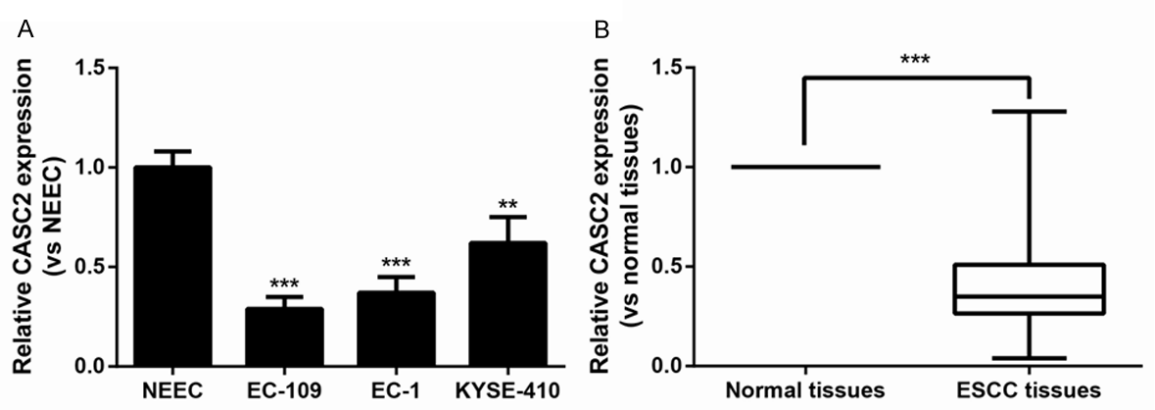

Figure 1. CASC2 is significantly down-regulated in ESCC tissues and cell lines. A. qRT-PCR was conducted to quantify the expression of CASC2 in ESCC cell lines. The results are presented as the fold-change in expression compared with normal esophageal epithelial cell (NEEC). The expression of GAPDH mRNA was used as a normalization control. B. qRT-PCR was conducted to quantify the expression of CASC2 in ESCC tissues. The results are presented as the fold-change in expression compared with adjacent non-tumor tissues. The expression of GAPDH mRNA was used as a normalization control. Results are expressed as means ± SD. $^{**}P<0.01$; $^{***}P<0.001$ by Student's t-test.

GCACATTGGACGGTGTTTCC-3', CASC2 Reverse: 5'-CCCAGTCCTTCACAGGTCAC-3'; GAPDH Forward: 5'-TGTTGCCATCAATGACCCCTT-3', GAPDH Reverse: 5'-CTCCACGACGTACTCAGCG-3'.

Construct generation and transient transfection

To study the effects of CASC2 overexpression on cell activity, the CASC2 full-length sequence was amplified from human genome, and then subcloned into a pCDNA3.1 expression vector (Invitrogen), named as pcDNA3.1-CASC2. Cells were transfected with the aforementioned pCDNA3.1 vectors for 48 h using Lipofectamine 3000 (Invitrogen) following the manufacturer's protocol. After transfection for 48 h, total RNA from the harvested cells was isolated, and the efficiency of overexpression was determined by qRT-PCR. The empty pcDNA3.1 vector was used as the negative controls (NC).

Cell proliferation assay

EC-109 cells placed in a 96-well plate (5×10^3/well) were transfected with pcDNA3.1-CASC2 or NC, and the effects of CASC2 on the proliferation of ESCC cells were investigated every 24 h using a Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan). Cell viability was assessed every 24 h for 3 days by measuring the absorbance at 450 nm using a microplate reader (Bio-Rad, Hercules, CA).

Wound healing assay

A total of 5×10^5 EC-109 cells were seeded on a 6-well plate. After the growing cell layers had reached ~80% confluence, a straight line wound was inflicted by scraping off the cells and then incubated for 48 h. The migration of cells was monitored and pictured under a microscope.

Transwell assay

Cell migration and invasion were assessed using Costar chambers containing transwell inserts (8 μm pore size; Corning Incorporated, USA). The upper chambers were coated with Matrigel (Invitrogen) for the invasion assay. After transfection, EC-109 cells (1×10^5) in 200 μl of serum-free medium were seeded in the upper chamber, while the bottom chambers were filled with 600 μl of 10% FBS-containing medium. After 48 h of incubation, cells in the upper chamber were removed using a cotton swab, whereas the cells that had migrated or invaded through the membrane were fixed in paraformaldehyde and stained with 0.1% crystal violet, imaged, and counted under an inverted microscope (Olympus, Japan) from five different fields.

Western blot analysis

Cells were lysed in cell lysis buffer containing phosphatase inhibitor cocktail and proteinase

Table 1. Association between CASC2 expression and clinicopathological characteristics in ESCC patients (n=133)

Characteristic	Total (n=133)	CASC2 expression		*P* value
		High (n=47)	Low (n=86)	
Age (yr)				0.557
<60	64	21	43	
≥60	69	26	43	
Gender				0.198
Male	86	27	59	
Female	47	20	27	
Tumor size (cm)				0.104
<4 cm	81	33	48	
≥4 cm	52	14	38	
Tumor location				0.729
Upper	32	13	19	
Middle	63	22	41	
Lower	38	12	26	
Lymph node status				0.016
Absence	55	26	29	
Presence	78	21	57	
TNM stage				0.012
I+II	74	33	41	
III+IV	59	14	45	

inhibitor cocktail (Sigma-Aldrich), and protein concentration was quantified using the BCA Protein Assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Protein samples were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% fat-free milk, and incubated with primary antibodies (anti-p65, anti-IκBα and anti-p-IκBα antibodies, Santa Cruz Biotechnology, USA) followed by secondary antibody. The signals were determined using an enhanced chemiluminescence, and the anti-GAPDH antibodies were used as a control.

Animals

A total of 10 male BALB/c-nude mice (4-5 weeks old), purchased from the Shanghai Experimental Animal Center, were housed five per cage in an air-conditioned room under specific pathogen-free conditions. The experimental protocols were in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee of Ningbo Medical Center Lihuili Eastern Hospital.

Tumor xenografts in nude mice

EC-109 cells (1×10^6 in 100 μl PBS) were injected subcutaneously into either side of the posterior flank of each nude mouse. The mice were randomized into two treatment groups with 5 mice per group when the implantations arose, and injected intratumorally with 30 μg of Lipofectamine 3000-encapsulated pcDNA3.1-CASC2 or NC every 3 days. Tumor volume (V) was monitored every 3 days by caliper measurements of the two perpendicular diameters, and calculated following the formula $V=(\text{width}^2 \times \text{length} \times 0.5)$. At 30 days post injection, the mice were sacrificed, and the tumors were excised and weighed.

Statistical analysis

All statistical tests were conducted with SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA). Data were expressed as mean ± standard deviation (SD) or frequencies and percentages if necessary. The differences between two groups were analyzed using the Student's *t* test. The correlation between CASC2 expression and clinicopathological features were evaluated by the χ^2 test. Differences were regarded statistically significant when two-sided *P*-values <0.05.

Results

Expression of CASC2 in human ESCC cells and tissue samples

To validate whether CASC2 was differentially expressed in ESCC, qRT-PCR assay was performed to firstly investigate the expression level of CASC2 in normal esophageal epithelial cell (NEEC) and three ESCC cell lines (EC-109, EC-1 and KYSE-410). According to the results of qRT-PCR, the relative level of CASC2 expression in ESCC cell lines was remarkably downregulated than that in NECC (all *P*<0.01, **Figure 1A**). We next explored the expression of CASC2 in 133 cases of ESCC tissues and corresponding non-tumor tissues. As demonstrated in **Figure 1B**, CASC2 expression level was re-

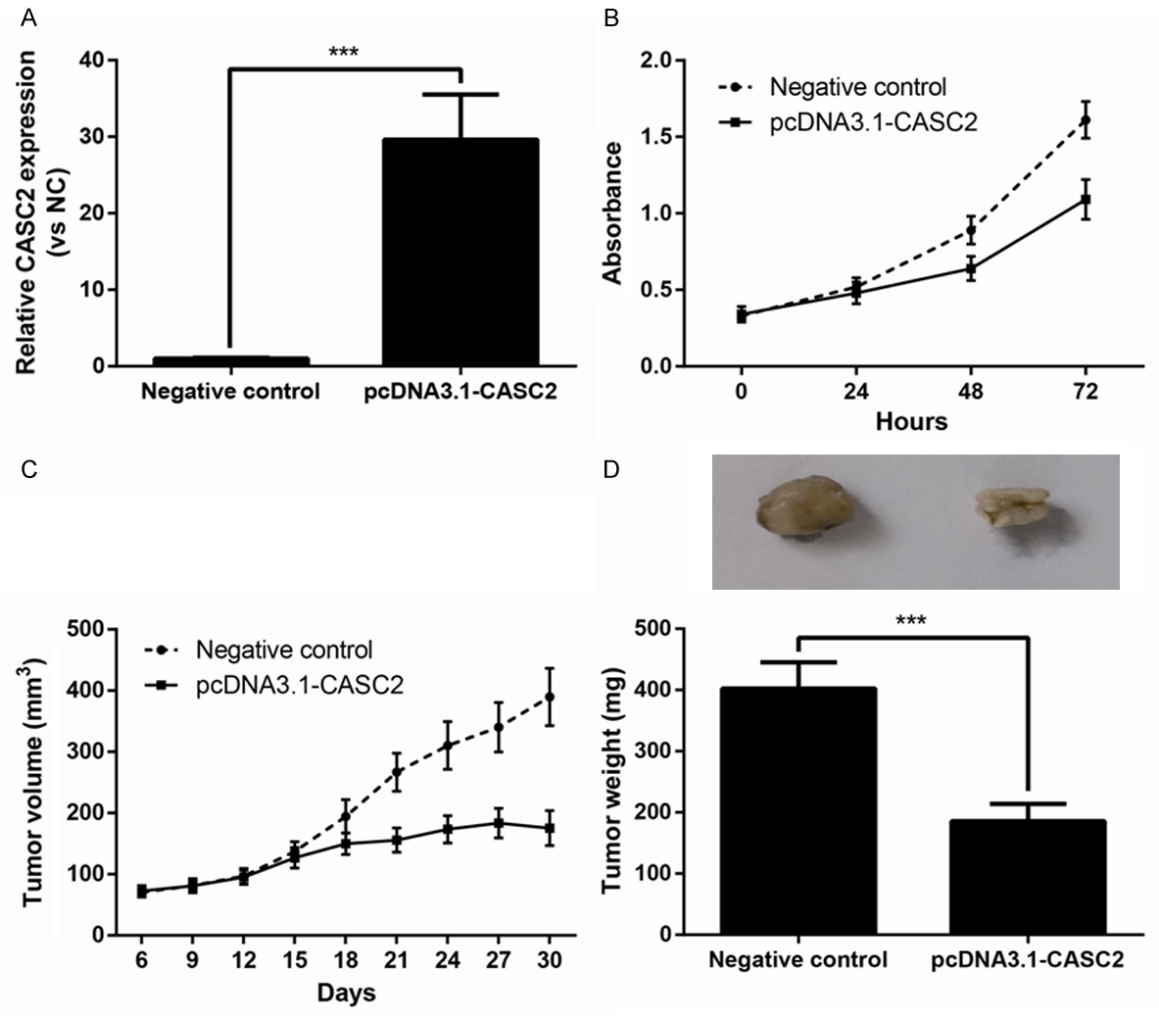

Figure 2. Overexpression of CASC2 in ESCC cells suppressed cell growth *in vitro* and *in vivo*. A. Transfection of pcDNA3.1-CASC2 successfully overexpressed CASC2 in EC-109 cells. B. CCK-8 assays were performed to determine *in vitro* cell proliferation of EC-109 cells transfected with NC and pcDNA3.1-CASC2 at indicated time points. Xenograft tumor models were developed in nude mice by EC-109 cells transfected with NC and pcDNA3.1-CASC2. C. Tumor volumes were measured on the indicated days. D. Representative photographs and average weights of the tumors from each group. Results are expressed as means ± SD. ***P<0.001 by Student's t-test.

markably lower in ESCC tissues than that in adjacent non-tumor tissues (P<0.001).

Associations of CASC2 expression with clinicopathologic features of ESCC patients

Next, we evaluated the correlations of CASC2 expression with clinicopathologic characteristics of patients with ESCC. All 133 ESCC patients were subsequently allocated into two groups: High CASC2 expression group (n=47, CASC2 expression ratio ≥ mean ratio) and Low CASC2 expression group (n=86, CASC2 expression ratio ≤ mean ratio). Then, the correlations of CASC2 expression with clinicopathologic characteristics of patients were statistically analyzed. As shown in **Table 1**, CASC2 expression was observed to be significantly associated with lymph node status (P=0.016) and TNM Stage (P=0.012). However, there were no remarkable correlations between CASC2 levels and other clinicopathologic features, including age, gender, tumor size and tumor location (all P>0.05).

Overexpression of CASC2 in ESCC cells suppressed cell growth in vitro and in vivo

Because of the aberrant expression of CASC2 in ESCC cells and tissues, we considered that it might exert potential regulatory functions in ESCC progression. To assess the role of CASC2

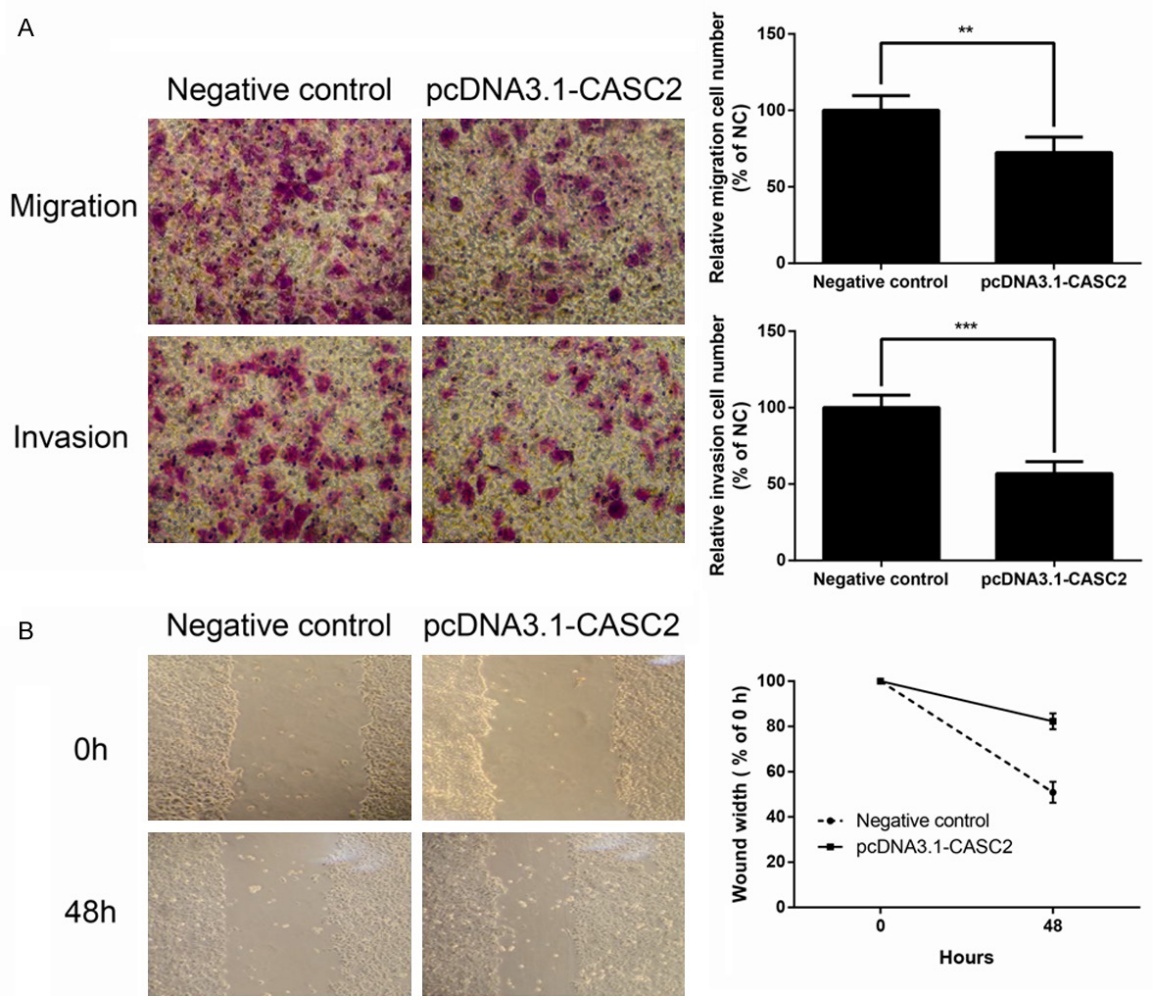

Figure 3. Overexpression of CASC2 inhibited cell migration and invasion *in vitro*. A. Cell migration and invasion of EC-109 cells transfected with NC and pcDNA3.1-CASC2 were investigated through transwell assays. Representative photographs of the transwell assays were shown. B. Cell migration of EC-109 cells transfected with NC and pcDNA3.1-CASC2 was investigated through wound healing assay. Representative photographs of the wound healing assay were shown. Results are expressed as means ± SD. ***P*<0.01; ****P*<0.001 by Student's t-test.

in ESCC growth, ESCC cell line was cultured and transfected with pcDNA3.1-CASC2 or negative control. At 48 h after transfection, the relative levels of CASC2 was analyzed using qRT-PCR and the results showed that the relative levels of CASC2 in EC-109 cells were remarkably up-regulated (*P*<0.001, **Figure 2A**).

To investigate the possible effect of CASC2 on the growth of ESCC cells, the cell proliferation activities of EC-109 cells were determined through CCK-8 assay. Compared with the NC group, cell proliferation was significantly inhibited in EC-109 cells which were transfected with pcDNA3.1-CASC2 (**Figure 2B**). The inhibitory impact of CASC2 overexpression on ESCC proliferation was also found in a nude mice tumor growth model. Overexpression of CASC2 in the ESCC cell lines significantly decreased the size and weight of xenograft tumors in nude mice, compared with mice inoculated with NC EC-109 cells (**Figure 2C** and **2D**).

Overexpression of CASC2 inhibited cell migration and invasion in vitro

To determine the function of CASC2 in ESCC metastasis, we investigated the migrant and invasive capacity of EC-109 cells transfected with pcDNA3.1-CASC2 or negative control. By transwell and wound healing assay, we observed that the percentage of cells travelled across the micropore membrane was dramatically decreased (**Figure 3A**), and the relative

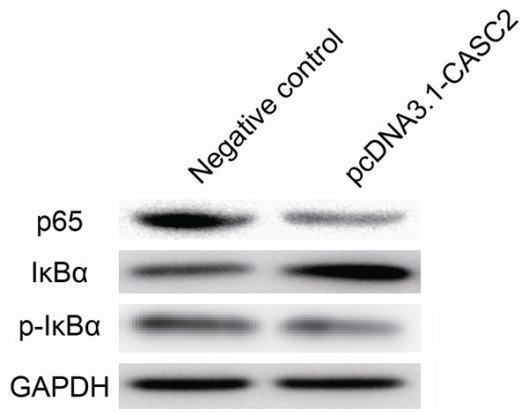

Figure 4. Overexpression of CASC2 inhibited the activation of NF-κB signaling pathway. Western blotting assay was performed to investigate the expressions of key components p65, p-IκBα, and total IκBα of NF-κB signaling pathway in EC-109 cells transfected with NC and pcDNA3.1-CASC2. The expression of GAPDH protein was used as a normalization control.

migrating distance of cells was remarkably shorter in pcDNA3.1-CASC2-transfected EC-109 cells as compared to the NC cells (**Figure 3B**). In addition, through matrigel-coated transwell assay, we found that the percentages of pcDNA3.1-CASC2-transfected EC-109 cells that invaded through the matrigel were dramatically down-regulated than those of NC cells (**Figure 3A**). These results indicated that overexpression of CASC2 suppressed the migration and invasion of ESCC cells.

Overexpression of CASC2 inhibited the activation of NF-κB signaling pathway

Due to the importance of NF-κB pathway in ESCC [21], in the present study, we explored the effect of CASC2 up-regulation on NF-κB pathway in EC-109 cells. We observed that levels of p65 and p-IκBα proteins in pcDNA3.1-CASC2-transfected EC-109 cells were dramatically lower than those in NC cells, but expression of IκBα protein in pcDNA3.1-CASC2-transfected EC-109 cells was evidently elevated than those in NC cells (**Figure 4**), indicating that CASC2 overexpression effectively blockades NF-κB pathway in ESCC cells.

Discussion

ESCC is one of the most common human malignancies in the world, so a comprehensive understanding of the mechanisms underlying ESCC progression is of great significance to identify more promising diagnostic biomarkers and effective therapeutic targets. To the best of our knowledge, this might be the first research of CASC2, a cancer-associated lncRNA, being involved in ESCC progression. In this report, we observed that CASC2 expression was significantly higher in ESCC tissues compared with paired-adjacent non-tumorous tissues and down-regulated CASC2 level was positively associated with greater lymph node metastasis and advanced TNM stage. In addition, overexpressed CASC2 could suppress the malignant phenotype of ESCC cells through regulating multiple cellular processes, such as migration, invasion and proliferation.

It has become clear that mammalian genomes encode thousands of lncRNAs [22]. Until now, roles of lncRNAs as oncogenic and tumor suppressive drivers in several cancer types have been reported in the literature [23, 24]. Recently, an lncRNA named CASC2, originally identified by Baldinu and coworkers in human endometrial cancer [14], has been demonstrated to exert extensive regulatory functions in a variety of human cancers [15-18]. In this article, we identified decreased CASC2 levels in ESCC tissues versus non-cancerous tissues through qRT-PCR. Similar results were also found in three ESCC cell lines. Additionally, the expression levels of CASC2 were reduced in samples from patients with higher tumor burdens, who were considered as those with advanced TNM stage and increased lymph node metastasis. Taken together, our results indicated that down-regulated expression levels of CASC2 might serve a critical role in the development and progression of ESCC.

To further assess the function of CASC2 in ESCC, we investigated the effects of gain of function of CASC2 on multiple aspects of ESCC biology. First, we observed that overexpression of CASC2 in ESCC cells led to a remarkable inhibition of *in vitro* migration and invasion. Besides, overexpression of CASC2 suppressed ESCC cell proliferation and tumor growth both *in vitro* and *in vivo*. Collectively, all of the results from the *in vitro* and *in vivo* experiments revealed that CASC2 inhibited the proliferation and invasion of ESCC; accordingly, CASC2 represents a novel promising target for ESCC treatment. We considered that this tendency should be validated in more tumor types, therefore strengthening the clinical value of CASC2.

NF-κB has been documented to be constitutively activated in various types of tumors, including ESCC, and blocking the NF-κB pathway results in the suppression of tumor growth, and metastasis of human ESCC, revealing the crucial role of NF-κB pathway in ESCC progression [25-27]. Activation of NF-κB pathway typically involves the phosphorylation of IκB by the IκB kinase (IKK) complex, which results in IκB degradation. To explore whether CASC2 can block the NF-κB signaling pathway, to this end, the phosphorylation level of IκBα and expressions of p65 subunit and total IκBα level of the NF-κB signaling pathway were detected, and the results demonstrated that CASC2 overexpression significantly decreased the phosphorylation level of IκBα and p65 expression, but elevated total IκBα level in ESCC cells, indicating that CASC2 can block the NF-κB signaling pathway in ESCC.

In summary, the observations of this study, for the first time, suggested that the expression of CASC2 correlates strongly with the clinical stages of ESCC patients and that the down-regulation of CASC2 exerts important regulatory functions in ESCC progression. However, further studies are required to explore the underlying mechanisms of CASC2 in the regulation of ESCC progression. Such studies might contribute to provide novel therapeutic strategies for ESCC treatment in the near future.

Disclosure of conflict of interest

None.

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