

Higher lncRNA CASC15 expression predicts poor prognosis and associates with tumor growth in cervical cancer

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Abstract. – OBJECTIVE: To investigate the role of long non-coding RNA Cancer Susceptibility Candidate 15 (CASC15) in cervical cancer and its potential molecular mechanism.

PATIENTS AND METHODS: The CASC15 expression was measured in cervical cancer tissues and cell lines by using quantitative Real-time polymerase chain reaction (qRT-PCR) analysis. Cell counting kit-8 (CCK8), flow cytometry analysis and transwell cell invasion assays were employed to detect the capacities of cell proliferation and cell invasion. Furthermore, Western blot analysis was applied to detect the E-cadherin and N-cadherin expression in EMT pathway.

RESULTS: We demonstrated that lncRNA CASC15 expression was higher in cervical cancer tissues compared to adjacent normal tissues. Higher lncRNA CASC15 expression associated with lymph node metastasis and FIGO stage. Moreover, our results showed that higher lncRNA CASC15 expression predicted poor prognosis of cervical cancer. Functional assays showed that knockdown of lncRNA CASC15 suppressed cell proliferation and cell cycle progression in cervical cancer. Moreover, we also found that knockdown of lncRNA CASC15 inhibited cell invasion ability and Epithelial-Mesenchymal Transition (EMT) signaling pathway by upregulating E-cadherin and downregulating N-cadherin expression in cervical cancer.

CONCLUSIONS: These results indicated that lncRNA CASC15 expression may be a prognostic biomarker and contributed to cell proliferation and invasion in cervical cancer.

Key Words:

Long noncoding RNA, CASC15, Tumor prognosis, Cell proliferation.

Introduction

Cervical cancer (CC) is one of the most common malignant gynecologic cancers and listed as

the leading reason of cancer-associated mortality for females¹. Although later advances in therapeutic methods including surgical resection, radiotherapy and chemotherapy, the overall survival is still unsatisfactory due to tumor recurrence². In clinical, DNA, protein and other metabolic markers are developed for the diagnosis of cervical cancer including SSC-Ag, CA-125, CEA, and cytokeratins³. However, no single screening bio-maker was used to detect this disease for highly sensitive and highly specific⁴. Thus, the underlying biological mechanisms in the progress of CC and develop a better biomarker for diagnosis and therapy of cervical cancer are urgent.

Long noncoding RNAs (lncRNAs) are identified as a class of noncoding RNA containing more than 200 nucleotides⁵. So far, emerging studies have showed the functional roles of lncRNAs in cervical cancer. Upregulation of lncRNA PANDAR predicts poor prognosis and promotes cell proliferation in cervical cancer⁶. Downregulation of long noncoding RNA MEG3 is associated with poor prognosis and promoter hypermethylation in cervical cancer⁷. Upregulation of long noncoding RNA TUG1 promotes cervical cancer cell proliferation and migration⁸. Long non-coding RNA CCHE1 overexpression predicts a poor prognosis for cervical cancer⁹.

lncRNA CASC15 acts as an oncogene in some tumors. Such as, lncRNA CASC15 overexpression promotes colon cancer cell proliferation and metastasis by regulating the miR-4310/LGR5/Wnt/ β -catenin signaling pathway¹⁰. High expression of lncRNA CASC15 is a risk factor for gastric cancer prognosis and promotes the proliferation of gastric cancer¹¹. Long non-coding RNA CASC15 is upregulated in hepatocellular carcinoma and facilitates hepatocarcinogenesis¹². However, the role of lncRNA CASC15 in cervical

cancer and its potential molecular mechanism remain unknown.

We found that lncRNA CASC15 was higher in cervical cancer tissues compared to adjacent normal tissues. Higher lncRNA CASC15 expression predicted a poor prognosis of cervical cancer. Furthermore, we showed that reduced lncRNA CASC15 expression suppressed cell proliferation, cell invasion and EMT pathway. Thus, our findings indicated that lncRNA CASC15 may be a target of lncRNA CASC15 treatment.

Patients and Methods

Tissue Samples

We collect 62 pairs of cervical cancer tissues and adjacent tissues from Department of Gynecology and Obstetrics, Chui Yang Liu Hospital Affiliated to Tsinghua University, age 45±8.5 years old. All tissues were confirmed by clinical pathological examination. The staging criteria for cervical cancer were in accordance with the 2014 FIGO staging criteria¹³. The study was approved by the Ethics Committee of Chui Yang Liu Hospital Affiliated to Tsinghua University. The tissues RNA were extracted and stored at -80°C for further analysis. The characteristics of patients with cervical cancer are shown in Table I.

Cell Culture

Three human cervical cancer cell lines including HeLa, Caski and C33A cell lines and a primary normal cervical squamous cells (NCSC), were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All of the cell lines were cultured in Roswell Park Memorial Institute(RPMI)-1640 medium supplemented with 10%, fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cells were maintained in humidified incubator at 37°C containing 5% of CO₂.

Cell Transfection

The two siRNAs against lncRNA CASC15 (siRNAs sequences: si-CASC15-1: sense, 5'-CCCTCAGGTGACTACAGAT-3' and antisense, 5'-GCTCAACCACATCTAATTT-3', si-CASC15-2: sense, 5'-GCAACATGCTTCACTGTCT-3' and antisense, 5'-GATCGCTGGGAATTCTCCAC-3') were purchased from Guangzhou Ribo Bio Co., Ltd., (Guangzhou, China). The cells were seeded into 6-well plates at a density of 1×10⁵ cells/well. The si-CASC15-1, si-CASC15-2 or si-negative control (si-NC) was transfected into HeLa or Caski cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA) according to the instructions provided by the manufacturer. Functional assays were performed 48 h post transfection.

Table I. The correlation between lncRNA CASC15 expression and clinicopathological features.

Features	lncRNA CASC15 expression			p-value
	Total (n=62)	Low (n=29)	High (n=33)	
Age				0.849
< 45	27	13	14	
> 45	35	16	19	
Tumor size				0.686
< 4 cm	24	12	12	
> 4 cm	38	17	21	
Differentiation				0.550
Highly and moderately	45	20	25	
Lowly	17	9	8	
Lymphatic metastasis				0.017*
No	35	21	14	
Yes	27	8	19	
HPV 16/18 infection				0.492
Positive	40	20	20	
Negative	22	9	13	
FIGO stage				0.016*
I	39	23	16	
II	23	6	17	

*p < 0.05.

Quantitative Reverse Transcriptase Polymerase Chain Reaction (QRT-PCR) Analysis

Total RNA was extracted from tissues and cell lines using TRIzol reagent (Beyotime Institute of Biotechnology, Shanghai, China). RNA was reverse transcribed to cDNA by Prime Script RT Master Mix (TaKaRa Bio, Inc., Dalian, China). QRT-PCR was performed with the SYBR® Premix Ex Taq™ kit (TaKaRa Bio, Inc., Dalian, China) on an ABI PRISM 7900 Real-Time system (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. The reaction temperature was listed as follows: initial denaturation at 95°C for 2 min, followed by 35 repeats of the three-step cycling program consisting of 30 s at 95°C (denaturation), 1 min at 53°C (primer annealing) and 30 s at 72°C (elongation), followed by a final extension step for 10 min at 72°C. The mRNA expression was normalized to GAPDH and was assessed using the $2^{-\Delta\Delta C_t}$ methods. The following primers were used: CASC15 forward, 5'-CACACGCATGGAAAACCCAG-3' and reverse, 5'-GAGGACCTGAGCTGTAAGCC-3'.

CCK-8 Analysis

Cell proliferation was detected with transfected cervical cancer cells, which were seeded in a 96-well plate (3000 cells/well) for 24 h. Next, cells were cultured at 0, 24, 48, and 72 h. A 10 μ l CCK8 solution (5 mg/ml; Beyotime Institute of Biotechnology, Shanghai, China) was added to each well and incubated for additional 2 h. After incubation for 2 h, cell proliferation was detected using the Multiskan Spectrum equipment (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and the optical density was measured at 450 nm.

Flow Cytometry Analysis

HeLa and Caski cells were transfected with si-CASC15-1 or negative control siRNA. After cell transfection at 48 h, cells were incubated with RNase A (TaKaRa Bio, Inc., Otsu, Shiga, Japan) at 100 μ g/ml for 20 min at 37°C. Then, cells were stained with propidium iodide (PI; BD Biosciences, Franklin Lakes, NJ, USA) at 50 μ g/ml for 20 min. Cells cycle were detected by using a BD FACS Calibur™ flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) and the results are expressed as

the mean \pm SD. Statistical difference was analyzed using Student's *t*-test (two-tailed) between two groups, or one-way analysis of variance followed by Tukey's post-hoc test for more than two groups comparisons. A $p < 0.05$ was considered as statistically significant.

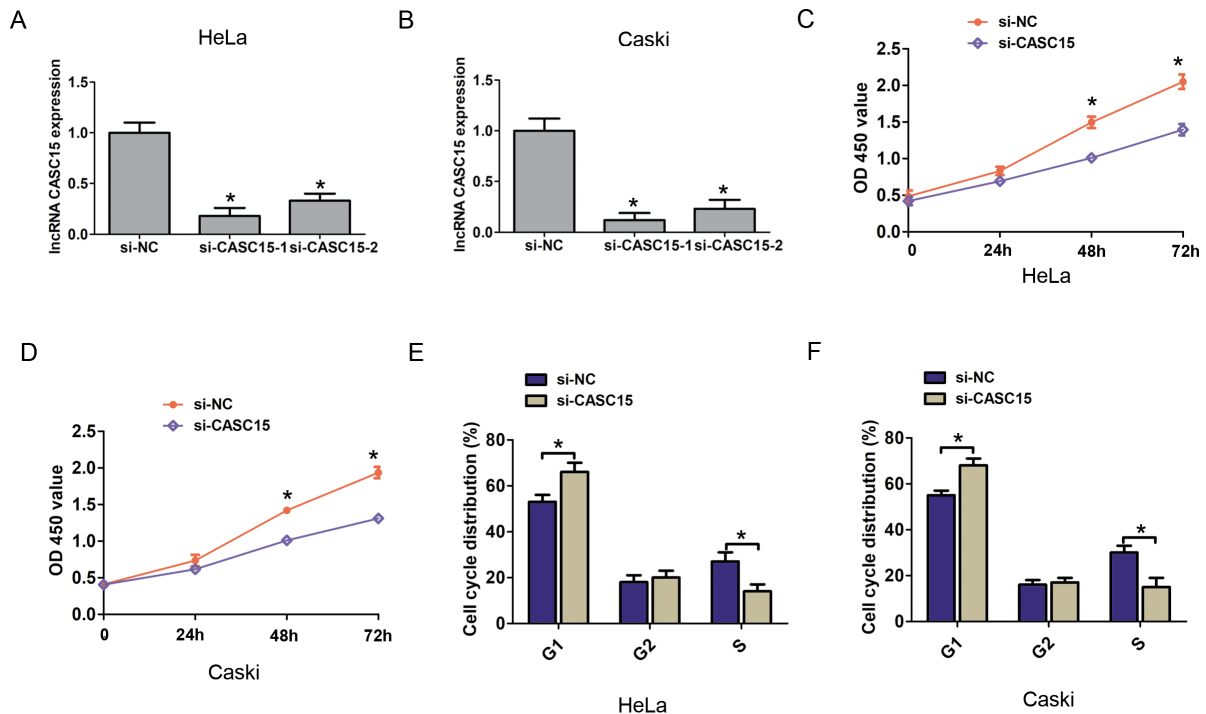
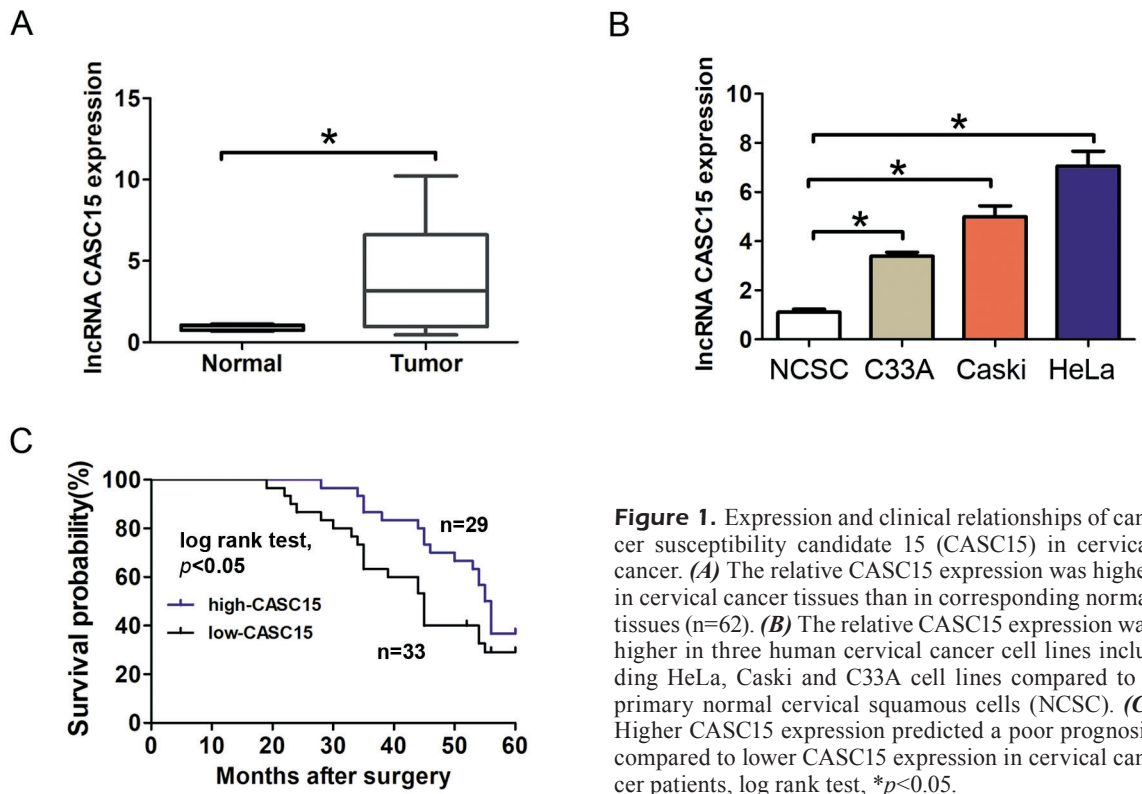
Results

lncRNA CASC15 Expression is Higher in Cervical Cancer Tissues and Cells

The lncRNA CASC15 expression was evaluated using qRT-PCR analysis from 62 cervical cancer patients. As shown in Figure 1A, lncRNA CASC15 expression was significantly upregulated in cervical cancer tissues compared with normal tissues ($p < 0.05$). Then, we examined the expression of lncRNA CASC15 in cervical cancer cells. We used the primary normal cervical squamous cells (NCSC) as the normal control. Among the cervical cancer cells, the lncRNA CASC15 expressions were notably upregulated in cervical cancer compared with NCSC cells (Figure 1B, $p < 0.05$). According to the mean expression of lncRNA CASC15 in cervical cancer tissues, we divided lncRNA CASC15 expression into two groups (high lncRNA CASC15 expression and low lncRNA CASC15 expression group). By Chi-square test, we found that higher lncRNA CASC15 expression associated with lymph node metastasis and FIGO stage (All of $p < 0.05$, Table I). Moreover, survival plots results showed that higher lncRNA CASC15 expression predicted a poor prognosis of cervical cancer compared to lower lncRNA CASC15 expression in cervical cancer patients (Figure 1C, log rank test, $p < 0.05$). Thus, these results indicated that higher lncRNA CASC15 expression showed an important role in cervical cancer.

Reduced lncRNA CASC15 Expression Suppresses Cervical Cancer Cell Proliferation and Cell Cycle Progression

As lncRNA CASC15 expression was significantly upregulated in cervical cancer tissues, it was hypothesized that it may be involved in tumor cell proliferation. We selected HeLa and Caski cells for CASC15 knockdown in the following experimental study (Figure 2A-2B). The results of CCK8 assay revealed that transfection with si-CASC15 in HeLa and Caski cells suppressed the proliferation of cervical cancer cells compared to si-NC groups (Figure 2C-2D). Cell cycle assays showed that HeLa and Caski CA33 cells transfected with si-CASC15 inhibited S phase cell number, but increasing the



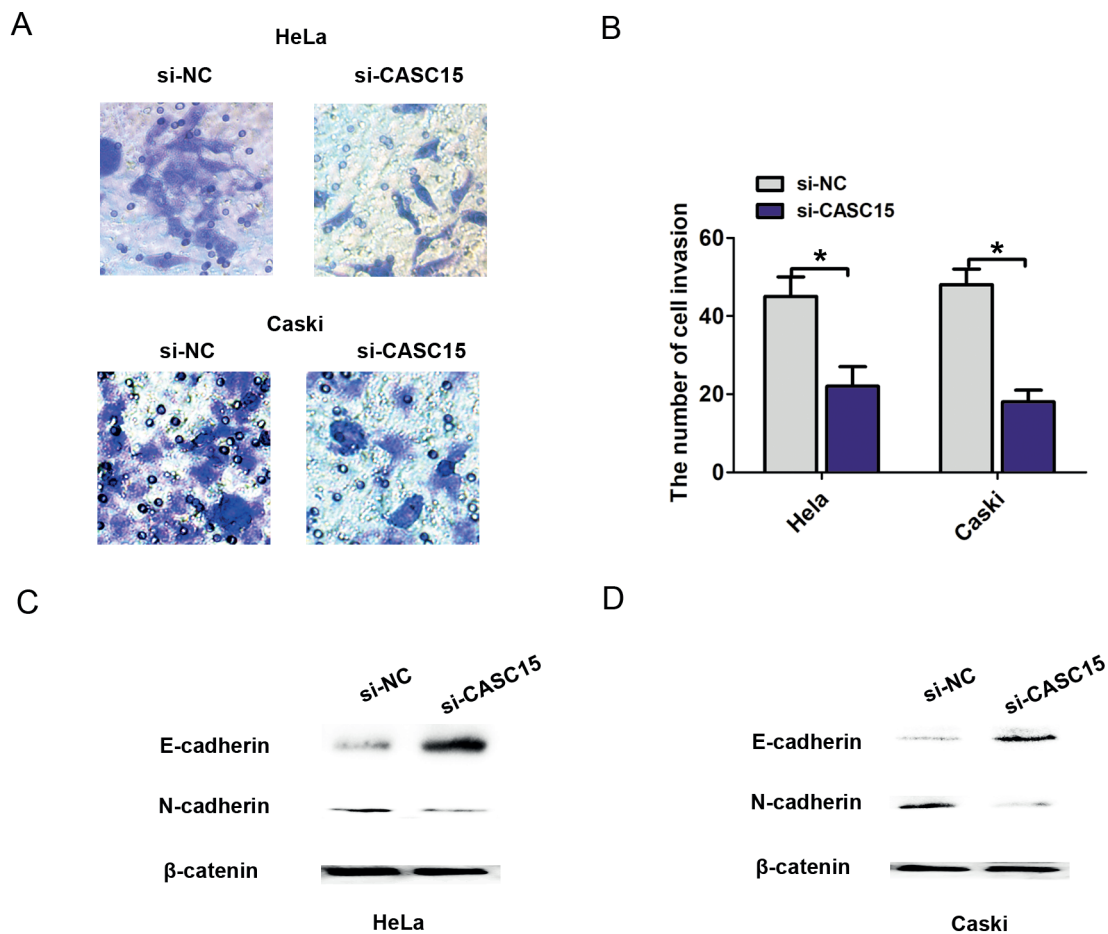


Figure 3. The effects of CASC15 knockdown on cervical cancer cell invasion and EMT pathway. (A-B) Transwell cell invasion assays results in si-CASC15-transfected and control siRNA-transfected HeLa and Caski cells. (C-D) Western blot analysis results showed the protein expression of E-cadherin and N-cadherin in si-CASC15-transfected and control siRNA-transfected HeLa and Caski cells. * $p < 0.05$.

G1 phase cell number compared to si-NC groups (Figure 2E-2F). These results indicated that lncRNA CASC15 may serve as an important regulator in cervical cancer cell proliferation.

Downregulation of lncRNA CASC15 Inhibits Cell Invasion and EMT Pathway in Cervical Cancer

Transwell invasion assay was carried out to investigate the effects of lncRNA CASC15 expression on cervical cancer cell invasion. HeLa and Caski cells transfected with si-CASC15 group exhibited decreased invasive abilities compared with those displayed by si-NC group (Figure 3A-3B). Furthermore, we found that HeLa and Caski cells transfected with si-CASC15 group exhibited decreased N-cadherin expression but increased the E-cadherin expression compared with those

displayed by si-NC groups (Figure 3C-3D). These results indicated that reduced lncRNA CASC15 expression could suppress cell invasion and EMT pathway in cervical cancer.

Discussion

Cervical cancer is ranked as one of the most common cancers causing mortality in females. Tumor targeted therapies have been advanced to interfere with the key signaling pathway components in cervical cancer¹⁴. lncRNAs have been considered as crucial regulatory molecules in various human cancers including cervical cancer¹⁵. To investigate the underlying clinical role and functional effects on cervical cancer progression could provide value for diagnosis and therapy of cervical cancer.

Cancer susceptibility candidate 15 (CASC15), a lncRNA, has been found to be involved in tumor progression. Higher long non-coding RNA CASC15 in hepatocellular carcinoma is correlated with a poor prognosis and facilitates hepatocarcinogenesis¹². Long non-coding RNA CASC15 regulates gastric cancer cell proliferation, migration and epithelial mesenchymal transition by targeting CDKN1A and ZEB1¹⁶. Long non-coding RNA CASC15 promotes tongue squamous carcinoma progression through targeting miR-33a-5p¹⁷. Long non-coding RNA CASC15 promotes melanoma progression by epigenetically regulating PDCD4¹⁸. However, the role of lncRNA CASC15 in cervical cancer and potential molecular mechanism remain unknown.

In the study, we demonstrated that lncRNA CASC15 was higher in cervical cancer tissues compared to adjacent normal tissues. Higher lncRNA CASC15 expression associated with lymph node metastasis and advanced FIGO stage. Moreover, our results showed that higher lncRNA CASC15 expression predicted a poor prognosis of cervical cancer. Furthermore, we investigated the effects lncRNA CASC15 expression on cell proliferation and invasion in cervical cancer. Functional assays showed that lncRNA CASC15 knockdown significantly suppressed cell proliferation, invasion, cell cycle progression and EMT signaling pathway. Together, these results indicated that lncRNA CASC15 may serve as a target of lncRNA CASC15 treatment.

Conclusions

We first demonstrated that lncRNA CASC15 expression was higher in cervical cancer tissues and cells. Higher promoted cell proliferation, invasion, cell cycle progression and EMT signaling pathway. Together, we showed that CASC15 may serve as a target of lncRNA CASC15 treatment.

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