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Independent prognostic Factor of low-expressed LncRNA ZNF667-AS1 for cervical cancer and inhibitory function on the proliferation of cervical cancer

L.-P. ZHAO[1], R.-H. LI[1], D.-M. HAN[1], X.-Q. ZHANG[1], G.-X. NIAN[1], M.-X. WU[1], Y. FENG[1], L. ZHANG[1], Z.-G. SUN[2]

[1]Department of Obstetrics and Gynecology, The 4th Affiliated Hospital of Baotou Medical College, Baotou, Inner Mongolia, China
[2]Department of Neurosurgery, The 4th Affiliated Hospital of Baotou Medical College, Baotou, Inner Mongolia, China

Abstract. – OBJECTIVE: To investigate the expression of long non-coding RNA ZNF667-AS1 in cervical cancer and its effect on the proliferation of cervical cancer cell line, SiHa cells.

MATERIALS AND METHODS: The expression level of ZNF667-AS1 from two microarray datasets (GSE63514 and GSE6791) and TCGA (The Cancer Genome Atlas) were selected to analyze the difference between cervical cancer tissues and normal cervical tissues with bioinformatics methods. Then, the prognosis of ZNF667-AS1 was calculated in TCGA. The expression of LncRNA ZNF667-AS1 in 30 normal cervical tissues and 60 cervical cancer tissue samples was explored using qRT-PCR. In addition, analysis of the clinical data found that the expression of LncRNA ZNF667-AS1 was correlated with the total survival, tumor size and International Federation of Gynecology and Obstetrics (FIGO) stage. At last, the proliferative ability was detected by CCK8 and colon formation assay.

RESULTS: Search the relevant microarray datasets using the keywords "cervical cancer" and "GPL570" from the Gene Expression Omnibus (GEO) database. Afterwards, two microarray datasets (GSE63514 and GSE6791) were selected to analyze the differentially expressed genes in cervical cancer tissues and normal cervical tissues using bioinformatics methods. The results showed that the expression of LncRNA ZNF667-AS1 in cervical cancer was significantly lower than that in normal cervical tissues. 30 normal cervical tissues and 60 cervical cancer tissue samples were selected to extract total RNA for qRT-PCR experiment, and found that the expression of LncRNA ZNF667-AS1 in cervical cancer tissues was lower than that in normal cervical tissues, which was consistent with that of TCGA. Analysis of the clinical data found that the expression of LncRNA ZNF667-AS1 was correlated with the total survival, tumor size and FIGO stage. Compared with the negative control group, the proliferation ability and cell cloning ability of cells with over-expressed LncRNA ZNF667-AS1 were significantly decreased ($p<0.001$), indicating that overexpression of ZNF667-AS1 inhibited the proliferation of SiHa cells.

CONCLUSIONS: Expression of LncRNA ZNF667-AS1 was significantly lower in cervical cancer tissues, and its expression was negatively correlated with the overall survival, tumor size and FIGO stage. ZNF667-AS1 inhibited the proliferation of cervical cancer cells and was expected to be the biomarker and potential therapeutic target for predicting cervical cancer and determining its prognosis.

Key Words:
Cervical cancer, ZNF667-AS1, lncRNA, TCGA.

Introduction

Cervical cancer is one of the most common malignancies in gynecology, with the second highest incidence of global malignancy, and the mortality rate ranks fourth worldwide[1-3]. 80% cases of cervical cancer occurred in developing countries, most of which were in middle and late stages when diagnosed; therefore, looking for reliable tumor biomarkers for early prevention and treatment of cervical cancer, diagnosis and treatment is of great significance. Long non-coding RNAs (LncRNAs) are a class of RNA molecules with over 200 nucleotides in length. With the improvement of

Corresponding Author: Zhigang Sun, MD; e-mail: sunzhigang1818@sina.com

gene sequencing techniques, many reports have revealed that long non-RNA can play a role in epigenetic regulation, transcriptional regulation, and post-transcriptional regulation[4]. Certain changes of specific lncRNAs are presented in tumor cells and this change can be used as a biomarker for diagnosing cancer and also serves as potential drug target, which makes them a new hot topic in the current research. A large number of studies[5-8] have found that lncRNA expression in various tumor cells, such as bladder cancer, lung cancer, and cervical cancer, was different. LncRNAs can be used as biological indicators in cancer diagnosis and prediction. However, only few lncRNAs, such as HOTAIR, MEG3, MALATl, GAS5, and EBIC[9-13] have been shown to be associated with the development of cervical cancer. Further investigations are needed to explore whether there are other unknown or less known lncRNAs playing an important role in the pathogenesis of cervical cancer[14]. Although LncRNA has great potential in studying the development and progression of tumors, the mechanism of LncRNA is very complex and has not yet been studied. ZN F667 (Zinc finger protein 667) is a member of the zinc finger protein C2H2 family, which was initially found to be significantly overexpressed in myocardial ischemia[15,16]. As a widely expressed protein in tissues, the ZNF667 protein has been reported in brain astrocytomas and hepatocellular carcinoma (HCC)[17]. However, the study of ZNF667-AS1 (ZNF667 antisense RNA 1) has not been reported. In our study, we first downloaded the common microarrays (GSE63514 and GSE6791) from the GEO database. Through comprehensive analysis, we found that expression of LncRNA ZNF667-AS1 was significantly lower in cervical cancer than in normal cervical tissues. Therefore, we suspected that ZNF667-AS1 was a candidate gene that inhibited the occurrence of cervical cancer. After that, we found that the downregulation degree of LncRNA ZNF667-AS1 was correlated with the overall survival time, tumor size and FIGO stage of cervical cancer patients through clinical information analysis. Results of proliferation experiments in cervical cancer cell line, SiHa cells showed that overexpression of LncRNA ZNF667-AS1 could inhibit the proliferation of SiHa cells. These results indicated that LncRNA ZNF667-AS1 was significantly lower in cervical cancer and inhibited the proliferation of cervical cancer cells, which was expected to be a predictor of cervical cancer, as well as a molecular biomarker for prognosis and a potential therapeutic target. At present, there is no study on LncRNA ZNF667-AS1 in cervical cancer.

Materials and Methods

Analysis of LncRNA ZNF667 - AS1 Expression

The prognostic information and expression profiles of cervical cancer patients were downloaded from the TCGA database and analyzed by the edger function. The common cervical cancer data set (GSE63514 and GSE6791) were downloaded from the GEO database, and then we re-annotated the probe with blast + 2.2.30 to exclude probes that correspond to multiple genes. Maximum homogeneous signal method was used to deal with multiple probes corresponding to one gene.

Cell Culture and siRNA Transfection

SiHa cells, a kind of cervical cancer cell line, were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA), and cells were incubated according to the conventional cell culture method in the sterile table. Cells were cultured in an incubator at 37°C, 5% CO_2. The cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) when the growth density was about 80% in the 6-well plates; 4 μL of Lipofectamine 2000 and 2 ug of pcDNA-ZNF667-AS1 dissolved in 500 μL of 1640 medium (Gibco, Grand Island, NY, USA) were added to each well. The same amount of Lipofectamine 2000 and pcDNA-NC were given in the experimental group, and the culture medium was changed 6 h after transfection.

RNA Extraction and qRT-PCR

After 24 h of transfection, 1 mL of TRIzol was used for collecting and extracting the total RNA. 1% agarose gel electrophoresis was used to detect the integrity of the RNA sample. The UV spectrophotometer was quantified and the reverse transcription kit was used for reversing it into cDNA according to the manufacture's protocol. The expression level of ZNF667-AS1 was detected by qRT-PCR. The reaction conditions were qPCR: 95°C for 2 min, 95°C for 10 s, 56°C for 10 s, 68°C for 12 s, 40 cycles; 95°C for 1 min; 55°C 1 min; 70°C 6 s; 70°C 6 s; GAPDH was used as the loading control, GAPDH-F: 5'-CCCACTCCTC-CACCTTTGAC-3', GAPDH-R: 5'-GGATCTC-

GCTCCTGGAAGATG-3'. PCR conditions were the same as the above, each experiment was performed in triplicate.

Cell Proliferation Assay by CCK8 Method

The transfection time point was 0 h, medium was changed 6 h later, cells were inoculated into 96-well plates at a density of 2×10^3/100 μL at 24 h and Cell Counting Kit-8 (CCK8) assay was performed after it was cultured for 24, 48, 72 and 96 h. The serum-free medium was replaced at the time of detection. 10 μL of CCK8 was added to each well. After incubation at 37°C and 5% CO_2 for 1 h, the OD value was measured at 450 nm. Each measurement was performed in quintuplicate.

Cloning Formation Assay

The transfection time point was 0 h, medium was changed 6 h later, cells were inoculated into the medium plate at a density of 3×10^3/100 μL at 24 h in an incubator at 37°C, 5% CO_2, medium was replaced every 2 d and the culture was terminated after 14 d. Medium was removed and cells were washed with the phosphate buffered saline (PBS) twice, and they wew fixed with 5% paraformaldehyde for 30 min. Remaining liquid was removed, 1 mL of 0.1% crystal violet solution per well was added, the crystal violet solution was removed after 30 min, cells were washed until the solution was clear with PBS; then, the visible colonies were counted.

Statistical Analysis

SPSS 22.0 statistical software (IBM, Armonk, NY USA) was used for data analysis, GraphPad Prism 5.0 (Version X; La Jolla, CA, USA) was used for picture editing. Kaplan-Meier survival curves were used for survival analysis and indicators with significant difference in the survival analysis were included in the COX regression analysis. Measurement data were compared with *t*-test and presented as mean ± standard deviation ($\bar{x}\pm s$), categorical data were compared with x^2 test. $p<0.05$ indicated significant difference; $^*p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$.

Results

Expression of LncRNA ZNF667-AS1 in Cervical Tissue of cervical Cancer and its Relationship with Clinical Data

The expression of LncRNA ZNF667-AS1 in cervical cancer tissues was significantly lower than that in normal cervical tissues (Figure 1A and 1B, $p<0.001$) through the analysis the data of TCGA expression by edger function. Lima function calculated two microarray datasets (GSE63514 and GSE6791) and found that the expression of LncRNA ZNF667-AS1 was lower in cervical tissues than in cervical normal tissues (Figure 1D, $p<0.001$ and Figure 1E, $p<0.05$). Afterwards, we selected 60 cervical cancer tissues from cervical cancer patients and 30 normal tissues; qRT-PCR was used to detect the expression of LncRNA ZNF667-AS1. The results further confirmed that expression of LncRNA ZNF667-AS1 in cervical cancer was low (Figure 2A, $p<0.001$). The results showed that the overall survival time analyzed in TCGA data and clinical samples was negatively correlated with the expression of ZNF667-AS1 (Figure 1C, $p=0.0281$, HR=0.5934 and Figure 2B, $p=0.0098$, HR=0.3536). In clinical data analysis, expression of ZNF667-AS1 was significantly reduced in patients with advanced tumors and large tumor volumes (Figure 2C and Figure 2D). In order to investigate the correlation between ZNF667-AS1 expression and clinical data, patients were divided into high expression group and low expression group based on the median expression of ZNF667-AS1. Results of x^2 test showed that FIGO staging and tumor size in low expression ZNF667-AS1 group were significantly higher than those of high expression group. However, the expression of ZNF667-AS1 was not correlated with age, tissue subtype, tissue typing, lymphatic metastasis and distant metastasis (Table I).

Screening Cell Lines

The normal cell line HcerEpic was used as the control cell line, total RNA was extracted in the cell lines (HcerEpic, HeLa, SiHa), expression of ZNF667-AS1 in each cell line was detected by qRT-PCR. As shown in Figure 3A, expression of ZNF667-AS1 in HeLa and SiHa cell lines were decreased significantly, especially in SiHa cells, as a consequence, SiHa cell line was chosen for the further investigation in this study. Corresponding plasmids were constructed, pcDNA-NC and pcDNA-ZNF667-AS1 were inserted into the cervical cancer cell line SiHa (Figure 3B).

Inhibitory Effect of ZNF667-AS1 on the Proliferation of SiHa Cells After Overexpression

The results of CCK8 showed that the D450 value in SiHa cells transfected with pcDNA-ZNF667-AS1 was decreased compared with that in the negative

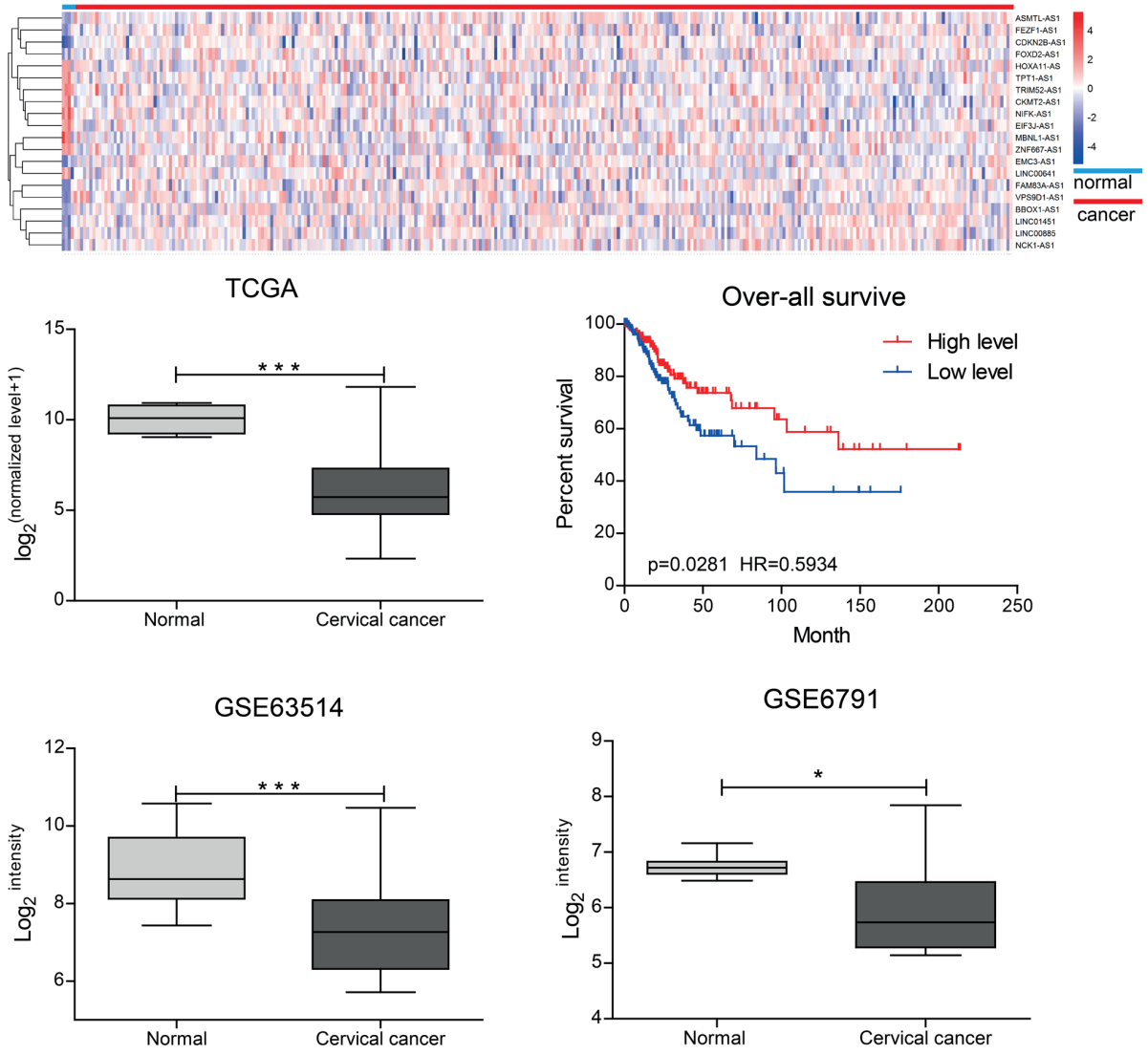

Figure 1. LncRNA ZNF667-AS1 expression is downregulated in cervical cancer. ***A,*** Heatmap of the differentially expressed probes between cervical cancer tissues and normal cervical tissues from analysis of two microarray datasets. ***B,*** LncRNA ZNF667-AS1 was downregulated in cervical cancer compared to the cervical normal tissues from TCGA. ***C,*** The patients were divided into two groups according to the median value of relative ZNF667-AS1 expression. ***D-E,*** LncRNA ZNF667-AS1 was downregulated in cervical cancer tissues in GSE63514 and GSE6791, respectively. ($^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$).

control transfected with pcDNA-NC, which indicated that overexpression of ZNF667-AS1 inhibited the proliferation of SiHa cells. The difference was significant ($p<0.01$, Figure 3C).

Inhibitory effect of ZNF667-AS1 on the Colony Formation of SiHa Cells After Overexpression

The results of colony formation showed that colonies of SiHa cells in normal control group (pcDNA-NC) were far more than those in overexpression group (pcDNA-ZNF667-AS1). This suggested that the ability of a single cell to form a cell mass after overexpression of ZNF667-AS1 in SiHa cells was reduced (Figure 3D). Colony formation assay and CCK8 assay results showed that ZNF667-AS1 was able to inhibit the proliferation of cervical cancer cells.

Discussion

Cervical cancer is one of the most common malignancies in women. Cervical cancer is a kind

Table I. Association between LncRNA ZNF667-AS1 expression and clinicopathological characteristics of patients with cervical cancer (n=60).

Clinicopathological features	Number of cases	ZNF667-AS1 expression		*p*-value
		Low (n=30)	High (n=30)	
Age (years)				
<50	34	16	18	0.6023
≥50	26	14	12	
Histological subtype				
Serous	43	23	20	0.3901
Others	17	7	10	
Tumor size				
<4 CM	37	23	14	0.0250*
≥4 CM	23	7	16	
FIGO stage				
I-II	37	25	12	0.0005*
III-IV	23	5	18	
Histological grade				
G1-G2	40	21	19	0.5839
G3	20	9	11	
Lymph node metastasis				
Absent	36	21	15	0.108
Present	24	9	15	

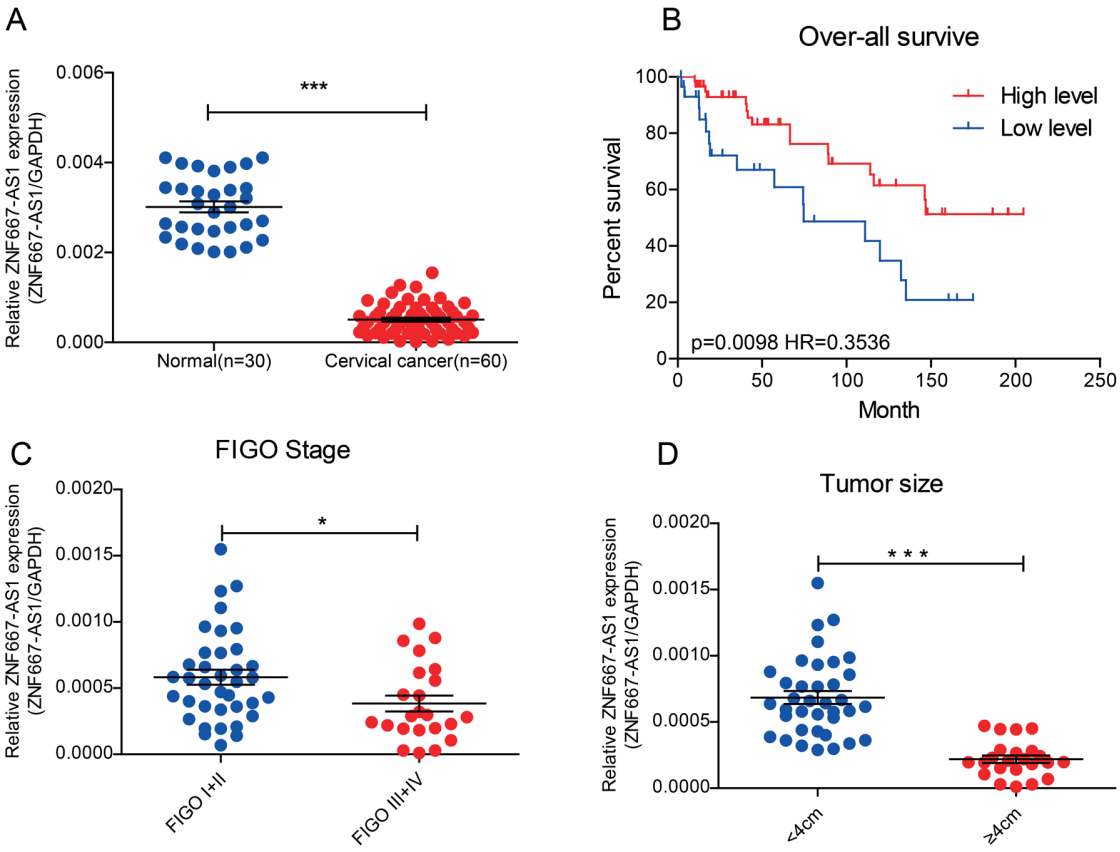

Figure 2. The expression of LncRNA ZNF667-AS1 was correlated with the total survival, tumor size and FIGO stage. ***A,*** Relative expression of ZNF667-AS1 in cervical cancer tissues (n=60) compared with cervical normal tissues (n=30). ***B,*** The patients were divided into two groups according to the median value of relative ZNF667-AS1 expression. ***C-D,*** Relative expression of ZNF667-AS1 in cervical cancer tissues related to FIGO stage and tumor size. ($^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$).

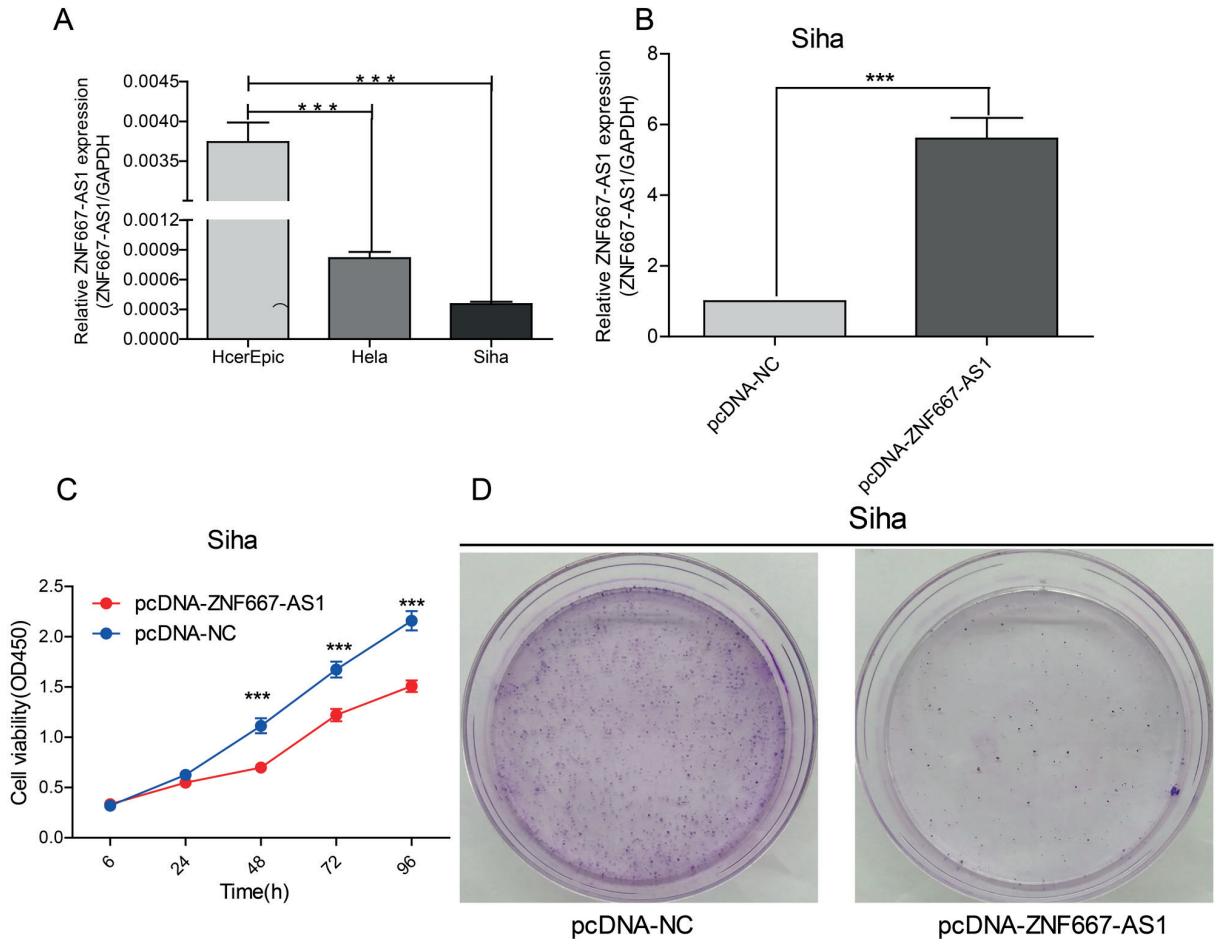

Figure 3. Overexpression of ZNF667-AS1 inhibits SiHa cells proliferation *in vitro*. *A*, Relative expression of ZNF667-AS1 expression in normal cervical cells (HcerEpic) and cervical cancer cells (HeLa and SiHa). Zhang Q. Prognostic role of HOTAIR in four estrogen-dependent malignant tumors: a meta-analysis. Onco Targets Ther 2015 Transfection efficiency was determined by qRT-PCR in SiHa cells. ***C,*** Overexpression of ZNF667-AS1 in SiHa cells reduced their proliferative capacities in CCK8 assay. ***D,*** Colony formation assay. (*p<0.05, **p<0.01, and ***p<0.001).

of disease caused by multi-step and multi-factor; however, its pathological mechanism is not yet clear. It is an important clinical prospect to find a new target for early diagnosis, treatment and prognosis of cervical cancer. Long-chain non-coding RNAs have become a hot topic in many tumor research, and play an important role in the development of tumor. LncRNAs are a class of RNA molecules whose transcribes are more than 200 nt. LncRNAs are initially considered to be "noises" of genomic transcription because of their lack of open reading frames without coding protein potential, which is a by-product of RNA polymerase II transcription without a biological function[18]. However, recent studies have shown that LncRNAs are involved in genomic imprinting, chromatin modification, transcriptional activation, post-transcriptional regulation and other multidimensional regulations, and its transcription and dysfunction are involved in tumorigenesis[17]. Some known lncRNAs, such as HOTAIR[19-21], MALAT1[22-24], GAS5[25, 26] and EBIC[13], have been shown to play important roles in the invasion and metastasis of cervical cancer. Through *in vitro* experiments, Kim et al[9] found that HOTAIR can up-regulate the expression of vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP-9) in cervical cancer cells, and can also lead to imbalance of epithelial mesenchymal transition (EMT) genes, thus affecting on the invasion and metastasis of cervical cancer. Li et al[27] found that HOTAIR could be an independent factor in the prognosis of patients with cervical cancer by meta-analysis. Yang et al[28] found that

after down-regulation of MALATl expression, proliferation and invasive ability of cervical cancer cell caSki were decreased significantly, whilst the apoptosis was increased. The study found that lncRNA-EBIC, which promoted cervical cancer cell infiltration, may be related to inhibition of E-cadherin expression[13]. lncRNA may be involved in the occurrence, metastasis and recurrence of cervical cancer.

LncRNA ZNF667-AS1 is a novel trans-long chain non-coding RNA located on human chromosome 19q13.43. Studies based on LncRNA ZNF667-AS1 related to human tumors are rarely reported.

In this study, we analyzed the data downloaded from the GEO database (GSE63514 and GSE6791). The results showed that the expression of LncRNA ZNF667-AS1 in cervical cancer was significantly lower than that in normal cervical tissues. Analysis results based on TCGA data were the same. In order to further explore the functional significance of LncRNA ZNF667-AS1 in cervical cancer, the differential expression of LncRNA ZNF667-AS1 in 60 cervical cancer tissues and 30 normal cervical tissues was detected by qRT-PCR. The results were consistent. This suggested that LncRNA ZNF667-AS1 may act as a tumor suppressor lncRNA involved in the pathophysiology of organelles. The clinical data of the patients were analyzed and found that the total survival time was negatively correlated with the expression of ZNF667-AS1. The results of x^2 test showed that the expression of LncRNA ZNF667-AS1 was related to tumor size and staging of cervical cancer. In order to further study whether LncRNA ZNF667-AS1 was involved in the proliferation of cervical cancer, the expression of LncRNA ZNF667-AS1, the proliferation ability and cell cloning ability of cervical cancer SiHa cells were significantly decreased in the cell model ($p<0.001$), which indicated that low expression of LncRNA ZNF667-AS1 may play an important role in maintaining the proliferation of cervical cancer. Compared with the negative control group, the proliferation ability and cell cloning ability of SiHa cells overexpressing long-chain non-coding RNA ZNF667-AS1 were significantly decreased ($p<0.001$), which indicated that the overexpression of ZNF667-AS1 inhibited the proliferation of SiHa cells.

Our data showed that LncRNA ZNF667-AS1 was down-regulated in cervical cancer tissues; at the same time, it negatively correlated with the overall survival, tumor size and FIGO stage of clinical patients. Overexpression of LncRNA ZNF667-AS1 significantly inhibited tumor cells, which indicated that LncRNA ZNF667-AS1 could play a role in the development of cervical cancer. It can be used as a molecular biomarker and potential therapeutic target for prediction of cervical cancer, however, transcriptional regulation mechanism of which still needs to be further investigated.

Conclusions

Expression of LncRNA ZNF667-AS1 was significantly lower in cervical cancer tissues, and its expression was negatively correlated with the overall survival, tumor size and FIGO stage. ZNF667-AS1 inhibited the proliferation of cervical cancer cells and was expected to be the biomarker and potential therapeutic target for predicting cervical caner and determining its prognosis.

Conflict of interest

The authors declare no conflicts of interest.

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