European Review for Medical and Pharmacological Sciences 2020; 24: 5404-5411

Expression of lncRNA AK058003 in esophageal carcinoma and analysis of its intervention effect

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Abstract. – OBJECTIVE: The aim of this study was to investigate the expression of long non-coding ribonucleic acid (lncRNA) AK058003 in esophageal carcinoma (EC) tissues, and to analyze its intervention effect.

PATIENTS AND METHODS: The expression of lncRNA AK058003 in EC tissues and para-carcinoma tissues from 130 EC patients was detected via quantitative Polymerase Chain Reaction (qPCR). EC cell lines were selected for exogenous interference in lncRNA AK058003. Subsequently, the expression of lncRNA AK058003 in normal esophageal epithelial cell line (Het-1A) and EC cell lines (EC109, EC9706, KYSE-150, KYSE-30, and TE-1) was detected by qPCR. EC9706 cell lines with the highest expression of lncRNA AK058003 were selected and transfected with lncRNA AK058003 siRNA and lncRNA AK058003 control, respectively. After transfection, the expression of lncRNA AK058003 was determined using PCR. The changes in cell growth and proliferation were analyzed via cell growth curve and cell cycle assay. Meanwhile, the changes in cell migration and invasion were analyzed through wound healing assay. Protein expressions of matrix metalloproteinase-1 (MMP1) and MMP2 were determined by Western blot. Clinical data were collected from EC patients, and the association between lncRNA AK058003 expression and tumor-node-metastasis (TNM) stage was finally analyzed.

RESULTS: LncRNA AK058003 was highly expressed EC tissues compared with para-carcinoma tissues ($p<0.01$). Compared with Het-1A cells, the expression of lncRNA AK058003 was significantly higher in EC109, EC9706, KYSE-150, KYSE-30, and TE-1 cells, with highest level in EC9706 cells ($p<0.05$). The expression of lncRNA AK058003 remarkably declined in lncRNA AK058003 siRNA group compared with lncRNA AK058003 control group ($p<0.001$). Compared with lncRNA AK058003 control group, the proliferation of EC cells was significantly weakened in lncRNA AK058003 siRNA group, with the greatest difference at 3 d. Flow cytometry results revealed that cell cycle was arrested in G0/G1 phase in lncRNA AK058003 siRNA group. Wound healing assay indicated that the intercellular distance became large, and cell migration ability was evidently enhanced in lncRNA AK058003 siRNA group with time ($p<0.05$). Besides, the protein expressions of MMP1 and MMP2 were remarkably lower in lncRNA AK058003 siRNA group than those in lncRNA AK058003 control group. This indicated remarkably declined invasion and metastasis ability. In addition, the postoperative prognosis was significantly worse in patients with higher expression of lncRNA AK058003 ($p<0.05$). All these findings suggested that lncRNA AK058003 could serve as a biomarker for EC prognosis.

CONCLUSIONS: LncRNA AK058003 is highly expressed in EC patients, which promotes proliferation, migration, invasion, and metastasis of EC cells. In addition, the postoperative prognosis of EC patients with high expression of lncRNA AK058003 is relatively poor.

Key Words:
Esophageal carcinoma (EC), LncRNA AK058003, Cell cycle, TNM stage.

Introduction

According to the latest statistics, esophageal carcinoma (EC) is among the top ten cancers worldwide. EC occurs in the esophagus, and reduces the living standard of patients, seriously threatening human health. The morbidity of EC is high, making it the 8th most common cancer in the world. Due to invasiveness and low survival rate, the early diagnosis of EC is difficult. Meanwhile, its 5-year survival rate is only about 15-25%[1]. Currently, the incidence rate of EC is gradually increasing globally, and the risk factors associated with EC also become increasingly complex[1]. Based on the histological type, EC can be divided into two types, including: squamous cell carcinoma and adenocarcinoma. The risk and type of EC vary greatly from re-

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gion to region. Esophageal squamous cell carcinoma (ESCC) is a major histological type of EC in some regions around the world[1,2]. Its risk is relatively high in China, especially in the West of Anyang City, Henan Province. There are no evident clinical symptoms for early EC. Despite great efforts made in the early diagnosis and treatment of EC, the practical and simple techniques in this field are still in evolution[3,4]. In recent years, chemotherapy is an important treatment method for EC. However, the multidrug resistance during treatment has become a great challenge. Therefore, exploring new therapeutic targets and searching for highly sensitive biomarkers can better facilitate the early diagnosis and effective treatment of EC.

Long non-coding ribonucleic acids (lncRNAs) are a kind of RNAs composed of about 200 nucleotides, with no protein-coding function. LncRNAs can bind to proteins and miRNAs to regulate gene expression, thereby participating in tumor progression[5-7]. LncRNAs are associated with tumor proliferation, invasion, and migration. Meanwhile, their abnormal expressions in tissues often act as the catalyst for tumor development, exacerbating the malignant phenotype of tumors, such as LINC00628 in gastric cancer[7], H19 in lung cancer[8], 12-lncRNA in breast cancer[9], UCA1 in bladder cancer[10], and 6-lncRNA in diffuse large B-cell lymphoma[11]. Researches on lncRNAs can not only help clarify the mechanisms of tumorigenesis and cancer invasion, but also provide valuable targets for the diagnosis and treatment of malignancies. Wang et al[12] have demonstrated that hypoxia upregulates lncRNA AK058003 and promotes metastasis of gastric cancer by targeting synuclein gamma. In breast cancer, unregulated lncRNA AK058003 can activate downstream synuclein gamma, eventually facilitating breast cancer growth, invasion, and migration[13]. However, there are few studies on the functional roles of lncRNA AK058003 and its prognostic value in EC. Therefore, the aim of this study was to explore the expression of lncRNA AK058003 in EC and its effect on prognosis.

Patients and Methods

Materials

Het-1A, EC109, EC9706, KYSE-150, KYSE-30, and TE-1 cell lines (American Type Culture Collection (ATCC; Manassas, VA, USA), matrix metalloproteinase-1 (MMP1), MMP2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies (Abcam, Cambridge, MA, USA), tissue cryotube (Promega, Beijing, China), polymerase chain reaction (PCR) amplification instrument (Eppendorf, Burlingame, CA, USA), TRIzol (Tiangen, Beijing, China), and PCR primers (Invitrogen, Carlsbad, CA, USA).

Research Objects and Grouping

Carcinoma tissues and para-carcinoma tissues were collected from 130 EC patients who received treatment in our hospital from April 2013 to July 2014. All patients underwent no chemotherapy but directly received operation. EC9706 cells were divided into lncRNA AK058003 control group and lncRNA AK058003 siRNA group. Based on the median expression of lncRNA AK058003 as the cut-off point, all patients were divided into two groups, including: low lncRNA AK058003 expression group and high lncRNA AK058003 expression group. This study was approved by the Ethics Committee of Tianjin Medical University General Hospital. Signed written informed consents were obtained from all participants before the study.

Fluorescence qPCR

Total RNA was extracted from EC tissues using TRIzol and chloroform and re-suspended in 50 μL of diethylpyrocarbonate (DEPC)-treated water (Beyotime, Shanghai, China). The concentration of RNA extracted was determined, and the absorbance $(A)_{260}/A_{280}$ was required to be 1.8-2.0. 3 μL of the total RNA which was reversely transcribed into cDNA. Reverse transcription reagents 1 and 2 were added for reaction at 65°C for 10 min. Subsequently, reagents 3, 4, 5, 6, and 7 were added for amplification using the general reverse transcription system containing forward and reverse primers, cDNA SYBR and enzyme-free water (20 μL in total), under the general conditions of fluorescence qPCR for 40 cycles. The relative expression level of gene was calculated using the $2^{-\Delta Ct}$ method. Three replicates were set for each sample. GAPDH was used as an internal reference. The primers used in this study were shown as follows: LncRNA AK058003-F: CAGATGGCTGAGGTGGAAGG; LncRNA AK058003-R: GACAAGGTCTCGCTCTTTTGCT. GAPDH-F: TGACTTCAACAGCGACACCCA; GAPDH-R: CACCCTGTTGCTGTAGCCAAA.

Transfection of LncRNA AK058003 SiRNA and LncRNA AK058003 Control

EC9706 cells were inoculated into 6-well plates and cultured in antibiotic-free medium. The complete medium and siRNA/control were mixed evenly (200:1) as solution A. Meanwhile, Dulbecco's Modified Eagle's Medium (DMEM) and Lipofectamine were mixed evenly (50:1) at 4°C as solution B. After growth for 12 h, the cells were transfected as follows. After the medium was discarded, the plate was gently washed twice with phosphate-buffered saline (PBS). Next, 500 μL of solution A and 500 μL of solution B were added. After incubation for 6 h under optimal conditions, the transfection reagent was replaced with complete medium.

Cell Proliferation Via MTS Assay

The cells were inoculated into 96-well plates at a density of 2500 cells/well, and the volume of medium in each well was 200 μL. Three parallel wells were set for each sample. After culture for 1 d, the medium was gently discarded, and normal medium and MTS reagent were mixed evenly (1:1). Next, 200 μL of mixture was added into each well for incubation for 2 h. Optical density (OD) value of each well at 490 nm was measured at 2 d, 3 d, 4 d, and 5 d, respectively. The growth curves were finally plotted.

Wound Healing Assay

The cells were inoculated into 6-well plates at a density of $3*10^5$ cells/well. The well bottom was scratched in a ''cross'' shape using the 200 μL pipette. To avoid contamination, the medium was replaced, and the cells were cultured in an incubator for 36 h. Subsequently, crystal violet dye was added, followed by incubation at 25°C for 30 min. Cell migration distance was observed under a microscope. The changes in cell migration rate were analyzed using ImageJ software.

Cell Cycle Via Flow Cytometry

After digestion with trypsin, cell suspension was collected into a centrifuge tube for centrifugation at 1000 rpm for 5 min. Subsequently, the precipitate was re-suspended with pre-cooled PBS, followed by centrifugation at 1000 rpm for 5 min to remove cell debris. After the supernatant was discarded, the cells were fixed with absolute ethanol for 30 min and centrifuged again under the same condition. Next, the supernatant liquid was discarded, and pre-cooled PBS was added, followed by centrifugation. PBS and Propidium Iodide (PI) dye were then added for incubation in the dark for 30 min. Finally, cell cycle was determined on the machine.

Western Blotting

Cell precipitate was collected, and residual PBS was aspirated. Radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) and PI were added and mixed evenly. The cell membrane and nuclear membrane were disrupted *via* ultrasonication for 3 times, followed by centrifugation at 14000 rpm for 30 min. Protein concentration was determined using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). After separation *via* sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the protein samples were transferred onto polyvinylidene difluoride (PVDF) membranes under a constant current of 300 mA. Next, the membranes were sealed with blocking buffer (prepared by buffer A and B at 1:1) for 10 min, and incubated with specific primary antibodies of GAPDH, MMP1, and MMP2 at 4°C for 16 h. On the next day, the membranes were incubated again with corresponding secondary antibodies for 1 h. Immuno-reactive bands were finally exposed and scanned.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA) was used all statistical analysis. The *t*-test and univariate analysis were performed when appropriate. $p<0.05$ was considered statistically significant.

Results

Expression of LncRNA AK058003 in EC Tissues and Cells

The expression of lncRNA AK058003 was significantly higher in EC tissues than that in para-carcinoma tissues ($p<0.01$). Compared with Het-1A cells, the expression of lncRNA AK058003 was significantly higher in EC109, EC9706, KYSE-150, KYSE-30, and TE-1 cells, with highest level in EC9706 cells ($p<0.001$) (Figure 1).

LncRNA AK058003 Promoted Proliferation of EC Cells

The expression of lncRNA AK058003 significantly declined in lncRNA AK058003 siRNA group when compared with lncRNA AK058003

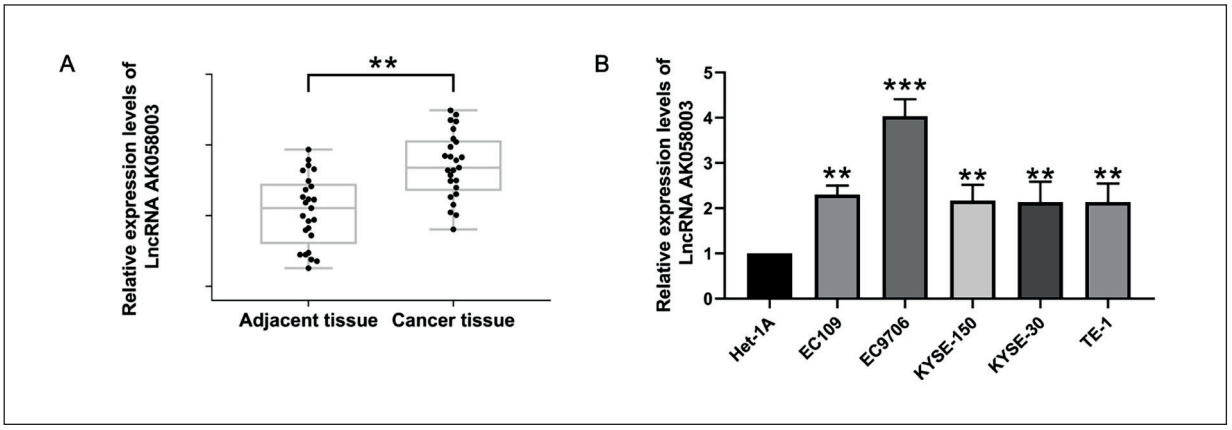

Figure 1. Expression of lncRNA AK058003 in EC tissues and cells detected *via* qPCR. **A,** The expression of lncRNA AK058003 is significantly higher in EC tissues than that in para-carcinoma tissues. **B,** Compared with Het-1A cells, the expression of lncRNA AK058003 is significantly higher in EC109, EC9706, KYSE-150, KYSE-30, and TE-1 cells, with the highest in EC9706 cells ($p<0.001$). (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

control group ($p<0.001$). OD value at 490 nm in lncRNA AK058003 siRNA group began to fall significantly at 3 d ($p<0.05$). It exhibited the greatest difference from that in lncRNA AK058003 control group at 4 d ($p<0.01$), which also remarkably declined at 5 d ($p<0.01$). Besides, G0/G1 phase in lncRNA AK058003 siRNA group was evidently prolonged and cell proliferation was inhibited when compared with lncRNA AK058003 control group (Figure 2).

LncRNA AK058003 Facilitated Migration of EC Cells

After culture for 24 h, the intercellular distance became larger ($p<0.05$). Moreover, the migration ability of EC cells was significantly enhanced in lncRNA AK058003 siRNA group compared with lncRNA AK058003 control group ($p<0.05$) (Figure 3).

LncRNA AK058003 Increased Protein Expressions of MMP1 and MMP2

Compared with lncRNA AK058003 control group, lncRNA AK058003 siRNA group showed evidently decreased protein expressions of MMP1 and MMP2 ($p<0.05$), indicating that lncRNA AK058003 promoted cell invasion (Figure 4).

EC Patients with Higher Expression of LncRNA AK058003 had Worse Postoperative Prognosis

According to the survival analysis of EC patients, the survival time of patients was remarkably shorter in high lncRNA AK058003 expression group ($p<0.05$). The receiver operating characteristic (ROC) curve demonstrated that the sensitivity and specificity of lncRNA AK058003 in the prognosis evaluation were 75% and 69.1%, respectively. Meanwhile, the area under the curve (AUC) was 0.715 (Figure 5).

Discussion

Mammalian genome encodes hundreds of lncRNAs through a variety of mechanisms of action. It is noteworthy that lncRNAs play important roles in dosage compensation, epigenetics, cell cycle, and differentiation[14]. The main functions of lncRNAs include genomic imprinting, chromatin remodeling, mRNA transcriptional regulation, and cell cycle regulation[15]. As a 1197 nt transcript, lncRNA AK058003 is located on the reverse strand of chromosome 10q22, with a weaker protein-coding ability. Currently, the mortality rate of EC is high in the world. ESCC accounts for 90% in all EC cases[16,17]. In recent years, great improvements have been made in the success rate of chemotherapy, radiotherapy, and surgery, and enormous progress in these techniques. However, the postoperative prognosis of EC remains poor, whose 5-year survival rate remains only 10-15%[18,19]. Therefore, the molecular mechanisms for the occurrence, development, and effective treatment of ESCC deserve further research.

In this study, we aimed to explore the role of lncRNA AK058003 in EC and its value in

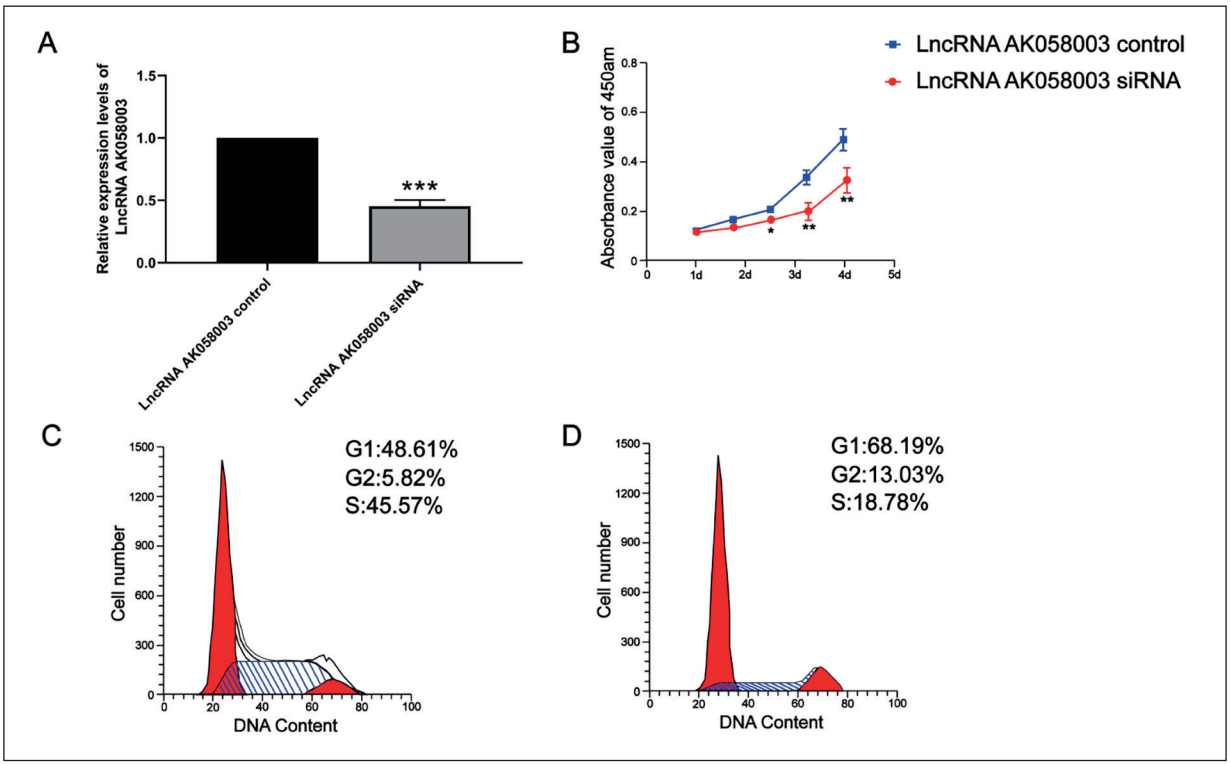

Figure 2. Cell growth curves in lncRNA AK058003 control group and lncRNA AK058003 siRNA group. **A,** The expression of lncRNA AK058003 significantly declines in lncRNA AK058003 siRNA group compared with that in lncRNA AK058003 control group (p<0.001). **B,** Compared with lncRNA AK058003 control group, the growth rate of EC cells in lncRNA AK058003 siRNA group significantly declines. It has a statistically significant difference at 3 d, and the difference is the most significant at 4 d and 5 d (p<0.05). **C,** and **D,** The G0/G1 phase in lncRNA AK058003 siRNA group is significantly prolonged compared with that in lncRNA AK058003 control group (p<0.05). (*p<0.05, **p<0.01, ***p<0.001).

prognosis evaluation. First, the difference in the expression of lncRNA AK058003 in EC tissues and para-carcinoma tissues was detected. QPCR results showed that the expression of lncRNA AK058003 was significantly higher in EC tissues. Previous studies have reported that lncRNA AK058003 can promote the growth and invasion of gastric cancer cells, and inhibit the proliferation of breast cancer cells, which may be related to tissue heterogeneity. To explore the function of lncRNA AK058003 in EC, EC-9706 cells were selected and transfected with lncRNA

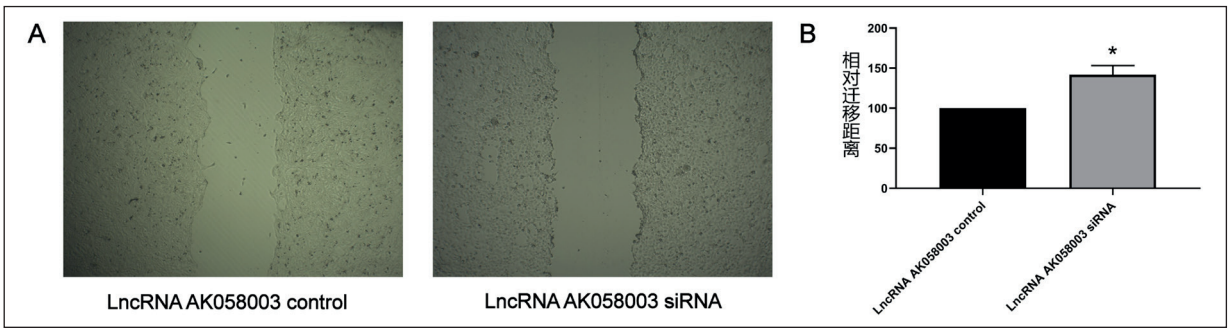

Figure 3. Changes in cell migration in lncRNA AK058003 control group and lncRNA AK058003 siRNA group at 24 h (magnification: 40×). **A,** and **B,** The intercellular distance becomes larger in lncRNA AK058003 siRNA group compared with that in lncRNA AK058003 control group (p<0.05). (*p<0.05).

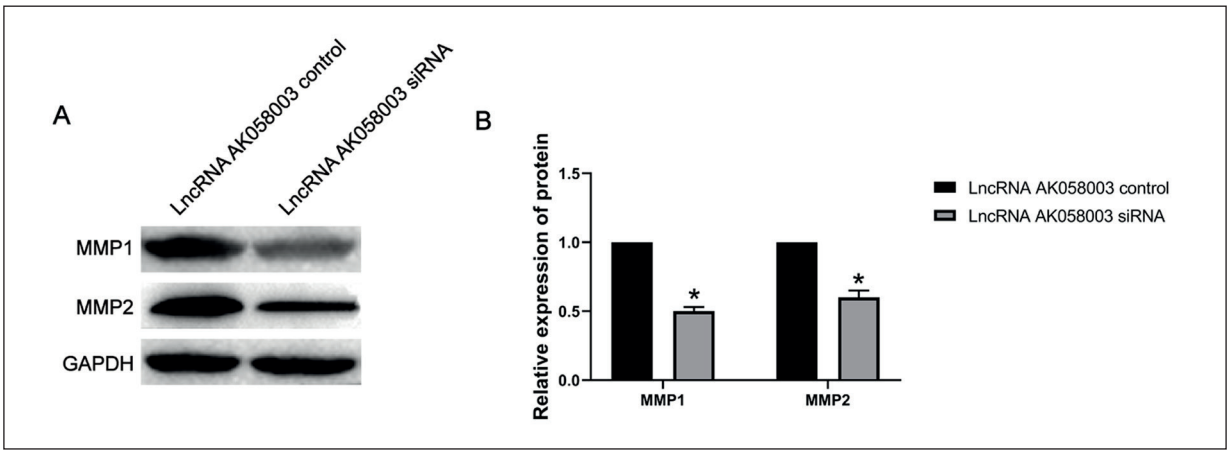

Figure 4. Changes in MMP1 and MMP2 expressions in lncRNA AK058003 control group and lncRNA AK058003 siRNA group. **A,** and **B,** Compared with lncRNA AK058003 control group, lncRNA AK058003 siRNA group has significantly decreased the protein expressions of MMP1 and MMP2 (p<0.05).

AK058003 siRNA and control, respectively. Cell proliferation was determined using MTS assay in cells of lncRNA AK058003 control group and lncRNA AK058003 siRNA group. The results showed that the proliferation rate of EC cells began to decline at 3 d in lncRNA AK058003 siRNA group compared with lncRNA AK058003 control group. Flow cytometry manifested that the G0/G1 phase was evidently prolonged, and the number of cells in S phase evidently declined in lncRNA AK058003 siRNA group compared with those in lncRNA AK058003 control group. This demonstrated that lncRNA AK058003 could promote cell proliferation. Both cell invasion and migration play important roles in tumor progression, which can facilitate distant metastasis of malignancies. Therefore, the effects of lncRNA AK058003 on cell migration and invasion were determined. The results showed that after culture for 24 h, the intercellular distance became larger, and cell migration ability was enhanced in lncRNA AK058003 siRNA group compared with lncRNA AK058003 control group. Western blotting results showed that the protein expressions of MMP1 and MMP2 were remarkably lower in lncRNA AK058003 siRNA group. Both MMP1

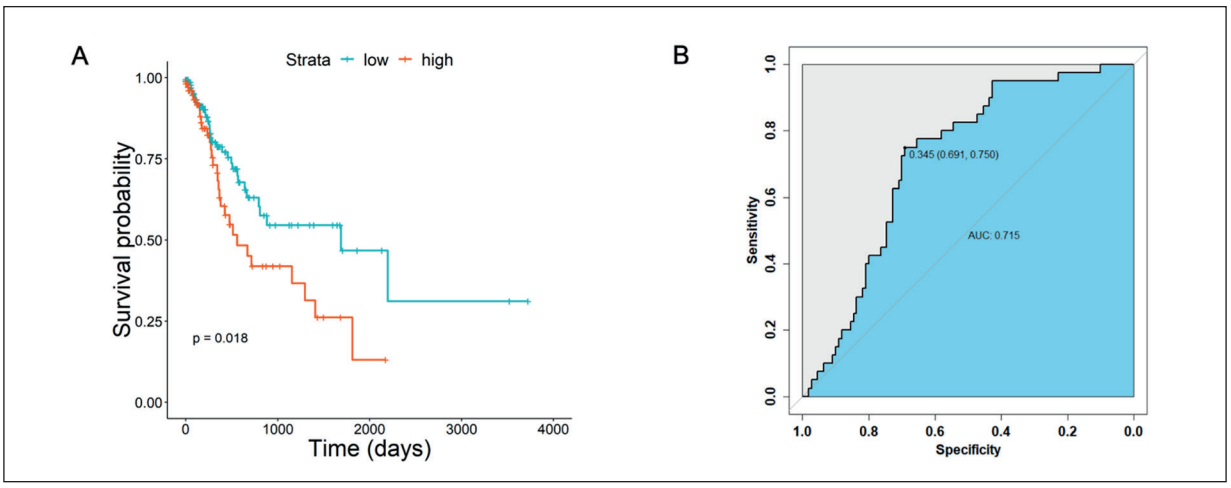

Figure 5. Survival curves in high expression group and low expression group. **A,** The prognosis of EC patients with higher expression is significantly worse (p<0.05). **B,** The ROC curve shows that the sensitivity and specificity of lncRNA AK058003 as the prognosis marker are 75% and 69.1%, respectively, and the AUC is 0.715.

and MMP2 are matrix metalloproteinases that mainly degrade proteins in the tissue matrix and destroy the integrity of matrix, thus promoting the distant metastasis of tumor cells. Our findings showed that lncRNA AK058003 could upregulate the protein expressions of MMP1 and MMP2 and destroy the matrix protein, thus accelerating the invasion and migration of EC cells. At present, the exact role of lncRNA AK058003 in EC has not been reported. Meanwhile, the association between lncRNA AK058003 and postoperative prognosis has not been studied either. To explore the guiding significance of lncRNA AK058003 in the prognosis of EC patients, survival analysis was performed. The prognosis was worse in EC patients with higher expression than those with lower expression, displaying a statistically significant difference. In addition, the ROC curve revealed that the sensitivity and specificity of lncRNA AK058003 as the prognosis marker were 75% and 69.1%, respectively. The AUC was 0.715, suggesting that lncRNA AK058003 could serve as a good biomarker for prognosis evaluation. In this study, the differential expression of lncRNA AK058003 was found between EC tissues and para-carcinoma tissues, whose expression was significantly high in EC tissues. LncRNA AK058003 could promote the growth and migration of EC cells. Meanwhile, patients with higher expression of lncRNA AK058003 exhibited significantly worse prognosis. These findings suggested that lncRNA AK058003 could be used as a biomarker for prognosis evaluation. However, the regulatory network of lncRNA AK058003 was not deeply explored in this study. In the future, the nucleic acids and proteins interacting with lncRNA AK058003 can be explored using bioinformatics. To sum up, lncRNA AK058003 is expected to be a new gene target for the treatment of EC.

Conclusions

LncRNA AK058003 is highly expressed in EC patients, which can promote proliferation, migration, invasion, and metastasis of EC cells. In addition, the postoperative prognosis of EC patients with high expression of lncRNA AK058003 is relatively poor.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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