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Expression and prognostic significance of long noncoding RNA AK001796 in esophageal squamous cell carcinoma

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Abstract. – OBJECTIVE: Previous study has reported that long noncoding RNA AK001796 (AK001796) functions as a tumor promoter in esophageal squamous cell carcinoma (ESCC). However, its clinical in ESCC patients remains largely unclear. The purpose of the present study was to evaluate the prognostic value of AK001796 in ESCC patients.

PATIENTS AND METHODS: The expression levels of AK001796 in ESCC tissues and matched normal tissues were detected by RT-PCR. Association between AK001796 levels and clinicopathological factors was also analyzed by chi-square test. Overall survival (OS) and disease-free survival (DFS) were analyzed using the Kaplan-Meier method and log-rank test. The predictors for OS and DFS were assessed by univariate analysis and multivariate analysis using Cox's proportional hazards model.

RESULTS: We found that AK001796 was elevated in human ESCC samples compared with the adjacent normal tissues ($p<0.01$), and the high level of AK001796 expression was significantly correlated with lymph node metastasis ($p=0.032$) and advanced UICC stage ($p=0.016$). Interestingly, Kaplan-Meier analysis indicated that patients with high AK001796 expression had a significantly lower OS ($p=0.010$) and DFS ($p=0.001$). Moreover, we showed that AK001796 was an independent poor prognostic factor for OS and DFS in ESCC patients through univariate and multivariate analysis.

CONCLUSIONS: Our data provide important evidence that AK001796 may be a useful biomarker of advanced progression and poor prognosis of ESCC.

Key Words
Long noncoding RNA, AK001796, Prognosis, Esophageal squamous cell carcinoma.

Introduction

Esophageal cancer is one of the most common and malignant cancers and the sixth leading cause of cancer mortality worldwide[1]. The incidence rates vary internationally, with the highest rates found in Eastern Asia[2]. The main sub-type of the disease is esophageal squamous-cell carcinoma (ESCC), which is common in China[3,4]. Currently, patients with ESCC are always treated with surgery, chemotherapy and/or radiation therapy, while the prognosis is still unsatisfactory, with a 5-year survival rate of less than 30% due to most patients diagnosed at an advanced stage and the high probability of metastasis and recurrence[5-7]. Thus, it is necessary to further explore the underlying molecular mechanisms and develop novel targets for the diagnosis, prognosis and treatment of ESCC. Non-coding RNAs have recently confirmed as important regulators of mRNAs expressions[8]. Long noncoding RNAs (lncRNAs) are a class of noncoding RNAs with over 200 nucleotides in length[9]. Growing data reveal that lncRNAs may act as critical regulators in cellular development, differentiation, and many other biological processes[10,11]. Recent evidences suggest that the dysregulation of lncRNAs occurs in numerous diseases, including cancers, and acts as an important regulator in development and progression of various tumors[12-14]. In addition, with the development of microchip analytical procedures, it become easy to detect the expression levels of lncRNAs, which highlighted the important application of lncRNAs as a potential diagnostic and prognostic biomarkers for tumor patients[15,16]. Although a number of cancer-specific lncRNAs have been identified, most of lncRNAs remains to be elucidated[17]. LncRNA AK001796 (AK001796), a cancer-related lncRNA, was recently functionally characterized. Several studies have identified AK001796 as a tumor promoter in esophageal squamous cell carcinoma[18] and non-small cell lung cancer[19]. Although AK001796 expression was reported to be up-regulated in ESCC and its clinical significance was also studied, the sample size was small and the prognosis value

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of AK001796 needed to be further studied. In this study, we provided more important evidence that AK001796 may be used as a potential prognostic biomarker for ESCC patients.

Patients and Methods

Patients and Clinical Specimens

Fresh samples from ESCC and corresponding normal adjacent tissue were obtained from 175 patients at The Affiliated Huaian No. 1 People's Hospital of Nanjing Medical University between January 2009 and November 2013 (Nanjing, China). All the samples were reviewed retrospectively by two pathologists to confirm histological diagnosis. After resection, all specimens were snap-frozen in liquid nitrogen immediately and then stored at -80°C. No patient received preoperative chemotherapy or radiotherapy. The clinical parameters of patients involved in the study are summarized in Table I. All 175 patients were followed up for 5 years and clinical assessment was performed in each patient at the end of study. Patients' informed consent was obtained for tissue acquisition, and this study was approved by our Ethics Committee.

RNA Extraction and Quantitative Real-Time PCR

Total RNA from tissues and matched normal tissues was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA) was synthesized using PrimeScript Reverse Transcriptase (Biosystems, Foster City, CA, USA). qPCR was conducted with SYBR Premix Ex Taq (TaKaRa, Dalian, Liaoning, China) on CFX96™ Real-Time PCR Detection System supplied with analytical software (Bio-Rad, Hercules, CA, USA), and the results were normalized to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression level. Each sample was run in triplicate, and fold changes were calculated using the relative quantification $2^{-\Delta\Delta Ct}$ method. Primer sequences used for RT-qPCR analysis in this study are shown in Table II.

Table I. AK001796 expression and clinicopathologic features in ESCC patients.

Variable	Number	AK001796 expression		*p*-value
		Low	High	
Age, years				0.485
< 60	75	40	35	
≥ 60	100	48	52	
Sex				0.496
Male	107	56	51	
Female	68	32	36	
Alcohol consumption				0.678
Ever and currently	63	33	30	
Never	112	55	57	
Smoking status				0.198
Ever and currently	76	34	42	
Never	99	54	45	
Tumor location				0.708
Cervical/upper	82	40	42	
Middle/lower	93	48	45	
Tumor size, cm				0.107
< 4	107	59	48	
≥ 4	68	29	39	
Differentiation status				0.074
Well or moderate	113	62	50	
Poor	62	26	37	
Lymph node metastasis				0.032
Absent	118	66	52	
Present	57	22	35	
UICC stage				0.016
I–II	112	64	48	
III	63	24	39	

Table II. Primer sequences used for RT-qPCR analysis in this study.

Name	Sequences (5′-3′)
AK001796: Forward	GCCCAGAUUUAAGGGCUAUTT
AK001796: Reverse	AUAGCCCUUAAAUCUGGGCTT
GAPDH: Forward	TGGCCTTCCGTGTTCCTAC
GAPDH: Reverse	GAGTTGCTGTTGAAGTCGCA

Statistical Analysis

SPSS software 16.0 (SPSS Inc., Chicago, IL, USA) was applied in the current work. Comparisons between groups for statistical significance were performed with a two-tailed paired Student's *t*-test. The x^2-test was applied to infer the relationship between AK001796 expression and clinicopathological characteristics of ESCC patients. Survival curve was drawn by Kaplan-Meier method and compared by Log-rank test. The Cox proportional hazards regression model was employed for multivariate analyses to estimate the prognostic factors. A *p*-value <0.05 demonstrated statistical significance.

Results

AK001796 is Strongly Upregulated in ESCC

Although previous study has reported that AK001796 expression was up-regulated in ESCC, the evidence is relatively limited. In order to further demonstrate whether AK001796 was dysregulated in ESCC, we performed RT-PCR to detect the expression level of AK001796 in ESCC tissues from 175 ESCC patients. As shown in Figure 1A, the results showed that AK001796 expression levels were significantly higher in the ESCC tumor tissues than in the adjacent non-tumor tissues ($p<0.01$). Furthermore, we also found that patients with advanced stages have a higher expression of AK001796 ($p<0.01$, Figure 1B). Together with previous study, our results confirmed that AK001796 was significantly overexpressed in ESCC and may act as a positive regulator.

Correlations of AK001796 Expression with Clinicopathological Features of ESCC Patients

Then, we further explored the clinical significance of AK001796 in ESCC patients. Patients were categorized as high or low AK001796 expression group according to the median value. As shown in Table I, the results of x^2-test indicated that high expression of AK001796 was significantly correlated with lymph node metastasis ($p=0.032$) and advanced UICC stage ($p=0.016$). However, AK001796 expression was not significantly related to gender, sex, alcohol consumption, smoking status, tumor location, tumor size and differentiation status (all $p>0.05$). Our data revealed that dysregulation of AK001796 may be involved in the clinical progression of ESCC.

Overexpression of AK001796 Predicts the Poor Prognosis of ESCC

In order to further explore the prognostic value of AK001796 in ESCC patients, we collected five-year clinical data and Kaplan-Meier analysis was performed. As shown in Figure 2, we found that glioma patients with high AK001796

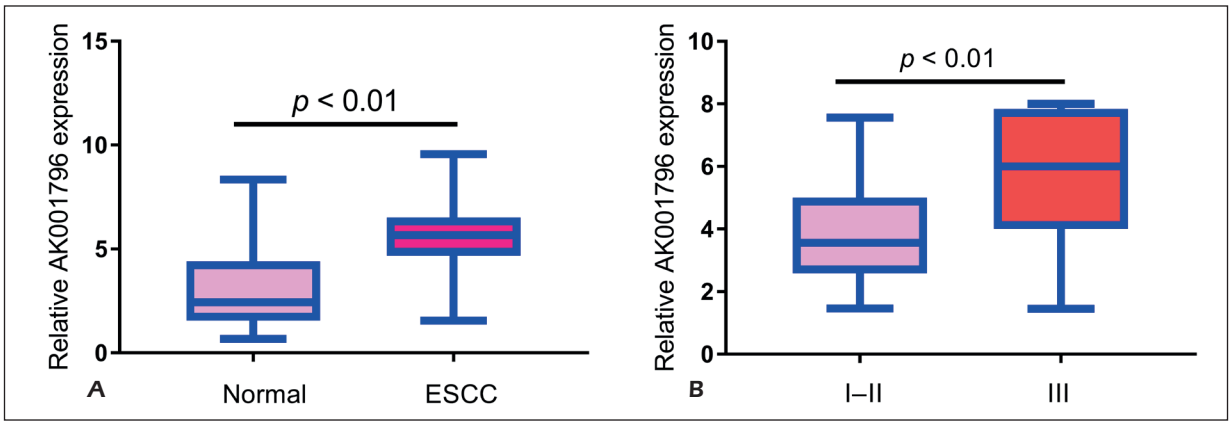

Figure 1. AK001796 expression in 175 ESCC tissues samples and corresponding normal tissues. **A**, AK001796 was upregulated in ESCC tissues compared to corresponding normal tissues ($p<0.01$). **B**, Higher expression of AK001796 was observed in ESCC tissues with advanced stages ($p<0.01$).

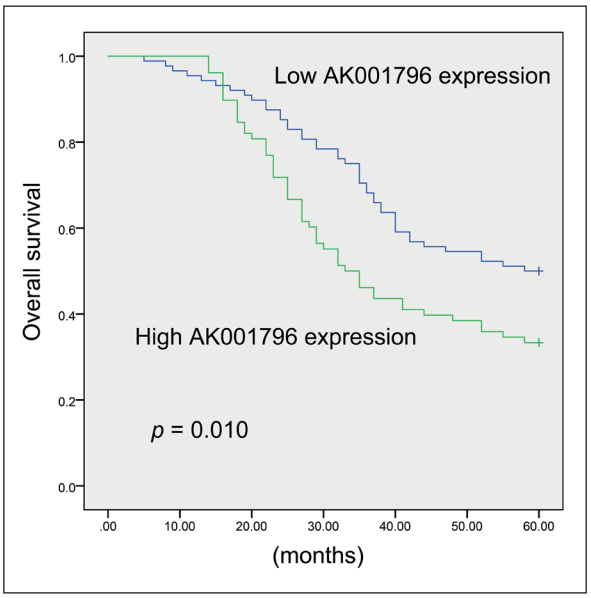

Figure 2. Overall survival curves for two groups defined by low and high expression of AK001796 in ESCC patients. The patients with high AK001796 expression had a significantly shorter overall survival than those with low AK001796 expression (p=0.010).

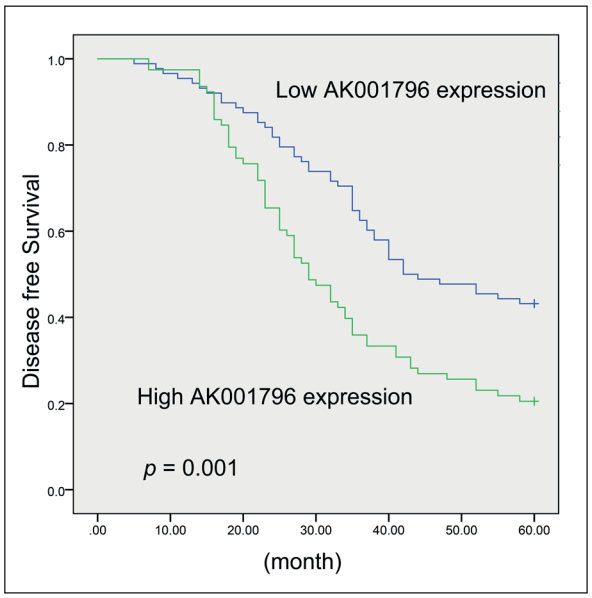

Figure 3. Disease-free survival curves for two groups defined by low and high expression of AK001796 in ESCC patients. The patients with high AK001796 expression had a significantly shorter disease-free survival than those with low AK001796 expression (p=0.001).

expression level had distinctly shorter overall survival (OS) than patients with high AK001796 expression level (p=0.010). In addition, according to the results of Figure 3, we also observed that patients with AK001796 higher expression have shown significantly poorer disease-free survival (DFS) than those with lower AK001796 expression (p=0.001). Our data suggested that AK001796 may influence the long-term survival of ESCC patients. In addition, multivariate analysis revealed AK001796 was an independent prognostic indicator for both OS (HR=3.347, 95% CI: 1.423-5.457, p=0.005) and DFS (HR=3.568, 95% CI: 1.537-5.778, p=0.003) in ESCC patients (Table III). Overall, our findings indicated that AK001796 may serve as a novel prognostic biomarker for ESCC patients.

Discussion

Overall incidence and mortality rates for ESCC are high in China. Predicting the prognosis of ESCC patients is very important for doctors to develop individual treatment plan[20]. Up to date, several clinicopathological factors have been used to predict the outcome of ESCC patients[21]. However, these factors have insufficient susceptibility. Thus, it is desirable to identify novel reliable biomarkers for ESCC. Recently, a large portion of overexpressed lncRNAs was identified in many human solid tumors, such as breast, lung, stomach and esophagus[22-25]. At the same time, more and more lncRNAs were reported to have potential to be prognostic biomarkers for ESCC patients[26,27]. However, more sensitive lncRNAs needed to be further identified. Recently, accumulating studies showed that lncRNAs were involved in the carcinogenesis of ESCC. For instance, Li et al[28] reported that lncRNA ATB was overexpressed in ESCC and its knockdown suppressed ESCC cells proliferation and metastasis by regulating miR-200b/Kindlin-2 axis. Yang et al[29] found that FTH1P3 was highly expressed in ESCC and associated with advanced clinical stages. Functionally, knockdown of FTH1P3 notably decreased the proliferation, migration, and invasion capacity of ESCC cells through SP1/NF-kB pathway. AK001796 was a newly identified lncRNA. Up to date, to our best knowledge, only three studies reported the expression pattern and function of AK001796 in cancers, including ESCC and lung cancer. Yang el[30] firstly reported that AK001796 was one of the most up-regulated lncRNAs in lung cancer by microarray analysis. Then, in their functional assay, it was found that knockdown of AK001796 inhibited the proliferation of lung cancer in both *in vitro* and *in vivo.*

Table III. Multivariate analysis of overall survival and disease-free survival in ESCC patients.

Variables	Overall survival			Disease-free survival		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Age	1.673	0.834-2.466	0.176	1.367	0.987-2.377	0.274
Sex	1.342	0.954-2.455	0.132	1.467	1.123-2.563	0.118
Alcohol consumption	1.436	1.132-1.987	0.256	1.674	1.326-2.327	0.129
Smoking status	1.523	1.328-2.328	0.148	1.237	1.238-2.457	0.178
Tumor location	0.953	0.534-1.782	0.326	1.232	0.784-2.326	0.277
Tumor size	0.873	0.623-1.895	0.216	1.123	0.856-2.231	0.156
Differentiation status	2.321	1.423-2.974	0.114	2.138	1.277-2.674	0.149
Lymph node metastasis	3.126	1.545-4.128	0.021	3.334	1.648-4.347	0.013
UICC stage	3.445	1.698-5.432	0.007	3.784	1.755-5.895	0.002
AK001796 expression	3.347	1.423-5.457	0.005	3.568	1.537-5.778	0.003

Liu et al[18] showed that the expression levels of AK001796 was significantly up-regulated in ESCC and its overexpression was positively associated with clinical progression. Furthermore, they confirmed AK001796 as a tumor promoter in ESCC because its knockdown could suppress ESCC cell growth by via regulating expression of p53, which revealed that AK001796 may affect the clinical prognosis of ESCC patients. Indeed, they have performed survival assay to explore the prognostic value of AK001796 in ESCC patients, finding that high AK001796 expression was associated with poor overall survival. However, the sample size was relatively small and multivariate analysis using Cox's proportional hazards model was not performed to further explore the possibility of AK001796 as an independent prognostic biomarker for ESCC patients.

In this work, we also observed that AK001796 expression was significantly up-regulated in ESCC tissues compared to matched normal tissues, which was consistent with previous results. Then, we further explored the correlation of AK001796 with clinicopathological factors, finding that high expression of AK001796 was significantly correlate with lymph node metastasis and advanced UICC stage, suggesting that AK001796 may be a metastasis-related lncRNA and contributed to the clinical progression of ESCC. Furthermore, we performed Kaplan-Meier analysis to analyze the association between AK001796 expression and long-term survival, finding that patients with higher expression levels of AK001796 had significantly shorter OS and DFS. Our results, together with previous study, indicated that AK001796 may affect the prognosis of ESCC patients. Finally, in order to explore the possibility of AK001796 as an independent prognostic biomarker for ESCC patients, we performed multivariate analysis and our results showed that high expression of AK001796 was a poor independent prognostic factor for both OS and DFS in patients with ESCC. Although we provided important evidence that AK001796 may be a novel biomarker for ESCC patients, the detailed mechanisms underlying its tumor suppressive role in this disease remain to be further elucidated.

Conclusions

We showed that high expression of AK001796 was significantly associated with tumor progression and decreased survival in patients with ESCC, suggesting that AK001796 could function as a novel prognostic marker for ESCC.

Conflict of Interests

The Authors declare that there are no conflicts of interest.

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