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The long non-coding RNA AK001796 contributes to poor prognosis and tumor progression in hepatocellular carcinoma

Q.-L. HAN[1], B.-T. CHEN[1], K.-J. ZHANG[2], S.-T. XIA[1], W.-W. ZHONG[1], Z.-M. ZHAO[3]

[1]Department of Gastroenterology, The No.1 People's Hospital of Jingmen, Jingmen, China
[2]Department of Traditional Chinese Medicine, The No.1 People's Hospital of Jingmen, Jingmen, China
[3]Department of Cirrhosis, Shuguang hospital, Shanghai University of traditional Chinese medicine, Pudong, Shanghai, China

Abstract. – OBJECTIVE: Increasing studies have indicated the important functions of long non-coding RNAs (lncRNAs) in tumorigenesis and progression including hepatocellular carcinoma (HCC). The study aims to explore the role of long non-coding RNA AK001796 in HCC progression.

PATIENTS AND METHODS: Quantitative Real-time polymerase chain reaction (QRT-PCR) analysis was performed to examine the lncRNA AK001796 expression in 73 cases of human HCC tissue samples and matched adjacent normal tissues. Besides, the relationship between lncRNA AK001796 expression and clinicopathologic characteristics was analyzed. Overall survival (OS) curves of patients were constructed by the Kaplan-Meier methods. Multivariate Cox regression analysis was used to evaluate independent risk factors affecting HCC prognosis. Cell proliferation and invasion abilities are analyzed by cell counting kit-8 (CCK-8) and transwell invasion assays.

RESULTS: We showed that the lncRNA AK001796 expression was significantly up-regulated in HCC tissues and cell lines, compared to their controls, respectively. Higher lncRNA AK001796 expression closely correlated with tumor size ($p<0.05$), TNM stage ($p<0.05$) and the poor overall survival (OS) rate of HCC patients ($p<0.05$). Besides, multivariate Cox regression analysis found that lncRNA AK001796 expression was identified as an independent risk factor for HCC prognosis. *In vitro*, we showed that lncRNA AK001796 knockdown markedly suppressed cell proliferation and cell invasion abilities.

CONCLUSIONS: Our results suggested that lncRNA AK001796 acts as a predictor of HCC prognosis and may provide an important clinical value for HCC treatment.

Key Words
Long non-coding RNA, Hepatocellular carcinoma, AK001796, Prognosis, Cell proliferation.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common causes of tumor-related mortality worldwide due to high incidence of tumor recurrence and metastasis[1]. Surgical resection is the major method for HCC treatment and patients who are diagnosed at early stage have a satisfactory 5-year HCC survival rate[2]. However, patients with diagnosed at late stage showed poor prognosis[3,4]. Thus, to identify novel diagnostic markers and therapeutic targets for HCC are urgent. Long noncoding RNAs play essential roles in cancer development and progression[5]. Emerging evidences suggest that some lncRNAs play positive function during hepatocarcinogenesis[6,7]. Such as, long non-coding RNA SPRY4-IT1 promotes development of hepatic cellular carcinoma by interacting with ERRα and predicts poor prognosis[8]. Long non-coding RNA NNT-AS1 promotes hepatocellular carcinoma progression and metastasis through miR-363/CDK6 axis[9]. LncRNA GHET1 predicts poor prognosis in hepatocellular carcinoma and promotes cell proliferation by silencing KLF2[10]. Knockdown of LncRNA ANRIL suppresses cell proliferation, metastasis, and invasion via regulating miR-122-5p expression in hepatocellular carcinoma[11]. LncRNA AK001796 is first reported to be involved in lung cancer progression. lncRNA AK001796 acts as an oncogene and is involved in cell growth inhibition by resveratrol in lung cancer[12]. However, the role of lncRNA AK001796 in HCC remains unknown. Our results showed that the lncRNA AK001796 expression was significantly up-regulated in HCC and closely correlated with tumor size, TNM stage and the poor prognosis of HCC. Besides, *in vitro*,

Corresponding Author: Bitao Chen, MD; e-mail: i33786936beng@163.com

we showed that lncRNA AK001796 knockdown suppressed markedly inhibited HCC cell proliferation and invasion. Thus, our results suggested that AK001796 had an important clinical value and may be a potential target for HCC treatment.

Patients and Method

Patient Tissue Samples

HCC tissues from 51 male and 22 female patients (age range, 16 to 82 years; median age, 52 years) who underwent surgical resection at the Department of Gastroenterology, The No.1 People's Hospital of Jingmen (Jingmen, China) between June 2008 and April 2012 were randomly obtained. The diagnosis of HCC was evaluated by two professional pathologists. The study was approved by the Ethics Committee of The No.1 People's Hospital of Jingmen (Jingmen, China). Written informed consent was obtained from all patients. After surgery, all of human HCC tissue samples and adjacent normal were immediately frozen in liquid nitrogen and stored at -80°C until further analysis. The clinical stage was determined by the Tumor-Node-Metastasis (TNM) classification system[13].

Cell Culture

The human normal liver cell line LO2 and four human HCC cell lines including SMMC-7721, Huh-7, MHCC-97H (97H), and MHCC-97L (97L) were purchased from the Shanghai Institute of Cell Biology (Shanghai, China). LO2 cells were cultured in Roswell Park Memorial Institute (RMPI)-1640 (Biological Industries, Kibbutz Beit-Haemek, Israel) and HCC cell lines including SMMC-7721, Huh-7, MHCC-97H (97H), and MHCC-97L (97L) were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Hyclone; GE Healthcare Life Sciences, South-Logan, UT, USA). All cells were incubated at 37°C in a 5% CO_2 atmosphere.

Quantitative Reverse Transcription Polymerase Chain Reaction (QRT-PCR)

Total RNA was extracted from HCC cells, LO2 cells or tissue samples by using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). RNA integrity was evaluated using a Nano Drop ND-1000 spectrophotometer (Nano Drop Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA). A total of 1 μg RNA was used to reverse transcription to cDNA by using a cDNA synthesis kit (Tian Gen Biotech Co., Ltd., Beijing, China). The qRT-PCR analysis was performed using SYBR® Green Master Mix according to the manufacturer's protocol. The mRNA expression was tested by an ABI 7500 (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The conditions were as follows: 95°C for 15 min; 40 cycles consisting of 95°C for 10 s and then 64°C for 31 s. The β-actin was used as the internal control. The mRNA expression was measured using the $2^{-\Delta\Delta Cq}$ methods [14]. The primer sequences were as follow: lncRNA AK001796 forward: 5'-GCCCAGAUUUAAGGGCUAUTT-3'; and lncRNA AK001796 reverse, 5'-AUAGCCCUUAAAUCUGGGCTT-3'. β-actin forward: 5'-AGCGAGCATCCCCCAAAGTT-3'; and β-actin reverse, 5'-GGGCACGAAGGCTCATCATT-3'.

Cell transfection

Two siRNAs targeting lncRNA AK001796 and a negative control were designed and synthesized by GenePharma Co., Ltd. (Shanghai, China). The siRNA sequences were as follow: siRNA-1, Forward: 5'-GGUCACUACUGCUUUAUAATT-3',Reverse:5'-UUAUAAAGCAGUAGUGACCTT-3';siRNA-2,Forward:5'-GGUGGCCUGUACCUAUAAUTT-3',Reverse:5'-AUUAUAGGUACAGGCCACCTT-3'. Cells were transfected with two siRNAs and the siRNA-negative control, respectively using Lipofectamine™ 2000 (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cells were harvested at 48 h after the transfection for qRT-PCR analysis.

Cell Proliferation Assay

Cell proliferation ability was detected using a Cell Counting Kit-8 assay kit (CCK8; Dojindo, Kumamoto, Japan). 97H and SMCC-7721 cells were seeded at a density of 2500 cells/well in 96-well plates. Cells were transfected with siRNA-NC or siRNA-1 oligos. Next, each plate was added with 10 μl of CCK-8 reagent. The absorbance of each well at 0, 12, 24, 48, and 72 hours was detected at 450 nm at Microplate Reader (Epoch; BioTek Instruments, Inc., Winooski, VT, USA). All experiments were performed three times.

Cell Invasion Assays

Cell invasion was detected using transwell assay with Matrigel (Clontech Laboratories, Inc., Mountainview, CA, USA). Transfected HCC cells (1×10^5) were seeded on the upper chamber. DMEM medium with 10% fetal bovine serum (FBS) was added to the lower chamber. The cells on the low chambers were removed using cotton

swab. HCC cells on the upper chamber were fixed with 100 % methanol, stained with 0.1 % crystal violet. Finally, the invaded cells were counted under an inverted microscope (IX31; Olympus Corporation, Tokyo, Japan) at ×200 magnification.

Statistical Analysis

Statistical analysis was performed using SPSS 19.0 (IBM Corp., Armonk, NY, USA) Two-tailed Student's *t*-test was used to evaluate differences between two groups. One-way analysis of variance followed Tukey's multiple comparisons test was used to examine differences between multiple groups. Multivariate Cox proportional hazard regression was performed to evaluate the risk factors. A $p<0.05$ was considered to indicate a statistically significant difference.

Results

LncRNA AK001796 Expression is Upregulated in HCC Tissues and Cells

In the study, lncRNA AK001796 expression was determined in 73 paired of HCC tissue samples and adjacent normal tissues using qRT-PCR analysis. Our results indicated that HCC tissues exhibited significantly higher expression of lncRNA AK001796, than adjacent normal HCC tissues (Figure 1A, $p<0.05$). Next, we analyzed the clinical significance of lncRNA AK001796 expression in HCC. Patients with HCC were divided into higher-lncRNA AK001796 expression and low-lncRNA AK001796 expression groups according to the mean value. By chi-square test, our results indicated that higher expression of lncRNA AK001796 dramatically associated with tumor size ($p=0.001$, Table I, Figure 1B) and advanced TNM stage ($p=0.010$, Table I, Figure 1C) in HCC. However, no significant association was identified between lncRNA AK001796 expression and age, gender, AFP (ng/ml) and so on (All of $p>0.05$, Table I).

LncRNA AK001796 Expression Associates with Prognosis of HCC Patients

Kaplan-Meier methods and log-rank analyses were used to examine the association between lncRNA AK001796 expression and patient overall survival. As presented in Figure 1D, Higher lncRNA AK001796 expression significantly associated with poor prognosis of HCC compared to lower lncRNA AK001796 expression in

Table I. Relationship between lncRNA AK001796 and clinicopathologic parameters in 73 HCC patients.

Clinicopathologic parameters	Number	lncRNA AK001796 expression		*p*-value
		Higher (n=37)	Lower (n=36)	
Age (year)				0.395
≤60	52	28	24	
>60	21	9	12	
Gender				0.665
Female	22	12	10	
Male	51	25	26	
Tumor size (cm)				0.001*
<5 cm	39	12	27	
>5 cm	34	25	9	
AFP (ng/ml)				0.262
<400	20	8	12	
>400	53	29	24	
HBV infection				0.832
Yes	56	28	28	
No	17	9	8	
Differentiation				0.308
Higher -Moderately	55	26	29	
Lower	18	11	7	
TNM stage				0.010*
I/II	46	18	28	
III/IV	27	19	8	

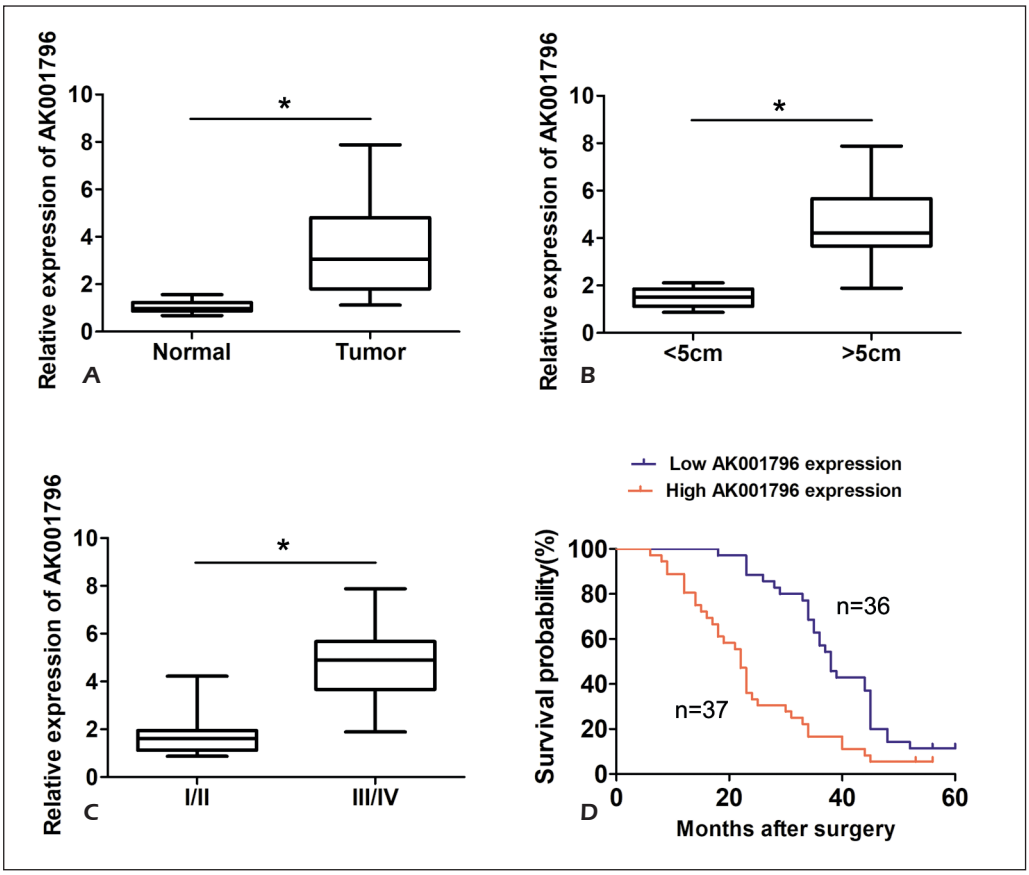

Figure 1. Relative expression of lncRNA AK001796 in patients with HCC. **A**, Higher relative lncRNA AK001796 expression was exhibited in HCC tissues compared with adjacent non-tumor tissues from patients by qRT-PCR analysis (n=73). **B**, The association of lncRNA AK001796 expression with tumor size was shown. **C**, The association of lncRNA AK001796 expression with TNM stage was shown. **D**, lncRNA AK001796 expression was classified into two groups: higher lncRNA AK001796 expression and lower lncRNA AK001796 expression. Kaplan-Meier methods and log-rank analyses showed that higher lncRNA AK001796 showed poor prognosis of HCC. *p<0.05. HCC, hepatocellular carcinoma.

HCC patients (p<0.05, log rank test). In particular, multivariate cox proportional hazard regression was performed to evaluate the risk factors of prognosis. Our results indicated that higher lncRNA AK001796 expression was identified as a risk factor for HCC prognosis (HR, 2.899, 95% CI,1.521-5.565, p<0.05). Thus, these data indicate that higher lncRNA AK001796 expression may indicate a poor prognosis for patients with HCC.

Reduced lncRNA AK001796 inhibits cell proliferation and invasion in HCC

Furthermore, we found that lncRNA AK001796 expression was also significantly upregulated in four human HCC cell lines, compared with LO2 cells (Figure 2A, p<0.05). As lncRNA AK001796 expression was shown to be higher in HCC cells, we selected 97H and SMCC-7721 cells for transfection with si-AK001796-1, si-AK001796-2 and si-NC. Following transfection, the AK001796

Table II. Multivariate analysis of over survival (OS) for prognosis in HCC patients (n=73).

Variables	HR	95%CI	*p*-value
Tumor size	2.455	1.125-5.121	0.001*
TNM stage	1.996	0.899-4.189	0.002*
lncRNA AK001796	2.899	1.521-5.565	0.001*

*, p-value <0.05 was considered as statistically significant, CI, confidence intervals.

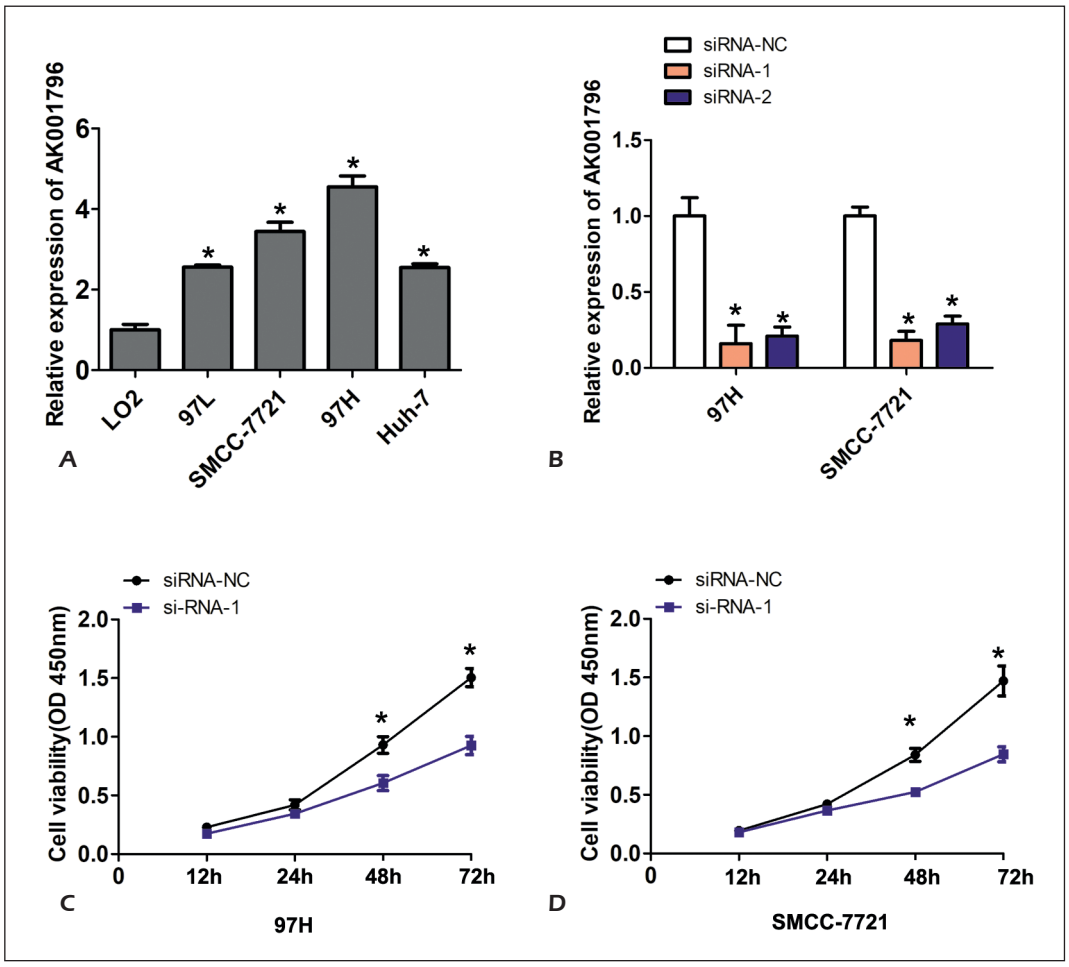

Figure 2. LncRNA AK001796 knockdown inhibited cell proliferation of HCC. **A**, lncRNA AK001796 expression was also significantly upregulated in four human HCC cell lines, compared with LO2 cells. **B**, The expression of lncRNA AK001796 was detected using qRT-PCR after 97H or SMCC-7721 cells were transfected with siRNA-1 or siRNA-NC. **C-D**, The cell proliferation was detected using CCK8 assays after 97H or SMCC-7721 cells were transfected with siRNA-1 or siRNA-NC. *p<0.05.

expression was significantly decreased compared with the control group in 97H and SMCC-7721 (Figure 2B). We selected si-AK001796-1 for following functional experiments due to its higher knockdown efficiency. The proliferative and invasive abilities of the transfected cells were examined using CCK8 and transwell invasion assays, respectively. As shown in Figure 2C-1D, lncRNA AK001796 silencing caused a significant reduced cell proliferation ability compared to control groups in 97H and SMCC-7721 cells, respectively (p<0.05). Moreover, lncRNA AK001796 silencing also caused a significant reduced cell invasion ability compared to control groups in 97H and SMCC-7721 cells, respectively (Figure 3A-3D). Thus, these data demonstrated that lncRNA AK001796 silencing could inhibit cell proliferation and invasion in HCC.

Discussion

LncRNAs play critical regulators in tumorigenesis and cancer progression[15]. However, the potential functional effects and regulating mechanisms for most lncRNAs in human HCC remain large unclear[16]. Evidence from previous studies shows some lncRNAs are involved in HCC progression. Such as, long noncoding RNA RUSC1-AS-N indicates poor prognosis and increases cell viability in hepatocellular carcinoma[17]. Long non-coding RNA SPRY4-IT1 promotes development of hepatic cellular carcinoma by interacting with ERRα and predicts poor prognosis[8]. Long non-coding RNA CASC15 is upregulated in hepatocellular carcinoma and facilitates hepatocarcinogenesis[18]. Long noncoding RNA HEIH promotes mela-

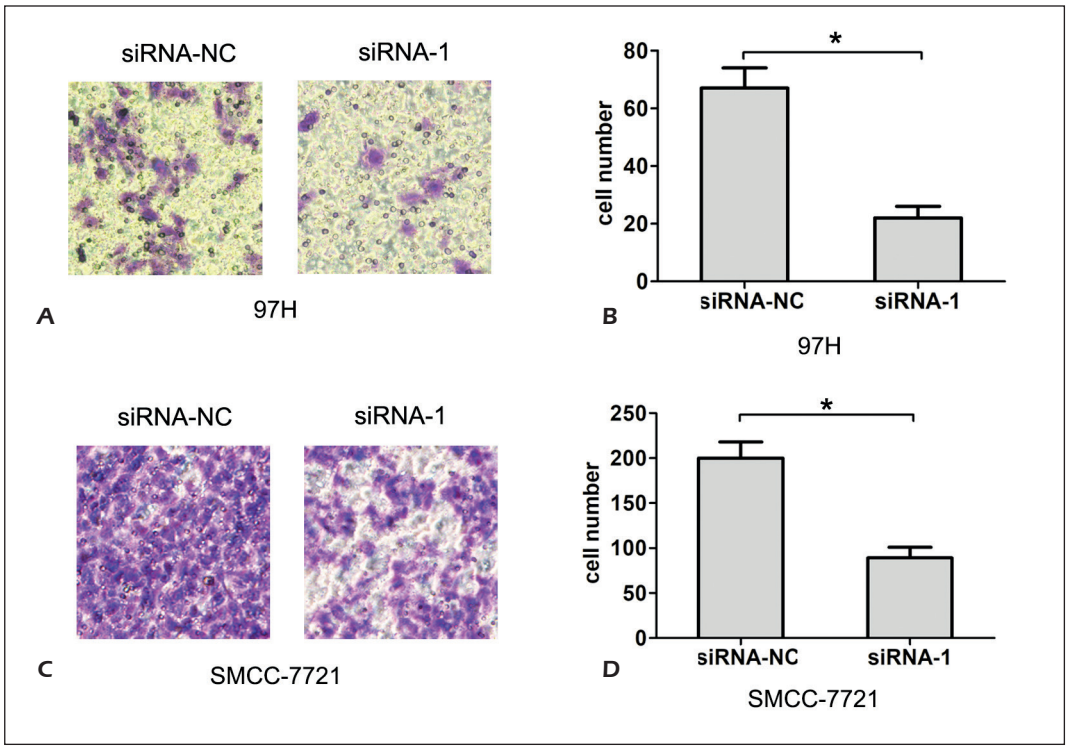

Figure 3. LncRNA AK001796 knockdown inhibited cell invasion of HCC. **A-B**, The cell invasion was detected using transwell assays after 97H cells were transfected with siRNA-1 or siRNA-NC. **C-D**, The cell invasion was detected using transwell assays after SMCC-7721 cells were transfected with siRNA-1 or siRNA-NC. *$p<0.05$.

noma cell proliferation, migration and invasion via inhibition of miR-200b/a/429[19]. However, the role of lncRNA AK001796 expression in HCC remains unknown. Here, we showed that lncRNA AK001796 expression was dramatically higher in human HCC tissues compared to adjacent normal tissues. Furthermore, we also showed that lncRNA AK001796 expression was higher in human HCC cells. By analyzing the association of lncRNA AK001796 with clinical data and tumor prognosis, we demonstrated that higher lncRNA AK001796 expression associated with large tumor size, advanced tumor stage and poor overall survival for HCC. These results indicated that lncRNA AK001796 expression acted as a predictor of HCC prognosis. Furthermore, we analyzed the functional effects of lncRNA AK001796 on cell proliferation and invasion, we found that reduced lncRNA AK001796 expression could inhibited cell proliferation and invasion ability. In previous study, lncRNA AK001796 was reported to be participated in some tumors, long noncoding RNA AK001796 acts as an oncogene and is involved in cell growth inhibition by resveratrol in lung cancer[12]. The long non-coding RNA AK001796 contributes to tumor growth via regulating expression of p53 in esophageal squamous cell carcinoma [20]. Here, we showed the functional significance of lncRNA AK001796 in HCC.

Conclusions

We demonstrated that lncRNA AK001796 expression was higher in HCC tissues and cells. Furthermore, higher lncRNA AK001796 expression associated with large tumor size, advanced tumor stage and poor overall survival for HCC. Knockdown of lncRNA AK001796 inhibited cell proliferation and invasion. Thus, lncRNA AK001796 may act as a predictor and target of HCC treatment.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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