Int J Clin Exp Med 2016;9(2):3033-3040
www.ijcem.com /ISSN:1940-5901/IJCEM0018119

Original Article

Long non-coding RNA AK021444 correlates with poor prognosis and promotes cell proliferation in colorectal cancer

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Received October 19, 2015; Accepted January 16, 2016; Epub February 15, 2016; Published February 29, 2016

Abstract: Long non-coding RNAs (lncRNAs) are aberrantly expressed in many diseases including cancer. Our previous studies suggested that lncRNA-AK021444 specificity expressed in Metastatic lymph node of colorectal cancer. However, the relationship between the clinical parameters and molecular mechanism of lncRNA-AK021444 in colorectal cancer (CRC) remain largely unknown. In the present study, we found that lncRNA-AK021444 was significantly related with CRC tumor size, lymph node metastasis, and increased death using in situ hybridization (ISH) method of 90 paired CRC specimens. Lentiviral vector-mediated lncRNA-AK021444 overexpression was employed in CRC cell line HCT116. Our study demonstrated that lncRNA-AK021444 overexpression regulated cell proliferation and apoptosis of CRC, which might be meaningful with regards to target therapy in CRC.

Keywords: LncRNA-AK021444, colorectal cancer, in situ hybridization

Introduction

In the global scope, colorectal cancer (CRC) is one of the most common causes of cancer-related deaths, leading to 600000 deaths each year [1, 2]. There is a great need to determine the relationships between clinical features and molecular changes in CRC to develop new diagnosis and treatment strategies [3, 4]. Long non-coding RNA (lncRNA), unable to be translated into proteins and greater than 200 nt in length, which can be used as one of the important regulatory molecules in the human genome and can exert its biological control function through a variety of ways [5, 6]. Recent studies have shown that lncRNAs were involved in chromatin modification and transcriptional activation and many other important biological processes [7, 8]. For instance, metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), acts as a factor indicating high metastatic potential and poor prognosis in several human neoplasms, and as a transcriptional regulator involved in cell proliferation, migration and metastasis [9-12].

In our previous works, lncRNAs were measured by lncRNA spectrum utilization America Array star lncRNA chip technology in metastatic lymph nodes, normal lymph node and CRC tissues. By comparing the difference of the expression of the lncRNA junction between the CRC tissue and metastatic lymph nodes and between metastatic lymph nodes and normal lymph node, screening out the shift of high specificity expression in lymph node lncRNA. Compared with normal lymph node and cancer tissues, AK021444 were confirmed to be specifically up-regulated in metastatic lymph node [13].

The aim of this study was to assess the relationships of lncRNA-AK021444 with clinical pathological parameters and prognosis of CRC and further explore the biological and molecular functions of lncRNA-AK021444. In situ hybridization (ISH) method of 90 paired CRC specimens revealed that increased expression of lncRNA-AK021444 was significantly correlated with CRC tumor size, lymph node metastasis, and increased death indicating that lncRNA-

AK021444 might be involved in the progression of CRC. Moreover, upregulation the expression of lncRNA-AK021444 could promote cell proliferation of HCT116 by arresting cell cycle and suppress cell apoptosis. Our results suggested that lncRNA-AK021444 might represent a novel biomarker of poor prognosis and be used as a basis and target for the diagnosis and gene therapy of CRC.

Materials and methods

Tissue microarray and in situ hybridization

Tissue microarrays (TMAs) containing 90 pairs of CRC tissues and the normal mucosa counterparts were constructed (Catalog no. HCol-Ade180Sur-08, Shanghai Biochip Company Ltd, China). In situ hybridization (ISH) was performed on the 4-μm-thick TMA sections. Briefly, sections were fixed and permeabilized for probe accessing with the help of xylenes, 100% ethanol, and protease, and then boiled in pretreatment buffer for 15 minutes. Hybridization was performed overnight at 37°C after the lncRNA-AK021444 probes were denatured. Finally, the lncRNA was visualized with enzymatic reaction of DAB (3, 3'-diaminobenzidine) and the sections were counterstained with hematoxylin.

German immunoreactive score (IRS) was used to evaluated the stained sections in three different areas by pathologists who were blinded to the clinical data. Briefly, staining intensity was assigned a score as "0 = negative", "1 = weak", "2 = moderate" and "3 = strong"; staining extent was assigned a score as "0 < 5%", "1 = 5-25%", "2 = 25-50%", "3 = 50-75%" and "4 > 75%". The final IRS of lncRNA-AK021444 expression was determined by multiplying the intensity score and the extent score, generating a score ranged from 0 to 12. For each sections, the median score ≥ 6 was defined as high lncRNA-AK021444 expression and the median score < 6 was defined as low lncRNA-AK021444 expression.

Cell culture

Human CRC cell line HCT-116, which were purchased from the Chinese Academy of Medical Sciences (Shanghai, China), was maintained in McCoy's 5A (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS). inoculated the HCT116 cells in CRC into the 96-well plates with (3-5) $\times 10^4$ per well, cultivated them for 24 hours with the McCOY'S 5A nutrient solution containing 10% of fetal calf serum into the 37°C incubator in which the volume fraction of CO_2 is 5%.

Lentiviral vectors construction and lentivirus infection

Lentiviral vectors containing green fluorescence protein (GFP) were employed in order to achieve high efficiency of introduction and subsequent stable expression of lncRNA-AK021444 in HCT116 cells. Recombined Ubi-MCS-3FLAG-SV40-EGFP vector with the lncRNA-AK021444 gene (LV-lncRNA-AK021444) and hU6-MCS-CMV-EGFP with a scrambled control sequence were constructed by Genechem Company (Genechem, Shanghai, China). HCT-116 cells were then infected with the above lentiviral vectors. A total of 5×10^5 HCT116 cells were seeded in a five well cell plate and further incubated for 12 hours to reach 30% confluent, and then infected with LV-lncRNA-AK021444 (AK021444 overexpression group, OE), LV-GV287 (negative control group, NC), and no infection (non-transfected control group, CON) by replacing the infection medium containing recombinant vectors at a multiplicity of infection (MOI) of 20 plaque-forming units (pfu) per cell. Plates were then incubated for 24 hours prior to having their media changed to fresh, virus-free media. Three days later, the GFP density contained by lentivirus was detected to evaluate the efficiency of infection, and cells were harvested for real-time quantitative polymerase chain reaction (qRT-PCR) analysis.

Total RNA extraction and real-time reverse transcription-polymerase chain reaction

Total RNA was extracted from colorectal cancer tissues and cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then cDNA was synthesized with M-MLV reverse transcriptase (Invitrogen). Real-time reverse transcription-polymerase chain reaction (RT-PCR) with SYBR Green assay (TaKaRa Biotechnology, Dalian, China) was performed to examine expression level of lncRNA-AK021444. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The assay was conducted using the ABI 7300 system (Applied Biosystems). All reactions were done in triplicate and expression level of lncRNA-AK021444 was calculated according to the equation $2^{-\Delta\Delta Ct}$.

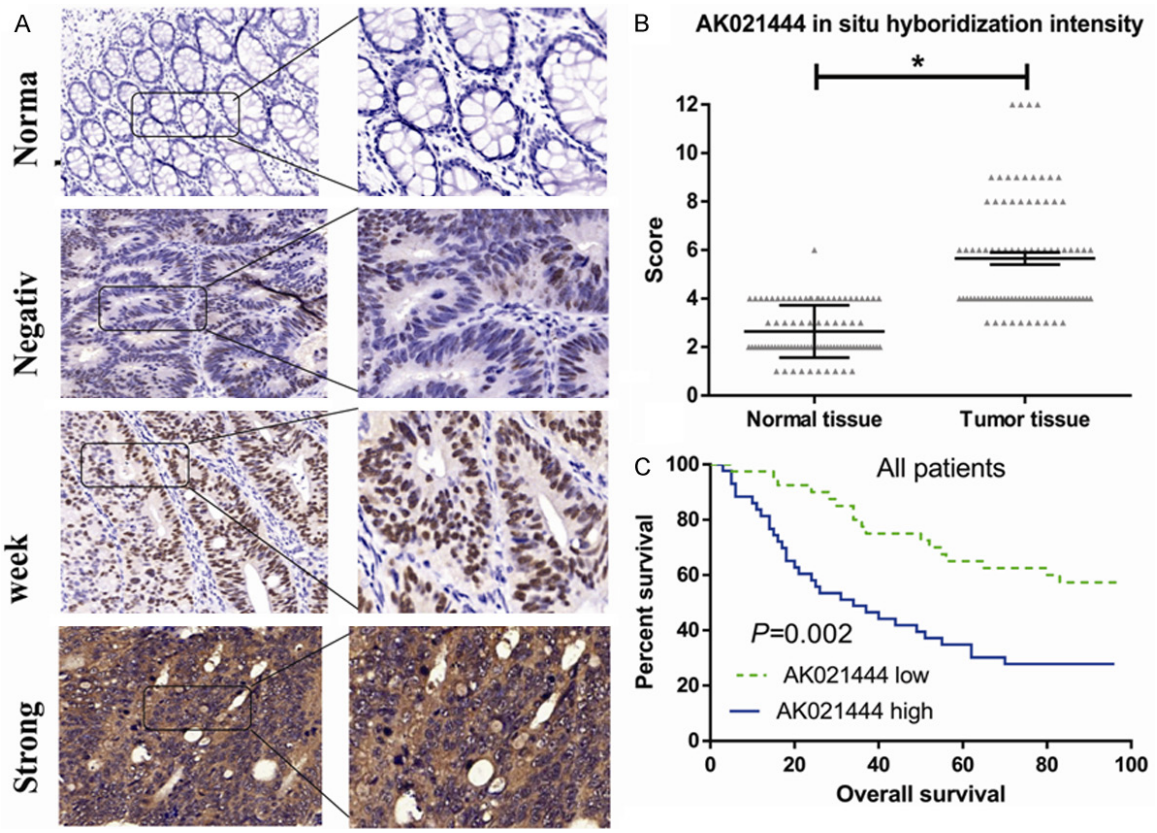

Figure 1. Expression of lncRNA-AK021444 in CRC tissues. A. ISH characteristics of lncRNA-AK021444 in tumor and adjacent normal tissues. Representative patterns of lncRNA-AK021444 expression were shown. (Magnification, left panel, ×100; right panel, ×200). B. The score (intensity* area) of lncRNA-AK021444 in the tumor or adjacent normal tissues in the study cohort ($P < 0.001$). C. Kaplan-Meier survival curves for overall survival (OS) of CRC patients with lncRNA-AK021444 low and high expression group.

Cell proliferation assay

Approximately 5.0 × 10^3 transfected HCT116 cells were plated in 96-well plates. After 24, 48 and 72 h of transfection, the proliferation rate of HCT116 cells was assessed using the Cell Counting Kit 8 (CCK8, Dojindo, Kumamoto, Japan). The absorbance value of each well was determined at 450 nm using the Infinite M200 spectrophotometer (Tecan, Germany). Each experiment was repeated three times.

Flow cytometric analysis

Transfected cells were harvested after transfection. Cells for cell cycle analysis were stained with propidium oxide by the Cycle TEST PLUS DNA Reagent Kit (BD Biosciences) following the protocol and analyzed by FACScan. The percentage of the cells in G0-G1, S, and G2-M phase were counted and compared.

For the cell apoptosis assay, the cells were treated with fluorescein isothio-cyanate-Annexin V and PI in the dark, according to the manufacturer's instructions. Then the cells were detected by flow cytometry with the help of Cell-Quest software. Cells were discriminated into dead cells, viable cells, early apoptotic cells, and late apoptotic cells. Next, the percentage of early apoptotic cells and late apoptotic cells was compared with empty vector from each experiment. Each assay was repeated in triplicate.

Cell wound scratch assay

Take in logarithmic growth phase, cells were plated at 10^5 per well in a 6 well plate. They were serum starved for 12 h when cells reached 90% confluency, and a "wound" was created using a 10-μl pipette tip. According to 2, 24 h hours sampling, pictures, data processing.

Statistical analysis

Two-tailed Student's *t* test was calculated to compare the significance between the CRC and

Table 1. Association between lncRNA-AK021444 expressionand clinical pathological parameter of CRC patients

Parameters	n	lncRNA-AK021444 expression		*P* value
		Low	High	
Age (years)				0.116
≤ 60	18	12	6	
> 60	72	39	33	
Sex				0.532
Female	43	20	23	
Male	47	25	22	
Tumor size (cm)				< 0.001*
≤ 5	45	35	10	
> 5	43	8	35	
Local invasion				0.729
T1 + T2	9	5	4	
T3 + T4	79	39	40	
Lymph node metastasis				< 0.001*
N0	56	36	20	
N1 + N2	34	9	25	
TNM stage				0.081
I	9	7	2	
II + III	81	38	43	
Death				0.009*
No	36	23	13	
Yes	48	17	31	

*Statistically significant (P < 0.05).

normal samples. Pearson or Fisher exact test was used to analyze the relationship between lncRNA-AK021444 expression and clinical characteristics. Kaplan-Meier survival analysis and log-rank test was performed for univariate analysis to compare the prognostic of colorectal cancer and lncRNA-AK021444 expression. The effect of each significant variable was included in the Cox multivariate regression analysis. All data are shown as the mean ± S.E.M. Statistical analyses were performed on SPSS software (version 18.0). A *p*-value of < 0.05 (*) was considered statistically significance.

Results

Upregulated lncRNA-AK021444 predict spoor prognosis of CRC patients

To test whether lncRNA-AK021444 expression is correlated with poor prognosis of CRC cancer, ISH was used to assess the expression level of lncRNA-AK021444 in 90 pairs of CRC tissues and pair-matched adjacent normal tissues as representatively shown in **Figure 1A**. The clinicopathological characteristics of the 90 CRC patients were summarized in **Table 1**. LncRNA-AK021444 level was markedly higher in cancer tissues compared with normal tissue based on ISH (**Figure 1B**). Furthermore, correlation analysis of lncRNA-AK021444 expression revealed a significant association between lncRNA-AK021444 expression and CRC tumor size ($P < 0.001$), lymph node metastasis ($P < 0.001$), and increased death ($P = 0.009$) (**Table 1**). However, the association between lncRNA-AK021444 expression and patient age, gender, local invasion or TNM stage was not found. Kaplan-Meier survival analysis and log-rank tests were performed to evaluate the effects of lncRNA-AK021444 on patient survival. The results showed that patients with high lncRNA-AK021444 expression had a significantly shorter OS than those with low lncRNA-AK021444 expression (**Figure 1C**, $P = 0.002$). By univariate and multivariable Cox regression analysis, we revealed that Lymph node metastasis and high lncRNA-AK021444 expression were risk factors for overall survival of CRC patients (**Table 2**). These results indicated that lncRNA-AK021444 expression could be an independent factor affecting overall survival.

Overexpression of lncRNA-AK021444 in HCT116 cells infected with lentiviral vectors

To investigate whether LncRNA-AK021444 had an effect on Colorectal Cancer Cell. The recombinant lentivirus vector LV-lncRNA-AK021444 was successfully constructed and infected HCT116 cells. The efficiency of the infection (averaged proportion of GFP-expressing cells on the total cell count) was approximately 70% at an MOI of 20 (**Figure 2A**). Consequently, an MOI of 20 was chosen for the next steps of this study. QRT-PCR analysis demonstrated that the relative expression level of lncRNA-AK021444-mRNA was markedly increased in the lncRNA-AK021444OE, compared with that in the NC and the CON (**Figure 2B**). There was no statistical difference of lncRNA-AK021444mRNA expression between the NC and the CON ($P > 0.05$).

Table 2. Univariate and multivariate Cox regression analyses of lncRNA-AK021444 expression and clinical variables for overall survival of CRC patients

Variables	Categories	Univariate analysis			Multivariate analysis		
		HR	95% CI	*P* value	HR	95% CI	*P* value
Age (years)	> 60/≤ 60	2.124	0.902-5.001	0.085			
Sex	Male/female	1.323	0.747-2.341	0.337			
Size (cm)	≥ 5/< 5	1.670	0.942-2.960	0.079			
Local invasion	1 + 2/3 + 4	1.232	0.487-3.114	0.659			
Lymph node metastasis	$N_0/N_1 + N_2$	2.569	1.449-4.557	0.001*	2.103	1.158-3.817	0.015*
TNM stage	I/II + III	1.816	0.652-5.061	0.254			
lncRNA-AK021444 expression	High/low	2.555	1.409-4.631	0.002*	2.108	1.135-3.913	0.018*

Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval. *Statistically significant (P < 0.05).

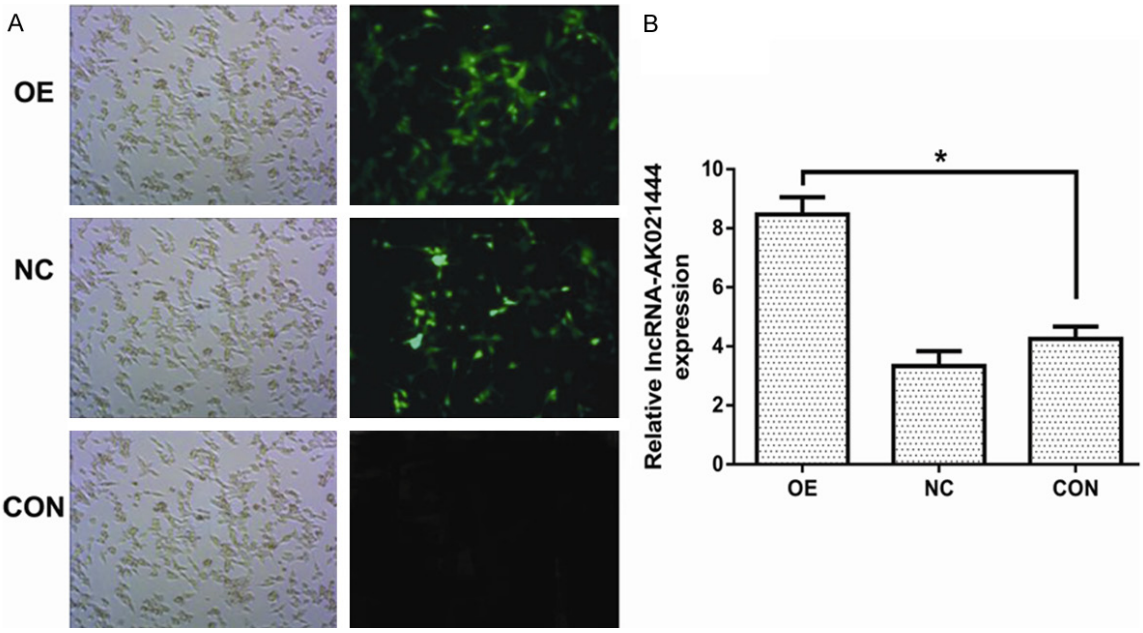

Figure 2. Overexpression of lncRNA-AK021444 in HCT116 cells by lentivirus-mediated gene expression system. A. Infection efficiency of LV-lncRNA-AK021444 in HCT116 cells by GFP detection (100×). (Left: bright field; Right: fluorescent field). B. Three days after infection, the relative lncRNA-AK021444 mRNA expression were determined by qRT-PCR analysis respectively ($P < 0.05$).

Effects of lncRNA-AK021444 on cell proliferation and apoptosis

We assessed the proliferation rate of OE, NC and CON, to determine the functional role of lncRNA-AK021444 in the carcinogenesis of colorectal cancer. After 24, 48 and 72 h of transfection, results of the CCK-8 assay revealed an evident increase in cell growth of HCT116 cells, indicating a growth-promoting role of lncRNA-AK021444 on CRC cells (**Figure 3A**). Flow cytometric analysis was performed to further examine whether the effect of lncRNA-AK021444 on proliferation of CRC cells by altering cell cycle progression. The results revealed that the cell cycle progression of HCT-116 cells was significantly stalled at the S-phase compared with cells transfected with empty vector (**Figure 3B**). Next, we investigated the effects of lncRNA-AK021444 over-expression on apoptosis. As shown, the percentages of apoptotic cells were significantly decreased in the OE compared to the NC (**Figure 3C**). These results suggested that lncRNA-AK02-1444 treatment could induce S-phase arrest and diminish colorectal cancer cell apoptosis.

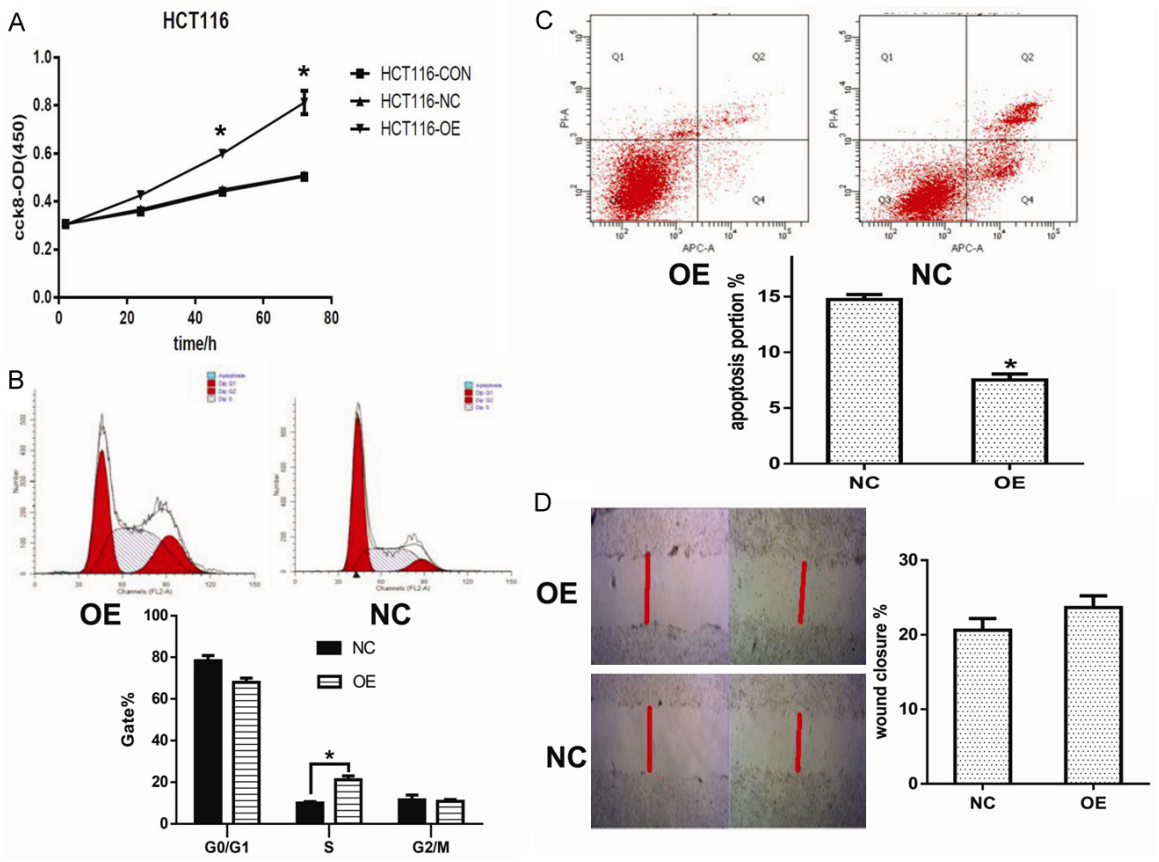

Figure 3. Effects of lncRNA-AK021444 on colorectal cancer cell in vitro. A. CCK8 assay was performed to determine the proliferation of HCT116 cells ($P < 0.05$). B. Cell cycle of HCT-116 was analyzed by flow cytometry. C. The percentage of apoptotic cells was determined by flow-cytometric analysis. D. The cell wound scratch assay on the cell migration process of HCT116.

Effect of lncRNA-AK021444 on colorectal cancer cell HCT116 migration

The cell wound scratch assay was used in a range of disciplines to study the coordinated movement of a cell migration. The purpose of this assay was to investigate the effects of lncRNA-AK021444 on the cell migration process in HCT116 cell. However, the wound closure percentage of NC and OE had no obviously difference ($P > 0.05$, **Figure 3D**).

Discussion

Tumor is a process of multiple factors, multiple steps of development, is the result of multiple gene interactions. In the whole genome transcription in lncRNA products, the proportion is far more than the proportion of coding RNA [14]. LncRNA through the interaction with DNA, RNA, protein, plays a very important role in the regulation of life activities in the network. It has been shown that lncRNA may play key roles in cell-cycle regulation and thus affect the malignant phenotypes of cancer cells [15, 16]. In addition to the expression and regulation of the function is very important in gene, lncRNA and the evolution of species, embryonic development, metabolism and tumorigenesis are closely linked to each other [17]. In clinical application, lncRNA can be a good biomarker, much research is still needed to be verified [18].

ViaTMA and ISH method, we attempted to evaluate the relationship between LncRNA-AK021444 expression and prognosis of CRC patients. Results showed that lncRNA-AK02-1444 was significantly associated with CRC tumor size, lymph node metastasis, and increased death. Kaplan-Meier analysis demonstrated that patients with high levels of LncRNA-AK021444 expression had remarkably shorter survival time than those with low levels. Multivariate analysis further revealed that

LncRNA-AK021444 expression was a significant independent predictor of poor survival of CRC patients.

Lentiviral vector as a vector used to often be used in gene therapy. This study successfully constructed stable lncRNA overexpression of lentivirus. This study found that over-expression of lncRNA-AK021444 in colorectal cancer cells can promote cell proliferation and inhibition apoptosis, but not for migration, laid a good foundation for the next research of colorectal carcinoma with regulation mechanism. However, the complexity is also should be fully aware of the regulatory mechanism of lncRNA molecules, clearly considering looking for lncRNA specific target gene or protein and to study its regulation with still needs a lot of experiments to clarify.

In conclusion, we demonstrated that lncRNA-AK021444 is upregulated in human CRC tumor tissues and can be considered an independent prognostic factor in patients with CRC. Overexpressed lncRNA-AK021444 could promote cell proliferation in vitro. Our study might supply a strategy and facilitate the development of lncRNA directed diagnostics and therapeutics against this deadly cancer.

Acknowledgements

This work was supported by Natural Science Foundation Project of Jiangsu Province (BK20-12872), the Science and Technology Projects, Health Department of Jiangsu Province (H20-1207) and the Research Innovation Program for College Graduates of Jiangsu Province (SJLX15_0427).

Disclosure of conflict of interest

None.

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