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Original Article

Long non-coding RNA LET is associated with prognosis and exhibits tumor-suppressive activity in laryngeal cancer

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Abstract: Introduction: Long non-coding RNAs (LncRNAs) are pervasively transcribed in the genome and potentially involved in progress of malignant tumors. The role of lncRNA LET in human laryngeal squamous cell cancer (LSCC) is not well understood. The aim of this study was to investigate whether lncRNA LET plays a role in LSCC. Methods: We examined the expression of lncRNA LET in 82 laryngeal cancer tissues and matched adjacent non-cancer tissues using quantitative real-time PCR and analyzed its correlation with the clinicopathological features. MTT was used to detected proliferation of LSCC cells after lncRNA LET transfection in vitro. The growth of mice xenografts was observed after lncRNA LET intratumoral injection. Results: The results showed that lncRNA LET expression in laryngeal cancer tissues was significantly down-regulated compared with the adjacent non-cancer tissues (P<0.01). Decreased lncRNA LET expression was significantly correlated with T stage, lymph node metastasis and advanced stage (P<0.05). Moreover, LSCC patients with lncRNA LET lower expression have shown significantly poorer overall survival than those with higher lncRNA LET expression (P<0.01). Cell proliferation was significantly inhibited after lncRNA LET transfection in vitro. The growth of xenografts was significantly suppressed by repeated injection of lncRNA LET lentivirus. Conclusions: These data suggest an important role for lncRNA LET in the progress of LSCC and the potential application of lncRNA LET in LSCC therapy.

Keywords: LncRNA LET, laryngeal squamous cell carcinoma, proliferation

Introduction

Laryngeal squamous cell carcinoma (LSCC) is a common malignancy of the upper respiratory tract. Current treatments including surgical intervention, radiation therapy and chemotherapy have a moderate effect on early stage cases, but are less effective for more advanced cases. A recent meta-analysis suggested an overall 5-year survival rate of LSCC was 64.2% [1]. Therefore, revealing the molecular mechanisms underlying LSCC carcinogenesis or progression is important for developing effective therapeutic strategies. In addition to the classical long protein coding mRNAs, there are a wide variety of noncoding RNA transcripts in mammalian genomes. As a kind of nocoding RNA, microRNAs have been identified critical roles for ncRNAs in cancer and microRNAs are associated with diagnosis, staging, progression, prognosis and treatment of malignant tumors [2]. Our previous study has shown that miRNAs play important role in the proliferation and apoptosis of LSCC [3, 4]. But in addition to the relatively well-known microRNAs, the growing knowledge of long non-coding RNA (lncRNA) has been revealed in the mammalian transcript. Similar to miRNA, lncRNA can also promote cellular pathways that lead to the development and progression of cancer [5, 6]. Recent studies have demonstrated that lncRNA LET is significantly downregulated in tumors such as cervical and gastric cancer [7, 8]. However, the biological roles of lncRNA LET in LSCC are still

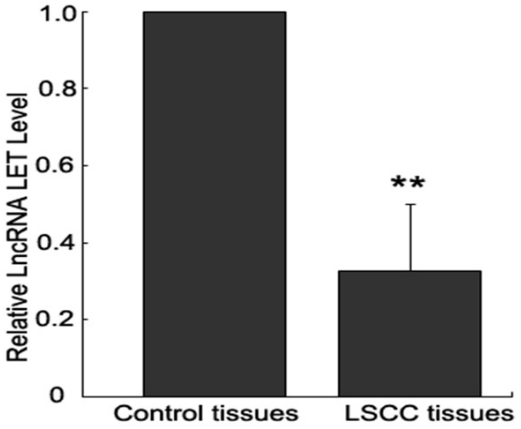

Figure 1. The expression of lncRNA LET in LSCC and adjacent non-neoplastic tissues. The lncRNA LET level in LSCC tissues was significantly lower than that in the adjacent non-neoplastic tissues (P<0.01).

Table 1. Relationship between lncRNA LET expression level and clinicopathologic parameters of LSCC

Characteristics (n)	Relative LET level	*P*
Sex		0.259
Male (55)	0.315±0.161	
Female (27)	0.344±0.199	
Age		0.168
≥58 (41)	0.307±0.158	
<58 (41)	0.344±0.189	
T classification		0.007
T1-2 (49)	0.362±0.183	
T3-4 (33)	0.270±0.124	
Differentiation		0.062
G1 (58)	0.308±0.182	
G2 (24)	0.368±0.148	
Lymph node metastasis		0.022
Negative (52)	0.353±0.183	
Positive (30)	0.277±0.148	
Primary location		0.102
Supraglottic (35)	0.304±0.177	
Glottic (47)	0.354±0.169	
Clinical stage		0.001
I-II (45)	0.375±0.184	
III-IV (37)	0.264±0.140	

poorly understood. Therefore, in this study we first detected the expression of lncRNA LET in LSCC by real-time PCR and found a significant downregulation of lncRNA LET in LSCC cancer tissue. Moreover, we found that lncRNA LET transfection can suppress proliferation and growth of LSCC. Our findings suggest that lncRNA LET plays a tumor-suppressive role in LSCC.

Materials and methods

Patients and samples

Patients were enrolled in the period between October 2006 and July 2008. Included in the study were 82 patients with laryngeal cancer who underwent partial or total laryngectomy at the Department of Otorhinolaryngology, the Second Affiliated Hospital of Harbin Medical University, under an approved protocol of Harbin Medical University. The patients had not received any therapy before admission. After surgery, the matched specimens of LSCC and the corresponding adjacent non-neoplastic tissues obtained from patients were preserved in liquid nitrogen within 5 min of excision, then transported frozen to the laboratory and stored at -80°C.

Real-time RT-PCR

Total RNA was extracted from cancerous/non-cancerous specimens or cell lines and expression level of lncRNA LET was determined by qPCR as described previously [3]. The primers for LncRNA LET detection were 5'-CCTTCCTGACAGCCAGTGTG-3' (sense) and 5'-CAGAATGGAAATACTGGAGCA-3' (reverse) [7]. To estimate the expression of LncRNA LET, the Ct values were normalized using 18S rRNA as internal control. The relative miRNA expression was calculated using the 2^{-DDCt}.

Lentivirus vectors for lncRNA LET

Human lncRNA LET lentivirus gene transfer vector harboring green fluorescent protein (GFP) sequence was constructed by Genechem (Shanghai, China). The recombinant lentivirus of lncRNA LET and the control lentivirus (GFP-lentivirus) were prepared and titered to 10^8 TU/mL (transfection unit).

Cell culture and virus transduction

The Hep-2 cells of human LSCC were kindly provided by the laboratory of cell pathology, Harbin Medical University. Cells were cultured in DMEM medium containing 10% fetal bovine serum (Gibco) and incubated in a humidified (37°C, 5% CO_2) incubator. Hep-2 cells were plated in 24-well plates ($2 10^4$ cells/well) overnight. The lentiviruses were diluted in 0.2 mL (10^7 TU/mL)

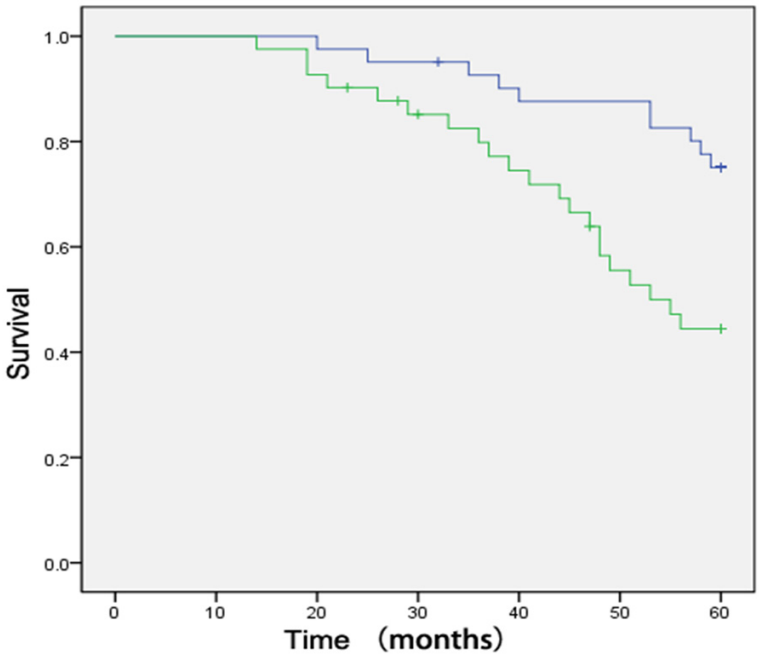

Figure 2. Kaplan Meier curves indicate significantly shorter 5-year overall survival for LSCC patients (n=82) in whose tumors lncRNA LET was low expressed (lower curve), compared with high expression (upper curve) (P<0.01).

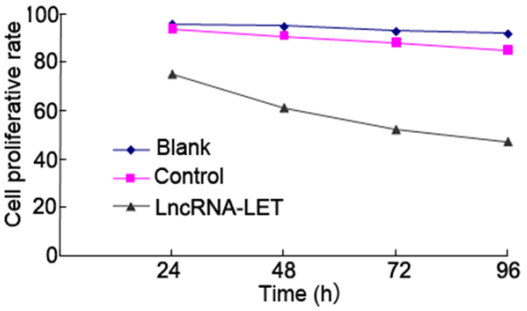

Figure 3. Curve of cell survival rate. After lncRNA LET transfection, the survival rate of Hep-2 cells was evidently decreased at each different time point (24, 48, 72, and 96 h, respectively) compared with the controls.

complete medium containing polybrene (8 mg/mL) and added to the cells for 1 h incubation at 37°C, followed by incubation in 0.3 mL of fresh prepared polybrene-DMEM for another 24 h, which was replaced with fresh DMEM medium and the cells were cultured for next 48 h.

MTT

After lncRNA LET transfection of Hep-2 cells for varying time periods: 20, 44, 68 and 92 h, 20 μL of sterile MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) dye (5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added and incubation continued for another 4 h at 37°C. Then, 150 μL of dimethyl sulfoxide was added to each well and the plates were thoroughly mixed for 10 min. Spectrometric absorbance at a wavelength of 492 nm was measured on an enzyme immunoassay analyzer (model 680; Bio-Rad Laboratories, Hercules, CA, USA).

Animal experiments

Sixteen BALB/c mice (provided by The Central Animal Facility of Harbin Medical University) were 5-6 weeks old and about 20 g in weight. They were bred in aseptic conditions according to standard guidelines under a protocol approved by Harbin Medical University. All mice were injected subcutaneously in the dorsal scapula region with 100 μl suspension (1×10^6) of Hep-2 cells. The size of the tumor was measured twice a week with calipers, and the volume of tumor was determined using the simplified formula of a rotational ellipsoid (length × width^2 ×0.5). Once tumors reached approximately 0.5-0.6 cm^3, the mice received an injection into the tumor once a week for 3 weeks. The 8 mice in the experimental group were treated with 100 μl lncRNA LET lentivirus, the remaining 8 mice, in the control group, received an injection of 100 μl GFP-lentivirus. Tumors were harvested 1 week after the end of treatment.

Statistical analysis

Data are expressed as means ± SD of three independent experiments, each performed in triplicate. Differences between groups were assessed by unpaired, two-tailed Student's t test. P<0.05 was considered significant.

Results

LncRNA LET is downregulated in LSCC

Total RNA was isolated from matched adjacent non-neoplastic tissues and LSCC tissues and

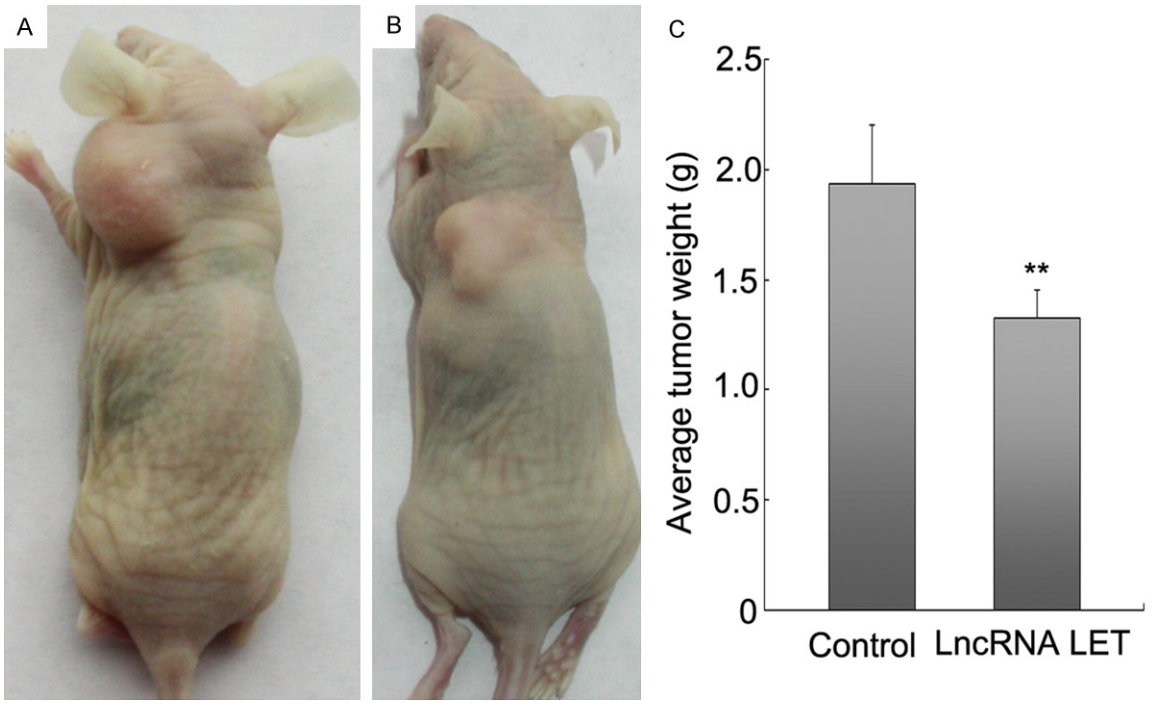

Figure 4. LncRNA LET suppresses Hep-2 tumor growth in vivo. Tumors from mice injected with Hep-2 cells were dissected 1 week after the treatment. A: Representative mouse in the control group; B: Representative mouse in lncRNA LET lentivirus treated group; C: Difference of tumor weight between lncRNA LET lentivirus treated-group and the control. $**P<0.01$.

the lncRNA LET levels were determined by real-time PCR. As shown in **Figure 1**, the lncRNA LET expression was significantly lower in LSCC tissues than that in adjacent non-neoplastic tissues ($P<0.01$). Next, the lncRNA LET expression was found to be statistically related with T grade, neck nodal metastasis and clinical stage (**Table 1**). Tumors with advanced clinical stages, with T3-4 grade or with lymph node metastasis expressed lower levels of lncRNA LET. Kaplan-Meier analysis showed that patients with lower lncRNA LET expression had significantly shorter overall survival than those with higher lncRNA LET expression ($X^2=8.605$, $P<0.01$) (**Figure 2**). These data suggest that decreased lncRNA LET level may have a function in the progression of LSCC.

LncRNA LET inhibits proliferation of Hep-2 cells

Lentiviral vector system that incorporates GFP was used as a reporter gene to upregulate lncRNA LET. We performed an MTT assay to examine the effect of upregulation of lncRNA LET on the proliferation of Hep-2 cells in vitro. After lncRNA LET transfection, the viability of the Hep-2 cells was evidently decreased at each different time-point (24, 48, 72, and 96 h). However, survival rates of the Hep-2 cells in the control groups did not show any obvious alteration during the time course (**Figure 3**) ($P<0.01$).

LncRNA LET suppresses tumor growth in vivo

All of the 16 mice developed detectable tumors after they were subcutaneously injected with Hep-2 cells. The growth of the LSCC xenografts was significantly inhibited in mice treated with lncRNA LET lentivirus compared with those treated with GFP-lentivirus (**Figure 4**). The average tumor weight (1.331±0.101 g) in the lncRNA LET treated LSCC xenografts was statistically lower ($P<0.01$) than the tumors in control group (1.936±0.272 g).

Discussion

Accumulating evidence has confirmed mammalian genome encodes thousands of lncRNA s that are pervasively transcribed. Although lncRNAs have frequently been disregarded as transcriptional 'noise', there is substantial evi-

dence to suggest that they are functional [9]. One of the primary functions of lncRNA s appears to be as epigenetic regulators of protein-coding gene expression [10]. Dysregulated expression of lncRNA s in cancer correlates to disease progression and the important function of lncRNA s in tumor has become a new field for cancer research [11, 12]. LncRNA LET was *primary* found to be decreased expression in hepatocellular carcinomas, colorectal cancers, and squamous cell lung carcinomas [13]. Recently, downregulation of lncRNA LET was further demonstrated in gastric, gallbladder and cervical cancer patients [7, 8, 14]. In this study, we examined the expression pattern of lncRNA LET in LSCC tissues and investigate its clinical implications. Quantitative PCR showed that the lncRNA LET was significantly decreased in primary LSCC compared with adjacent noncancerous tissues. Furthermore, the patients with lymph node metastasis or advanced clinical stages were detected with lower lncRNA LET expression. These data suggest that the downregulation of lncRNA LET may play an important regulative role in the carcinogenesis and malignant progression of LSCC. Previous study showed transfection of lncRNA LET suppressed the metastasis of hepatocellular and colorectal cancer cells in vivo through the modulation of NF90 (a double-stranded RNA-binding protein) [13]. Moreover, the ectopic expression of lncRNA LET led to the promotion of cell cycle arrest at G0/G1 phase and to the induction of apoptosis under hypoxic conditions in gallbladder cancer [14]. In agreement with these findings, our data showed significant decrease of proliferative ability of Hep-2 cells after lncRNA LET transfection. Furthermore, to investigate the function of lncRNA LET in LSCC in vivo, the LSCC xenografts of mice were treated by lncRNA LET lentivirus. The average tumor weight was significantly lower in the mice compared with control, thus suggesting that lncRNA LET could effectively suppress the progress of LSCC in vivo.

In summary, we have identified that lncRNA LET is down-regulated in LSCC tumor tissues and associated with metastasis and prognosis. Moreover, overexpression of lncRNA LET can inhibits proliferation and growth of LSCC. LncRNA LET may play a tumor suppressor role and should be investigated further as a potential therapeutical target in LSCC.

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Disclosure of conflict of interest

None.

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