Identification of the long non-coding RNA LET as a novel tumor suppressor in gastric cancer

JINGJING TIAN1, XIBAO HU2, WEI GAO1, JIE ZHANG1, MING CHEN1, XINRONG ZHANG1, JUNHONG MA1 and HONGXIA YUAN3

1Department of Gastroenterology, NanKai Hospital, Tianjin 300100; 2First Teaching Hospital and 3School of Management, Tianjin University of Traditional Chinese Medicine, Tianjin 300193, P.R. China

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Abstract. Long non-coding RNAs (lncRNAs) have emerged recently as important factors in regulating fundamental biological processes. Alterations in the expression and function of lncRNAs have been observed to promote tumor formation, progression and metastasis. Although downregulation of the expression levels of LET lncRNA in several tumors has been reported, its role in gastric cancer remains unknown. The aim of the present study was to investigate the expression and function of LET in gastric cancer development. The expression levels of LET in 37 pairs of gastric cancer and adjacent non-tumor tissues were detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). In addition, LET expression in gastric cancer cell lines was analyzed by RT-qPCR assay analysis. Furthermore, the impact of LET on cell proliferation, migration and apoptosis were detected using the cell counting kit-8, wound scratch and ELISA assays, respectively. The results demonstrated that the expression level of LET was downregulated in gastric cancer tissues and cell lines (SGC-7901 and MGC-803) compared with normal tissues and a normal human gastric epithelial cell line (GES-1). Restoration of LET expression using a synthesized recombinant overexpression vector transfected into SGC-7901 and MGC-803 cells, significantly inhibited cell proliferation and migration, and promoted cell apoptosis in vitro. The present study is the first to demonstrate that LET may function as a tumor suppressor in gastric cancer. The results indicate that LET may be a promising biomarker and/or a therapeutic target for gastric cancer.

Introduction

Gastric cancer is the second leading cause of cancer-associated mortality worldwide, and is the most common gastrointestinal tumor in East Asia (1,2). Gastrectomy remains the primary treatment for gastric cancer. Although the majority of patients at an early stage of gastric carcinoma may be cured by surgery, >50% of patients do not survive due to carcinoma recurrence at an advanced stage of the disease, despite undergoing a curative gastrectomy (3). Therefore, an improved understanding of the pathogenesis and identification of the molecular alterations is essential for the development of diagnostic markers that may aid novel effective therapies for gastric cancer (4,5).

Long non-coding RNA (lncRNAs), which are transcripts >200 bp in size with no protein-encoding function, represent a less investigated class of non-coding RNAs (6). Previous studies have reported that lncRNAs are crucial for the regulation of chromatin structure, gene expression and translational control (7-9). A significant number of studies have investigated small interfering RNAs and microRNAs, and their functions and molecular mechanisms have been illustrated in recent years (10,11). However, our understanding of the function of lncRNAs in different diseases remains limited.

An increasing number of studies are investigating the role of lncRNAs in the origin and development of cancers, particularly digestive system cancers. High expression levels of PVT1 was demonstrated to be a potential candidate biomarker for gastric cancer, and promoted cell proliferation through the epigenetic regulation of p15 and p16 (12). In addition, upregulation of LINC00152 is reportedly correlated with depth of tumor invasion in patients with gastric cancer (13). The lncRNA CCAT2 is highly expressed and associated with poor prognosis in gastric cancer (14). Although several lncRNAs have been reported to function in the development of gastric cancer, the function of <1% of the 30,000 lncRNAs identified to date has been reported (15). Therefore, identification of gastric cancer-associated lncRNAs and investigation of their molecular mechanisms and biological functions, is essential for understanding the molecular biology of gastric cancer development.

A previous study demonstrated that LET was downregulated in gastric cancer and was associated with poor prognosis (16). The aim of the present study was to verify the expression of LET in gastric cancer tissues and assess
its impact on gastric cancer cell proliferation, migration and apoptosis. The results demonstrated that LET may function as a tumor suppressor lncRNA in gastric cancer.

Materials and methods

Patients and specimens. A total of 37 cases of paired gastric cancer specimens used for the purposes of the present study, were collected from gastrectomy procedures at the Department of Gastroenterology, NanKai Hospital (Tianjin, China) between June 2008 and June 2014. Samples were collected from 17 male and 20 female patients. The average weight of male patients was 63.4±3.4 kg and the average weight (mean ± standard deviation) of female patients was 52.6±3.8 kg. The average age for male and female patients (mean ± standard deviation) was 48.5±5.3 years and 57.6±6.2 years, respectively. All tissue samples were reviewed and diagnosed as gastric cancer according to the American Joint Committee on Cancer staging manual based on histopathological evaluation (17). None of the patients received percutaneous ablation, chemoembolization or radiotherapy prior to the gastrectomy operation. Written informed consent was provided by all patients, and the clinical research was approved by the Institutional Review Board of NanKai Hospital. All specimens were immediately frozen in liquid nitrogen for downstream analysis.

Cell culture and transfection. The human gastric cancer cell lines, SGC-7901 and MGC-803, were purchased from the American Type Culture Collection (Manassas, VA, USA). The normal human gastric epithelial cell line, GES-1, was obtained from the Cell Line Resource Center, Shanghai Institute of Biochemistry and Cell Biology, the Chinese Academy of Sciences. (Shanghai, China). Cells were maintained in Dulbecco's Modified Eagle's medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum at 37˚C in a humidified atmosphere with 5% CO₂.

To investigate the function of LET in gastric cancer cells, an overexpression plasmid containing the LET lncRNA sequence was generated using gene recombination technology. The sequence of human LET was amplified by polymerase chain reaction (PCR) using the following primers: Forward, 5' -CGC GGA TCC CTC ACA GAC AAA GGA GAG TCT GAT G-3', and reverse, 5' -CCG GA ATT CTG GAT G-3'. The human gastric cancer cell line, GES-1, was cultured in normal medium for 24 h, before they were transfected with the pcDNA3.1(+)-LET vector or the pcDNA3.1(+)empty vector. At 0, 24, 48 and 72 h following transfection, the absorbance of each well at 450 nm (with 630 nm as the reference wavelength) was measured using an ELISA microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All assays were repeated at least three times.

Cell proliferation assay. The migratory ability of gastric cancer cells (SGC-7901 and MGC-803) was analyzed using an in vitro wound scratch assay. Cells (~5x10⁵) were cultured in a 6-well dish for 24 h, before they were transfected with the pcDNA3.1(+)LET vector or the pcDNA3.1(+)empty vector using Lipofectamine 2000. Vertical horizontal wounds were generated with a sterile 10 µl pipette tip at 6 h following transfection. The cells were then washed with phosphate-buffered saline and maintained at 37˚C in the incubator. The wound images were acquired with a digital camera system at 0 and 24 h after the wounds were generated. The width of wounds was measured using a standard caliper. All experiments were performed in triplicate and repeated at least three times.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA from gastric cancer tissues and cells (1x10⁶) was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. cDNA was synthesized from 1 µg RNA using the PrimeScript RT kit (Takara Biotechnology Co., Ltd.). RT-qPCR reactions were performed using the SYBR® Premix EX Taq™ II PCR kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's instructions, with the Roche LightCycler® 480 Instrument II (Roche Diagnostics, Indianapolis, IN, USA). All primers were obtained from Invitrogen (Thermo Fisher Scientific, Inc.). The primer sequences were as follows: LET, forward, 5'-GGGAGTTAAAAGGGAAGATGGTG-3', and reverse, 5'-AGGCTAGGAAGTTGTTGGTGATGG-3'; GAPDH, forward, 5'-CATCAGAAGGTGTTGAAGCGG-3', and reverse, 5'-AAAAGTTGAGGATGGGTGTCG-3'. Data were collected and analyzed using Roche LightCycler® 480 software (version 1.5; Roche Diagnostics). The expression of LET was normalized internally using the quantification cycle of the GAPDH housekeeping gene. The relative quantitative value was calculated using the 2^ΔΔCq method (18). Each experiment was performed in quintuplicate and repeated five times.
density at 405 nm was measured using an ELISA microplate reader (Bio-Rad Laboratories, Inc.). The ratio of cell apoptosis was calculated according to the formula: (OD × dilution factor) / (10.5 × volume of sample mL × reaction time min). The apoptosis of cell was also detected by a Zeiss LSM 710 laser scanning confocal microscopy (Carl Zeiss AG, Oberkochen, Germany) and analyzed using the Image-Pro Plus software program (version, 5.10; Media Cybernetics, Inc., Rockville, MD, USA). All experiments were performed in triplicate.

Statistical analysis. Statistical analysis of data was performed using SPSS software (version, 18.0; SPSS, Inc., Chicago, IL, USA). The paired t-test was used to compare LET expression in gastric cancer tissues with matched non-tumor tissues. The independent samples t-test was used to analyze the remaining data. P<0.05 was considered to indicate a statistically significant difference.

Results

LET expression is downregulated in gastric cancer tissues and cell lines. A previous study reported that LET is downregulated in gastric cancer tissues (17). To assess the function of LET in gastric cancer in the present study, the expression level of LET in 37 gastric cancer tissues and matched adjacent non-tumor tissues was examined by RT-qPCR analysis. As showed in Fig. 1A, the expression of LET in gastric cancer tissues was significantly lower when compared with matched adjacent non-tumor tissues (P<0.01). LET expression in gastric cancer cell lines and the human gastric epithelial mucosa cell line, GES-1 (P<0.05 vs. GES-1). The 2^−ΔΔCq method was used to quantify the expression levels of LET relative to GAPDH. Data are expressed as the mean ± standard deviation (n=3). RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

Restoration of LET expression suppresses gastric cancer cell proliferation in vitro. To investigate the functional role of LET in gastric cancer cell proliferation, the pcDNA3.1(+) vector or pcDNA3.1(+) empty vector was transfected into SGC-7901 and MGC-803 cells, and cell proliferation was examined using a CCK-8 assay. As showed in Fig. 2A, SGC-7901
and MGC-803 cells transfected with the pcDNA3.1(+)-LET vector demonstrated a significant increase in LET expression when compared with pcDNA3.1+ empty vector-transfected cells (P<0.01; Fig. 2A). The CCK-8 assay was then performed and the optical density values of the pcDNA3.1(+)-LET vector or pcDNA3.1(+)-empty vector-transfected cells were measured at 0, 24, 48 and 72 h following transfection. The results demonstrated that the relative cell proliferation of pcDNA3.1(+)-LET vector-transfected SGC-7901 cells was significantly decreased at 24, 48 and 72 h when compared with pcDNA3.1(+)-transfected cells (5.43, 6.07 and 9.21%, respectively; P<0.01; Fig. 2B). Similarly, the relative cell proliferation of pcDNA3.1(+)-LET vector-transfected MGC-803 cells was significantly decreased at 24, 48 and 72 h following transfection when compared with pcDNA3.1(+)-transfected cells (4.09, 3.88 and 7.47%, respectively; P<0.01; Fig. 2C). These results indicate that overexpression LET may significantly inhibit cell proliferation of gastric cancer cells in vitro.

Restoration of LET expression inhibits gastric cancer cell migration in vitro. The effect of LET overexpression on gastric cancer cell migration was determined using a wound healing assay. As shown in Fig. 3A and B, the wound widths of SGC-7901 and MGC-803 cells transfected with the pcDNA3.1(+)-LET vector or pcDNA3.1(+)-empty vector were markedly greater when compared with the pcDNA3.1(+)-empty vector-transfected cells. The results demonstrate that restoration LET expression may inhibit gastric cancer cell migration.

Restoration of LET expression induces gastric cancer cell apoptosis in vitro. LncRNAs have been reported to serve a crucial role in cell apoptosis, particularly in mediating escape from apoptosis in cancer cells (19). To determine the effect of LET on gastric cancer cell apoptosis, a caspase 3 ELISA assay was used to measure the rate of apoptosis. As revealed in Fig. 4, the apoptosis rate of SGC-7901 cells transfected with pcDNA3.1(+)-LET vector was markedly higher when compared with the pcDNA3.1(+)-empty vector-transfected cells (16.53% vs. 3.35%; P<0.01). Similarly, the apoptosis rate...
of pcDNA3.1(+)-LET vector-transfected MGC-803 cells was significantly higher when compared with pcDNA3.1(+)-empty vector-transfected cells (13.30% vs. 2.19%; P<0.01; Fig. 4B). The results demonstrate that overexpression of LET may promote apoptosis of gastric cancer cells. The results presented thus far, identify LET IncRNA as a novel tumor suppressor in gastric cancer.

**Discussion**

Gastric cancer is one of the most common types of digestive tumors (20). It is the second most frequent cause of cancer-associated mortality worldwide and presents a major public health issue (21). Despite significant advances in cancer therapy, major limitations in the management of gastric cancer remain. A large number of patients are diagnosed with advanced gastric cancer and have a poor prognosis.

Emerging evidence suggests that IncRNAs serve essential roles in the modulation of tumor behavior through various complex mechanisms, such as modulating gene transcription and epigenetic signaling pathways (22,23). A recent study suggested that the HOX transcript antisense RNA IncRNA may promote the malignant growth of human liver cancer stem cells through downregulation of SET domain containing 2 (24). In addition, the intronic prostate cancer antigen 3 IncRNA regulates the prune homolog 2 suppressor in prostate cancer (25). Furthermore, increased HOXA transcript at the distal tip IncRNA expression is correlated with progression and metastasis in tongue squamous cell carcinoma (26). Silencing prostate cancer associated transcript-1 induces cell growth arrest and apoptosis in human bladder cancer (27). However, limited data are available regarding the expression and function of IncRNAs in gastric cancer.

The LET IncRNA gene is located on chromosome 15q24.1. LET exhibits differential expression patterns in various tumors (16,28-30). Previous studies have demonstrated that LET expression is downregulated in cervical and gastric cancer (16,28). However, to the best of our knowledge, the precise function of LET has not yet been reported in gastric cancer. In the present study, the expression of LET in gastric cancer tissues and cell lines was first examined. Consistent with previously reported results, the expression of LET was downregulated in gastric cancer tissues when compared with matched adjacent normal tissues. In addition, LET expression was decreased in two gastric cancer cell lines (SGC-7901 and MGC-803) when compared with a human gastric epithelial mucosa cell line (GES-1). These results provided a strong rationale for subsequent functional experiments.

To further understand the biological functions of LET in gastric cancer cells, cell proliferation, migration, and apoptosis was examined by applying a gain-of-function approach. Transfection of SGC-7901 and MGC-803 cells with the pcDNA3.1(+)-LET vector led to a significant reduction in cell proliferation and migration, and increased apoptosis when compared with pcDNA3.1(+)-empty vector-transfected cells. These results suggest that LET may function as a tumor suppressor gene in the occurrence and development of gastric cancer.

In conclusion, the results of the present study confirm that LET is significantly downregulated in human gastric cancer tissues and cells. In addition, this study is the first to demonstrate that LET serves a tumor suppressive role in gastric cancer by influencing cellular migration, proliferation and apoptosis. Therefore, LET presents a promising biomarker and/or a therapeutic target for gastric cancer.

**References**


