Long non-coding RNA-Low Expression in Tumor inhibits the invasion and metastasis of esophageal squamous cell carcinoma by regulating p53 expression

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Abstract. Long non-coding RNAs (lncRNAs) are involved in governing fundamental biological processes, and, in many lncRNAs, the expression level is altered and likely to have a functional role in tumorigenesis, including apoptosis, migration and invasion. The lncRNA-Low Expression in Tumor (LET), a recently identified IncRNA, was demonstrated to be downregulated in hepatocellular and gallbladder cancer. However, its role in esophageal squamous cell carcinoma (ESCC) requires investigation. The expression level of lncRNA-LET mRNA in primary ESCC and matched healthy tissues (48 cases) was determined by reverse transcription-quantitative polymerase chain reaction. In addition, the effects of lncRNA-LET on cell apoptosis were evaluated by flow cytometric analysis, the regulatory effect of lncRNA-LET on migration was detected using a wound healing assay and cellular invasion was analyzed by Matrigel-coated transwell assay. Furthermore, the effect of lncRNA-LET on cell proliferation was investigated by 5-ethynyl-2'-deoxyuridine cell proliferation assay and protein levels of lncRNA-LET targets were analyzed by western blotting. IncRNA-LET expression was decreased in primary ESCC tissues when compared with paired healthy tissues, and was identified to be associated with the clinical features. Overexpression of lncRNA-LET was observed to inhibit the migration and invasion of ESCC cells, and modulate p53 expression levels in human ESCC cell lines in vitro. These results establish that lncRNA-LET is significant in the regulation of tumor progression and metastasis, and serves as a tumor suppressor in, and therefore has therapeutic potential for, the treatment of human ESCC.

Introduction

Esophageal squamous cell carcinoma (ESCC), a highly lethal malignancy, is the eighth most common cancer worldwide and the sixth most common cause of cancer-associated mortality (1). Furthermore, ESCC has become one of the most common types of malignant tumor in China, Japan and Southeast Africa (2,3). In China, ESCC is the predominant subtype and contributes to ~90% of all esophageal cancers (ECs) (4,5). Despite the use of multimodal treatments, such as radical surgery, chemotherapy and radiotherapy, the overall prognosis for ESCC remains poor, with 5-year survival rates of 5-45% (6-8). Although previous studies have demonstrated that alterations of numerous oncogenes and tumor-suppressor genes are involved in ESCC, the underlying molecular and genetic mechanism of esophageal carcinogenesis remains largely unknown (9).

Long non-coding RNAs (lncRNAs), with transcripts >200 nt in length, which were initially recognized to represent random transcriptional noise, have been implicated in numerous biological behaviors, such as epigenetic regulation, chromatin modification, transcription and post-transcriptional processing (10-12). Increasing evidence has revealed the contribution of lncRNAs as proto-oncogenes, tumor suppressor genes and drivers of metastatic transformation (13-15).

lncRNA-Low Expression in Tumor (IncRNA-LET), a recently identified IncRNA located at chromosome 15q24.1, was initially established to be downregulated in hepatocellular carcinoma (16). Recently, it was demonstrated to be vital in the development and progression of gallbladder cancer (GBC) (17). However, the prognostic role of IncRNA-LET in cancer remains unknown and to date, to the best of our knowledge, no data were available regarding the IncRNA-LET expression level and biological role in human ESCC.

In the present study, the expression level of IncRNA-LET was demonstrated to be significantly decreased in ESCC tissues when compared with that of adjacent healthy tissues. Its correlation with clinicopathological factors in ESCC patients...
was also evaluated. Using ESCC cell lines, overexpression of lncRNA-LET by lentivirus-mediated gene transfection was investigated and observed to induce apoptosis, and inhibit invasion and proliferation. In addition, the present study verified that overexpression of lncRNA-LET induced the activation of p53. Thus, the current study indicates that lncRNA-LET has a significant role in ESCC development and may be considered as a potential prognostic factor for the prediction of clinical outcomes in ESCC patients.

Materials and methods

ESCC specimens. A total of 48 ESCC patients that underwent esophagectomy at The First Affiliated Hospital of Nanjing Medical University (Nanjing, China) between 2012 and 2013 were enrolled in the present study. Tumor specimens and paired healthy esophageal tissue specimens, obtained from a site distant to the cancerous lesion, were immediately snap-frozen in liquid nitrogen and stored at -80°C until total RNA was extracted. No radiotherapy or chemotherapy was conducted in these patients prior to surgery. The clinical data, including age, gender, pathological stage, grade, tumor location and lymph node metastasis were acquired from the medical records. Patients were classified according to criteria set by the World Health Organization (18) and were staged according to the tumor-lymph node-metastasis (TNM) classification system, in which T refers to the size of the ESCC and whether it has invaded nearby tissue, N refers to whether or not regional lymph nodes are involved, and M refers to distant metastasis (19). The study was approved by the Research Ethics Committee of Nanjing Medical University. Informed consent was obtained from all of the patients.

Cell culture. Human ESCC cell lines, Eca109 and TE-1 were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium (both purchased from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) and 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.), within a humidified atmosphere containing 5% CO₂, incubated at 37°C in a 5% CO₂ incubator, in culture medium with 10% FBS.

RNA extraction and reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA from the tissues and cells was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Briefly, 1 ml Trizol was used to lyse cells (5x10⁶ cells/well), then 0.2 ml of chloroform was added and the cells were incubated at room temperature for 3 min. After centrifugation at 12,000 x g at 4°C for 15 min, the RNA aqueous phase was transferred to a fresh tube. Then, 0.5 ml isopropanol per 1 ml TRIzol was added, and cells were incubated for 10 min at room temperature, followed by centrifugation at 10,000 x g at 4°C for 10 min. After washing the RNA pellet with 75% ethanol, the RNA was dissolved in 0.03 ml RNase-free water and incubated for 10 min at 55°C. RNA was reverse transcribed into cDNA using the PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China). The following thermal cyclic protocol was used for reverse transcription: 37°C for 15 min for 3 cycles, followed by 85°C for 5 sec. The cDNA template was amplified by qPCR using the SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd.) according to the manufacturer's instructions. Briefly, 5 µl 10-fold cDNA was mixed with 10 µl SYBR Premix Ex Taq and 4 nmol of primer in a volume of 20 µl. The following thermal cycle was followed: 30 sec at 95°C, followed by 40 cycles of 95°C for 5 sec, and 60°C for 35 sec.

The relative levels of LET were determined by RT-qPCR using gene specific primers. Ornithine decarboxylase antizyme (OAZ-1) served as an internal control, and the lncRNA-LET values were normalized to OAZ-1. The RT-qPCR reactions and data collection were performed on a StepOne Plus™ Real-Time PCR system (Thermo Fisher Scientific, Inc.). The relative expression fold change of mRNAs was calculated using the 2⁻ΔΔCT method (20). The primer sequences (Sangon Biotech Co., Ltd., Shanghai, China) were as follows: Forward, 5'-CGAGGACAGAGCAGCCTT-3' and reverse, 5'-GACAA CCCAGGCAGATGA-3' for OAZ-1; forward, 5'-GTTGTGT GTGGCAATTGGGT-3' and reverse, 5'-AAGATGGAGAGTG AGACCT-3' for lncRNA-LET.

Plasmid and transfection. The sequence of LET was synthesized and subcloned into PLL3.7-EF-1a-SV40pA (Geneziv, Inc., Suzhou, China), and the expression level of LET was detected by RT-qPCR.

Cells were grown on six-well plates to 70% confluence and then transfected with the PLL3.7-EF-1a-SV40pA using 10 µg/ml polybrene (HanBio, Shanghai, China) according to the manufacturer's instructions. Cells were harvested after 48 h for RT-qPCR and western blot analysis.

Cell migration and invasion assay. Cells (5x10⁴ cells/well) were seeded in six-well plates and cultured in RPMI-1640 medium. After 48 h, cell layers were wounded using the tip of 200 µl pipette. After washing cells 3 times with PBS, the serum-free RPMI-1640 medium was added to the plates and incubated within a humidified atmosphere containing 5% CO₂ at 37°C for 48 h. Wound closure was observed under a light microscope (DFC500; Leica, Wetzlar, Germany) and measured using AxioVision version 4.7 software (Carl Zeiss Meditec, Dublin, CA, USA).

For the invasion assays, transwell apparatus was used with a polycarbonate membrane (pore size, 8 µm) Boyden chamber insert (EMD Millipore, Billerica, MA, USA) to measure cell motility. The transfected cells and wild-type cells were treated with trypsin/EDTA solution (Sigma-Aldrich) and washed once with serum-containing RPMI-1640 medium. A total of 1x10⁵ cells in 0.2 ml serum-free RPMI-1640 medium were seeded on transwell apparatus. Each insert was precoated with 45 µg Matrigel (BD Biosciences; San Jose, CA, USA). Prior to examination, the chambers were incubated for 24 h at 37°C in a 5% CO₂ incubator, in culture medium with 10% FBS in the lower chambers. The cells on the upper surface were scraped using cotton buds and washed away with PBS, whereas the invaded cells on the lower surface were fixed in 100% precooing methanol (Sigma-Aldrich) for 10 min, stained with 0.05% crystal violet (Sigma-Aldrich) for 30 min,
then rinsed in phosphate-buffered saline (PBS; Thermo Fisher Scientific, Inc.) and subjected to microscopic inspection (DFC500; Leica). Finally, the values for invasion were obtained by counting three fields per membrane. Experiments were independently repeated in triplicate.

**Cell proliferation assay.** The 5-ethyl-2-deoxyuridine (EdU) detection kit (Guangzhou RiboBio Co., Ltd., Guangzhou, China) was used to evaluate cell proliferation. According to the manufacturer's instructions, cells were grown on six-well plates to 70% confluence, treated with 50 µM EdU for 2 h at 37°C and fixed with PBS containing 4% paraformaldehyde (Sigma-Aldrich) for 30 min at room temperature. After cells were incubated with 2 mg/ml glycine (Abcam, Cambridge, UK) for 5 min at room temperature, they were treated with 0.5% Triton X-100 (Sigma-Aldrich) for 10 min and stained with 1X Apollo reaction cocktail (Guangzhou RiboBio Co., Ltd.) for 30 min at room temperature. After one wash with 0.5% Triton X-100 in PBS, 1X Hoechst 33342 (Thermo Fisher Scientific, Inc.) was used to incubate cells at room temperature for 30 min. Images were captured under a confocal laser scanning microscope (LEXT OLS3100; Olympus America, Inc., Center Valley, PA, USA). The assay was repeated in triplicate.

**Flow cytometric analysis.** The effect of LET treatment on cell apoptosis was determined by flow cytometry. Cells transfected with PLL3.7-EF-1a-SV40pA were plated in six-well plates for 48 h. The cells harvested and fixed in 70% ice-cold ethanol for 24 h were collected and analyzed for cell apoptosis using a flow cytometer (FACSCalibur; BD Biosciences). In addition, the cells were harvested and stained with Annexin V/propidium iodide (PI), using the Annexin V–fluorescein isothiocyanate apoptosis detection kit (KGI Biotechnology Co., Ltd., Nanjing, China).

**Western blot analysis.** Cells harvested from six-well culture plates were lysed using mammalian protein extraction reagent RIPA (Beyotime Institute of Biotechnology, Haimen, China) supplemented with protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland) and phenylmethylsulfonyl fluoride (Roche Diagnostics). The lysates were then collected and subjected to ultracentrifugation (Q700 Sonicator; Misonix, Inc., Farmingdale, NY, USA) and centrifugation at 14,000 x g for 20 min. The supernatants were collected, and protein content was determined using a Bradford assay (Abcam). Protein extractions (50 µg) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (90 min at 100 V; Abcam), then transferred to Immobilon-P polyvinylidene fluoride membranes (EMD Millipore) and incubated with specific antibodies. A GAPDH antibody served as a control. An enhanced chemiluminescence chromogenic substrate (SignalFire ECL Reagent; Cell Signaling Technology, Inc., Danvers, MA, USA) was used to visualize the bands with a chemiluminescent detection system (Pierce ECL Substrate Western Substrate; Thermo Fisher Scientific, Inc.) and then exposed in a Molecular Imager ChemiDoc XRS system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The intensity of the bands was quantified using Image J software (version 1.42; National Institutes of Health, Bethesda, MD, USA). The rabbit anti-human GAPDH monoclonal antibody (1:5,000; cat. no. 14C10) and rabbit anti-human p53 polyclonal antibody (1:1,000; cat. no. 9282) were purchased from Cell Signaling Technology, Inc.. The secondary goat anti-rabbit horseradish peroxidase-conjugated polyclonal antibody (1:2,000; cat. no. ab6721) was purchased from Abcam. All experiments were performed in triplicate.

**Statistical analysis.** Statistical analysis was performed using SPSS 20.0 (IBM SPSS, Armonk, NY, USA). Statistical significance was evaluated by Student's t-test or a χ² test as appropriate. Survival analysis was performed using the Kaplan-Meier method, and the log-rank test was used to compare the differences between patient groups. P<0.05 was considered to indicate a statistically significant difference.

### Results

**lncRNA-LET expression level is downregulated in ESCC specimens, and is correlated with pathological grade, tumor stage and lymph node metastasis.** The expression levels of lncRNA-LET in 48 pairs of human ESCC and adjacent non-tumor tissue samples were examined by RT-qPCR and normalized to GAPDH. The lncRNA-LET expression level was identified to be significantly downregulated in tumor tissue samples when compared with non-tumor tissue samples. Furthermore, correlation analysis of lncRNA-LET expression levels with clinicopathological features of ESCC patients indicated that the expression level of lncRNA-LET was significantly correlated with the pathological grade (P=0.043), clinical stage (P=0.034) and lymph node metastasis (P=0.024); while there was no significant correlation between lncRNA-LET expression level and gender, age or tumor location (Table I). These results indicate that lncRNA-LET may be involved in the progression and metastasis of ESCC.

**lncRNA-LET inhibits ESCC cell migration and invasion in vitro.** To investigate the biological role of lncRNA-LET in ESCC progression, Eca109 and TE-1 cells were transfected with PLL3.7-EF-1a-SV40pA-LET. The transfection efficiency was validated using RT-qPCR (Fig. 1). Using a wound healing assay, the relative migrating distance of cells was identified to be significantly reduced in wild-type ESCC cells as compared with the PLL3.7-EF-1a-SV40pA-LET-infected cells (Fig. 2). In addition, a Matrigel-coated transwell assay demonstrated that the numbers of PLL3.7-EF-1a-SV40pA-LET-infected Eca109 (104 cells) and TE-1 (208 cells) cells that invaded through the Matrigel were significantly less than those of the wild-type Eca109 (200 cells) and TE-1 (509 cells) cells (P<0.05; Fig. 3). These results indicate that lncRNA-LET suppresses the invasive phenotype of ESCC cells in vitro.

**lncRNA-LET inhibits ESCC cell proliferation in vitro.** To determine whether LET overexpression affected ESCC growth, the EdU assay was performed on ESCC cells infected with PLL3.7-EF-1a-SV40pA-LET. The percentages of EdU-positive cells (cells in the S phase of the cell cycle) in the PLL3.7-EF-1a-SV40pA-LET-infected groups were not identified to be significantly lower than those of the wild-type groups (P>0.05; Fig. 4).
Table I. IncRNA-LET expression and clinicopathological characteristics in esophageal squamous cell carcinoma.

| Characteristic                  | Cases | Low | High | P-value |
|---------------------------------|-------|-----|------|---------|
| Gender                          |       |     |      | 0.883   |
| Male                            | 27    | 16  | 11   |         |
| Female                          | 21    | 12  | 9    |         |
| Age (years)                     |       |     |      | 0.915   |
| <60                             | 34    | 20  | 14   |         |
| ≥60                             | 14    | 8   | 6    |         |
| Histological grade              |       |     |      | 0.043   |
| Well differentiated             | 15    | 5   | 10   |         |
| Moderately differentiated       | 16    | 10  | 6    |         |
| Poorly differentiated           | 17    | 13  | 4    |         |
| T stage                         |       |     |      | 0.034   |
| T1-2                           | 18    | 7   | 11   |         |
| T3-4                           | 30    | 21  | 9    |         |
| Lymph node metastasis          |       |     |      | 0.024   |
| Negative                       | 22    | 9   | 13   |         |
| Positive                       | 26    | 19  | 7    |         |
| Tumor location                  |       |     |      | 0.575   |
| Upper and middle 1/3           | 31    | 19  | 12   |         |
| Lower 1/3                       | 17    | 9   | 8    |         |

P<0.05 is considered to indicate a statistically significant difference. *Tumor was found in the mucosa, submucosa or superficial muscle; **Tumor was in the deep muscular or outer layer; †tumor was identified in the esophageal lymph nodes; ‡T1, tumor invades lamina propria, muscularis mucosa or submucosa; T2, tumor invades muscularis propria; ‡T3, tumor invades adventitia; T4, tumor invades adjacent structure. IncRNA-LET, long non-coding RNA-Low Expression in Tumor.

**IncRNA-LET inhibits ESCC cell proliferation via inducing apoptosis.** To evaluate the effect of IncRNA-LET on tumor cell apoptosis, Eca109 and TE-1 cells were employed as the model system. It was hypothesized that IncRNA-LET overexpression induced cell apoptosis in ESCC cells. The apoptotic rate of Eca109 and TE-1 cells transfected with PLL3.7-EF-1a-SV40pA-LET was identified to be significantly increased when compared with those of the wild-type ESCC cells (Fig. 5). This indicates that upregulation of LET induces ESCC cell apoptosis in vitro.

**IncRNA-LET induces activation of the p53 protein.** Recent studies have demonstrated that numerous IncRNAs may participate in the regulation of cell growth by modulating the p53 signaling pathway (21-24). To further investigate whether, and the mechanism by which, LET induces ESCC cell growth arrest and apoptosis, the protein level of p53 was examined following transfection of PLL3.7-EF-1a-SV40pA-LET in wild-type ESCC cells. The results of western blot analysis indicated that the expression level of p53 was significantly increased in ESCC cells transfected with PLL3.7-EF-1a-SV40pA-LET when compared with that of wild-type ESCC cells (Fig. 6). These data indicate that LET functions as a tumor suppressor gene by activating p53 in ESCC cells.

**Discussion**

Recent studies have revealed that IncRNAs participate in a multitude of biological processes, such as chromatin modification, transcription and post-transcriptional processing (10,12,25,26). In addition, previous studies have demonstrated that dysregulation of IncRNAs may also affect epigenetic information and provide a cellular growth advantage, resulting in a wide range of effects.
of diseases, particularly in progressive and uncontrolled tumor growth (27,28). HOX transcript antisense RNA, a well known lncRNA involved in tumor pathogenesis, has been consistently upregulated and identified as a strong prognosis marker of patient outcomes in various types of human cancer (14,28-30). H19, encoded by an imprinted gene, has been verified to be upregulated in tumors and to possess oncogenic properties (31-33). Maternally expressed gene 3 (MEG3) is located at chromosome 14q32, and a loss of MEG3 expression has been observed in a number of primary human tumors, including glioma, hepatocellular cancers, non-small cell lung cancer, and gastric cancer (34-37). Although evidence of the carcinogenicity of these lncRNAs is strong, the molecular mechanism regarding tumor development and the promotion of metastasis is not fully understood.

lncRNA-LET, as a novel lncRNA molecule, was initially well known for its downregulation in primary hepatocellular carcinoma, wherein lncRNA-LET suppresses cancer invasiveness and metastasis (16). Furthermore, downregulation of lncRNA was examined in squamous-cell lung carcinoma

Figure 2. Overexpression of long non-coding RNA-LET inhibits the migration of ESCC cells. Wound healing assay was used to assess the migration ability of ESCC cells. Images represent the cells that have migrated into the wounded area and the histogram demonstrates the relative migration distance of cells. Magnification x200. (A) Eca109 cells; (B) Eca109 cells transfected with PLL3.7-EF-1a-SV40pA-LET; (C) TE-1 cells; (D) TE-1 cells transfected with PLL3.7-EF-1a-SV40pA-LET; (E) percentage of wound closure. *P<0.05. ESCC, esophageal squamous cell carcinoma; LET, Low Expression in Tumor.
and colon carcinoma tissues and compared with their paired healthy primary tissues (16). In addition, there were findings indicating that lncRNA-LET may represent a prognostic marker and potential therapeutic target for GBC (17). Due to the observation that lncRNA-LET is involved with dysregulation during cancer progression, the biological role of lncRNA-LET in ESCC progression was investigated in the present study and its clinical significance was analyzed.

The present study indicated that the expression level of lncRNA-LET was downregulated in ESCC tissue samples when compared with adjacent healthy tissue samples. Furthermore, the low expression level of IncRNA-LET in ESCC tissue samples was demonstrated to be closely associated with clinicopathological features. Low expression levels of IncRNA-LET were correlated with poorly differentiated histology, higher tumor grade and positive nodal status. Similarly, overexpression of IncRNA-LET expression was identified to inhibit the migration and invasion of ESCC cells, and also significantly increased the response of ESCC cells to cell apoptosis induction.

Figure 3. Overexpression of long non-coding RNA-LET inhibits the invasion of ESCC cells. Matrigel-coated transwell assay was used to assess the invasive ability of ESCC cells. The images represent the cells that invaded the matrigel and the histogram demonstrates the number of invasive cells. Magnification x200; 0.05% crystal violet stain. (A) Eca109 cells; (B) Eca109 cells transfected with PLL3.7-EF-1a-SV40pA-LET; (C) TE-1 cells; (D) TE-1 cells transfected with PLL3.7-EF-1a-SV40pA-LET; (E) number of invasive cells. *P<0.05. ESCC, esophageal squamous cell carcinoma; LET, Low Expression in Tumor.
p53, as a master regulator for gene expression, directly or indirectly regulates the expression of numerous target genes, which leads to the suppression of tumor development and growth by blocking cell proliferation or by activating cell death programs (36,38). The present study examined whether IncRNA-LET affects the expression level of p53 protein to further investigate the underlying mechanisms by which IncRNA-LET induced cell growth arrest and apoptosis. Overexpression of IncRNA-LET was identified to significantly increase the level of p53 protein when compared with that of the controls.

In conclusion, the loss of IncRNA-LET expression was demonstrated to be a common occurrence underlying EC, suggesting that IncRNA-LET may perform a key functional role in suppressing the invasive and metastatic behavior of ESCC cells. These findings indicate that IncRNA-LET may function as a tumor suppressor and its deficiency or decreased expression may contribute to ESCC development. Furthermore, IncRNA-LET may be exploited in a promising therapeutic approach for the treatment of EC, and potentially be useful as a novel prognostic marker for EC.

Figure 4. Long non-coding RNA-LET inhibits esophageal cancer cell proliferation in vitro. (A) The EdU assay was performed on esophageal squamous cell carcinoma cells infected with PLL3.7-EF-1a-SV40pA-LET. (B) Percentage of EdU-positive cells. EdU-positive cells in all groups were detected by confocal laser scanning microscopy. The cells were counted in five random fields. Eca109 LET, Eca109 cells transfected with PLL3.7‑EF‑1a‑SV40pA‑LET; TE‑1 LET, TE‑1 cells transfected with PLL3.7‑EF‑1a‑SV40pA‑LET. EdU, 5-ethynyl-2-deoxyuridine; LET, Low Expression in Tumor.
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