MiR-1275 promotes non-small cell lung cancer cell proliferation and metastasis by regulating LZTS3 expression

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Introduction

Lung cancer is a malignant tumor with the highest fatality rate among cancers. Most patients are already in the advanced stage when diagnosed, and more than 50% have had distant metastases. The incidence rate of non-small cell lung cancer (NSCLC) accounts for almost 80-85% of lung cancer, and pathological types of NSCLC mainly include squamous cell carcinoma (SCC) and adenocarcinoma (AD). Although current diagnosis and treatment of NSCLC have been greatly improved, the 5-year survival rate of lung cancer patients is still below 18%, which is mainly caused by the lack of specific biomarkers for early diagnosis and detailed occurrence and development mechanisms of NSCLC. Therefore, to find new biological markers that can increase diagnostic rate, improve prognosis and serve as individualized treatment means in clinical practice, is urgently needed.

Micro-ribonucleic acids (miRNAs) are a class of small single-stranded non-coding RNAs with about 19-22 nucleotides in length, which are considered as important components of epigenetic regulation system. They mainly regulate the expression levels of genes after transcription by degrading target messenger RNA (mRNA) or inhibiting the translation of target gene. Literature reported that miR-339-5p inhibits lung cancer cell invasion and migration by regulating the epithelial-to-mesenchymal transition via BCL6 in vitro. Lin et al. found that miR-133b can reverse the sensitivity of NSCLC cells to cisplatin by regulating the expression of glutathione S-transferase pi-1 (GSTP1). However, the expression and role of miR-1275 in NSCLC have not been reported.
Recent studies have shown that miR-1275 has up-regulated expressions in many tumors and plays a role similar as “oncogene” to promote the occurrence and development of tumors. MiR-1275 can promote the proliferation, invasion and metastasis of head and neck SCC cells by regulating the expressions of insulin-like growth factor 1 receptor (IGF-1R) and C-C chemokine receptor (CCR7)\textsuperscript{10}. Besides, miR-1275 promotes the proliferation of hepatocellular carcinoma cells by binding to IGF2 mRNA\textsuperscript{11}. In this work, miR-1275 was first found to be elevated in NSCLC tissues. Statistics also discovered that highly expressed miR-1275 was positively correlated with tumor size, tumor node metastasis (TNM) staging and lymph node metastasis in NSCLC patients, and it was confirmed by \textit{in vitro} researches that highly expressed miR-1275 promoted the proliferation and metastasis of NSCLC cells.

**Patients and Methods**

**Tissues**

A total of 70 pairs of NSCLC tissues and corresponding adjacent tissues (5 cm away from tumor tissues) were derived from specimens surgically resected in our hospital from June 2012 to December 2015. All tissues were confirmed by two pathologists, and all patients received no treatment before surgery. After specimens were obtained by clinical operation, they were isolated on ice for material collection within 0.5-1.0 h. This study was approved by the Ethics Committee of the First Affiliated Hospital of Chengdu Medical College, and patients signed informed consent.

**Cell Culture**

Normal 16 human bronchial epithelial (16HBE) cells and NSCLC cell lines A549, SPCA-1, PC-9, H292, and H460 were obtained from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China), cultured in Roswell Park Memorial Institute (RPMI)-1640 medium or Dulbecco’s modified Eagle medium (DMEM) (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA), and incubated at 37°C with pH of 7.2-7.4, a humidity of 95%, and CO\textsubscript{2} volume fraction of 5%.

**Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)**

Total RNA was extracted from cells using TRIzol (Invitrogen Corporation, Carlsbad, CA, USA) assay, and then complementary deoxyribonucleic acid (cDNA) was synthesized based on the instructions of reverse transcription kits (Invitrogen Corporation, Carlsbad, CA, USA). Then, amplification was performed according to qRT-PCR instructions (Invitrogen Corporation, Carlsbad, CA, USA), and amplification curve and solubility curve were confirmed after completion of the reaction. Above procedures were repeated 3 times for each experimental sample, and the average cycle threshold (Ct) value was taken. Data of miR-1275 and leucine zipper putative tumor suppressor 3 (LZTS3) mRNA expression levels were calculated using 2\textsuperscript{-ΔΔCt} method. Primers involved: miR-1275 F 5’-GTGCAGGGTCCGAGGT-, R 5’-GCCGCTAGCTTATCGACTACG-3’, U6 F 5’-CTCGCT'I"CGGCAGCACA-3’, R 5’-AAC-GCT TCACGAATTTGCGT-3’ and interference sequences: si-miR-1275 5’-UCAACAUCAGUAUAGC-3’. Primers and interference sequences were designed and synthesized by Aksonics (Shanghai, China).

**Cell Clone Detection**

After cells were transfected with small interference (si)-miR-1275 and control sequence for 6 h, cells from experimental group and control group were inoculated into a 6-well plate at 1000/well and cultured in the 5% CO\textsubscript{2} incubator at 37°C for 14 d. When clone formation was macroscopic, culture was terminated. Then, cells were fixed with methanol for 15 min, stained for 20 min and photographed, and the number of macroscopic colonies was counted.

**Flow Cytometry**

NSCLC cells were seeded in the 6-well plate, transiently transfected with si-miR-1275 and si-normal control (si-NC) and then cultured for 48 h. After that, cells were digested with ethylene diamine tetraacetic acid (EDTA)-free pancreaticin, collected, re-suspended with precooled 75% ethanol and placed in a refrigerator at -20°C for fixation overnight. Lastly, the intracellular DNA content was detected by flow cytometry propidium iodide (PI) staining.

**Cell Wound Healing Assay**

After 48 h of transfection, cells were taken from each group and seeded in the 6-well plate. When 80% cells were fused, 10 μL pipette were used to draw a horizontal stroke (in Chinese characters) on the bottom of the culture plate. Then, phosphate-buffered saline (PBS) was added to
elute suspended cells. After that, cells were photographed at 0 and 36 h after culture, respectively, according to the experimental design.

**Transwell Assay**

Cells were treated and collected as described above, and cell density was adjusted to $2.5 \times 10^5$/mL. 200 µL above cell suspension were added in the upper chamber of a transwell chamber (50 mg/L BD matrigel diluted at 1:7 were added in the transwell chamber), and 700 µL RPMI-1640 medium or DMEM containing 10% fetal bovine serum (FBS) were added in the lower chamber at the same time. The transwell chamber was incubated for 24 or 48 h, followed by formaldehyde fixation, crystal violet staining, observation and counting.

**Dual Luciferase Reporter Gene**

Wild-type and mutant LZTS3-3′-untranslated region (3′UTR) reporter gene plasmids were constructed. Then, reporter gene plasmids, Renilla luciferase-thymidine kinase promoter (pRL-TK) plasmids, miR-1275 mimics and their negative control plasmids were transfected into HEK293T cells. After 48 h, assay was carried out according to instructions of dual luciferase detection (Beyotime, Shanghai, China), and the ratio of firefly fluorescence intensity to Renilla fluorescence intensity was used to reflect the relative fluorescence intensity of different treatment groups.

**Western Blotting**

Total protein was extracted from cells in experimental group and control group, and protein concentration was measured via bicinchoninic acid (BCA) assay (Beyotime, Shanghai, China). Samples in the same amount were taken from each group and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then, protein was transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After that, the membrane was blocked in 5% skimmed milk, incubated with LZTS3 antibody and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody at 4°C overnight, and washed with Tris-buffered saline and Tween-20 (TBST-20) for three times (10 min/time). Next, the membrane was added with secondary antibody, incubated at room temperature for 1 h, followed by washing with TBST for three times (10 min/time). Lastly, enhanced chemiluminescence (ECL) solution (Thermo Fisher Scientific, Waltham, MA, USA) was added, followed by exposure, development and fixing.

<table>
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<th>Characteristics</th>
<th>miR-1275 Low no. case</th>
<th>miR-1275 High no. case</th>
<th>$x^2$-test</th>
<th>$p$-value</th>
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$p < 0.05$. 
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Statistics Analysis
Statistical product and service solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA) were used for analysis. Data were expressed as mean ± standard deviation ( x±s). Comparison between groups was done using One-way ANOVA test followed by Least Significant Difference (LSD). Correlation between two ordinal variables was detected via Spearman correlation analysis. Pearson’s test was employed for comparison between two classified variables. p<0.05 suggested that the difference was statistically significant.

Results

MiR-1275 was Highly Expressed in NSCLC Tissues and Cells
QRT-PCR showed that among 70 cases of NSCLC tissues, 52 cases had up-regulated miR-1275 expressions compared with those in adjacent tissues (Figure 1A). Statistical analysis indicated that highly expressed miR-1275 was positively correlated with tumor size, TNM staging, and lymph node metastasis in patients with NSCLC (Table I). To further investigate the biological function of miR-1275, qRT-PCR was applied to detect its relative expression levels in NSCLC cells and 16HBE, and the results showed that miR-1275 expression in NSCLC cells was increased (Figure 1B). Next, miR-1275 interference sequence was designed, synthesized and transfected into NSCLC cells, and the efficiency of the interference was examined after 48 h (Figure 1C-D).

Effect of miR-1275 on the Proliferation of NSCLC Cells
Experimental and control sequences were transfected into NSCLC cells. The results of

Figure 1. MiR-1275 is highly expressed in NSCLC. A, Relative expression levels of miR-1275 in 70 cases of NSCLC tissues are measured by qRT-PCR assay, in which increased expression is found in 52 cases of tissues, with U6 as an internal reference. B, Relative expression levels of miR-1275 in NSCLC cells and 16HBE are measured by qRT-PCR assay. C-D, Transfection efficiency is detected after NSCLC cells are transfected with si-miR-1275 and miR-NC for 48 h (**p<0.01, *p<0.05).
MiR-1275 Regulated LZTS3 Expression

To investigate the potential molecular mechanisms of miR-1275 in exerting biological functions in NSCLC cells, bioinformatics (http://www.mirdb.org/) was utilized to predict the downstream target genes of miR-1275 (Figure 4A), and qRT-PCR and Western blotting were carried out to verify that the expression of LZTS3 was regulated by miR-1275 (Figure 4B-C). Then, dual luciferase reporter gene assay was applied, and the results indicated that
miR-1275 was able to bind directly to LZTS3 mRNA (Figure 4D).

**Discussion**

More and more miRNAs have been reported and confirmed to play important roles in cell proliferation, apoptosis, angiogenesis, epithelial-mesenchymal transition (EMT) and tumor stem cell maintenance by directly or indirectly regulating the expressions of genes, thus promoting/inhibiting the occurrence and development of NSCLC.

Researchers have confirmed that miRNA-let-7, the first miRNA discovered in human, can regulate the growth and proliferation of tumor cells by inhibiting the expressions of oncoproteins such as rat sarcoma (RAS), myelocytomatosis (Myc) and cyclin-dependent kinase 6 (CDK6). In NSCLC cell and animal experiments, it is also proved that let-7 is a typical tumor suppressor. In addition, miR-338-3p has been proved to have a down-regulated expression in highly metastatic NSCLC cells, and it can inhibit the migration and invasion of NSCLC cells via the targeted regulation of sex-determining region Y (SRY)-related HMG-box 4 (SOX4).
expression\textsuperscript{16}. MiR-34a and miR-34b/c, members of the miR-34 family, are induced and transcribed by p53 upon DNA damage, thus regulating cell cycle arrest and apoptosis of tumor cells. MiR-34a has a down-regulated expression in lung cancer and inhibits cell proliferation as a tumor suppressor gene in a p53-independent manner\textsuperscript{17}. Shi et al\textsuperscript{18} confirmed that miR-34a can inhibit the colony-forming ability of NSCLC cells, implying that it has the function of anti-tumor stem cell. Moreover, in this work, it was found via in-vitro experiments for the first time that interference in miR-1275 could inhibit the proliferation and metastasis of NSCLC cells.

Literature\textsuperscript{19,20} reported that the regulation mechanisms of miRNAs in NSCLC include genomic abnormalities, epigenetic changes, miRNA sequence single nucleotide polymorphisms, and interactions between miRNA and competing endogenous RNA (ceRNA) and protein. Among them, ceRNAs contain multiple miRNA binding sites, which act by competitively binding miRNA to mRNA. In this study, potential downstream target genes of miR-1275 were first predicted using bioinformatics and verified via qRT-PCR and other experiments. Finally, it was found through dual luciferase reporter gene assay that miR-1275 could directly bind to LZTS3 mRNA.

**Conclusions**

We showed that miR-1275 expression is increased in NSCLC. Highly expressed miR-1275 may play a similar role as “oncogene” and promotes the proliferation and metastasis of NSCLC cells through the targeted regulation of LZTS3 expression. In addition, targeted treatment of miR-1275/LZTS3 may provide an important basis for the reversal of the formation of NSCLC malignant phenotype in clinic.

**Conflict of Interest**

The Authors declare that they have no conflict of interest.

**Fund**

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**Figure 4.** MiR-1275 regulates the expression of LZTS3. A, Bioinformatics show that miR-1275 can bind to LZTS3 mRNA. B, LZTS3 mRNA expression changes after interfering in miR-1275 expression are detected via qRT-PCR. C, LZTS3 protein expression changes after interfering in miR-1275 expression are detected via Western blotting. D, Direct binding of miR-1275 to LZTS3 mRNA is proved through dual luciferase reporter gene. (**p<0.01, *p<0.05).
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References