Circular RNA_LARP4 inhibits the progression of non-small-cell lung cancer by regulating the expression of SMAD7

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Abstract. – OBJECTIVE: Researchers have uncovered the importance of circular RNAs (circ) in malignant tumors. Circ LARP4 has been found to serve as a tumor suppressor gene in gastric cancer. However, the exact function of circ LARP4 in non-small-cell lung cancer (NSCLC) has not been fully elucidated. The aim of this study was to uncover the role of circ LARP4 in the tumorigenesis of NSCLC.

PATIENTS AND METHODS: Expression level of circ LARP4 in NSCLC tissues was detected through Real Time-quantitative Polymerase Chain Reaction (RT-qPCR). Subsequently, the association between expression and patients' prognosis was analyzed. Circ LARP4 lentivirus was constructed and transfected into NSCLC cells. The effect of circ LARP4 on NSCLC cell migration and invasion was detected by function assays. Furthermore, Western blot was performed to analyze the expression of predicted protein of circ LARP4.

RESULTS: Compared with adjacent tissues, circ LARP4 was lowly expressed in NSCLC tissues. Meanwhile, expression of circ LARP4 was associated with the prognosis of NSCLC patients. Downregulated circ LARP4 was found in NSCLC cell lines as well. The migration and invasion abilities of NSCLC cells were significantly inhibited *via* overexpression of circ LARP4. SMAD7, the predicted protein of circ LARP4, increased remarkably *via* overexpression of circ LARP4.

CONCLUSIONS: Circ LARP4 could suppress the metastasis of NSCLC by up-regulating SMAD7.

Key Words

Circular RNA, Circ LARP4, Non-small-cell lung cancer (NSCLC), SMAD7.

Introduction

As the completion of human genome sequencing, researchers have found that about 75% of the human genome can be transcribed into RNAs. Only 2% of those RNAs encode proteins. The rest of RNAs are called non-coding RNAs (ncRNAs), with no protein-encoding function. Circular RNAs (circRNAs) are a kind of ncRNAs widely existing in mammals. They have been found widely involved in gene regulation. CircRNA is mostly derived from the exon region of the gene. Unlike long noncoding RNA (lncRNA) or microRNA (microRNA), circRNAs do not have 5' or 3' terminal structures, which are formed by covalently closed ring structures. CircRNAs are widely involved in human physiological and pathological regulation, acting as a "sponge" of microRNAs. Meanwhile, they can bind with proteins or translate into polypeptides or other ways. Consistently, by regulating miR-1324/FZD5/Wnt/β-catenin signaling, circ_0067934 facilitates the growth and migration of hepatocellular carcinoma[1]. Up-regulation of circRNA_102004 enhances the proliferation of prostate cancer cells, serving as a potential biomarker of prostate cancer[2].

Currently, the incidence and mortality of lung cancer rank 1st globally. Non-small cell lung cancer (NSCLC), the major subgroup of lung cancer, remains a public threat even for the next several decades[3,4]. NSCLC can be refined into three types, including large cell carcinoma (LCC), lung squamous cell carcinoma (LSCC), and adenocarcinoma (LAD). Compared with SCC, the growth, proliferation and metastasis of NSCLC

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are relatively slower. Unfortunately, due to the lack of sensitive and effective early detection, most patients have already been in local advanced stage or metastasis stage when first diagnosed. Therefore, these patients have missed the best time of treatment. Traditional treatment of lung cancer has achieved considerable progress in recent years. Meanwhile, targeted therapy has been widely applied in clinical practice, such as tyrosine kinase inhibitors (TKIs) and immunological checkpoint inhibitors[5,6]. However, the prognosis of NSCLC patients remains poor[7]. Therefore, further exploring the mechanism underlying lung cancer and searching for biomarkers related to the occurrence and development of lung cancer are of great significance.

In our study, circ LARP4 was found significantly downregulated in NSCLC tissues and cell lines. Low expression of circ LARP4 was closely related to poor disease-free survival of NSCLC patients. Circ LARP4 significantly inhibited tumor metastasis of NSCLC *in vitro*. Furthermore, circ LARP4 overexpression down-regulated SMAD7 expression in NSCLC cells.

Patients and Methods

Tissue Samples

Totally, 52 pairs of NSCLC tissues and para-cancer tissues were obtained at Beijing Chest Hospital. The prognosis of patients was analyzed. No radiotherapy or chemotherapy was performed for any patient before the surgery. This investigation was approved by the Ethics Committee of Beijing Chest Hospital. Informed consent was obtained from patients and their families before the study.

Cell Culture

Normal human bronchial epithelial cell line (16HBE) and human NSCLC cell lines (A549, SPCA1, and H1299) were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) in an incubator at 37°C with 5% CO_2.

Cell Transfection

NSCLC cells were first seeded into 6-well plates for 24 h of culture. Later, the cells were transfected with lentivirus targeting specifically targeting circ LARP4 (lentivirus) and control (GenePharma; Shanghai, China) according to the instructions of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Only GFP-positive cells were chosen for the following experiments.

RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA in tissues and cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The synthesis of complementary deoxyribose nucleic acids (cDNAs) was conducted through reverse Transcription Kit. Primer sequences used for RT-qPCR were as follows: circ LARP4, forward: 3'- GGGCATCAGGAGCAAACTTA-5'; circ LARP4, reverse: 3'- GTGTTGGGCTGGAAGCCATC-5'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers forward: 5'-CCAAAATCAGATGGGGCAATGCTGG-3' and reverse5'-TGATGGCATGGACTGTGGTCATTCA-3'. The expression level of mRNA was normalized to GAPDH.

Scratch Wound Assay

Cells were first seeded into 6-well plates and cultured in DMEM medium overnight. After scratched with a plastic tip, the cells were cultured in serum-free DMEM. Wound closure was viewed at 48 h. Each assay was independently repeated for three times.

Transwell Assay

Transwell chambers with 8 μm pores were provided by Corning (Corning, NY, USA). Transfected cells were seeded into 24-well plates of upper chambers. Meanwhile, 20% FBS-DMEM was added to lower chambers of culture inserts. After culture for 24 h, these inserts were fixed with methanol for 30 min and stained with hematoxylin for 20 min. Number of migrated cells was finally counted under a light microscope (Olympus, Tokyo, Japan).

Matrigel Assay

Transwell chambers with 8 μm pores were provided by Corning (Corning, NY, USA). The membrane was pre-coated with 50 μL Matrigel. Transfected cells were seeded into 24-well plates of upper chambers. Meanwhile, 20% FBS-DMEM was added to lower chambers of culture inserts. After culture for 24 h, these inserts were fixed with methanol for 30 min and stained with hematoxylin for 20 min. Number of invasive cells was finally counted under a light microscope (Olympus, Tokyo, Japan).

Western Blot

Radio-immunoprecipitation assay (RIPA) was used to extract total protein in cells. Subsequently, protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Then, the membranes were incubated with primary antibodies of rabbit anti-GAPDH and rabbit anti-SMAD7 (Cell Signaling Technology; CST, Danvers, MA, USA). On the next day, the membranes were incubated with goat anti-rabbit secondary antibody. Image J software (NIH, Bethesda, MD, USA) was applied for the assessment of protein expression.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 18.0 (SPSS Inc., Chicago, IL, USA) was conducted for all statistical analysis. GraphPad Prism 5.0 (San Diego, CA, USA) was used for image editing. The difference between two groups were compared by Kaplan-Meier method and Student's *t*-test. $p<0.05$ was considered statistically significant.

Results

Expression Level of Circ LARP4 in NSCLC Patients

RT-qPCR was used to detect circ LARP4 expression in 52 pairs of NSCLC tissue samples and corresponding normal tissues. As shown in Figure 1A, circ LARP4 expression in NSCLC tissues was significantly lower than that in corresponding normal tissues. Then, NSCLC patients were divided into two groups, including high circ LARP4 expression group and low circ LARP4 expression group. Kaplan-Meier method was used to analysis disease-free survival of patients after surgery. As shown in Figure 1B, compared with those in high circ LARP4 expression group, NSCLC patients in low circ LARP4 expression group showed significantly worse disease-free survival. The results suggested that dysregulated circ LARP4 might participate in NSCLC progression.

Expression of Circ LARP4 in NSCLC Patients

Circ LARP4 was lowly expressed in NSCLC cells (A549, SPCA1, and H1299) when compared with normal human bronchial epithelial cell (16HBE), which was shown in Figure 2.

Circ LARP4 Overexpression Depressed Invasion and Migration of NSCLC Cells

To explore the effect of circ LARP4 on NSCLC metastasis, scratch wound assay, transwell assay, and Matrigel assay were conducted. SPCA1 cells were selected for transfection of circ LARP4 lentivirus. RT-qPCR was used to verify the transection efficiency (Figure 3A). As shown in Figure 3B, scratch wound assay indicated that the growth ability of SPCA1 cells was significantly repressed after circ LARP4 was overexpressed. As shown in Figure 3C, transwell assay showed that the number of migrated cells was remarkably reduced after circ LARP4 overexpression. As shown in Figure 3D, the number of invaded SPCA1 cells decreased significantly after circ LARP4 was overexpressed.

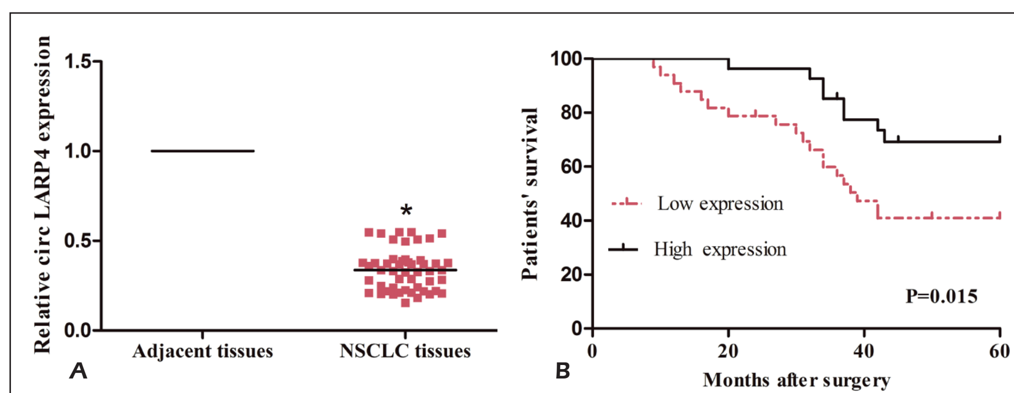

Figure 1. Low expression level of circ LARP4 was associated with poor prognosis of NSCLC patients. **A**, Circ LARP4 expression decreased significantly in NSCLC tissues compared with adjacent tissues. **B**, Low level of circ LARP4 was associated with poor disease-free survival of NSCLC patients. Data were expressed as mean ± standard error of the mean. *$p<0.05$.

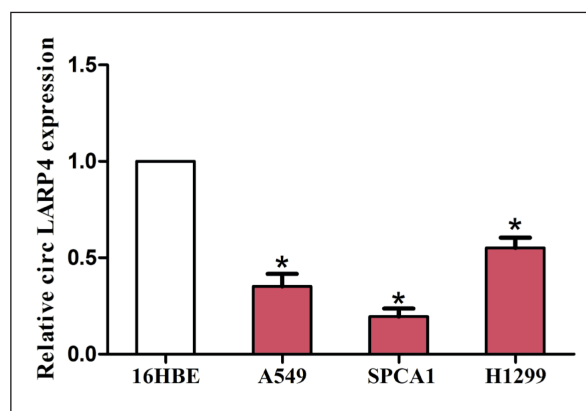

Figure 2. Expression levels of circ LARP4 increased significantly in NSCLC cell lines. Expression levels of circ LARP4 relative to GAPDH were determined in human NSCLC cell lines and normal human bronchial epithelial cell line (16HBE) by RT-qPCR. GAPDH was used as an internal control. Data were presented as mean ± standard error of the mean. *$p<0.05$.

Circ LARP4 Overexpression Up-regulated SMAD7 Expression in NSCLC

SMAD7 has been detected to suppress the proliferation of numerous cancers, including NSCLC. In our study, we first determined the interaction between SMAD7 and circ LARP4. RT-qPCR was utilized to measure the expression of SMAD7 in SPCA1 cells transfected with circ LARP4 lentivirus or control. The results showed that circ LARP4 overexpression up-regulated the mRNA expression of SMAD7 (Figure 4A). The protein level of SMAD7 was measured through Western blot assay. The results demonstrated that circ LARP4 overexpression significantly upregulated the protein expression level of SMAD7 (Figure 4B). Linear correlation analysis revealed that the expression of circ LARP4 was positively related to the expression level of SMAD7 in NSCLC tissues (Figure 4C).

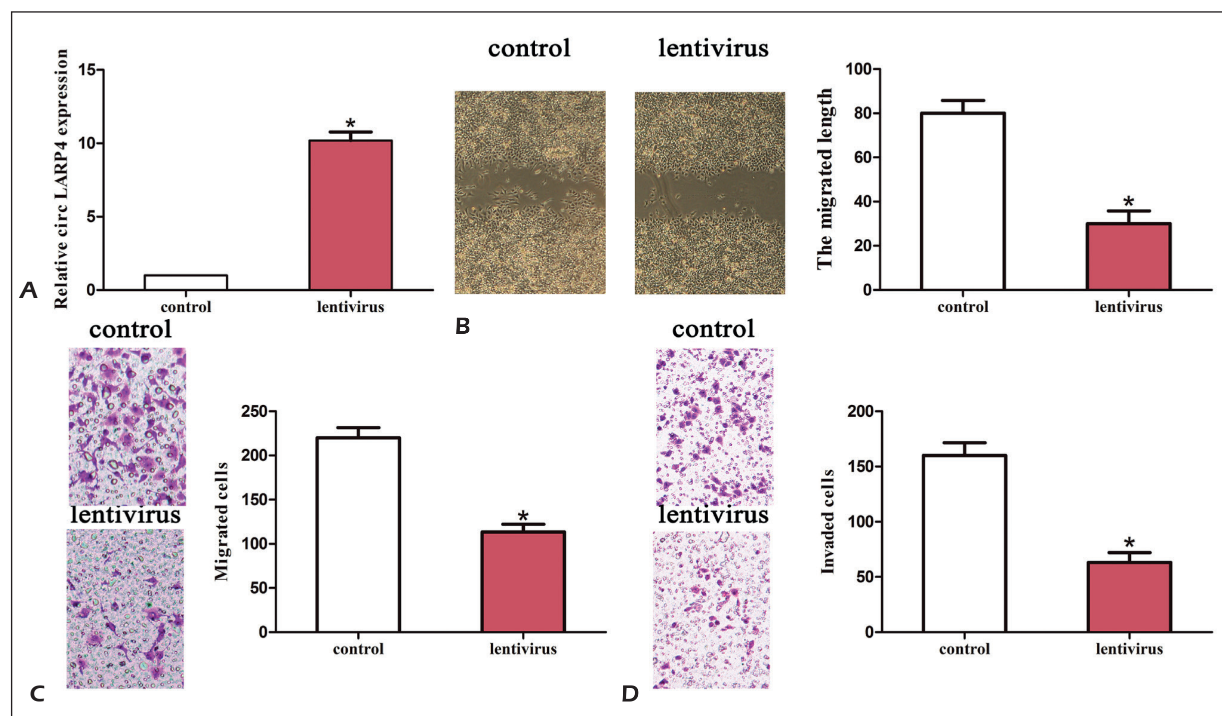

Figure 3. Overexpression of circ LARP4 inhibited NSCLC cell migration and invasion. **A**, Circ LARP4 expression in NSCLC cells transfected with circ LARP4 lentivirus and control was detected by RT-qPCR. **B**, Scratch wound assay showed that overexpression of circ LARP4 significantly repressed the migration of NSCLC cells (magnification: 40×). **C**, Transwell assay showed that the number of migrated cells decreased remarkably via overexpression of circ LARP4 in NSCLC cells (magnification: 40×). **D**, Matrigel assay showed that the number of invaded cells decreased significantly after circ LARP4 was overexpressed in NSCLC cells (magnification: 40×). The results represented the average of three independent experiments (mean ± standard error of the mean). *$p<0.05$, as compared with control cells.

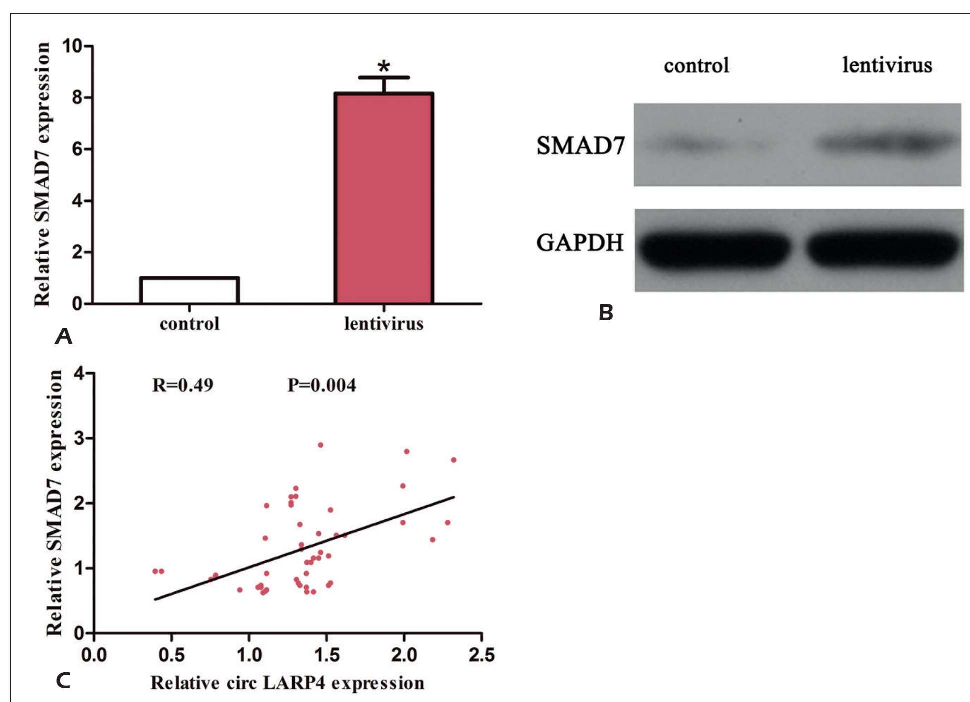

Figure 4. Circ LARP4 overexpression up-regulated SMAD7 expression in NSCLC. **A**, RT-qPCR results showed that SMAD7 expression increased markedly in circ LARP4 lentivirus group compared with control group in NSCLC cells. **B**, Western blot results showed that the protein expression of SMAD7 was significantly up-regulated in circ LARP4 lentivirus group compared with control group. **C**, Linear correlation between the expression levels of SMAD7 and circ LARP4 in NSCLC tissues. The results represented the average of three independent experiments. Data were expressed as mean ± standard error of the mean. $*p<0.05$.

Discussion

Lung cancer has the highest morbidity and mortality in the world. Although many researchers have focused on exploring the pathogenesis of lung cancer, the relevant mechanisms have not been fully elucidated. Meanwhile, the effect of treatment measures is still far from satisfactory[8]. Current studies have observed that circRNAs play crucial roles in pathophysiological processes of various tumors. It has also become the focus of researches in the exploration of the pathogenesis and treatment strategy of NSCLC. With the application and development of technologies such as circRNAs microarray and RNA sequencing, increasing circRNAs have been found abnormally expressed in NSCLC. These circRNAs can function as oncogenes or tumor suppressor genes. Consistently, upregulation of hsa_circ_100395 dramatically inhibits cell proliferation and reduces cell migration and invasion in lung cancer by targeting TCF21[9]. By sponging miR-338-5p and miR-331-3p, circ_0001649 inhibits the progression of NSCLC, serving as a prognostic biomarker[10]. Mediated by miR-503/LARP1 signaling, silencing of circ-BANP suppresses the migration and proliferation of lung cancer cells[11].

Circ LARP4 derives from LARP4 gene locus. By serving as a La-related RNA-binding protein, circ LARP4 has been revealed to restrain cell invasion and migration in malignancies. By sponging miR-424-5p and modulating LATS1 expression, circ_LARP4 suppresses the proliferation and invasion of gastric cancer cells[12]. In addition, it has been reported that circ LARP4 is significantly down-regulated in ovarian cancer. It serves as a potential biomarker for the prognosis of ovarian cancer patients as well[13]. To determine the function of circ LARP4 in NSCLC proliferation, circ LARP4 lentivirus was transfected into NSCLC cells. Function assays showed that circ LARP4 overexpression significantly repressed the migration of NSCLC cells. Furthermore, we explored the effect of circ LARP4 on the invasion of NSCLC cells. The results indicated that overexpression of circ LARP4 contributed to decrement of cell invasion in NSCLC. All these results suggested that circ LARP4 inhibited tumor metastasis of NSCLC.

Later, related proteins of circ LARP4 were explored. SMAD7 is an important inhibitor of transforming growth factor β (TGFβ) receptor signaling. Slattery et al[14] have revealed that SMAD7 influences the risk of sporadic colorectal cancer. Deletion of SMAD7 is significantly associated with better prognosis[15]. Overexpression of SMAD7 promotes the progression of skin cancer and pancreatic cancer. However, SMAD7 up-regulation suppresses the metastasis of human melanoma cells and breast cancer cells[16]. Through heme oxygenase-1 inhibition, SMAD7 enhances the sensitivity of lung cancer cells to cisplatin-induced apoptosis[17]. MiR-21-5p accelerates the progression of NSCLC *via* modulating the expression of SMAD7[18]. In the present study, the potential interaction between SMAD7 and circ LARP4 was explored. The results showed that circ LARP4 overexpression up-regulated SMAD7 expression *in vitro* and was positively correlated with SMAD7 expression in NSCLC tissues. All the above data indicated that circ LARP4 might inhibit the growth of NSCLC by up-regulating SMAD7.

Conclusions

In summary, circ LARP4 was remarkably down-regulated in NSCLC tissues. Down-regulation of circ LARP4 indicated poor prognosis of NSCLC patients. By up-regulating SMAD7, circ LARP4 inhibited cell migration and invasion in NSCLC. All these findings suggested that circ LARP4 might contribute to therapy for NSCLC as a prospective target.

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Conflict of Interests

The Authors declare that they have no conflict of interests.

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