

LPAR5 stimulates the malignant progression of non-small-cell lung carcinoma by upregulating MLLT11

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Abstract. – OBJECTIVE: This study aims to explore the diagnostic and prognostic values of Lysophosphatidic acid receptor 5 (LPAR5) in non-small-cell lung cancer (NSCLC) and its regulatory effects on biological functions of NSCLC cells.

PATIENTS AND METHODS: NSCLC and adjacent non-tumoral tissues were collected for analyzing differential levels of LPAR5 by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Clinical information of recruited NSCLC patients was collected for assessing the diagnostic and prognostic values of LPAR5. *In vitro* regulation of LPAR5 on proliferative and migratory potentials of H1299 and SPC-A1 cells was examined by Cell Counting Kit-8 (CCK-8) and transwell assay, respectively. In addition, *in vivo* regulation of LPAR5 on the growth rate of NSCLC in nude mice was detected by tumorigenicity assay. The interaction between LPAR5 and its downstream target MLLT11 was determined by rescue experiments.

RESULTS: LPAR5 was upregulated in NSCLC tissues than adjacent non-tumoral ones. High level of LPAR5 predicted higher rates of lymphatic metastasis and distant metastasis, as well as worse overall survival and progression-free survival in NSCLC. Knockdown of LPAR5 not only attenuated *in vitro* proliferative and migratory abilities in H1299 and SPC-A1 cells, but also slowed down *in vivo* growth of NSCLC in nude mice. MLLT11 was upregulated in NSCLC tissues, and displayed a positive correlation to LPAR5. Overexpression of MLLT11 was able to reverse the attenuated *in vitro* proliferative and migratory abilities, and the suppressed *in vivo* growth of NSCLC because of LPAR5 knockdown.

CONCLUSIONS: LPAR5 stimulates proliferative and migratory potentials in NSCLC by positively regulating MLLT11, which can be served as an effective diagnostic marker for early stage NSCLC.

Key Words:

LPAR5, MLLT11, NSCLC, Malignancy

Introduction

Lung cancer has the highest incidence in the world and it is the major cause of tumor-related death^{1,2}. According to the estimated data released in 2018, lung cancer is the most common reason of both male and female cancer death in the United States, accounting for 25% of cancer deaths^{3,4}. In China, the incidence and mortality of lung cancer both rank the first place^{5,6}. Based on the histological classification, non-small-cell lung cancer (NSCLC) covers 80-85% of lung cancer, including adenocarcinoma and lung squamous cell carcinoma⁷. Although recent studies have achieved massive progresses on lung cancer treatment, the 5-year survival is far away from satisfy^{8,9}. Clarifying molecular mechanisms of NSCLC progression is of significance to enhance therapeutic efficacy and to develop drug targets^{10,11}.

Lysophosphatidic acid receptor (LPAR) is a vital regulator responsible for lipid signal transduction¹². LPAR family contains six members, that is, LPAR1-6¹². LPAR5 is involved in tumor progression, which is a novel therapeutic target with a promising application^{13,14}. Using on-line bioinformatic software, it is considered that LPAR5 can directly target MLLT11. MLLT11 is a heterotopic fusion gene located on chromosome 1q21, and it is also known as AF1q¹⁵⁻¹⁷. Overactivated MLLT11 is found in many types of solid tumors^{18,19}. This study aims to explore the co-reg-

ulation of LPAR5 and MLLT11 on NSCLC progression, and thus provides theoretical references for developing effective biomarkers.

Patients and Methods

NSCLC Samples

NSCLC and paracancerous tissues were collected from 50 NSCLC patients who did not have preoperative chemotherapy or radiotherapy. Paracancerous tissues were harvested at least 5 cm away from tumor lesions. Tumor Node Metastasis (TNM) staging of NSCLC was diagnosed based on the Union for International Cancer Control (UICC) criteria. Inclusion criteria of NSCLC patients were as follows: (1) no severe diseases in other organs; (2) none of patients had preoperative chemotherapy/radiotherapy or molecular targeted therapy. In addition, the exclusion criteria of ccRCC patients were as follows: (1) distant metastasis; (2) other malignancies; (3) mental disease; (4) myocardial infarction; (5) heart failure or other chronic diseases, or those previously exposed to radioactive rays. This study was approved by the research Ethics Committee of Shanghai Pulmonary Hospital and complied with the Helsinki Declaration. Informed consent was obtained from patients. Telephone follow-up or outpatient review was conducted for recording general conditions, clinical symptoms and imaging examinations.

Cell Lines and Reagents

NSCLC cell lines (A549, H1299, PC-9, H358, SPC-A1) and the lung epithelial cell line (BE-AS-2B) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA) at 37°C with 5% CO₂. Medium was replaced every 2-3 days. Cell passage was conducted at 90% confluence, and those in the logarithmic growth phase were collected for experiments.

Transfection

LPAR5 shRNA (sh-LPAR5), sh-NC, pcDNA-MLLT11 and pcDNA-NC were synthesized by GenePharma (Shanghai, China). Cells were cultured to 40-60% density in a 6-well plate, and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h cell

transfection, cells were collected for verifying transfection efficacy and functional experiments.

Cell Proliferation Assay

Cells were inoculated in a 96-well plate with 2×10^3 cells/well. At 24, 48, 72 and 96 h, optical density at 450 nm of each sample was recorded using the Cell Counting Kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Transwell Migration Assay

Cell suspension was prepared at 5×10^5 cells/mL. 200 μ L of suspension and 700 μ L of medium containing 20% FBS was respectively added on the top and bottom of a transwell insert, and cultured for 48 h. Migratory cells on the bottom were induced with methanol for 15 min, crystal violet for 20 min and captured using a microscope. Ten random fields per sample were selected for capturing and counting cells.

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

Cells were lysed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for isolating RNAs. Qualified RNAs were reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using AMV reverse transcription kit (TaKaRa, Otsu, Shiga, Japan), followed by qRT-PCR using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was the internal reference. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. LPAR5: forward: 5'-GGCCCTGAGGAGGTCTCTG-3'; reverse: 5'-TCATGGCATGGCATTACCT-3'; MLLT11: forward: 5'-AGGAGTGAGAAGACAAAGCCG-3', reverse: 5'-GGTCCCTCATAGCTTCCTGTT-3'; GAPDH: forward: 5'-CCTGGCACCCAGCACAAT-3', reverse: 5'-TGCCGTAGGTGTCCCTTTG-3'.

Western Blot

Cells were lysed in radioimmunoprecipitation assay (RIPA: Beyotime, Shanghai, China) on ice for 15 min, and the mixture was centrifuged at $14000 \times g$, 4°C for 15 min. The concentration of cellular protein was determined by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). Protein samples with the adjusted same concentration were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-

PAGE), and loaded on polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was cut into small pieces according to the molecular size and blocked in 5% skim milk for 2 h. They were incubated with primary and secondary antibodies, followed by band exposure and grey value analyses.

In Vivo Xenograft Model

This study was approved by the Animal Ethics Committee of Tongji University Animal Center. Twenty male nude mice with 8 weeks old were classified into 4 groups, with 5 in each group. They were administrated with SPC-A1 cells transfected with sh-NC, sh-LPAR5, sh-LPAR5+pcDNA-NC, or sh-LPAR5+pcDNA-MLLT11 in the armpit. Tumor width and length were recorded every 5 days. Mice were sacrificed at 30 days for collecting tumor tissues. Tumor volume was calculated using the formula: Tumor width²×tumor length/2. Positive expression of LPAR5 in xenografted tumor sections was detected by immunoprecipitant staining.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM Corp., Armonk, NY, USA) was used for statistical analyses and data were expressed as mean ± standard deviation. Differences between groups were compared by the *t*-test. The influence of LPAR5 on clinical data of NSCLC patients was analyzed by Chi-square test. Kaplan-Meier survival curves were depicted, followed by log-rank test for comparing differences between curves. *p*<0.05 was considered as statistically significant.

Results

Expression Pattern and Clinical Significance of LPAR5 in NSCLC

Differential level of LPAR5 in clinical samples of NSCLC was first examined. Compared with normal tissues, positive expression rate of LPAR5 was much higher in NSCLC tissues (Figure 1A). Consistently, LPAR5 was upregulated in NSCLC cell lines (Figure 1B). In particular, higher level of LPAR5 was detected in NSCLC cases with lymphatic metastasis or distant metastasis than those non-metastasis ones (Figure 1C). Chi-square test also obtained that LPAR5 level was closely linked to rates of lymphatic and distant metastasis in

NSCLC (Table I). Kaplan-Meier survival analysis showed that high level of LPAR5 predicted poor overall survival and progression-free survival in NSCLC patients (Figure 1D). The AUC value of 0.782 (95% CI=0.895-0.904) by the ROC curve was obtained, suggesting the diagnostic value of LPAR5 in patients with NSCLC.

Knockdown of LPAR5 Weakened Proliferative and Migratory Abilities of NSCLC

We constructed *in vitro* LPAR5 knockdown models in H1299 and SPC-A1 cells by transfection of sh-LPAR5 (Figure 2A). As CCK-8 assay revealed, the knockdown of LPAR5 reduced viability in H1299 and SPC-A1 cells (Figure 2B). Moreover, migratory cell number was declined after transfection of sh-LPAR5 in NSCLC cells (Figure 2C). Notably, the growth rate and weight of xenografted NSCLC in nude mice were alleviated by knockdown of LPAR5 (Figure 2D).

For conducting the *in vivo* tumorigenicity assay, nude mice were administrated with SPC-A1 cells transfected with sh-NC or sh-LPAR5. Thirty days later, mice were sacrificed for harvesting NSCLC tissues and prepared into tumor sections. Compared with controls, positive expression of LPAR5 was much lower in NSCLC tissues harvested from mice with *in vivo* knockdown of LPAR5, confirming the successful modeling (Figure 2E).

LPAR5 Positively Regulated MLLT11

Previously we have predicted that MLLT11 is a target binding LPAR5. Here, protein level of MLLT11 in H1299 and SPC-A1 cells was markedly downregulated by transfection of sh-LPAR5 (Figure 3A). MLLT11 presented a similar expression pattern as LPAR5 in NSCLC, which was upregulated in NSCLC tissues and cell lines (Figure 3B, 3C). Furthermore, it is found that LPAR5 was positively correlated to MLLT11 level in clinical samples of NSCLC (Figure 3D).

Co-Regulation of LPAR5 and MLLT11 on NSCLC

Subsequently, we focused on the biological function of MLLT11 in NSCLC progression. Transfection efficacy of pcDNA-MLLT11 was firstly examined in H1299 and SPC-A1 cells with LPAR5 knockdown (Figure 4A). Interestingly, co-transfection of sh-LPAR5 and pcDNA-MLLT11 in NSCLC cells resulted in higher viability and migratory cell number

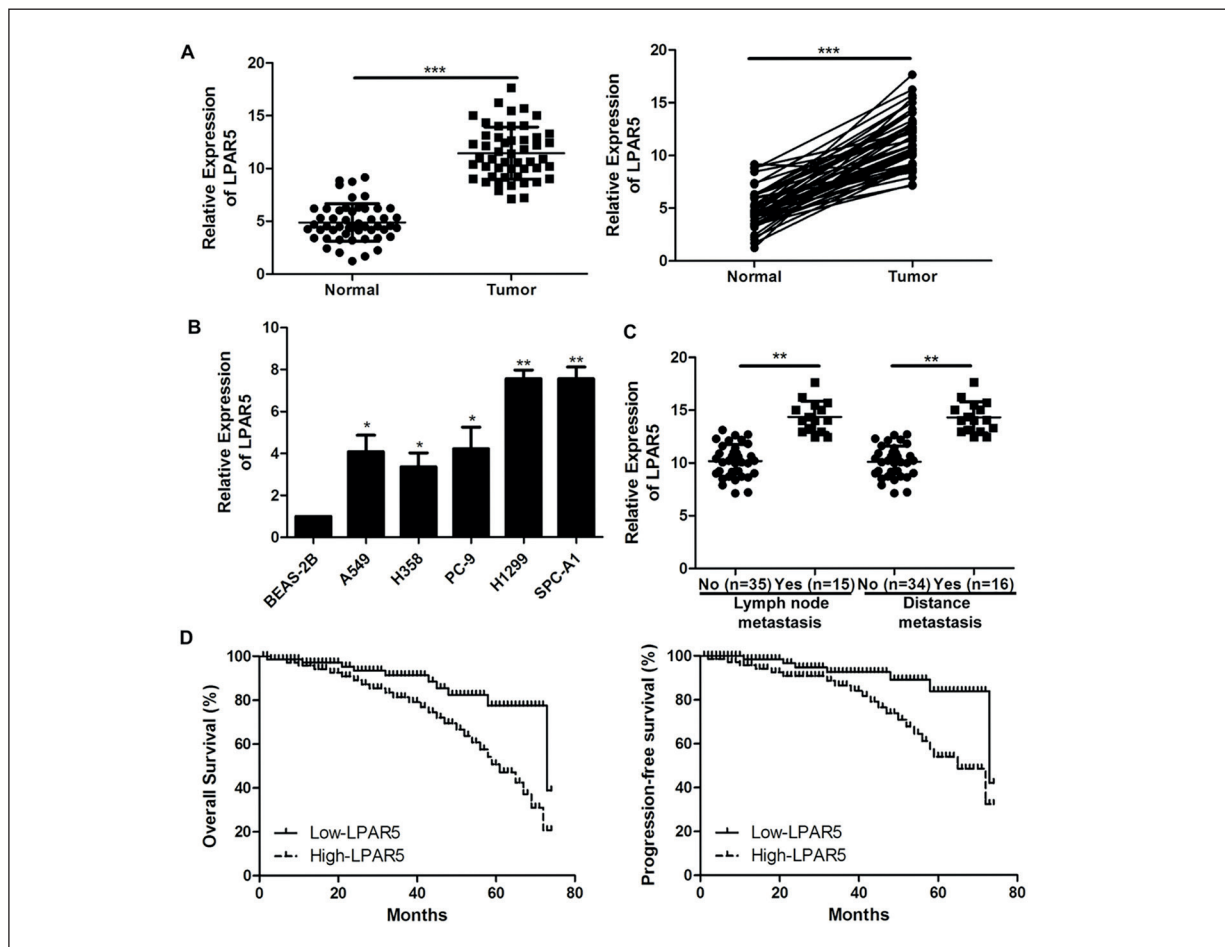


Figure 1. Expression pattern and clinical significance of LPAR5 in NSCLC. **A**, Differential level of LPAR5 in clinical samples of NSCLC and normal tissues. **B**, LPAR5 level in NSCLC cell lines. **C**, LPAR5 level in NSCLC cases with lymphatic metastasis, distant metastasis or not. **D**, Overall survival and progression-free survival in NSCLC patients classified by LPAR5 level. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table I. Association of LPAR5 expression with clinicopathologic characteristics of non-small cell lung cancer.

Parameters	Number of cases	LPAR5 expression		p-value
		Low (%)	High (%)	
Age (years)				0.945
< 60	22	12	10	
≥ 60	28	15	13	
Gender				0.586
Male	24	12	12	
Female	26	15	11	
T stage				0.297
T1-T2	30	18	12	
T3-T4	20	9	11	
Lymph node metastasis				0.011
No	35	23	12	
Yes	15	4	11	
Distance metastasis				0.005
No	34	23	11	
Yes	16	4	12	

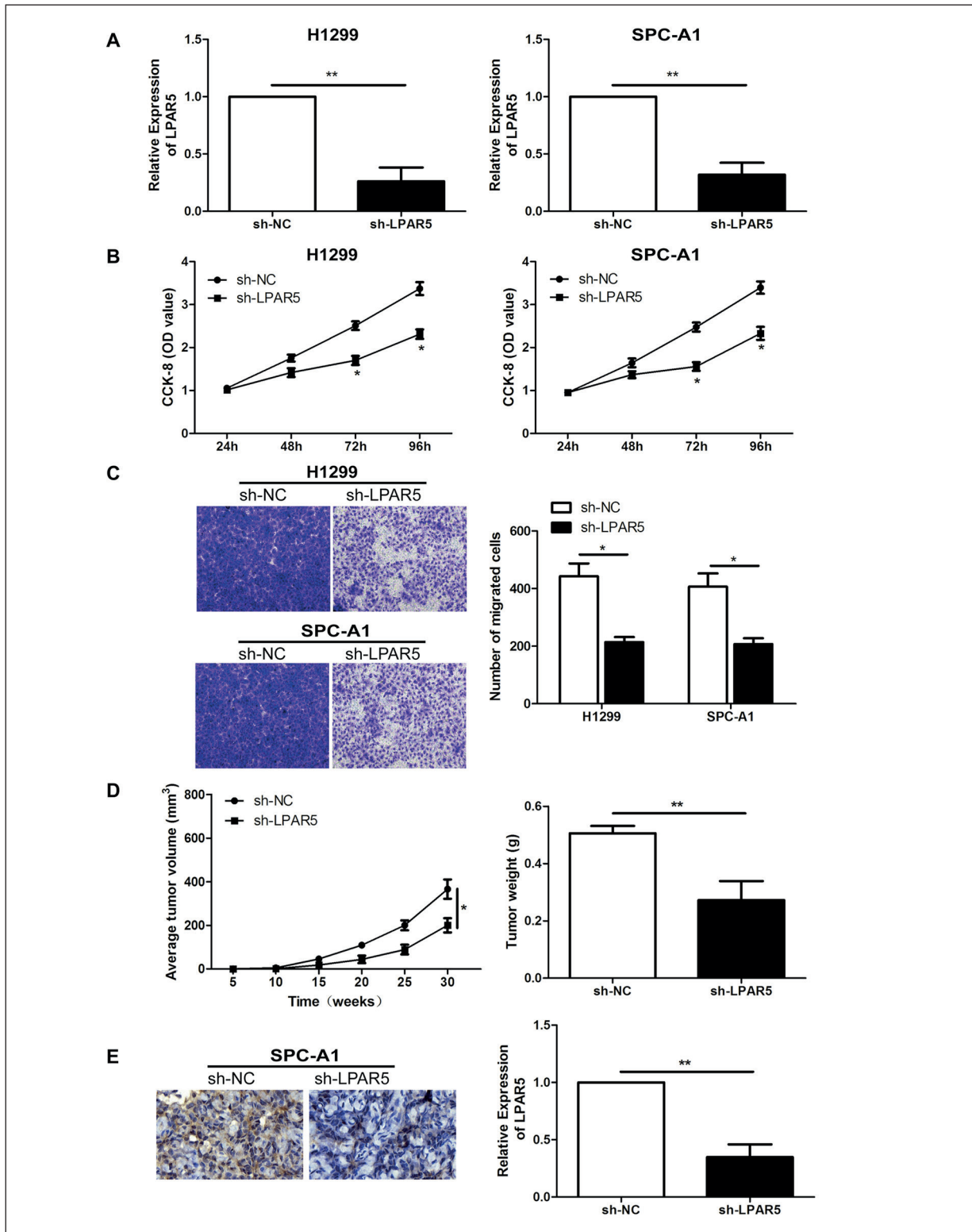


Figure 2. Knockdown of LPAR5 weakened proliferative and migratory abilities of NSCLC. **A**, Transfection efficacy of sh-LPAR5 in H1299 and SPC-A1 cells. **B**, Viability in H1299 and SPC-A1 cells with LPAR5 knockdown. **C**, Migration in H1299 and SPC-A1 cells with LPAR5 knockdown; (magnification: 40×). **D**, Tumor volume and tumor weight of nude mice with xenografted NSCLC. **E**, Positive expression of LPAR5 in xenografted NSCLC of nude mice (magnification: 40×). * $p < 0.05$, ** $p < 0.01$.

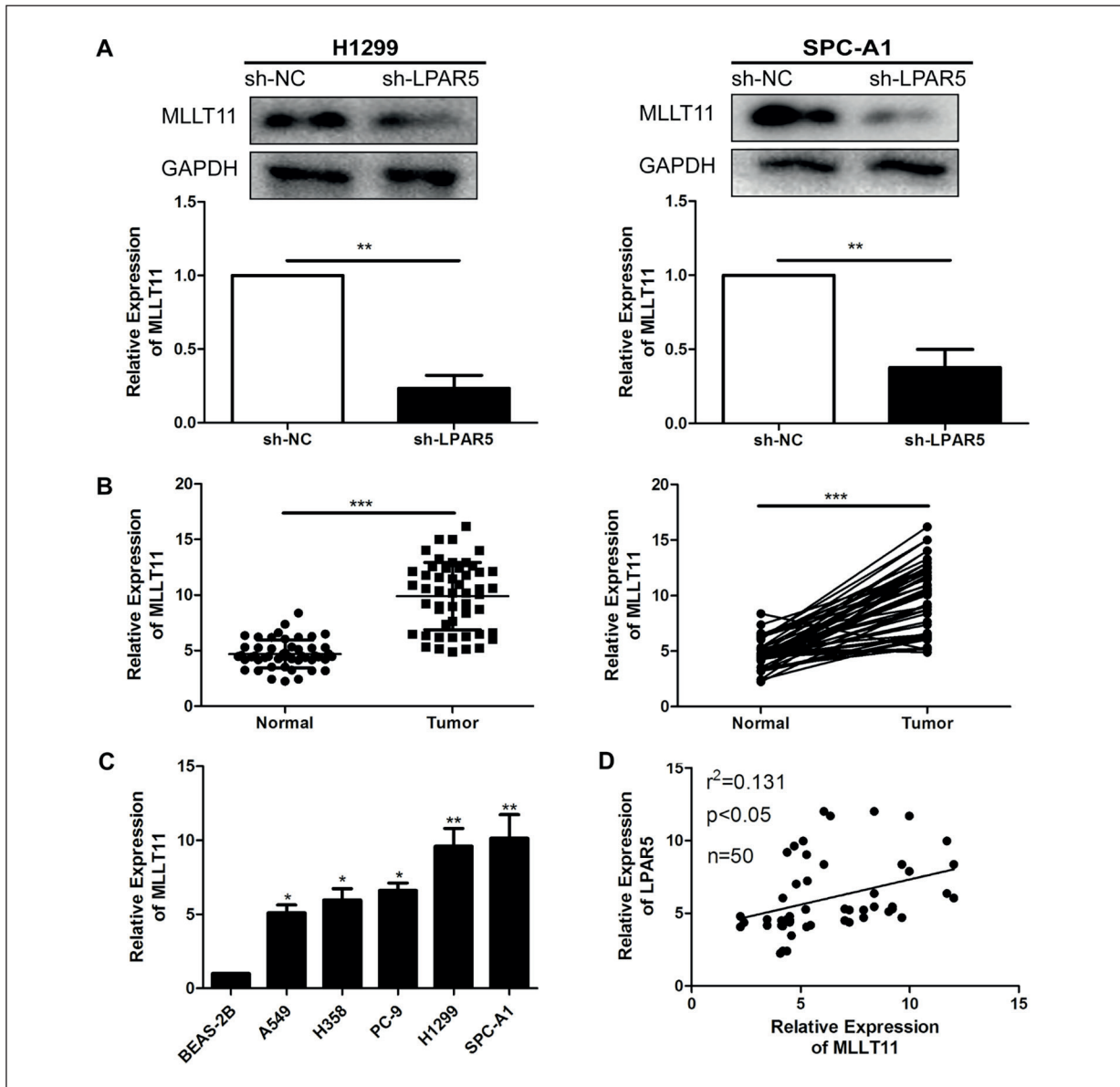


Figure 3. LPAR5 positively regulated MLLT11. **A**, Protein level of MLLT11 in H1299 and SPC-A1 cells with LPAR5 knockdown. **B**, Differential level of MLLT11 in clinical samples of NSCLC and normal tissues. **C**, MLLT11 level in NSCLC cell lines. **D**, A positive correlation between LPAR5 and MLLT11 in NSCLC tissues. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

compared to those with solely knockdown of LPAR5 (Figure 4B, 4C). As expected, retarding of NSCLC growing in nude mice due to silenced LPAR5 was reversed by MLLT11 overexpression (Figure 4D). A higher positive level of LPAR5 was examined in NSCLC sections collected from mice administrated with SPC-A1 cells co-transfected with sh-LPAR5 and pcDNA-MLLT11 than those with LPAR5 knockdown only (Figure 4E).

Discussion

Lung cancer-associated deaths account for 28% of male cancer death, and 26% of female cancer death¹⁻³. Generally speaking, NSCLC exceeds 85% of the clinical cases of lung cancer, and is featured by the poor prognosis⁴⁻⁷. About 30-55% of NSCLC patients experience cancer recurrence after surgery, due to the sustained proliferation and metastasis of NSCLC cell lines^{7,8}. Therefore, we are making efforts to clarify the key molecular mechanisms un-

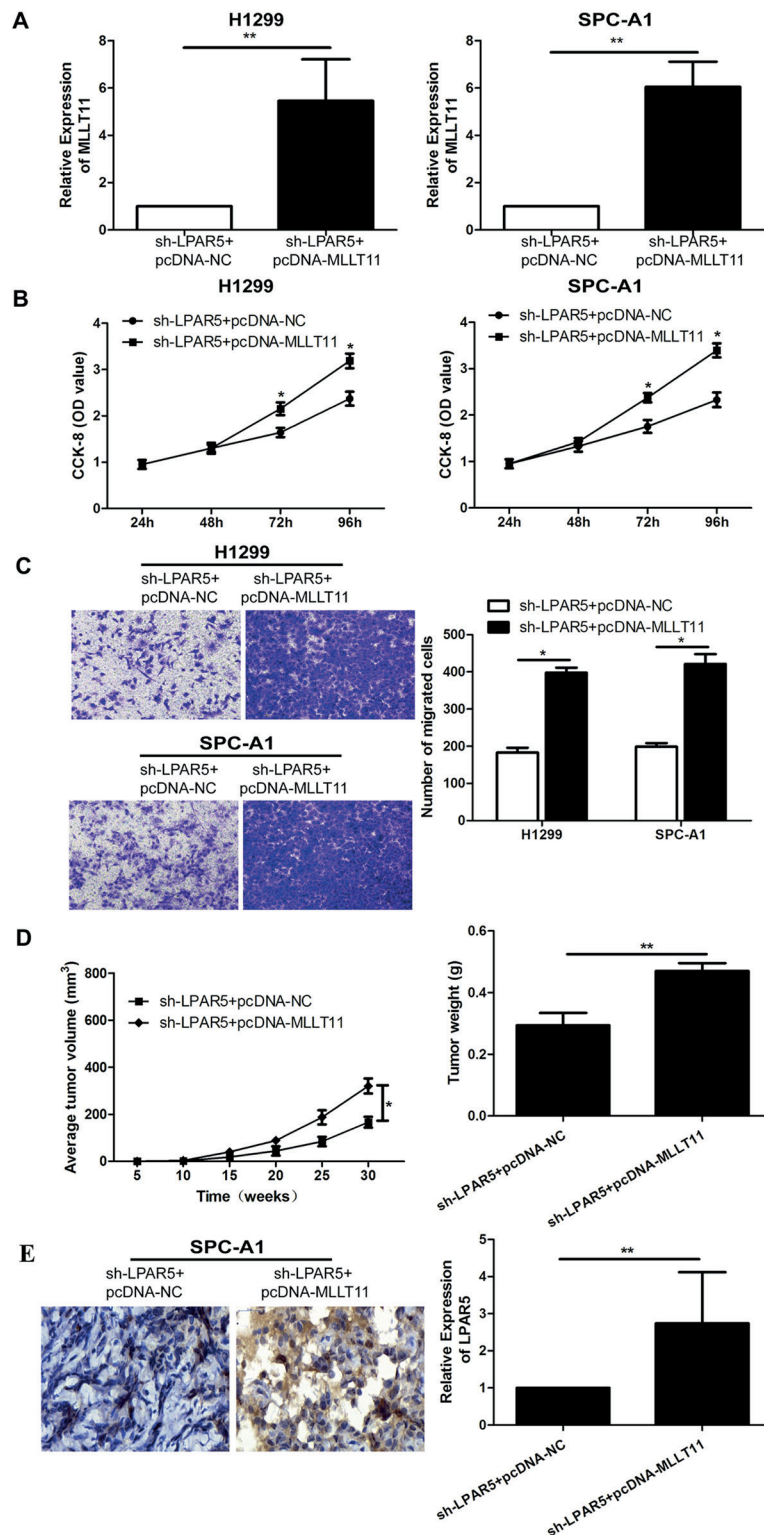


Figure 4. Co-regulation of LPAR5 and MLLT11 on NSCLC. **A**, Transfection efficacy of pcDNA-MLLT11 in H1299 and SPC-A1 cells with LPAR5 knockdown. **B**, Viability in H1299 and SPC-A1 cells regulated by LPAR5 and MLLT11. **C**, Migration in H1299 and SPC-A1 cells regulated by LPAR5 and MLLT11 (magnification: 40 $\times$). **D**, Tumor volume and tumor weight of nude mice with xenografted NSCLC. **E**, Positive expression of LPAR5 in xenografted NSCLC of nude mice (magnification: 40 $\times$) * p < 0.05, ** p < 0.01.

derlying NSCLC deterioration⁹⁻¹¹. It has been suggested that upregulated LPAR5 promoted the progression of tumors through adsorption mechanisms. However, the association about LPAR5 and NSCLC is unclear. In this paper, the findings uncovered that LPAR5 was up-regulated in NSCLC samples, and its level was correlated to rates of lymphatic and distant metastases in NSCLC patients. In addition, Kaplan-Meier curves uncovered that LPAR5 was unfavorable to the overall survival and progression-free survival of NSCLC. Knock-down of LPAR5 not only attenuated *in vitro* proliferative and migratory abilities in H1299 and SPC-A1 cells, but also slowed down *in vivo* growth of NSCLC in nude mice.

Bioinformatic analysis demonstrated that MLLT11 is a potential target binding LPAR5. So far, the biological function of MLLT11 and its pathological regulation remain largely unclear¹⁷⁻¹⁹. Unlike other MLL fusion genes, MLLT11 has a unique biological structure^{18,19}. MLLT11 is fused into MLL gene in its complete open reading frame (ORF), rather than the truncated ORF as seen in other fusion genes¹⁹. Here, Western blot analyses showed that protein level of MLLT11 was downregulated in NSCLC cells with LPAR5 knockdown. Compared with normal tissues, MLLT11 was upregulated in NSCLC samples and positively correlated to LPAR5. Notably, overexpression of MLLT11 was capable of reversing the regulatory effect of LPAR5 on NSCLC proliferation and metastasis. To sum up, a positive feedback loop identified that LPAR5 aggravated the process of NSCLC by positively regulating MLLT11, and the LPAR5/MLLT11 regulatory network might be a new target for the diagnosis and treatment of NSCLC.

Conclusions

Briefly, LPAR5 stimulates proliferative and migratory potentials in NSCLC by positively regulating MLLT11, which can be served as an effective diagnostic marker for early stage NSCLC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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