

Inhibition of cell proliferation and migration in non-small cell lung cancer cells through the suppression of LYPLA1

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Abstract. Lysophospholipase1 (LYPLA1) also known as acyl-protein thioesterase1 (APT1) belongs to the superfamily of α/β hydrolase. It has been found to have the properties of a homodimer by manifesting depalmitoylation as well as lysophospholipase activity. LYPLAs are under the control of both microRNAs, miR-138 and miR-424. They were observed to be significantly overexpressed in chronic lymphocytic leukemia cells. To date, LYPLAs are the sole enzymes recognized to activate depalmitoylation. In this study, we provide the expression pattern of LYPLA1 in non-small cell lung cancer (NSCLC) using four different NSCLC cell lines. Western blot analysis and RT-PCR were performed to detect the protein expression and mRNA expression of LYPLA1 in NSCLC cell lines. We detected the highest LYPLA1 protein expression level in SPC-A-1 cells followed by A549 cells, and the highest LYPLA1 mRNA expression level was detected in the SPC-A-1 cells followed by the H1299 cell line. We found that suppression of LYPLA1 expression using small-interfering RNA significantly inhibited proliferation, migration and invasion of the LYPLA1-transfected NSCLC cells. Furthermore, we explored the involvement of LYPLA1 in the regulation of epithelial-mesenchymal transition (EMT). The epithelial marker E-cadherin was significantly increased, while mesenchymal markers N-cadherin, vimentin and SNAIL were markedly decreased in the LYPLA1-silenced cells.

Collectively the results of the present study suggest that the LYPLA1 gene plays a tumor-promotor role in NSCLC cells *in vitro*.

Introduction

Lung cancer is one of the leading causes of cancer-related mortality in developing as well as developed countries (1). The cancer registration report of China published in 2013 affirmed that the incidence and death caused by lung cancer ranked as first in urban as well as rural areas of China (2). Cigarette smoking either by active or passive inhalation is considered as the most significant cause of various types of cancers, and it is well recognized that cigarette smoking leads to a high risk of lung cancer (3-5). The two main pathological subtypes of lung cancer are small cell lung cancer and non-small cell lung cancer (NSCLC), the latter accounts for approximately 85% of all clinically diagnosed cases of lung cancer (6). In recent decades, there have been great improvements and achievements in the field of lung cancer treatment which have led to the understanding of interactions between cancer cells and the immune system and the development of new therapeutic strategies that reinforce the body's immune response to mediate antitumor immunity (7). The mainstay of treatment comprises surgery, chemotherapy, radiation therapy and targeted therapy. The progress in the field of molecular profiling and the utilization of next generation sequencing in the evaluation of patients with advanced stage NSCLC has changed the approach of NSCLC treatment. The discovery of driver mutations that interact with the epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK) genes, has already been successfully implemented in clinical practice (8,9). Consequently effective treatments are available for novel targets such as HER2, ROS1, RET, BRAF and MET (10). Nevertheless, the prognosis still remains poor, with a 5-year survival rate of approximately 15%. Unfortunately, the survival rate further declines to 5% in the case of metastatic conditions, which reflect the limitations in the outcome of current treatment modalities (11). Most patients seek profession health care only upon the presentation of intensified symptoms. Hence early diagnosis is deferred. The stage at the time of diagnosis determines the

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Abbreviations: NSCLC, non-small cell lung cancer; LYPLA1, lysophospholipase1; EMT, epithelial-mesenchymal transition; FBS, fetal bovine serum

Key words: NSCLC, LYPLA1, non-small cell lung cancer cell lines, epithelial-mesenchymal transition, transfection, invasion, migration

prognosis for lung cancer. It is very essential to interpret the factors influencing the screening outcomes of lung cancer in order to provide the most appropriate treatment (12-15). Therefore, it is necessary to identify and consider the factors associated with the initiation and progression of NSCLC. The transformation of normally functioning lung tissues into malfunctioning cancerous tissues involves many genetic as well as epigenetic factors.

Lysophospholipases (LYPLAs) also known as acyl-protein thioesterases (APTs) are enzymes identified to have action on biological membranes to regulate lysophospholipids. The protein encoded by LYPLAs is known to hydrolyze lysophosphatidylcholine in both monomeric and micellar structures. Moreover, LYPLAs are cytosolic enzymes that are capable of catalyzing depalmitoylation (16). LYPLAs are under the control of both microRNAs, miR-138 and -424; hence, LYPLAs were observed to be significantly overexpressed in chronic lymphocytic leukemia (CLL) cells. To date, only LYPLAs are recognized to activate depalmitoylation. Some studies have revealed that LYPLAs can directly interact with CD95 to stimulate depalmitoylation, thereby regulating apoptosis through CD95. The specific inhibition of LYPLA activity by siRNAs, their combined treatment with miR-138/-424 and therapeutic access cause increased CD95-mediated apoptosis in CLL cells and other cancer cells, which may explain a pivotal regulatory character of LYPLAs in CD95 apoptosis (17). The process involved in protein palmitoylation is understood as a potent post-translational conversion, where the addition of a 16-carbon saturated fatty acid (palmitate) to cysteines of proteins modulates protein sorting, targeting and signaling (18). LYPLAs are well known to mediate the palmitate removal from proteins. LYPLAs are known to be involved in several potential mechanisms. To date, palmitoylation is the only identified reversible lipid modification. Until now, proteomic analyses have recognized 1,000 proteins as palmitoylated. These proteins were observed to be involved in specific biological mechanisms, such as differentiation, activation, immune responses, proliferation and migration (19,20). When palmitate is added to the specific protein, it takes control of the membrane transition and trafficking, and it can also regulate protein-protein interaction as well as enzyme activity. Since palmitoylation is a reversible transformation, the mechanism is crucial for the regulation of protein activity. Protein acyltransferases and protein acyl-thioesterases are known to regulate palmitoylation. LYPLA1 is the first cytosolic thioesterase to be characterized, which is capable of catalyzing depalmitoylation of the α -subunit of G-proteins and proto-oncogene H-Ras product *in vitro* (21). A study on protein acyltransferases Erf2/Erf4 and Akr1 in yeast provided more information regarding the palmitoylation reaction. These molecules interact with thioesterases such as lysophospholipase1, where it can alter the condition of palmitoylation in several signaling molecules, finally affecting their activity (22).

Lung cancer cell lines have been widely used in lung cancer translational research and biomedical discovery. Previously, it was misconstrued that tumor cells might lose their differentiation properties during cell culture. Surprisingly, it was later proven that the differentiation results from stromal cell overgrowth, and the cancer cell cultures

often retain this property (23). Thus, cancer cell line cultures accurately represent cancer cells *in vivo* without the complex *in vivo* environment. The entire driver mutations present in lung cancer cells are also represented in the large bank of lung cancer cell lines that are available for the study of lung cancer pathogenesis. The relevance of cell lines for biomedical studies is dependent on how closely they resemble the tumors from which they were derived (24,25).

However, the biological mechanisms of LYPLA1 in NSCLC are widely unknown. In this present study, we explored the role of the *LYPLA1* gene in NSCLC. We explored the expression pattern of LYPLA1 in NSCLC cell lines *in vitro*. Furthermore, we demonstrated that suppression of LYPLA1 using specific shRNA inhibited cell proliferation, migration, and invasion in NSCLC cell lines *in vitro*.

Materials and methods

Cell lines and cell culture. Four human NSCLC cell lines, SPC-A-1, NCI-H1299, NCI-H1650 and A549, were purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Science (CBTCCAS, Shanghai, China). These cell lines were cultured using RPMI-1640 basic medium (cat. no. 72400047; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) along with 10% fetal bovine serum (FBS; cat. no. 10099141; Gibco; Thermo Fisher Scientific, Inc.), 2 mM L-glutamine (cat. no. G3126; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 100 U/ml penicillin/streptomycin (cat. no. 0513; ScienCell Research Laboratories, Carlsbad, CA, USA) combination and were maintained under a humidified atmosphere of 5% CO₂ at 37.8°C.

Extraction of RNA and real-time PCR (RT-PCR). The cells were harvested and the total RNA was extracted using RNAiso Plus (Takara Bio, Inc., Otsu, Japan) and further reverse transcribed into cDNA using the Prime Script RT reagent kit (Takara Bio, Inc.), using procedures executed according to the manufacturer's instructions. Then we performed RT-PCR using ABI 7500 FAST Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) and an SYBR Green Master Mix (Takara Biotechnology Co., Ltd., Dalian, China). The gene expression levels were determined using the 2^{- $\Delta\Delta C_q$} method (26) after normalizing to a standard reference GAPDH. The experiment was performed thrice to eliminate any experimental inaccuracy. The primer sequences in RT-PCR were as follows: LYPLA1 forward, 5'-ATACTGCCC TTACCACACAG-3' and LYPLA1 reverse, 5'-GTCACATTG GCTGGATTTCAC-3'; GAPDH forward, 5'-GGTAGACAA GTTCCCTT-3' and GAPDH reverse, 5'-ATATGTTCTGGA TGATTCT-3'.

Western blot analysis. The cells were harvested and total protein was extracted from the cells using RIPA lysis buffer (P0013B; Beyotime Institute of Biotechnology, Nantong, China) after 48 h following transfection and the protein concentration was determined using the BSA method (Beyotime Institute of Biotechnology). A total of 50 μ g of protein was extracted and placed into each wells of 10% SDS-polyacrylamide gels. Following electrophoresis, the gels were transferred to polyvinylidene fluoride (PVDF) membranes. The membranes

were then blocked using 5% skim milk powder in TBST and incubated overnight with the appropriate primary antibody. On the following day, the membranes were washed thrice with TBST and incubated with the appropriate secondary antibody (cat. nos. A0208 and A0216; Beyotime Institute of Biotechnology) at 1:5,000 dilution for 1 h at room temperature. Protein detection was performed using the enhanced chemiluminescence (ECL) system (Millipore, Bedford, MA, USA). Primary antibodies used were as follows: Anti-GAPDH (dilution 1:1,000; cat. no. 8884; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-LYPLA1 (dilution 1:1,000; cat. no. ab91606; Abcam, Cambridge, UK), anti-E-cadherin (dilution 1:50; cat. no. ab1416; Abcam), anti-vimentin (dilution 1:1,000; cat. no. ab92547; Abcam), anti-N-cadherin (dilution 1:5,000; cat. no. ab76011; Abcam) and anti-SNAIL (dilution 1:2,000; cat. no. ab85936; Abcam). Image Lab™ software (version 5.0; Bio-Rad Laboratories, Hercules, CA, USA) was used for densitometry.

Transfection and construction of stable cell lines. For silencing the expression of the LYPLA1 gene, we implemented three small-interfering RNAs targeting human LYPLA1 mRNA, designated as follows: shLYPLA1: shRNA-1 sense: 5'-CGG TGGTGCTAATAGAGATAT-3'; shRNA-2 sense: 5'-CAAGAA GTGAAGAATGGCATT-3'; shRNA-3 sense: 5'-CTATGC CTTTCATGGTTTGATA-3' and the negative control duplex, shControl, sense: 5'-TTCTCCGAACGTGTCACGT-3'. The RNA duplexes were purchased from Biomics Biotechnologies Co., Ltd. (Nantong, China). The Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used for transfection.

All three shRNAs exhibited a notable magnitude of silencing efficiency; however, shRNA-1 exhibited the highest silencing efficiency as determined by western blot analysis. Thus we conducted further experiments with shRNA-1 alone. shRNA-1 sequence was bound with the pGPH1/GFP/NEO vector. Furthermore, we cloned full-length LYPLA1 cDNA into the pCMV6/AC/GFP vector. GFP testing was performed to screen all the cell lines. Cell culture was carried out using medium contained 200 mg/ml G418 (Invitrogen; Thermo Fisher Scientific, Inc.). We further isolated and replicated cell clones after 30 days. Stable cell lines with suppressed LYPLA1 expression were constructed and designated as A549 shLYPLA1 and SPC-A-1 shLYPLA1. Their corresponding controls were SPC-A-1 shControl and A549 shControl, respectively.

Cell proliferation/cell viability assay. The two NSCLC cell lines, SPC-A-1 and A549, were seeded into the 96-well plates at $\sim 5.3 \times 10^3$ cells/well and were incubated for 24 h. The cells were transfected using RNA duplex (shLYPLA1 or shControl) for 4 days at an ultimate concentration of 50 nM. Every 24 h, the medium was replaced with WST-8/CCK-8 (Dojindo Laboratories, Kumamoto, Japan). Every time, after incubating for 1 h at 37.8°C, the absorbance was quantified using a spectrophotometer at 450 nm with MRX II absorbance reader (Dynex Technologies, Inc., Chantilly, VA, USA).

Cell migration and invasion assays. Transwell chambers (Millipore) were used to perform cell migration and invasion

assays. For the invasion assay, cell culture inserts were nested in the culture plates, and each insert was pre-coated on the upper-surface using Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). After completing cell transfection, $\sim 8.3 \times 10^4$ cells from each group were collected and placed in serum-free medium (0.2 ml) and added to the upper-surface of the gel. After that, 0.6 ml RPMI-1640 with 10% FBS was added to the lower compartment to function as a chemoattractant. After incubation at 37.8°C for 24 h, the remaining cells on the upper surface of the membrane were carefully removed using a clean cotton swab, and cells that reached the lower surface of the membrane were fixed using 100% methanol, and 0.3% crystal violet was used for staining. To quantify the magnitude of invasion and migration, we captured images from five random visual fields (total magnification, x200) for each insert and cell counting was performed under an Olympus BX41 light microscope (Olympus Corp., Tokyo, Japan). The representative images are displayed.

Statistical analysis. All data are expressed as the mean \pm standard deviation (SD). We applied Student's t-test (two-tailed) to calculate the significance between groups, and ANOVA was performed to evaluate the difference in shRNA1, shRNA2 and shRNA3 in regards to the Control followed by a Turkey's post hoc test. SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA) was used for data analyses and a two-tailed value of $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of LYPLA1 in NSCLC cell lines. To determine the expression of the LYPLA1 gene in NSCLC, we first examined the expression of LYPLA1 at the protein level using western blot analysis in four different NSCLC cell lines (SPC-A-1, H1299, H1650 and A549). As demonstrated in Fig. 1A and B, the highest LYPLA1 expression level was detected in SPC-A-1 cells followed by A549 cells. Furthermore, we examined the expression of LYPLA1 at the mRNA level by RT-PCR in the four NSCLC cell lines (Fig. 1C). The highest LYPLA1 expression level was detected in the SPC-A-1 cells followed by the H1299 cell line. However, LYPLA1 expression was lacking in the H1650 cell line at both the protein and mRNA levels.

Efficiency of cell transfection. SPC-A-1 cells were cultured and further transfected using three different types of shLYPLA1 (shRNA-1, shRNA-2 and shRNA-3) and shControl cells. Western blot analysis was performed to examine the shLYPLA1 expression at the protein level, which confirmed reduced LYPLA1 protein expression in all three shLYPLA1-transfected cells compared to the shControl cells (Fig. 2A and B). shRNA-1 was detected to have the highest silencing efficiency among the three shRNAs (fold change 0.63 vs. 1.52, shRNA-1 vs. shControl cells). Thus, shRNA-1 was used for the further loss-of-function experiments.

Suppression of LYPLA1 inhibits cell proliferation, migration and invasion in vitro. To ascertain the role of LYPLA1 in cell proliferation, we performed the CCK-8 assay using

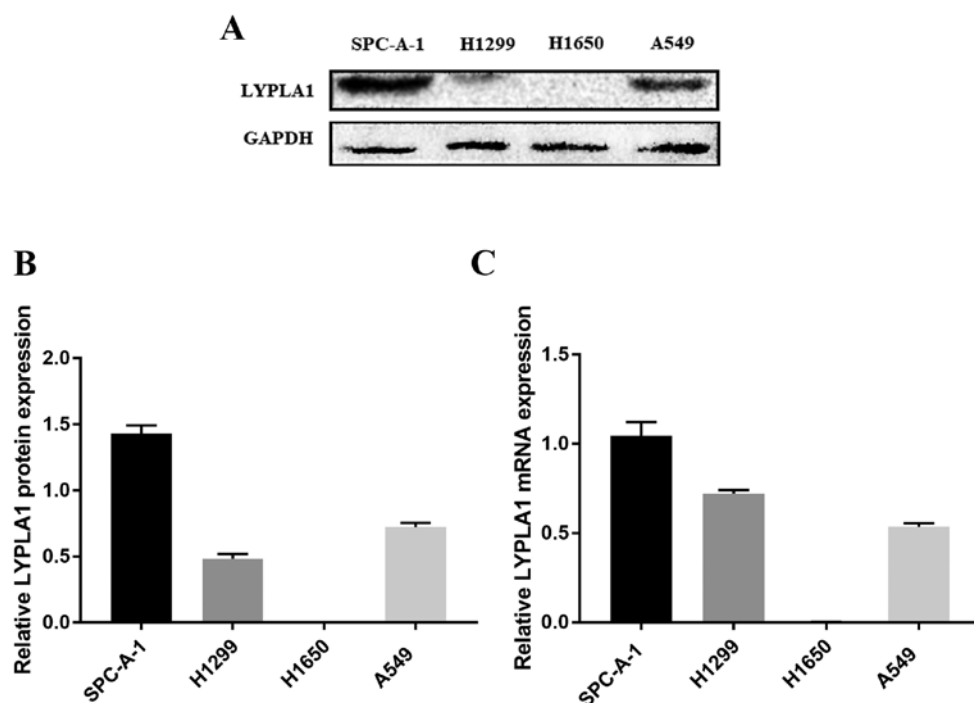


Figure 1. Expression of LYPLA1 in NSCLC cell lines. (A) Western blot analysis was utilized to determine the relative protein expression levels of LYPLA1 in four NSCLC cell lines, SPC-A-1, H1299, H1650 and A549. (B) A graph representing the relative protein expression levels of LYPLA1 in four NSCLC cell lines. (C) A graph representing the relative mRNA expression levels of LYPLA1 in four NSCLC cell lines. NSCLC, non-small cell lung cancer; LYPLA1, lysophospholipase 1.

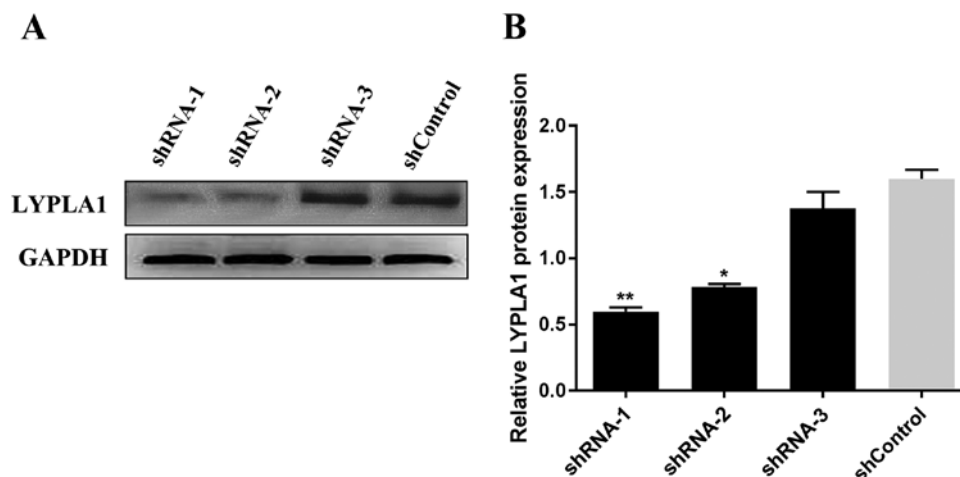


Figure 2. Assessment of the silencing efficiency of the shRNAs in the shLYPLA1-transfected cells. (A) Western blot analysis was utilized to identify the most efficient shRNA in the SPC-A-1 cells transfected with the three shRNAs against LYPLA1. (B) A graph representing the relative protein expression levels of LYPLA1 in the three different shLYPLA1-transfected cells compared with the shControl cells. The data are presented as mean \pm SD; n=5-8/group. *P<0.05, **P<0.01 vs. the shControl group. LYPLA1, lysophospholipase 1.

SPC-A-1 and A549 cells. As demonstrated in Fig. 3A and B, compared with the control and normal cells, the CCK-8 assay revealed that suppression of LYPLA1 by shLYPLA1 effectively inhibited the proliferation ability in the transfected cells in a time-dependent manner. Furthermore, to understand whether LYPLA1 plays a role in cell migration and invasion we performed Transwell assays with SPC-A-1 and A549 cells. These two cell lines were transfected either with shControl or shRNA-1. G418 was used to construct the two stable LYPLA1-silenced cell lines. Matrigel invasion assay confirmed that invasiveness was markedly decreased in the

shLYPLA1-transfected SPCA-1 and A549 cells compared with the control cells and normal cells (Fig. 3C and D). Likewise, migration assays also revealed that cell motility was markedly decreased in the shLYPLA1-transfected SPCA-1 and A549 cells compared with the control and normal cells (Fig. 3E and F).

Suppression of LYPLA1 regulates EMT markers in vitro. Western blot analysis was performed to understand the effects of LYPLA1 on EMT markers (E-cadherin, N-cadherin, vimentin and SNAIL) in SPC-A-1 cells. Results of the

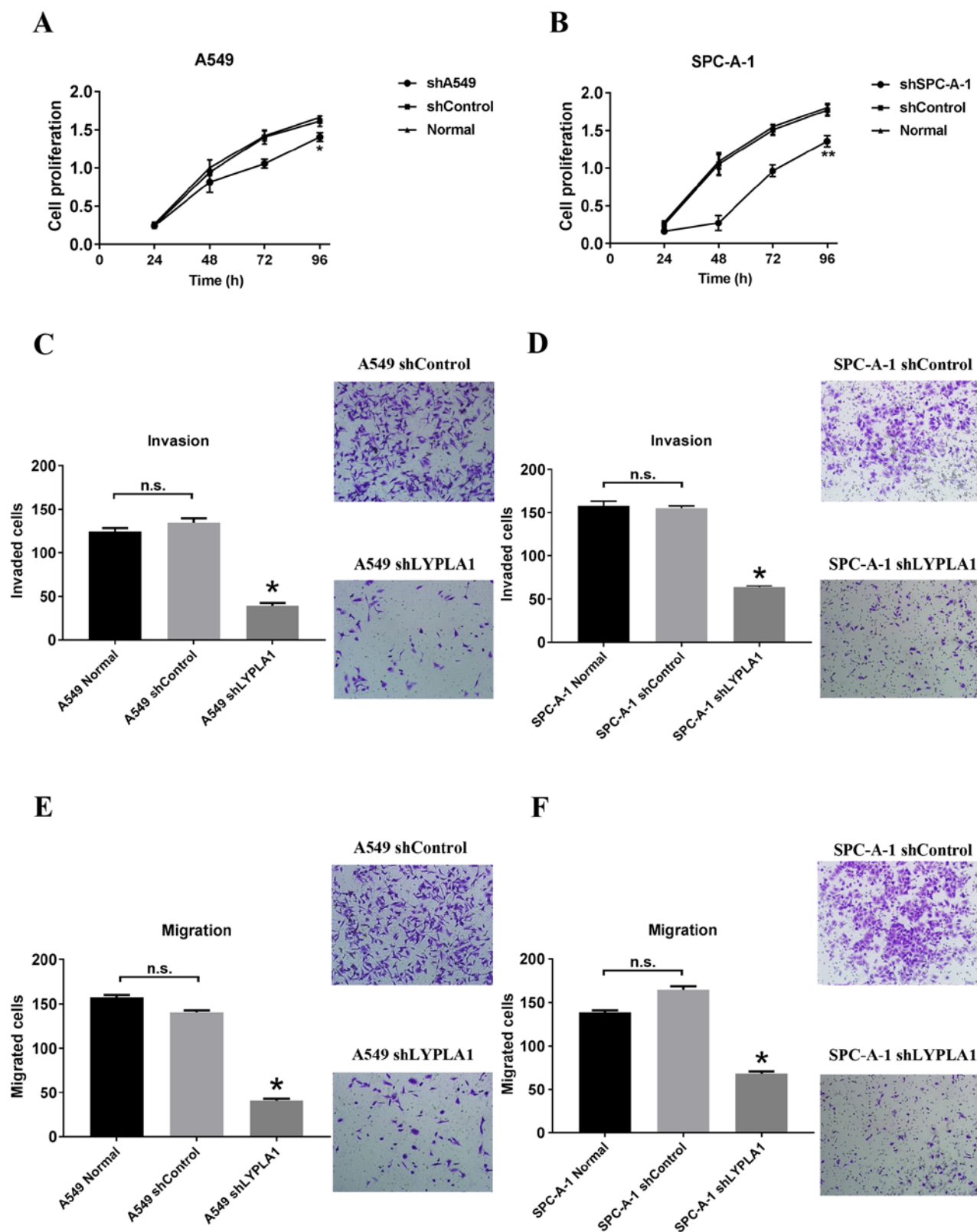


Figure 3. Effect of LYPLA1 in regulating NSCLC cell proliferation and motility *in vitro*. (A and B) CCK-8 assay demonstrated that suppression of LYPLA1 by shLYPLA1 effectively inhibited the tumorigenic ability in SPC-A-1 and A549 cells, respectively. Transwell assays showing the (C and D) cell invasion and (E and F) cell migration in shLYPLA1-transfected (C and E) A549 and (D and F) SPC-A-1 cells compared with the control and normal cells. The data are presented as means \pm SD; n=5-8/group. *P<0.05, **P<0.01 vs. the shControl group. n.s., not significant; NSCLC, non-small cell lung cancer; LYPLA1, lysophospholipase 1.

western blot analysis revealed that, following shLYPLA1 transfection using shRNA-1 in the SPC-A-1 cells, the protein levels of E-cadherin (epithelial marker) was significantly

increased, while the protein levels of N-cadherin, vimentin, and SNAIL (mesenchymal markers) were decreased in the LYPLA1-silenced cells compared with the control and normal

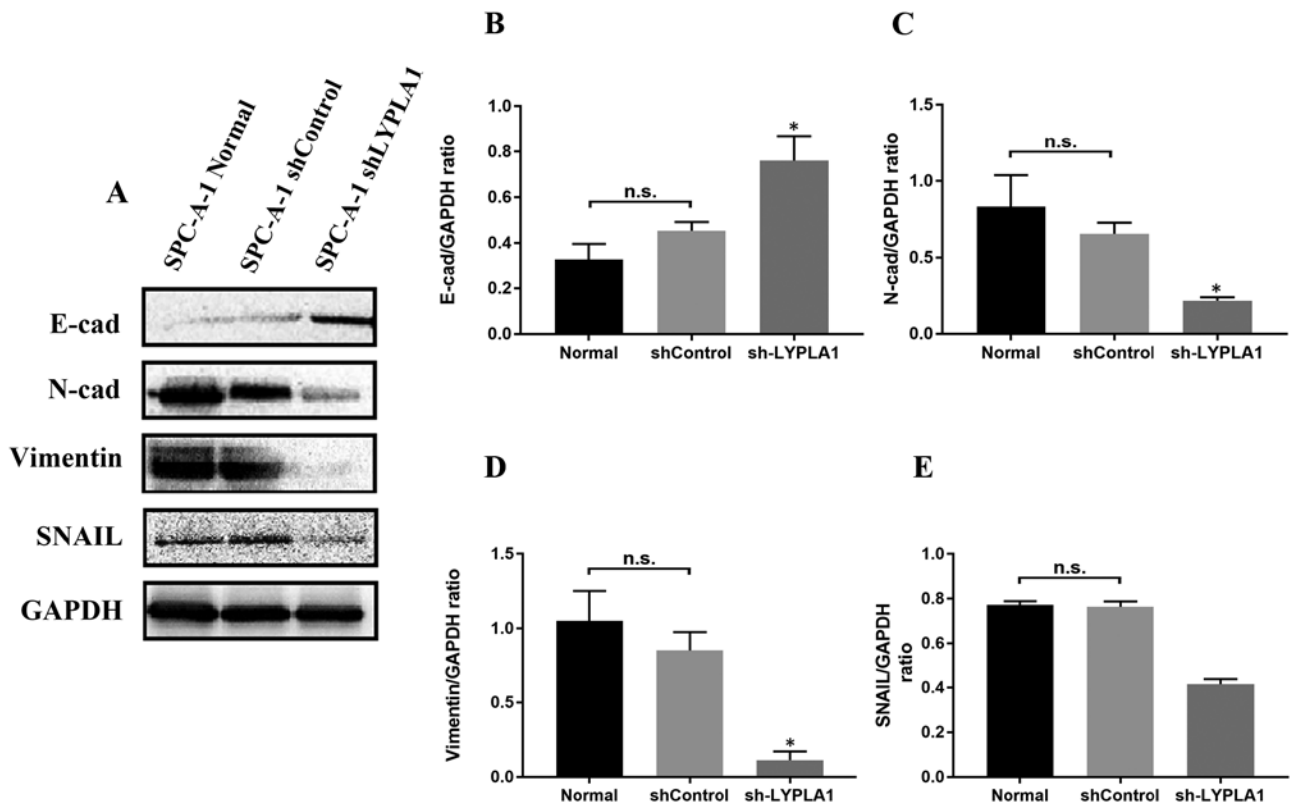


Figure 4. Suppression of LYPLA1 regulates EMT markers. (A) Western blot analysis of the expression of EMT markers (E-cad, N-cad, vimentin and SNAIL) in LYPLA1-silenced SPC-A-1 cells. (B-E) Graphs representing relative protein expression levels of each EMT marker compared with the control and normal cells. The internal standard used for loading was GAPDH. The data are presented as means \pm SD; n=5-8/group. *P<0.05 vs. shControl group. E-cad, E-cadherin; N-cad, N-cadherin; n.s., not significant; EMT, epithelial-mesenchymal transition; LYPLA1, lysophospholipase 1.

cells *in vitro* (Fig. 4A-E). The protein expression levels were determined to check the magnitude of outcomes.

Discussion

Acyl-protein thioesterase 1 otherwise known as lysophospholipase1 (LYPLA1) belongs to the superfamily of the α/β hydrolase. The encoded protein has been identified to undertake the function of a homodimer by exhibiting depalmitoylation as well as lysophospholipase activity (27). Despite the fact that palmitoylation is necessary for membrane localization as well as the functioning of certain proteins, depalmitoylation is conjointly necessary for lysosomal hydrolases for recycling or degradation. Therefore, dynamic palmitoylation has been recognized as a crucial mechanism for controlling the function of several significant proteins, such as the α -subunit of G-proteins and the product of the proto-oncogene H-Ras (28-31).

Even though the current approach to lung cancer, especially NSCLC, is very advanced and effective, most of the lung cancers exhibit a high incidence of recurrence and have been recognized to develop resistance (32). Radiotherapy and chemotherapy have been extensively employed in the treatment of many cancers. Radiotherapy uses high-energy ionizing radiation to shrink and eventually execute cancerous cells, and likewise, chemotherapeutic drugs induce apoptosis and promote cell death. However, the efficacy of radiotherapy is limited due to the survival of concealed cancer cells or

metastasis to distant organs, subsequently resulting in recurrence (33). Thus, the invention of potent drugs to alleviate such chemo- or radio-resistance is essential for the successful treatment and recovery of NSCLC. The process of transformation of normally functioning lung tissues into malignant tissues in NSCLC involves many different factors and numerous steps with the involvement of multiple genes. Such activities are characterized by alteration at the cellular, genetic, and epigenetic levels and abnormal cell division (34). The underlining cause for tumorigenesis is to date understood either as the downregulation of tumor-suppressor genes or the upregulation of oncogenes, which involves single or multiple genes (35).

The genetic involvement of LYPLA1 in human cancer remains unexplored. In the present study, we demonstrated that the suppression of LYPLA1 gene expression inhibited cell proliferation, migration and invasion *in vitro* of NSCLC cell lines. Furthermore, we explored the role of LYPLA1 in the regulation of epithelial-mesenchymal transition (EMT) markers. EMT is considered as a crucial phenotypic conversion which has been identified to promote metastasis in epithelium-derived cancers (36-38). During this process, cancer cells are observed to give up their epithelial structures and adopt plastic and high motile mesenchymal properties. Likewise, during the process of oncogenesis, epithelial cancer cells experience EMT and exhibit highly invasive and metastatic changes. Henceforth, EMT is considered as a unique phenotypic key for invasion and metastasis (39). The hallmark of EMT includes the decrease in expression of epithelial

markers such as E-cadherin and the increase in expression of mesenchymal markers such as vimentin (40). We observed that the expression of E-cadherin (epithelial marker) was significantly increased in the shLYPLA1-transfected cells. Loss of E-cadherin is considered as a significant response in EMT, where it modulates cellular dimensions and cell shape (41,42). We also found that the silencing of LYPLA1 markedly decreased the expression of N-cadherin, vimentin and SNAIL (mesenchymal markers). Taken together these results suggest that the LYPLA1 gene plays a tumor-promotion role in NSCLC cells *in vitro*. In recent years, several prognostic and predictive markers have been discovered to diagnose and predict the severity of NSCLC patients. To the best of our knowledge, this is the first study to identify the role of LYPLA1 in NSCLC.

Suppression of LYPLA1 using a specific shRNA was found to inhibit cell proliferation, migration and invasion *in vitro*, suggesting that LYPLA1 regulates NSCLC cell proliferation and motility. We believe that in our future research we can further elucidate the mechanisms and functions of the LYPLA1 gene, its involvement *in vivo* and whether it can be considered as a prognostic marker. In the present study, we observed that in the CCK-8 assays the suppression of LYPLA1 by shLYPLA1 successfully inhibited the cell proliferation in transfected cells at various time points, suggesting that LYPLA1 regulates NSCLC cell proliferation. Collectively, based on the findings of the present study, LYPLA1 might be an effective therapeutic target for NSCLC treatment.

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Availability of data and materials

All data and materials that were used in this study are included in this manuscript.

Authors' contributions

AM, CZ, SZ, QS and JL performed the experiments; ZT and HL conceived and designed the study and AM prepared and wrote the study. All authors read the manuscript in its entirety and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

All authors declare that they have no competing interests.

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