

# LYAR promotes the proliferation of non-small cell lung cancer and is associated with poor prognosis

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## Abstract

**Introduction.** The aim of the study was to investigate the clinical significance of Ly-1 antibody reactive clone (LYAR) in non-small-cell lung cancer (NSCLC).

**Material and methods.** The expressions of LYAR at the protein level in representative paired NSCLC tumor tissues and adjacent non-tumor tissues were measured by Western blot and immunohistochemistry. Kaplan-Meier method was used to calculate the survival curve of patients with NSCLC. Cell Counting Kit-8 assay and flow cytometry were used to estimate the cell proliferation and cell cycle, respectively. Terminal-deoxynucleotidyl-transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay was performed to detect cell apoptosis.

**Results.** LYAR was dramatically overexpressed in NSCLC tissues which were closely related to the survival of patients with NSCLC. In clinical studies, the expression of LYAR was related to the clinical stage, histological differentiation, and Ki-67 expression. A positive correlation was found between LYAR and Ki-67 expression by Spearman's correlation test. After serum starvation for 72 h, serum re-addition significantly increased the expression of LYAR, PCNA, and Cyclin A and promoted the cell cycle progression. LYAR knockdown inhibited the proliferation and induced the G0/G1 cell cycle arrest and apoptosis of A549 cells.

**Conclusions.** The present study revealed the clinical significance of LYAR in NSCLC. LYAR might serve as a tumor promoter in NSCLC progression by promoting the proliferation and inhibiting the apoptosis of NSCLC cells. Inhibiting the expression of LYAR was considered as a potential novel therapeutic strategy for NSCLC. (*Folia Histochemica et Cytophiologica* 2021, Vol. 59, No. 4, 282–290)

**Key words:** non-small-cell lung cancer; Ly-1 antibody reactive clone; A549 cells; cell cycle; prognosis

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## Introduction

Lung cancer is the leading cause of cancer-related mortality globally has an estimated 1.4 million deaths each year [1]. Non-small cell lung cancer (NSCLC) accounts for about 70–80% of all lung cancer types [2, 3]. Nearly 50% of patients with stage I NSCLC die within ten years after initial diagnosis [4]. The five-year survival rate for patients with NSCLC is merely 15% due to the clinical complexity [5, 6]. Therefore, a comprehensive understanding of the molecular mechanisms regarding NSCLC will contribute to the development of diagnostic technologies, new treatment methods, and preventive approaches [7].

Ly-1 antibody reactive clone (LYAR), a 45 kD nucleolar zinc finger protein, was firstly obtained from the T cell leukemia line cDNA library of a mouse [8]. It has been reported that LYAR overexpressing in fibroblast cells has increased the ability to form tumors in nu/nu mice [8]. In addition to its high expression in a variety of leukemia cell lines [8], LYAR was found to be a candidate gene for human neuroblastoma by using comparative genomic hybridization [9]. The previous study has shown that LYAR is a key regulator of cell migration and invasion in colorectal cancer [10]. A gene expression profile study in patients with ovarian cancer revealed that LYAR is a potential prognostic biomarker for ovarian cancer treatment and postoperative supportive therapies [11]. However, the potential function of LYAR in NSCLC is still unclear. Thus, the present study was performed to investigate the clinical significance of LYAR in NSCLC.

## Materials and methods

**Patients and tissue samples.** One hundred and twenty-one NSCLC samples were collected from patients who underwent salvage ectomy in Nantong Tumor Hospital and The Affiliated Suqian First People's Hospital of Nanjing Medical University from 2016 to 2017. The fresh samples were frozen in liquid nitrogen immediately and maintained at  $-80^{\circ}\text{C}$  until Western blot analysis. None of the patients was treated with preoperative therapies. Samples were classified according to the tumor-node-metastasis classification system [12]. This study was approved by the ethics committee of The Affiliated Suqian First People's Hospital of Nanjing Medical University. Written informed consents were obtained from each patient.

**Western blot.** Proteins were prepared from tumor tissue and cell cultures using RIPA lysis buffer.  $25\mu\text{g}$  total proteins were separated on polyacrylamide gels and then transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA). The membranes were blocked with 5% non-fat milk for about 2 h at room temperature and then were incubat-

ed with primary antibodies at  $4^{\circ}\text{C}$  overnight. The primary antibodies were as follows: anti-LYAR (Abgent, 1:1000), anti-PCNA (Santa Cruz Biotechnology, 1:1000), anti-GAPDH (Santa Cruz Biotechnology, 1:1000), anti-Cyclin A (Santa Cruz Biotechnology, 1:1000), and anti-Caspase 3 (Santa Cruz Biotechnology, 1:500). Subsequently, the membrane was incubated with corresponding rabbit horseradish peroxidase-linked secondary IgG antibodies (Amersham, Arlington Heights, IL, USA) for 2 h at room temperature, followed by detection with the enhanced chemiluminescence (Thermo Fisher Scientific, Inc.). The band density was measured by a computer-assisted image-analysis system (Imaging Technology, Ontario, Canada).

**Immunohistochemistry.** Tumor tissues and adjacent non-tumor tissues samples were fixed in 4% paraformaldehyde and embedded in the paraffin. Thereafter, tissues were cut into  $4\mu\text{m}$ -thick sections. Tissue sections were deparaffinized with xylene, rehydrated with graded ethanol followed by antigen retrieval. After the slices were cooled at room temperature, tissue sections were incubated with LYAR and Ki-67 primary antibodies at  $4^{\circ}\text{C}$  overnight and secondary antibodies for 2 h at room temperature, respectively. Following a final washing in phosphate-buffered saline (PBS), LYAR and Ki-67 signals were visualized with chromogen diaminobenzidine, counterstained with hematoxylin, and mounted in Kayser's glycerine gelatine. For semi-quantitative measurements of LYAR and Ki-67 density, the slides were photographed and analyzed using a computer-aided image-analyzing system (Motic Images Advanced 3.2).

**Immunohistochemical evaluation.** Immunostained sections were evaluated blinded without knowing the patient's clinical and pathological parameters. For the assessment of LYAR and Ki-67, more than 1000 cells from five high-power fields in each section were chosen randomly. Staining was measured by the percentage of the immunostained cells related to the total number of cells. For the evaluation of the LYAR protein immunoreaction, staining intense was classified as 1 (negatively or poorly staining), 2 (moderately staining), or 3 (strongly staining). Percentage scores were assigned as 1 (negatively or poorly stained), 2 (moderately stained), and 3 (strongly stained). Percentage scores were assigned as 1 (1–25%), 2 (26–50%) and 3 (51–100%) for univariate and multivariate analyses. After multiplying the two scores, they were divided into two groups (high expression group and low expression group) according to the scores (average = 4.5) [13].

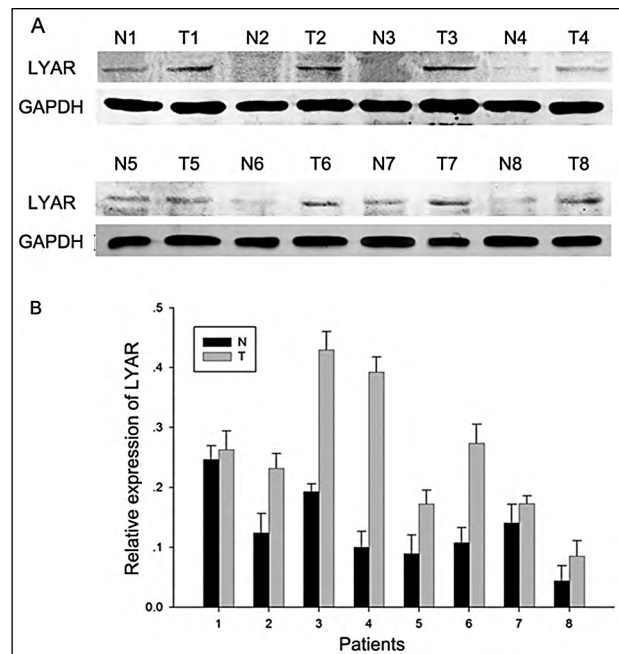
**Cell culture and transient transfection.** NSCLC cell line (A549) was purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in Nutrient Mixture F-12 Ham (Sigma-Aldrich, USA) supplemented with 2 mM glutamine, 10% fetal bovine serum (FBS), and 100 U/mL

penicillin-streptomycin mixture (GibCo BRL, Grand Island, NY) at 37°C and 5% CO<sub>2</sub>. Human bronchial epithelial cell line (BEAS-2B) was obtained from the Department of Pathology of Nantong University (Nantong, China) and were cultured in high-glucose DMEM (GibCo BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin-streptomycin mixture at 37°C and 5% CO<sub>2</sub>. Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to perform cell transfection according to the manufacturer's protocol. The knockdown of LYAR in A549 cells was induced using 2 µg short hairpin RNA (shRNA, Gene Chem technology) against LYAR, using stable non-specific shRNA (2 µg) as the negative control (shRNA-NC). The shRNA-NC sequence was 5'-GAAGCCTAAGAAGCGCAA-3' and 5'-AACAGCGAACCAGTCAATA-3'. The LYAR-shRNA3# sequence was 5'-AGAACCCTACCTTTAAGTT-3' and 5'-TGGTCATCTTTAACAAGAA-3'. Cells were transfected for 24 h at 37°C in an atmosphere containing 5% CO<sub>2</sub> to obtain stably transfected cells for future use. The knockdown of LYAR was confirmed using Western blotting.

**Cell viability assay.** According to the manufacturer's introduction, a cell viability assay was carried out using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Briefly, cells (2 × 10<sup>4</sup> cells/well) were seeded in a 96-well plate with 100 µL culture medium and incubated in a CO<sub>2</sub> incubator at 37°C overnight. Then, the Counting Kit-8 reagent (Dojindo, Japan) was added and cells were incubated at 37°C for 2 h. Finally, absorbance was recorded at 450 nm using a microplate reader (BioRad, Hercules, CA, USA).

**Flow cytometry.** For cell cycle analysis, A549 cells were seeded at a density of 2.5 × 10<sup>5</sup> cells per well in 24-well plates. After incubation for 24 h, the cells were collected and fixed with cold ethanol (70% v/v, -20°C, 30 min). Then, cells were incubated with RNase A (1 mg/mL, 30 min at 37°C). Subsequently, cells were stained with 50 µg/mL propidium iodide and analyzed using a Becton-Dickinson flow cytometer BD FACScan (CA, USA).

**Terminal-deoxynucleotidyl-transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay.** TUNEL assay was performed as described previously [14]. A549 cells were seeded at a density of 2.5 × 10<sup>5</sup> cells per well in 24-well plates and incubated for 24 h. Then, cells were transfected with shRNA-LYAR or shRNA-NC for another 48 h. Subsequently, cells were subjected to TUNEL staining by using an In Situ Cell Death Detection Kit (Roche, Mannheim, Germany). Cells were fixed in paraformaldehyde (4%, 4°C for 30 min), and then treated with 0.1% TritonX-100, labeled with fluorescein-12-dUTP using terminal deoxynucleotidyl transferase. Images were



**Figure 1.** LYAR protein expression was increased in NSCLC tissues. **A.** Western blot was performed to determine the expression of LYAR in eight representatives paired NSCLC tumor tissues (T) and adjacent non-tumor tissues (N). **B.** Quantification of the expression of LYAR in eight representatives paired NSCLC tumor tissues (T) and adjacent non-tumor tissues (N). NSCLC, non-small cell lung cancer; LYAR, Ly-1 antibody reactive clone.

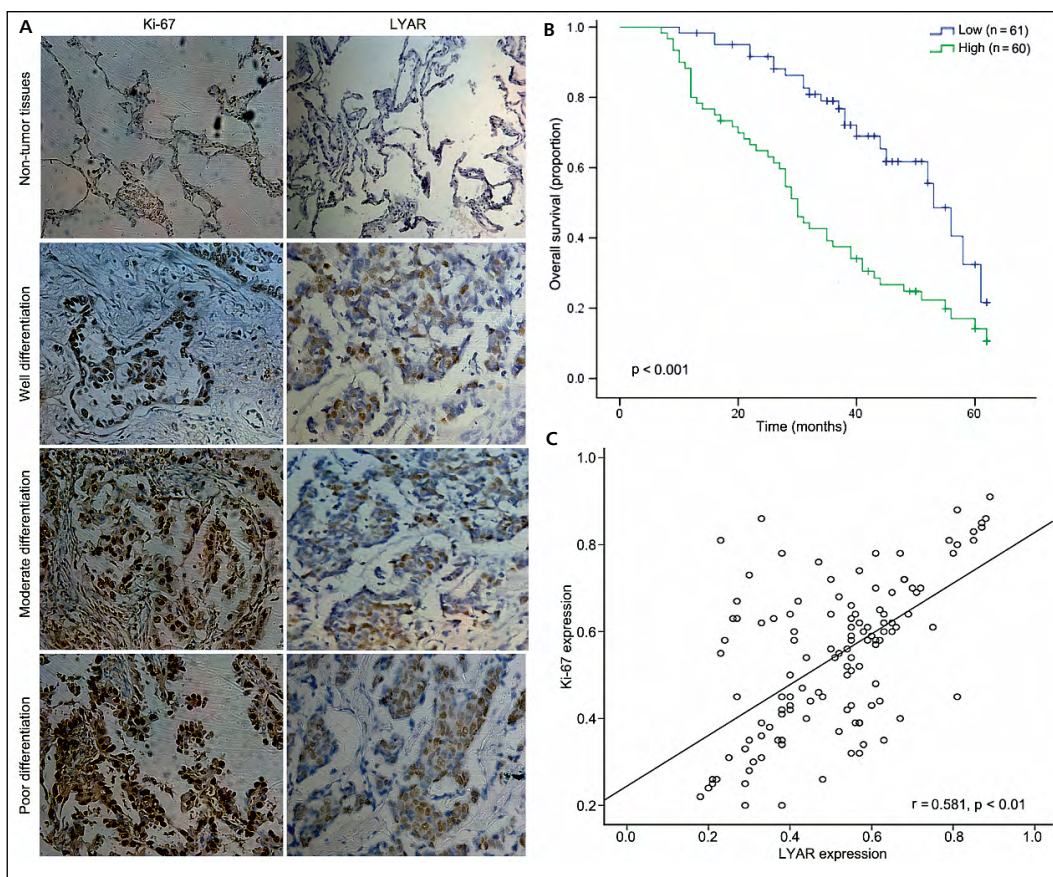
detected by fluorescence microscopy (Leica, DM 5000B; LeicaCTR 5000; Wetzlar, Germany).

**Statistical analysis.** SPSS version 13.0 (SPSS Institute, IL, USA) was used for statistical analyses. Quantitative data were expressed as means ± standard deviations (SD) and compared by Student's *t*-test. Qualitative data were expressed as numbers and percentages, and were compared using the  $\chi^2$  test. Kaplan-Meier method was used to calculate the survival curve. Spearman's correlation test was used for the correlation analysis among the LYAR and Ki-67 expression. Cox's proportional hazards model was used to perform multivariate analysis. Statistical significance was set at  $p < 0.05$ .

## Results

### *LYAR expression was increased in NSCLC tissues*

To explore the relationship between LYAR and NSCLC, we first examined the expression of LYAR at protein levels at eight NSCLC tissues and their adjacent noncancerous tissues by Western blot. As shown in Figure 1, the expression of LYAR was dramatically



**Figure 2.** LYAR immunoreactivity was correlated with Ki-67 and NSCLC patients’ survival. **A.** Representative images of immunohistochemical staining of LYAR and Ki-67 in 121 NSCLC tissues and adjacent non-tumor tissues (200×). **B.** LYAR protein overexpression predicts a poor prognosis for NSCLC patients. **C.** Correlation analysis between LYAR and Ki-67 expression. Data were presented as mean ± SD. NSCLC, non-small cell lung cancer; LYAR, Ly-1 antibody reactive clone.

increased in NSCLC tissues compared with normal noncancerous tissues.

**LYAR expression was correlated with Ki-67 and NSCLC patients’ survival**

To explore the clinic significance of LYAR in NSCLC, 121 NSCLC samples and their adjacent noncancerous tissues were selected. The expression of LYAR and Ki-67 (a cell proliferation-associated nuclear marker) was determined by immunohistochemistry (Fig. 2A). Immunoreactivity of both LYAR and Ki-67 were seen predominantly in the nucleus. As expected, the expression of LYAR and Ki67 were up-regulated in NSCLC tissues compared with their adjacent noncancerous tissues. In addition, the expression of LYAR in poorly differentiated samples was higher than that in well-differentiated samples, which was consistent with Ki-67.

The histopathological and clinical characteristics of the 121 NSCLC patients were shown in Table 1. In

consistence with the results shown in Figure 2A, the expression of LYAR was related to the clinical stage, histological differentiation, and Ki-67 expression (all  $p < 0.05$ , Table 1). In addition, Kaplan-Meier analysis showed that the overall survival rate in patients with LYAR high expression was significantly lower than that with LYAR low expression ( $p < 0.05$ , Fig. 2B). Cox’s proportional hazard regression model showed that LYAR expression, tumor size, histological differentiation, and Ki-67 expression were independent prognostic factors for overall survival (Table 2).

In order to further confirm the correlation between LYAR and Ki-67 expression, Spearman’s correlation test was performed by a percentage of positive malignant cells. As shown in Figure 2C, a positive correlation was found between LYAR and Ki-67 expression ( $r = 0.581, p < 0.01$ ). Taken together, these results indicated that LYAR could predict poor prognosis of NSCLC, and may be involved in the proliferation of NSCLC cells.

**Table 1.** The characteristics and histopathological data of patients

Characteristic	Total	LYAR expression		p-value
		Low	High	
Age (year, n/%)				0.935
< 60	54 (44.63)	27 (22.31)	27 (22.31)	
≥ 60	67 (55.37)	34 (28.10)	33 (27.27)	
Gender (n, %)				0.085
Male	64 (52.89)	37 (30.58)	27 (22.31)	
Female	57 (47.11)	24 (19.84)	33 (27.27)	
Smoking (n, %)	20 (16.53)	1 (0.83)	19 (15.70)	0.150
Clinical stage (n, %)				0.007
I	77 (63.64)	47 (38.84)	30 (24.79)	
II	28 (23.14)	10 (8.26)	18 (14.88)	
III	16 (13.22)	4 (3.31)	12 (9.92)	
Tumor size (n, %)				0.000
< 3 cm	52 (42.98)	36 (29.75)	16 (13.22)	
≥ 3 cm	69 (57.02)	25 (20.66)	44 (36.36)	
Differentiation (n, %)				0.001
Well	14 (11.57)	1 (0.83)	13 (10.74)	
Moderate	81 (66.94)	43 (35.54)	38 (31.40)	
Poor	26 (21.49)	17 (14.05)	9 (7.44)	
Lymph node status (n, %)				0.003
0	80 (66.12)	48 (39.67)	32 (26.45)	
> 0	41 (33.88)	13 (10.74)	28 (23.14)	
Ki-67 expression (n, %)				0.000
Low	52 (42.98)	38 (31.40)	14 (11.57)	
High	69 (57.02)	23 (19.01)	46 (38.02)	

LYAR — Ly-1 antibody reactive clone

**Table 2.** Contribution of various potential prognostic factors to survival by Cox regression analysis in 121 NSCLC specimens

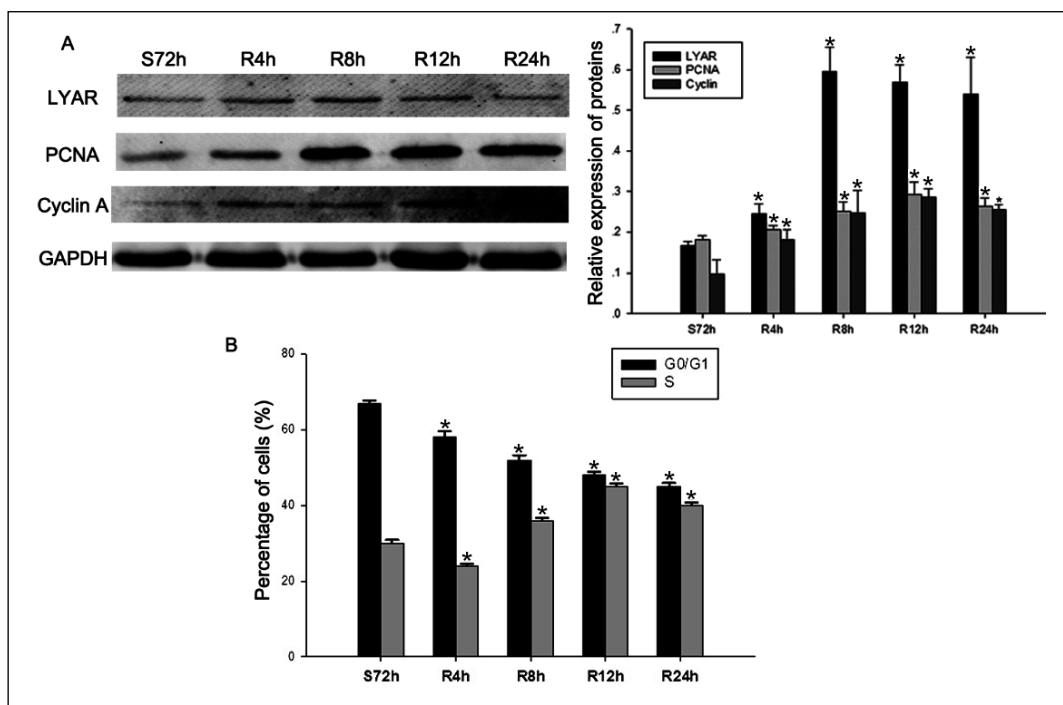
Parameters	Hazard ratio	95% confidence interval	p-value
Age	1.037	0.632–1.701	0.886
Gender	1.700	1.004–2.879	0.480
Tumor size	3.600	1.524–8.503	0.003
Clinical stage	1.513	0.905–2.528	0.114
Lymph node metastasis	1.475	0.851–2.555	0.166
Pathology grade	0.485	0.280–0.839	0.010
LYAR expression	1.931	1.084–3.439	0.026
Ki-67 expression	0.382	0.161–0.906	0.029

LYAR — Ly-1 antibody reactive clone

### ***LYAR affected the cell cycle progression of NSCLC cells***

Considering the correlation between LYAR and Ki-67 expression, we hypothesized that LYAR may be involved in the cell cycle progression of NSCLC

cells. A549 cells were cultured in a serum-free conditioned medium for 72 h and then recovered by adding a medium containing 10% FBS. Following that, a Western blot was performed to evaluate the expression of LYAR, along with PCNA (a marker



**Figure 3.** LYAR affected the cell cycle progression of NSCLC cells. **A.** Cells were synchronized at G0/G1 after serum starvation for 72 h (S72h), then progressed into the cell cycle by adding a medium containing 10% FBS for the indicated times (R4h, R8h, R12h, R24h). The expression of LYAR, PCNA, and cyclin A was analyzed by Western blot. **B.** Cell cycle distribution of A549 cells was analyzed by flow cytometry assay. Data were presented as mean  $\pm$  SD. NSCLC, non-small cell lung cancer; LYAR, Ly-1 antibody reactive clone; S, serum starvation; R, serum re-addition; \* $p < 0.05$ , compared with S72h.

of proliferation) and Cyclin A (a marker of the cell cycle) during cell cycle progression. As expected, the expression of LYAR significantly increased upon serum re-addition after serum starvation for 72 h, with a concomitant up-regulation of PCNA and Cyclin A (all  $p < 0.05$ , Fig. 3A).

Furthermore, a flow cytometric assay was used to analyze the cell cycle progression after serum deprivation and re-addition. The results showed that A549 cells were arrested in the G1 phase after serum deprivation for 72 h (Fig. 3B). After serum re-addition for 24 h, the number of A549 cells at the S phase increased gradually from 28.11% to 40.08% ( $p < 0.05$ ). The above results indicated that LYAR might have an impact on the cell cycle progression of NSCLC cells.

#### ***LYAR promoted the proliferation and inhibited the apoptosis of NSCLC cells***

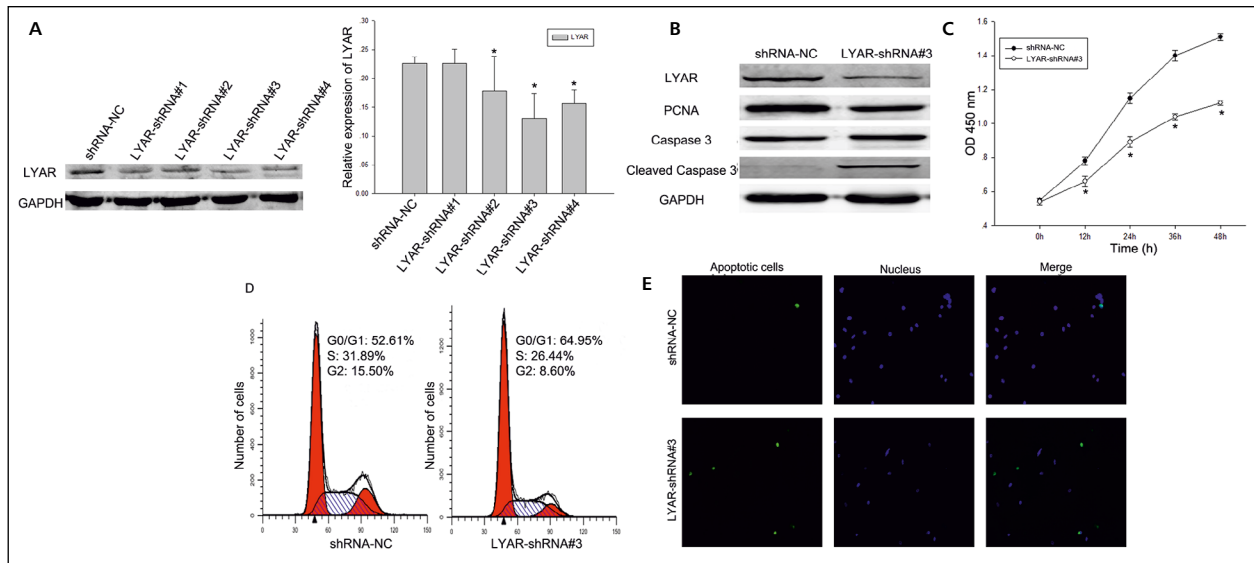
To further confirm the role of LYAR in the proliferation of NSCLC cells, A549 cells were selected to construct LYAR knocked-down cells. The efficiency of transfection was confirmed by Western blot. As shown in Figure 4A, LYAR-shRNA#3 transfected A549 cells showed the most significant decrease in LYAR expression ( $p < 0.05$ ). Therefore, the LYAR-shR-

NA#3 was used for the following experiments. Western blot showed that the knockdown of LYAR with shRNA#3 dramatically decreased the expression of PCNA and increased cleaved-Caspase3, compared with the shRNA-NC (Fig. 4B). CCK-8 assays were used to determine the effect of LYAR on cell proliferation. As shown in Fig. 4C, the proliferative ability of A549 cells transfected with LYAR-shRNA#3 was significantly inhibited compared with cells transfected with shRNA-NC ( $p < 0.05$ ). Furthermore, flow cytometric assay of A549 cells which were transfected with LYAR-shRNA#3 showed the number of cells at the G0/G1 phase increased from 52.61% to 64.95%, and the number of cells at the S phase decreased from 31.89% to 26.44% (Fig. 4D). TUNEL assay was used to explore the effect of LYAR on cell apoptosis. The result showed that the knockdown of LYAR induced the apoptosis of A549 cells (Fig. 4E). These results indicated that LYAR may promote the proliferation and inhibit the apoptosis of NSCLC cells.

#### **Discussion**

The 5-year survival rate of patients with NSCLC is still very low (about 15%), despite the advances in





**Figure 4.** LYAR promoted the proliferation and inhibited the apoptosis of NSCLC cells. **A.** Expression levels of LYAR in A549 cells transfected with shRNA-NC or LYAR-shRNA were determined using Western blot. **B.** Western blot analysis of LYAR, PCNA, Caspase 3, and cleaved-Caspase 3 expressions in A549 cells transfected with shRNA-NC or LYAR-shRNA#3. **C.** Cell proliferation was measured using a Cell Counting Kit-8 assay in A549 cells transfected with shRNA-NC or LYAR-shRNA#3. **D.** Cell cycle distribution was analyzed by flow cytometric assay in A549 cells transfected with shRNA-NC or LYAR-shRNA#3. **E.** Cell apoptosis was analyzed using a TUNEL assay in A549 cells transfected with shRNA-NC or LYAR-shRNA#3. Data were presented as mean  $\pm$  SD. NSCLC, non-small cell lung cancer; LYAR, Ly-1 antibody reactive clone; NC, negative control; shRNA, short hairpin RNA; \* $p < 0.05$ , compared with shRNA-NC.

the treatment of NSCLC recently [5, 6]. Therefore, detailed NSCLC progression researches are necessary for improving the prognosis of NSCLC. LYAR is a nuclear protein that consists of 379 amino acids with three copies of nuclear localization signals and a zinc-finger motif [15]. Evidence has shown that LYAR can increase the tumorigenicity of the fibroblast cells in nu/nu mice, and may function as a novel nucleolar oncoprotein in regulating cell growth [8]. In addition, several reports have shown that LYAR is involved in various types of cancer, such as colorectal cancer [10], ovarian cancer [11], and leukemia [8]. However, to the best of our knowledge, the role of LYAR in lung cancer is still unknown. The present study aimed to investigate the clinical significance of LYAR in NSCLC.

In the current study, we found that the expression level of LYAR in NSCLC tissues was higher than that in their adjacent noncancerous tissues. Kaplan-Meier analysis revealed that the high expression of LYAR predicted poor survival of NSCLC. These results were consistent with the report by Sun *et al.*, which demonstrated that high levels of LYAR expression in human neuroblastoma tissues predicted poor event-free and overall survival of patients with neuroblastoma [16]. The present study also showed that the LYAR expression showed a positive correlation

with the Ki-67 expression. Ki-67 was a nuclear protein expressed in the M, G1, S, and G2 phases of the cell cycle, but not in the G0 phase [17]. Thus, Ki-67 was usually used as a marker for the proliferation of various tumor cells [18]. Interestingly, evidence had shown that the LYAR gene was not expressed during the proliferation of some normal cells [8], suggesting that LYAR was associated with abnormal cell growth. Taken together, our results revealed that LYAR may be involved in the proliferation of NSCLC cells.

To further confirm the role of LYAR in cell proliferation, a series of *in vitro* experiments were designed. Serum starvation and re-addition experiments showed that LYAR expression was associated with cell proliferation, and LYAR knockdown in A549 cells inhibited the cell proliferation and induced the G0/G1 cell cycle arrest. Similarly, LYAR overexpression was found to promote the proliferation of NIH-3T3 cells (a fibroblast-like line derived from a murine embryo) [19]. These results indicated that LYAR may promote the proliferation of NSCLC cells. One possible mechanism for the role of LYAR in cell proliferation was maintaining the homeostasis level of nucleolin, which is a multifunctional RNA-binding protein [20]. It has been reported that LYAR could form a complex with nucleolin and prevent its self-cleavage to promote cell proliferation [21]. However, the mechanism linking

LYAR-mediated increase in cell proliferation needs to be determined in further investigations.

In conclusion, the present study revealed the clinical significance of LYAR in NSCLC. The results showed that LYAR was highly expressed in NSCLC tissues and closely correlated to the survival of patients with NSCLC. Additionally, a significantly positive correlation was found between LYAR and Ki-67 expressions. Finally, LYAR knockdown inhibited the proliferation and induced the G0/G1 cell cycle arrest and apoptosis of NSCLC cells. Taken together, our study demonstrated that LYAR might serve as a tumor promoter in NSCLC progression by promoting the proliferation and inhibiting the apoptosis of NSCLC cells. Inhibiting the expression of LYAR was considered as a potential novel therapeutic strategy for NSCLC.

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## Author contributions

Conception and design: Q-W Zang, X-N Lu, and G-J Ju; Administrative support: Q-W Zang; Materials and samples provided: Y-X Wang and Y-L Wang; Data collection and collation: K Wang and J-L Chen; Data analysis and interpretation: W Cai; Drafting article, X-N Lu and G-J Ju. All the authors have read and approved the final manuscript.

## Data availability statements

The data underlying this article are available in the article.

## Experimental ethics

This study was approved by the ethics committee of The Affiliated Suqian First People's Hospital of Nanjing Medical University.

## Conflict of interests

The authors declare that they have no conflict of interest.

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