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Original Article

Effect of lysozyme on invasion and migration of human lung carcinoma A549 cells

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Abstract: Objective: To investigate the effect of lysozyme (LYZ) on invasion and migration of the human lung carcinoma A549 cells. Methods: The LYZ knockdown lentivirus (LV3-LYZ) was used to silence the expression of LYZ gene. GFP fluorescence detector and Western bolting were used to detect the efficiency and effectiveness of gene silencing. Effect of LYZ expression silence on invasion ability of the A549 cells was detected by Transwell invasion assay; effect of LYZ expression silence on migration ability of the cells was detected by wound scratch assay and Transwell migration assay; effect of LYZ expression silence on cytoskeleton was detected by the phalloidin-labelled cytoskeleton assay; and effect of LYZ expression silence on the expression of RhoA and ROCK was detected by Western blotting. Results: Transfection with LV3-LYZ lentivirus could effectively inhibit the expression of LYZ protein. Silencing the expression of LYZ could inhibit invasion and migration of the human lung carcinoma A549 cells. The cytoskeleton staining results showed that compared with the LV3-NC group, F-actin staining of the A549 cells transfected with LV3-LYZ decreased significantly, and cell membrane ruffle and pseudopod formation were significantly reduced. Silencing the expression of LYZ could down-regulate the expression of RhoA and ROCK. Conclusion: Silencing the expression of LYZ inhibits invasion and migration ability of the lung carcinoma A549 cells by influencing cytoskeleton and down-regulating the expression of RhoA and ROCK.

Keywords: LYZ, lung carcinoma, western blotting, transwell, wound scratch assay, cytoskeleton

Introduction

Lung carcinoma is a malignancy with the highest morbidity and mortality across the world [1]. In recent years, although the effect of chemotherapy and radiotherapy is constantly enhancing with the improvement of surgical procedures, the five-year survival rate of patients with lung carcinoma, especially advanced lung carcinoma is still very low. Tumor metastasis is the main cause of death in the patients [2]. When tumor metastasis occurs, it will firstly reduce the adhesion of tumor cells and enhance cell mobility, and then infiltrate the adjacent tissues and induce distant metastasis [3]. At present, the molecular mechanisms of tumor metastasis is still unclear, which is a great obstacle for the treatment of tumors.

Lysozyme (LYZ) is a kind of alkaline enzyme encoded by LYZ gene that digests the mucopolysaccharides in pathogens. Enzymes are generally divided into five classes: (1) N-acetyl hexosaminidase. It is similar to hen egg white lysozyme and destroys the β-1.4 glucosidic bonds of peptidoglycan in bacterial cell wall; (2) lactamase. It cuts off the N-acetylmuramic acid-L-alanine bond between N-acetylglucosamine and peptide "tail" in peptidoglycan of bacterial cell wall; (3) endopeptidase. It cleaves the peptide bond at the peptide "tail" and peptide "bridge"; (4) β-1.3, β-1.6 glucanase and mannanase. This enzyme decomposes the walls of yeast cells; (5) chitinase. It is a lysozyme that decomposes the cell walls of molds.

Lysozyme has a strong antibacterial ability. It has better effect for killing G^+ bacteria, *bacillus subtilis*, etc., and dissolves to a certain extent G^- bacteria like *E. coli*. A number of studies showed that [4] the expression level of lysozyme increased in many chronic inflammations. Some studies also revealed that the expression level of lysozyme increased dramatically in gastric cancer, colorectal cancer and other malignancies, which was associated with tumor

invasion and metastasis [5, 6]. In this study, we investigate the effect of lysozyme in migration and invasion of lung carcinoma and explore its possible mechanism.

Materials and method

Cell line and main reagents

The human lung carcinoma cell line A549 was purchased from Shanghai Institute of Cells. The cell culture conditions were RPMI 1640 containing 10% fetal calf serum, cultured at 37°C, 5% CO_2. The fetal calf serum and RPMI 1640 medium were obtained from Gibco (US). RhoA, ROCK and LYZ rabbit monoclonal antibodies were from Abcam (US) (ab-187027, ab45171 and ab108508). Transwell chamber was purchased from Millipore (US), and Matrigel from BD (US). The LYZ silencing and control lentiviruses were purchased from Shanghai GenePharma Technology Co., Ltd. Rhodamine-labeled phalloidin was obtained from Sigma (US).

Lentivirus transfected into A549 cells

One day prior to the experiment, 5×10^3 A549 cells were inoculated to 96-well plates respectively, to make the cell confluency at 40%~60%. According to the GenePharma lentivirus operating manual, the appropriate MOI (Multiply of Infection) of LYZ lentivirus was detected at the concentration gradients of 0, 10 and 100.

1) Prepare two sterile EP tubes, draw 10 μl of 1×10^8 TU/ml virus to the first tube, gently mix well without foams. Similarly, draw 10 μl of virus from the first tube to the second tube and mix well, to get virus of three different gradients: stock solution, 10-fold dilution, and 100-fold dilution.

2) Add virus solutions at the three different gradients to three holes in each group, 10 μl each. Calculation showed that MOI of the three holes were 100, 10 and 1 respectively, and the optimal MOI measured was 100.

3) The experiment included three groups: silence group, control group and blank control group. In the silence group, LYZ silencing lentivirus (LV3-LYZ) was transfected; in the control group, the negative control (LV3-NC) was transfected; and in the blank control group, no treatment. The 100-fold diluted virus stock solution was added in the LV3-LYZ group. The 100-fold diluted negative control virus was added in the LV3-NC group. 24 h later, the GFP fluorescence expression was observed.

Expression of LYZ, RhoA and ROCK kinase proteins detected by western blotting

72 hours after cell transfection, the total proteins in the LV3-LYZ group, LV3-NC group and the blank control group were extracted, and the protein concentrations were determined by BCA method, and then loading buffer was added for protein denaturation. 8% and 12% SDS-PAGE were prepared, and 20 μg protein sample was added to each hole, then transferred to a PVDF membrane using the electric wet transfer method, sealed 2 h with 5% skim milk, and the primary antibody was diluted by 1:1000 TBST (LYZ, RhoA, ROCK), overnight at 4°C; then 1:5000 dilution of goat anti-rabbit secondary antibody was added, incubated at room temperature for 2 h; and ECL was performed. The experiment was performed in triplicate.

Effect of LYZ expression on lung carcinoma cell invasion ability by transwell invasion assay

All reagents and equipments were pre-cooled on ice. The Transwell chambers were placed in a 24-well plate. 50 μl Matrigel gel was evenly applied to inner membrane of Transwell chamber (0.2 μg/μl), incubated for 15 min at 37°C to solidify the gel; when digested, centrifuged and counted, cells were diluted with 2.5×10^4/mL serum-free medium to prepare cell suspension; the cell suspension was added to the upper Transwell chamber at 20 μL each well, and 500 μL of 10% FBS and medium were added to the lower Transwell chamber, placed in a 37°C incubator for culture; fixed with formalin, stained by crystal violet for 15 min, and then the cells on the inner membrane were wiped with a cotton swab, counted under a microscope, to count the cells that passed through the membrane under 4 high power fields (×40). The experiment was performed in triplicate.

Effect of LYZ expression on lung carcinoma cell migration ability by wound scratch assay and transwell migration assay

Wound scratch assay: The A549 cells were inoculated to a 6-well plate, and when cell con-

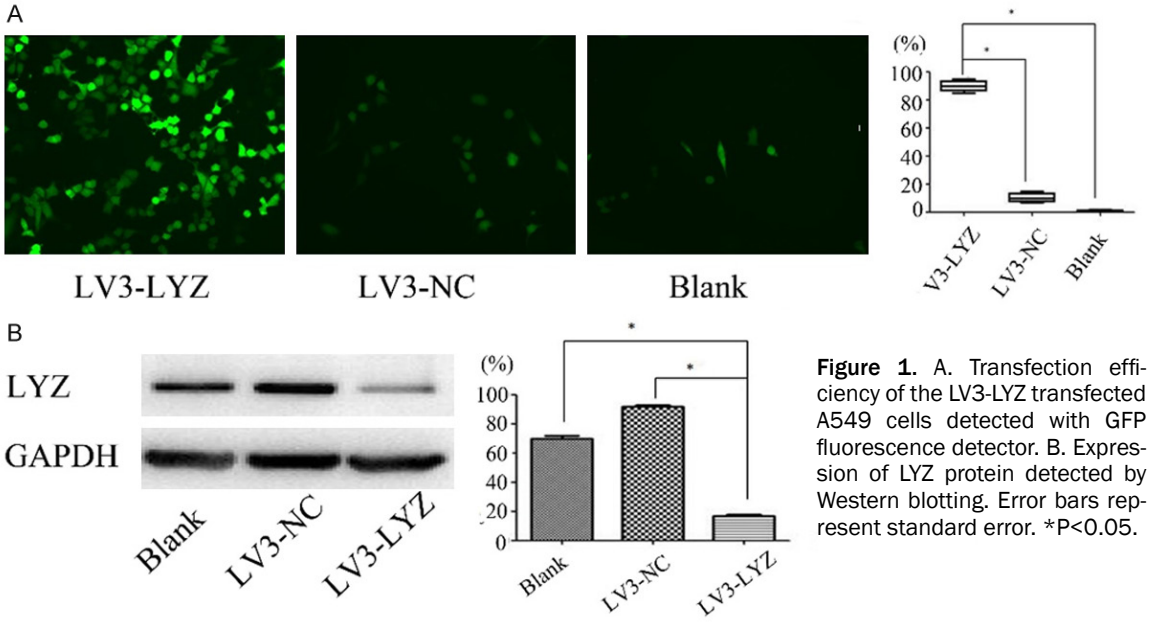

Figure 1. A. Transfection efficiency of the LV3-LYZ transfected A549 cells detected with GFP fluorescence detector. B. Expression of LYZ protein detected by Western blotting. Error bars represent standard error. *P<0.05.

fluence reached 90%, scratch from up to bottom using a 200 μl sterile pipette tip, observe under a microscope, to measure the initial distance of scratch (0 time); at 24 h, 48 h and 72 h, the distances of scratch were measured respectively and photographed, to calculate the cell migration rate. The experiment was performed in triplicate.

Transwell migration assay: All reagents and equipments were pre-cooled on ice. The Transwell chambers were placed in a 24-well plate. When digested, centrifuged and counted, cells were diluted with 2.5×10^4/mL serum-free medium to prepare cell suspension; the cell suspension was added to the upper Transwell chamber at 20 μL each well, and 500 μL of the whole culture medium was added to the lower Transwell chamber, placed in a 37°C incubator for culture; then fixed with formalin for 24 h, stained with crystal violet for 15 min, and then the cells on the inner membrane were wiped with a cotton swab, counted under a microscope, to count the cells that passed through the membrane under 4 high power fields (×40). The experiment was performed in triplicate.

Phalloidin-labeled cytoskeleton

The cell climbing slice was prepared, and 24 h later, fixed with 4% paraformaldehyde for 5-10 min; then the membrane was ruptured using 0.1% Triton X-100/PBS at room temperature for 3-5 min; 10 μl of FITC-Phalloidin stock solution was added to 150 μl of PBS to prepare the working solution (5 μg/ml) for staining cells for 30-60 min; after cleaning, the excess water was absorbed, and fluorescence sealing liquid was used for sealing, and then observed under a fluorescence microscope.

Statistical analysis

The SPSS 19.0 software was used for statistical analysis, measurement data were expressed in ($\bar{x}\pm s$), t-test was employed for comparison of means between groups, and P<0.05 indicated statistically significant difference.

Results

Down-regulation of LYZ protein expression in the A549 cells after transfected with LV3-LYZ

24 h after transfection with LV3-LYZ in the A-549 cells, GFP fluorescence showed that (**Figure 1A**), compared with the LV3-NC and blank control groups, the expression of green fluorescence in LV3-LYZ transfected cells was significantly increased [(90.94±1.46)% vs (15.02±2.03)%, (15.02±2.03)%, P<0.05]. Results showed that LV3-LYZ lentivirus could be well incorporated into the lung carcinoma A549 cells.

The Western blotting results (**Figure 1B**) showed that compared with the LV3-NC and blank control groups, the LYZ expression level

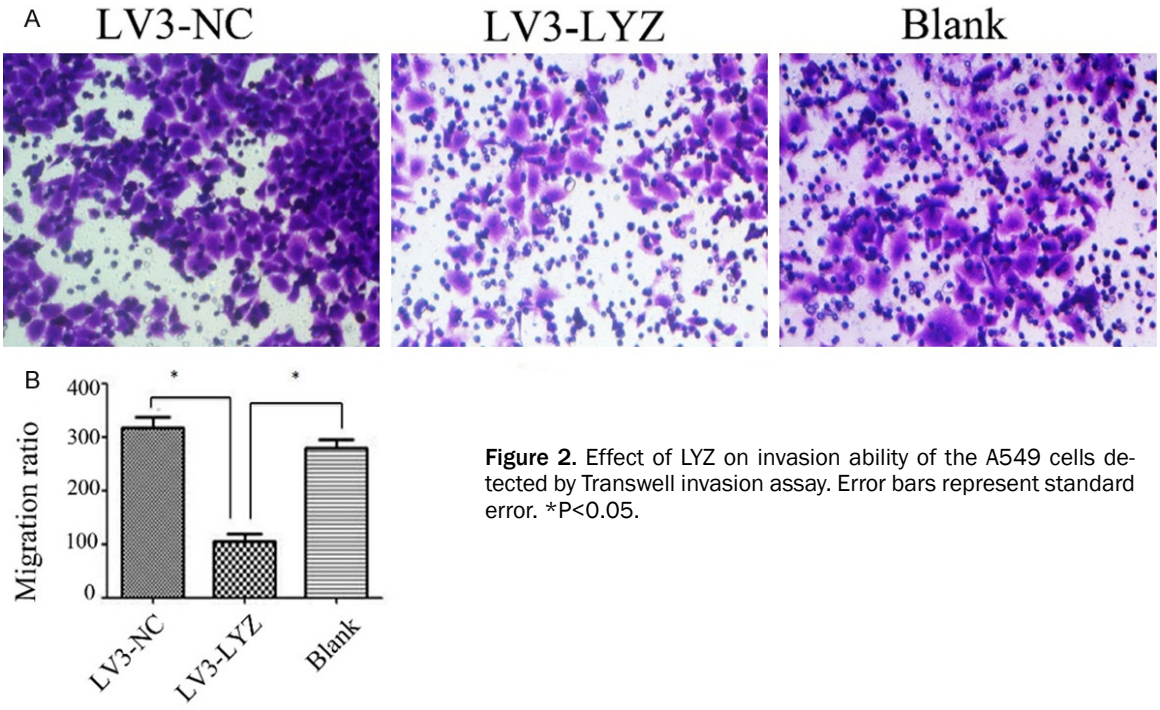

Figure 2. Effect of LYZ on invasion ability of the A549 cells detected by Transwell invasion assay. Error bars represent standard error. *P<0.05.

in the LV3-LYZ group decreased significantly [(16.00±1.58)% vs (86.80±5.16)%, (68.80±3.27)%, P<0.05], suggesting that transfection of the A549 cells with LV3-LYZ could significantly reduce the LYZ expression.

Invasion ability of the A549 cells inhibited by silencing the LYZ expression

The ability of cells to pass through the Matrigel gel reflects their invasion ability. Transwell results showed (**Figure 2A** and **2B**) that the number of cells passing through the gel was 88.54±6.76 in the LV3-LYZ group, significantly less than that in the LV3-NC and blank control groups (323.92±16.88, 295.68±13.20), with statistically significant difference (P<0.05). This showed that silencing the LYZ expression could inhibit invasion ability of the A549 cells.

Migration ability of the A549 cells inhibited by silencing the LYZ expression

The width of scratches in any three parts of cells in each group was measured under a microscope at the time of 0 h, 24 h, 48 h and 72 h. The migration rate was calculated according to the formula: Migration rate = $[D_{(t = 24\ h,\ 48\ h,\ 72\ h)} - D_{(t = 0\ h)}]/D_{(t = 0\ h)}$. Results of the wound scratch assay (**Figure 3A** and **3B**) showed that compared with the LV3-NC and blank control groups, at 24 h, 48 h and 72 h, migration rate in the LV3-LYZ group decreased significantly [24 h (0.16±0.03)% vs (0.37±0.04)%, (0.41±0.04)%, P<0.05; 48 h (0.25±0.04)% vs (0.57±0.05)%, (0.59±0.05)%, P<0.05; 72 h (0.37±0.04)% vs (0.81±0.06)%, (0.88±0.07)%, P<0.05], with statistically significant difference. Transwell migration assay showed (**Figure 3C** and **3D**) that the number of cells passing through Transwell holes in the LV3-LYZ group was 92.54±7.12, significantly less than that in the LV3-NC group and the control group (267.42±15.72 and 311.25±14.81, respectively), with statistically significant difference (P<0.05). The wound scratch assay and Transwell migration assay showed that silencing the expression of LYZ could inhibit migration of the A549 cells.

Expression of cytoskeleton-associated proteins inhibited by silencing LYZ expression

The cell invasion and migration is associated with the change in cell adhesion, degradation of extracellular matrix and the morphological changes, and all these processes are completed via constantly changing cytoskeletons [7]. The experiment described above showed that silencing LYZ could inhibit invasion and migration of the A549 cells. Thus, it could be inferred that LYZ might induce cytoskeleton reconstruc-

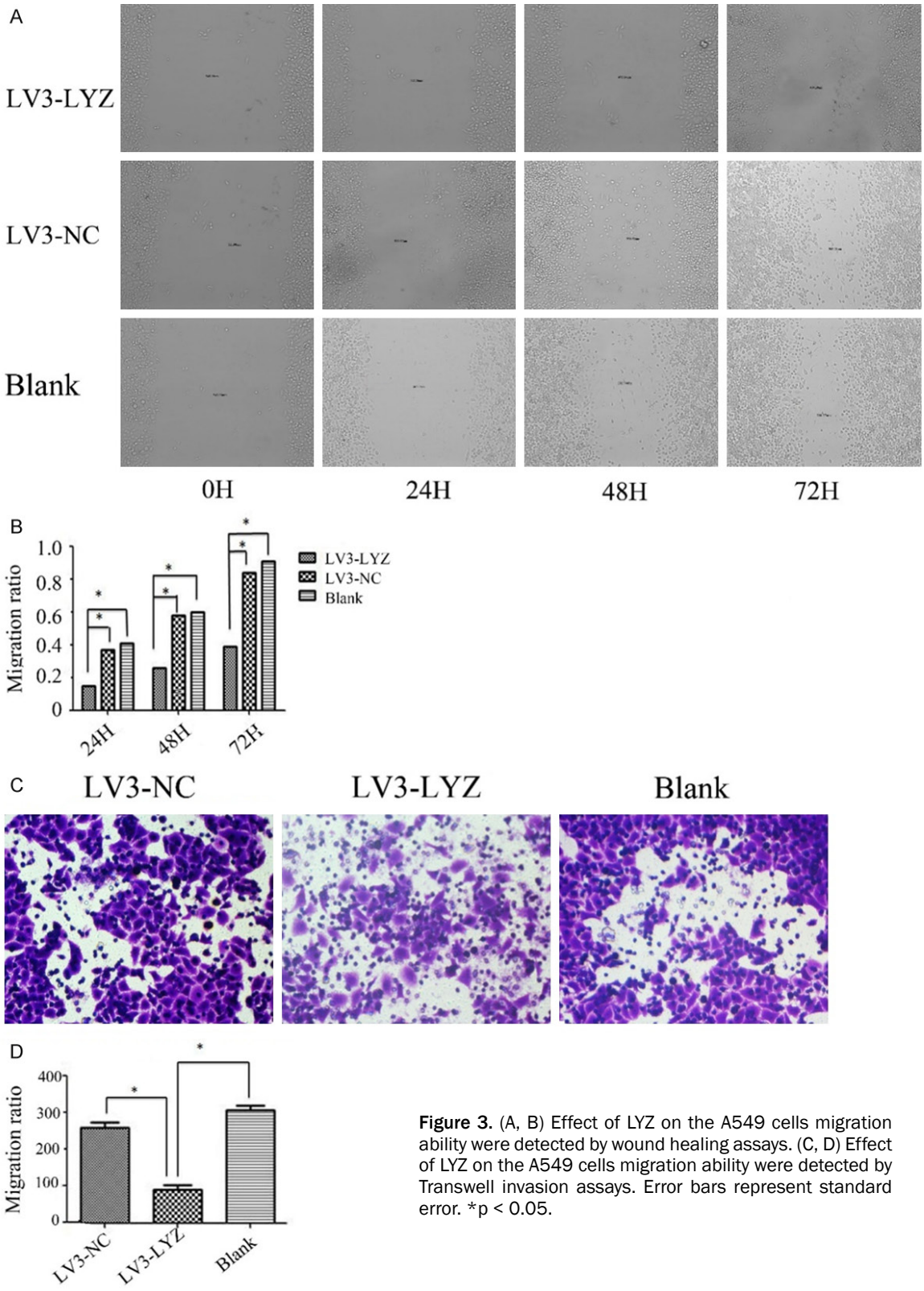

Figure 3. (A, B) Effect of LYZ on the A549 cells migration ability were detected by wound healing assays. (C, D) Effect of LYZ on the A549 cells migration ability were detected by Transwell invasion assays. Error bars represent standard error. *$p < 0.05$.

tion by altering the cytoskeleton, thereby leading to cell invasion and migration. Rho protein family is an important regulatory protein of cytoskeleton actin [8]. We speculate that LYZ

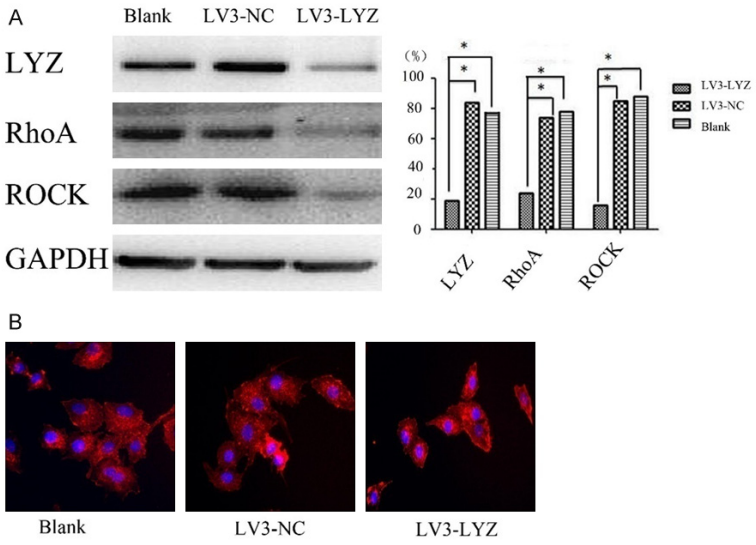

Figure 4. A. Expression of RhoA and ROCK detected by Western blotting. B. Phalloidin used to label the F-actin. Original magnification, 400X. Error bars represent standard error. *P<0.05.

may activate the signaling pathways of associated cytoskeleton by regulating the Rho family proteins, to promote the invasion and migration of lung carcinoma cells.

The Western blotting results (**Figure 4A**) showed that compared with the LV3-NC and blank control groups, the expression level of RhoA and ROCK in the LV3-LYZ group decreased significantly [RhoA (22.6±1.92)% vs (72.34±3.96)%, (77.00±4.19)%; ROCK (14.6±1.22)% vs (82.17±4.25)%, (86.60±4.50)%, P<0.05], suggesting that silencing LYZ expression could down-regulate the expression of RhoA and ROCK.

The phalloidin staining of cytoskeleton showed (**Figure 4B**) that compared with the LV3-NC and blank control groups, F-actin staining of the A549 cells, the formation of membrane ruffles and the pseudopod formation in the LV3-LYZ group were significantly reduced.

Discussion

LYZ is widely distributed in a variety of tissues and secretions of human and animals and some plants and microorganisms. According to different sources, LYZ is generally divided into 6 types: C type, G type, invertebrate type, bacteriophage, bacteria and plant-derived lysozyme [9]. LYZ is a thermolabile basic protein with low molecular weight, of which, the content of arginine is maximum. It is a peptide chain consisting of 129 amino acids, with a slightly oval-shaped three-dimensional configuration by twisting. There is a gap in the middle of configuration, and one end of the gap is connected with the surface which is the site of LYZ acting on the substrate. Through the induction adaptation of enzyme molecules, LYZ binds with the substrate to form an enzyme-substrate complex and hydrolyze the substrate catalytically. In clinical practice, lysozyme has a wide range of applications in fighting against inflammation, monitoring renal tubular function and judging the extent of burns, etc. [10].

The function of LYZ is relatively specific, and its substrate can be divided into two categories: chitin and oligosaccharides synthesized by N-acetyl glucosamine (GLcNAc); mucopolysaccharide *peptide and* oligosaccharides formed alternatively by N-acetyl glucosamine and N-acetyl muramyl acid (MurNAC), and both of which are composed of N-acetyl hexosamine residues connected by β (1-4), and they are cellulosic substances. When LYZ shows the activity of N-acetyl glucosamine, it acts on the shell polysaccharides. However, when LYZ shows only the activity of N-acetyl muramidase, it splits the glycosidic bond of MurNAc residues. Length of the gap in the middle of LYZ is equivalent to the connection of 6 glycosylation furan rings (indicated by A, B, C, D, E and F), therefore, it is suitable for the hydrolysis of polysaccharides. LYZ has more active catalytic groups on enzyme molecules equivalent to glycosyls D and E. The -COOH at the 35th glutamic acid residue and -COOH at the 52nd aspartate residue are located at both sides of the gap, thus, the most suitable sites for hydrolysis of the substrate are located at the connection between glycosyls D and E. Taking the hydrolysis of mucopolysaccharide *peptides by* LYZ as an example, it splits the glycosidic bond of the MurNAC residues, resulting in destruction of the cell wall structures of bacteria.

Studies both at home and abroad have shown that LYZ is also strongly associated with occurrence and progression of tumor. Carlos Serra *et al.* [11] carried out a study on 75 patients with breast cancer and the immunohistochemical staining showed that LYZ expression level was significantly increased in the patients and the LYZ expression level and lymph node metastasis were the independent prognostic factors. Carlos *et al.* [12] found out that compared with the normal tissues, the LYZ expression level in the Barrett esophageal columnar epithelial cells was significantly increased, suggesting that LYZ might be involved in the formation of Barrett's esophagus. Researches performed by Tahara *et al.* [13] revealed that the LYZ expression level was significantly increased in the tissues of gastric cancer and was associated with poor prognosis of the patients, besides, the serum LYZ level in patients with gastrointestinal malignancies was correlated to growth speed and invasion and migration ability of tumors. The increase of serum lysozyme in patients with tumor may be related to the type of tumor, extent of lesion and the body's resistance. Determination of LYZ may be of important significance for diagnosis, staging and prognosis of tumors.

In this study, by down-regulating the LYZ gene with lentivirus and detecting the changes in invasion and migration ability of lung carcinoma cell lines, the effects of LYZ in invasion and migration of the cell lines and the related mechanism are investigated. Results showed that with decrease in LYZ expression, invasion and migration of the cells decreased, suggesting that LYZ played an important role in tumor invasion and metastasis. Cui Huaibo *et al.* [14] performed an immunohistochemical assay on the tissues of 50 patients with lung carcinoma and found that with increase in tumor size, the LYZ positive rate was increased, and with the lymph node metastases, it increased significantly, indicating that LYZ was correlated with invasion of lung carcinoma. The immunohistochemical results are consistent with those in this study.

Tumor invasion and metastasis is a complex, multi-step process and the role of protein degradation enzymes is one of the causes that cannot be ignored. In normal circumstances, LYZ is secreted to the glandular cavity and natural tubes, etc. Since these structures have covering epithelia, LYZ will not cause hydrolysis on the body's own structure. However, due to disorder in tumor growth, the secreted LYZ may enter the mesenchyma to act with the substrate, to break through the basement membrane, thereby invade deeper tissues [15]. Results of the present study showed that compared with the LV3-NC group, F-actin staining of the A549 cells transfected with LV3-LYZ decreased significantly, and the formation of membrane ruffles was significantly reduced. Silencing the expression of LYZ could reduce the expression of RhoA and ROCK, hence suggesting that LYZ could regulate the Rho family proteins and affect remodeling of cytoskeleton, leading to migration of the tumor cells.

This study revealed that LYZ played an important role in invasion and migration of the lung carcinoma cell lines. Further exploration on the related mechanisms indicated that LYZ might play a role in invasion and migration by regulating cytoskeleton, suggesting that LYZ is possibly involved in progression and metastasis of lung carcinoma, and it may become a protein marker for predicting progression and prognosis of the disease and the therapeutic effect. Further study is needed for clarification since there are few studies on LYZ and on the interaction between LYZ and other proteins or signaling pathways.

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Disclosure of conflict of interest

None.

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