

Original Article

KRT14 promoting invasion and migration of lung cancer cells through ROCK-1 signaling pathway

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Abstract: Objective: To explore the effects of KRT14 on the invasion and migration abilities of lung cancer cells and the relevant mechanism. Methods: The expression of KRT14 was measured in different lung cancer cell lines by Western blotting; lentivirus silencing KRT14, GFP fluorescence and Western blotting were used to detect the LV3-KRT14 silencing efficiency and efficacy; the effect of silencing KRT14 on invasion of lung cancer cells was detected by Transwell invasion assay; the effect of silencing KRT14 on migration of lung cancer cells was detected by wound scratch assay; the expression of the proteins ROCK-1 and RhoA was detected by Western blotting; the interaction between KRT14 and RhoA was detected by CoIP assay; the effects of silencing KRT14 on size and volume of lung cancer were detected in the subcutaneous tumor formation experiment in nude mice; and the expression of KRT14 and RhoA was measured by immunohistochemistry in the LV3-NC and LV3-KRT14 groups. Results: The expression of KRT14 was the highest in the A549 cells; LV3-KRT14 lentivirus could effectively inhibit the expression of KRT14; silencing KRT14 inhibited the invasion and migration abilities of the lung cancer A549 cells; silencing KRT14 could down-regulate the expression of ROCK-1 and RhoA; KRT14 directly interacted with RhoA; the tumor volume and weight of tumor-bearing mice decreased significantly in the LV3-KRT14 group as compared to the LV3-NC group; and IHC results showed that compared to the LV3-NC group, the expression of KRT14 and RhoA decreased significantly. Conclusion: KRT14 influences the invasion and migration abilities of lung cancer cells through the RhoA/ROCK-1 signaling pathway.

Keywords: KRT14, lung cancer, ROCK-1, A549, invasion, migration

Introduction

Lung cancer is currently one of the malignant tumors with the highest incidence worldwide, and in China, morbidity and mortality of the disease has been on the rise due to aging of the population, worsening environment pollution and impacts of tobacco [1]. Occurrence and development of lung cancer is a complicated multi-step process with many genes, involving activation of oncogenes and mutation of antioncogenes. The main reason for death of patients with the disease is tumor invasion and metastasis, and distant metastasis is already present in most of the patients at initial diagnosis, therefore, investigating the related mechanisms in lung cancer and exploring the relevant tumor markers in metastasis to facilitate diagnosis, predict prognosis and guide treatment has become the focus in research on the disease.

Abundant and stable in epithelial cells, with high specificity in tissues and cells, cyokeratin (CK/KRT) is an ideal biological indicator for identifying tumor origin and predicting prognosis [2]. KRT14 is not expressed in normal cells, on the contrary, its expression increases significantly in many tumors [3]. It was revealed in several studies that KRT14 was expressed in squamous cell carcinoma of various origins and degrees of differentiation. The study by Huang et al [4] found out that KRT14 had no or low expression in normal cervical tissues, but its expression increased gradually in CIN1, CINII and CINIII, and it was believed that KRT14 could be used as an indicator of auxiliary diagnosis for early cervical cancer. The study by Xue et al [5] revealed that the expression of KRT14 was low in early esophageal cancer, but it increased remarkably in severe atypical hyperplasia and cancer in situ, and it increased gradually with progression in tumor stage and differentiation

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degree; in invasive esophageal cancer, the deeper tumor infiltrated the esophageal wall, the higher the expression of KRT14. In this study, we intend to explore the role of KRT14 in invasion and migration of lung cancer, which has not been reported in literature, and probe into the related mechanism.

Materials and methods

Cell lines and main reagents

The human lung cancer cell lines A549 and H1975 were purchased from Chinese Representative Culture Collection Center; and 95D and MBC-5 were from ATCC. Cell culture conditions: cultured in RPMI DMEM or RPMI 1640 containing 10% fetal bovine serum at 37°C, 5% CO₂. Fetal bovine serum (FBS), RPMI DMEM and RPMI 1640 were purchased from Gibco. The primary antibodies to KRT14 and ROCK-1 were obtained from Abcam (ab51054 and ab45171); the primary antibody to RhoA was from Santa Cruz (sc-166399). Transwell chambers were from Corning; KRT14 silencing and control lentiviruses were purchased from Shanghai Genechem Co., Ltd.; and the Matrigel gel was from BD (US). The nude mice aged 6 weeks were provided by Laboratory Animal Center, Academy of Military Medical Science; the Universal Immunohistochemical Kit was from Beijing Zhongshan Golden Bridge Biotech Co., Ltd.; Trizol was from Ambion (US); the FSQ-101 ReverTra Ace qPCR RT Kit was from TOYOBO (Japan); and the PCR kit was from Kapa (US).

Western blotting

The proteins were extracted from different lung cancer cell lines, and the protein concentrations were determined by BCA method, and then loading buffer was added for protein denaturation. 10% SDS-PAGE was prepared, and 20 µg protein sample was added into each well, then transferred to a PVDF membrane using the electric wet transfer method, sealed for 2 h with 5% skim milk, and the primary antibody (KRT14) was diluted by 1:1000 TBST, 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.

72 h after transfection, the total proteins were extracted from the cells in the experimental and control groups, and the protein concentrations were determined by BCA method, and then loading buffer was added for protein denaturation. 8% and 12% SDS-PAGE was prepared, and 20 µg protein sample was added into each well, then transferred to a PVDF membrane using the electric wet transfer method, sealed for 2 h with 5% skim milk, and the primary antibodies (RhoA and ROCK-1) were diluted by 1:1000 TBST, 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.

Expression of KRT14 in different lung cancer cell lines detected by PCR

Total RNA was extracted from tissues in accordance with Trizol instructions, RNA concentration was determined with ultramicrospectrophotometer, and the KRT14 primers were designed by FuleGen Co., Ltd. The primer sequences were as follows: upstream, 5'-TACCCGAGCACCTTCTCTTC-3'; downstream, 5'-TGCTGGAGAACAAGTAGCTGC-3'. 100 ng of total RNA was used as template for reverse transcription of cDNA, and the reaction conditions were 37°C for 15 min and 98°C for 5 min. Then, PCR reaction was carried out as per the instructions of Kapa PCR kit. The data obtained were used to calculate the expression of mRNA according to the formula: $RQ=2^{-\Delta\Delta CT}$. The experiment was performed in triplicate.

Lentivirus transfected into the A549 cells

One day before the experiment, 5×10^3 A549 cells were inoculated into 96-well plates, to make the cell fusion degree at 40%~60%. According to the GenePharma lentivirus operating manual, the appropriate MOI (multiplicity of infection) of KRT14 lentivirus was determined using the gradients of 0, 10 and 100.

Two sterile EP tubes were prepared. 10 µl of viruses at 1×10^8 TU/ml were drawn to the first tube and mixed gently, producing no foams. Similarly, 10 µl of viruses were drawn from the first tube to the second and mixed well, to get virus solutions at three different concentrations: stock solution, 10× dilution, and 100× dilution.

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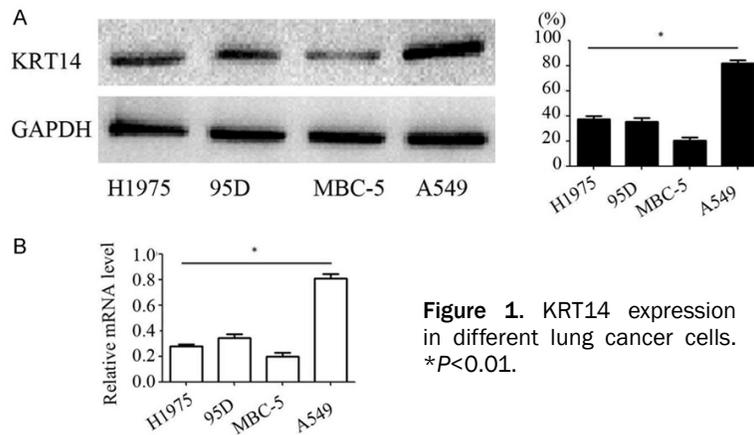


Figure 1. KRT14 expression in different lung cancer cells. * $P < 0.01$.

Effects of silencing KRT14 on the migration ability of lung cancer cells detected by wound scratch assay

The A549 cells were inoculated into a 6-well plate, and when cell confluence reached 90%, scratch from up to bottom using a 200 μ l 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.

Co-immunoprecipitation assay

An appropriate amount of cell lysis buffer containing protease inhibitor was added into the A549 cells, lysed for 30 min on ice and centrifuged for 30 min at maximum rotation rate to obtain supernatant. Added with 1 μ g of KRT14 antibodies, the supernatant was incubated overnight at 4°C. 10 μ l of the pre-treated protein A agarose beads were added into the cell lysis buffer that was incubated overnight with antibodies to incubate for 3 h at 4°C, for coupling of the antibodies with protein A agarose beads. The lysis buffer was washed 3 times, 10 min each time, and the resulted precipitate complex was added with 2 \times SDS loading buffer and boiled for 5 min. Western blotting assay was performed. The experiment was performed in triplicate.

Effect of silencing KRT14 on tumor growth in subcutaneous tumor formation experiment in nude mice

Lung cancer cells in the logarithmic growth phase in the LV3-NC and LV3-KRT14 groups were taken and digested in culture flasks, with concentration adjusted to 2 \times 10⁸ cells/ml. 0.1 ml of cell suspension was injected subcutaneously into each nude mouse (10 in total) by the left forelimb armpit to observe subcutaneous tumor growth daily. One week after inoculation, the tumor grew to about 5-6 mm. The mice were divided into 2 groups, 5 in each group. The animals were sacrificed 28 days after inoculation of tumor cells. The tumor diameter (a) and the vertical orthogonal diameter (b) of

10 μ l of virus solutions at three different gradients were added to three wells in each group, to calculate the MOI of three wells, which were 100, 10 and 1 respectively. The most appropriate MOI was determined to be 100.

The experiment consisted of two groups: the silencing group, transfected with KRT14 silencing lentivirus (LV3-KRT14), and the control group, transfected with the negative control lentivirus (LV3-NC). 100-fold diluted virus stock solution was added to the LV3-KRT14 group, and 100-fold diluted negative control virus solution was added to the LV3-NC group. The expression of GFP fluorescence was observed 24 h later.

Effects of KRT14 expression on the invasion ability of lung cancer cells detected by Transwell invasion assay

All reagents and equipment were pre-cooled on ice. The Transwell chambers were placed in a 24-well plate. 50 μ l (0.2 μ g/ μ l) Matrigel gel was evenly applied to inner membrane of Transwell chamber, incubated for 15 min at 37°C to solidify the gel; when digested, centrifuged and counted, the cells were diluted with 2.5 \times 10⁴/mL serum-free medium to prepare cell suspension; the cell suspension was added to the upper Transwell chamber at 200 μ L each well, and 500 μ L 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 (\times 40). The experiment was performed in triplicate.

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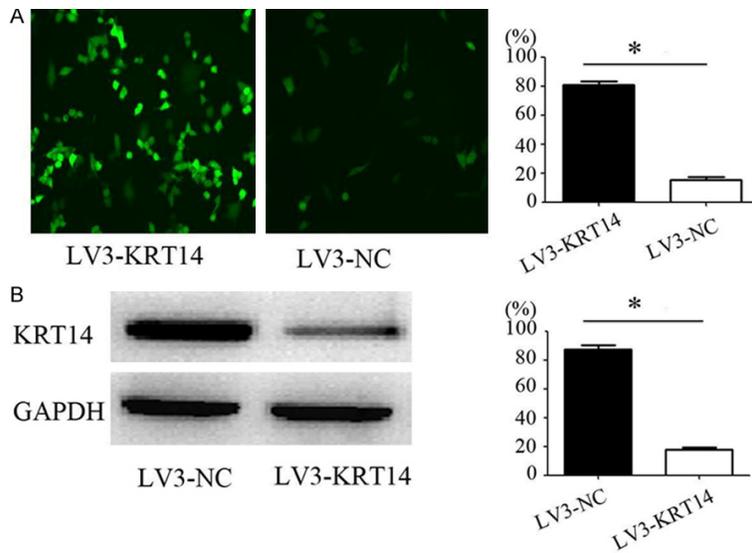


Figure 2. A. Transfection efficiency after LV3-KRT14 was transfected into the A549 cells detected with GFP fluorescence; B. Expression of KRT14 was detected by Western blotting. Error bars represent standard error. * $P < 0.05$.

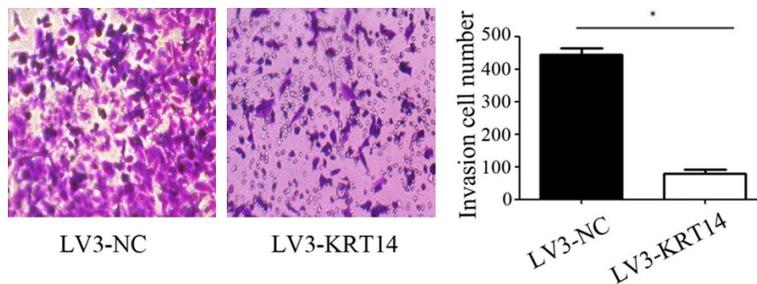


Figure 3. Effect of KRT14 on invasion ability of the A549 cells detected by Transwell invasion assay. Error bars represent standard error. * $P < 0.05$.

tumor-bearing mice were measured in each group. The tumor size V (mm^3) = $a \times b^2 / 2$; and the weight was measured. The tumor tissues were fixed in 4% paraformaldehyde.

Expression of KRT14 and RhoA in tumor tissues of nude mice detected by immunohistochemistry

The tumor tissues were embedded in paraffin and cut into sections of 4 μm in thickness. The immunohistochemical operations were performed according to the immunohistochemical S-P kit (Beijing Zhongshan Golden Bridge Biotech Co., Ltd.): dewaxing of the tissues and then hydrating. Antigen retrieval was performed in microwave with citrate buffer solution for 30 min, cooled down to room temperature, then washed 3 times with PBS, 3 min each time,

incubated with 3% H_2O_2 at 37°C for 15 min, then washed 3 times with PBS, 3 min each time, then the primary antibody was placed overnight, washed 3 times with PBS, 3 min each time; the horseradish peroxidase-labeled donkey anti-rabbit IgG, washed 3 times with PBS, 3 min each time; the horseradish peroxidase labeled streptavidin avidin working solution (Beijing BLOSS Bio-tech Co., Ltd.) was placed in 37°C water bath for 20 min, then washed 3 times with PBS, 3 min each time, and then stained with diaminobenzidine (DAB). In the negative control group, the primary antibody was replaced by PBS. All sections were reviewed independently by two pathologists, and 22 representative high power fields (10 \times 40 folds) were selected by each pathologist. The percentage of positive cells in each specimen was counted and the positive results were judged and scored according to the method described by De Falco M et al [8]: 0 (positive cells less than 1%); 1 (between 1% and 20%), 2 (between 21% and 40%); 3 (between 41% and 60%); and 4 (more than 61%).

Statistical analysis

The SPSS 22.0 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

Expression of KRT14 in different lung cancer cell lines

The Western blotting results (Figure 1A) showed that compared to other lung cancer cell lines, the expression of KRT14 was the highest in the A549 cells [(39.31 \pm 2.12)% vs (37.80 \pm

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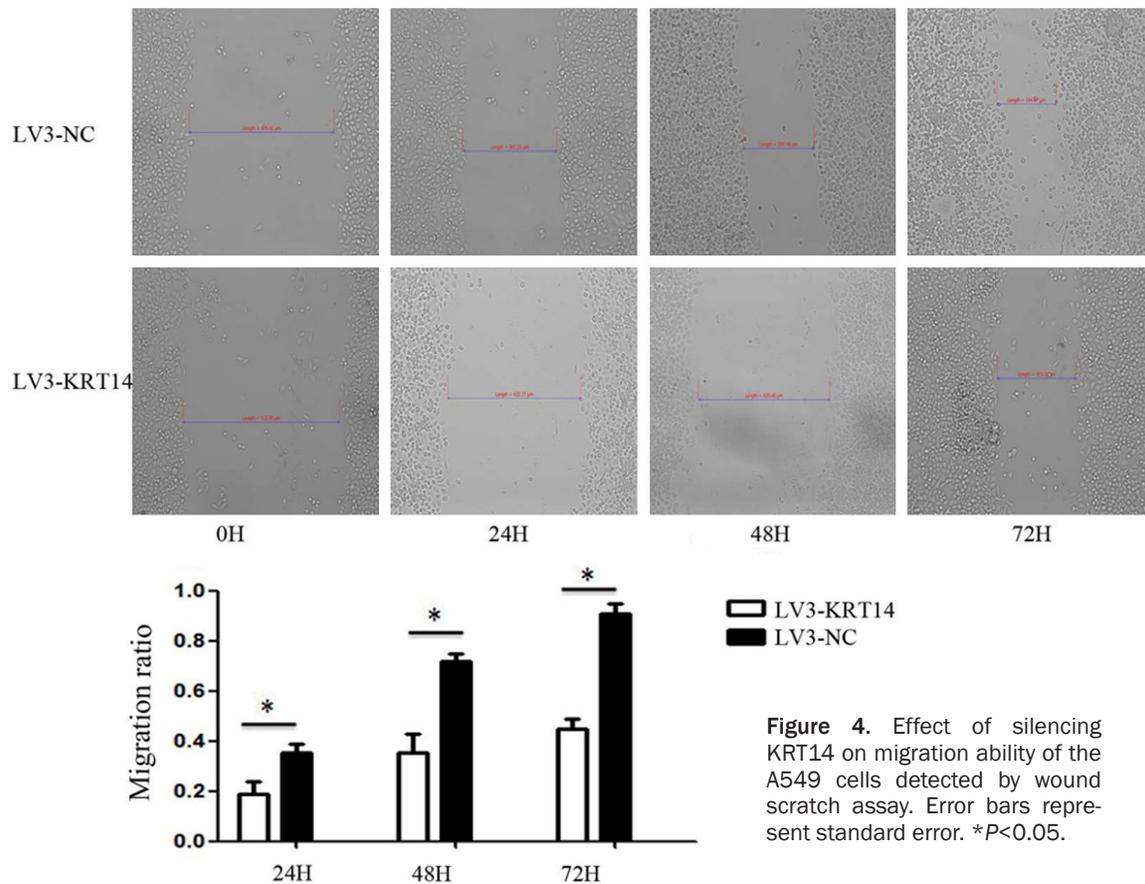


Figure 4. Effect of silencing KRT14 on migration ability of the A549 cells detected by wound scratch assay. Error bars represent standard error. * $P < 0.05$.

2.19%) vs (18.41±1.27)% vs (79.84±3.80)%, $P < 0.01$], with statistically significant difference.

The qPCR results (**Figure 1B**) showed that in the four lung cancer cell lines, the expression of KRT14 mRNA was the highest in the A549 cells [(0.25±0.01) vs (0.31±0.02) vs (0.19±0.01) vs (0.78±0.03), $P < 0.01$], thus the cells were selected for KRT14 silencing for further functional study.

Low expression of KRT14 after LV3-KRT14 was transfected into the A549 cells

24 h after LV3-KRT14 was transfected into the A549 cells, it was shown under the GFP fluorescence microscope (**Figure 2A**) that compared with the LV3-NC group, expression of green fluorescence in the LV3-KRT14 transfected cells was significantly increased [(80.24±3.46) vs (15.02±2.03), $P < 0.05$], suggesting that LV3-KRT14 could be well incorporated into the A549 cells.

The Western blotting results (**Figure 2B**) showed that compared with the LV3-NC group, the

KRT14 expression level in the LV3-KRT14 group was significantly decreased [(84.80±5.16) vs (18.80±2.27), $P < 0.05$], suggesting that KRT14 expression could be effectively reduced after LV3-KRT14 was transfected into the A549 cells.

Invasion ability of the A549 cells inhibited by silencing KRT14

The Transwell results (**Figure 3**) showed that the number of cells passing through the Matrigel gel was 71.54±6.73 in the LV3-KRT14 group, obviously less than that in the LV3-NC group (443.92±22.88), with statistically significant differences ($P < 0.05$). Results from the Transwell invasion assay indicated that silencing the expression of KRT14 could inhibit invasion ability of the A549 cells.

Migration ability of the A549 cells inhibited by silencing KRT14

The width of scratches in any three parts of cells in each group was measured under a microscope at the time points of 0 h, 24 h, 48 h and 72 h. The migration rate was calculated

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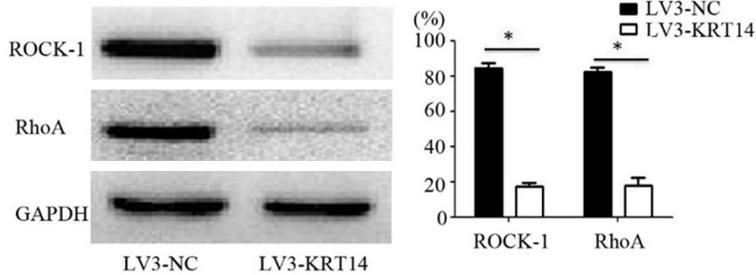


Figure 5. Expression of ROCK-1 and RhoA detected by Western blotting. Error bars represent standard error. * $P < 0.05$.

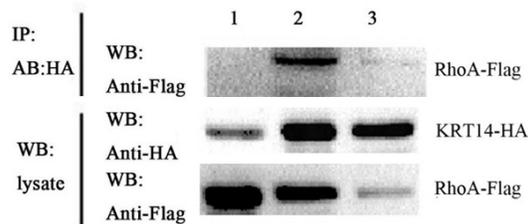


Figure 6. Interaction between KRT14 and RhoA detected by CoIP assay.

according to the formula: Migration rate = $[D_{(t=24h, 48h, 72h)} - D_{(t=0h)}] / D_{(t=0h)}$. Results of the wound scratch assay (**Figure 4**) suggested that compared to the LV3-NC group, migration rate in the LV3-KRT14 group was significantly reduced at 24 h, 48 h and 72 h [24 h (0.19 ± 0.03)% vs (0.35 ± 0.04)%], $P < 0.05$; 48 h (0.34 ± 0.04)% vs (0.72 ± 0.05)%], $P < 0.05$; 72 h (0.42 ± 0.04)% vs (0.89 ± 0.06)%], with statistically significant differences. It was revealed in the wound scratch assay that silencing the expression of KRT14 could inhibit migration ability of the A549 cells.

Expression of ROCK-1 and RhoA inhibited by silencing KRT14

Invasion and migration of cells involves changes in the adhesion ability and morphology of cells and degradation of extracellular matrix, which are completed through constant changes in cytoskeleton [7]. Results of the experiment mentioned above suggested that silencing KRT14 could inhibit the invasion and migration abilities of the A549 cells. It is therefore speculated that KRT14 may lead to invasion and migration of cells by changing cytoskeleton to result in its reconstruction. The Rho protein family is a group of important regulators for actin cytoskeleton [8], and KRT14 may promote invasion and migration of lung cancer cells by

regulating the proteins to activate the signaling pathway of relevant cytoskeleton.

The Western blotting results (**Figure 5**) showed that compared to the LV3-NC group, the expression of RhoA and ROCK-1 decreased significantly in the LV3-KRT14 group [RhoA (19.6 ± 3.92)% vs (80.34 ± 2.96)%; ROCK-1 (18.9 ± 1.22)% vs (83.15 ± 2.25)%], $P < 0.05$,

indicating that silencing the expression of KRT14 could down-regulate the expression of RhoA and ROCK-1.

Interaction of KRT14 with RhoA

To clarify if there is direct interaction between KRT14 and RhoA, the CoIP assay was designed. RhoA-Flag and KRT14-HA were co-transfected into cells and samples were collected at 24 hr. The results showed **Figure 6** that Flag zone was undetected in the cells transfected with RhoA-Flag (zone 1) or KRT14-HA (zone 3) alone, but it was detected in the cells co-transfected with RhoA-Flag and KRT14-HA (zone 2), suggesting a direct interaction between KRT14 and RhoA.

Subcutaneous tumor formation experiment in nude mice indicated that silencing KRT14 could inhibit tumor growth

The survival time of the tumor-bearing mice was 4-9 weeks, with a median of 6.5 weeks. The autopsy showed that tumor grew in the left armpit, and the tumor was gray, solid, round or oval, with nodular projection on the surface and fish-like sections; and the tumor formation rate was 100%.

Tumor growth in nude mice (**Figure 7A**): tumor size in the LV3-KRT14 group was significantly reduced compared with that in the LV3-NC group.

Comparison of tumor weight and volume (**Figure 7B and 7C**): compared to the LV3-NC group, the tumor volume and weight of the mice decreased significantly in the LV3-KRT14 group [volume (1.83 ± 0.08) cm³ vs (0.23 ± 0.02) cm³, $P < 0.05$; weight (2.12 ± 0.12) g vs (0.38 ± 0.02) g, $P < 0.05$].

The immunohistochemical (IHC) results showed that compared to the LV3-NC group, the expres-

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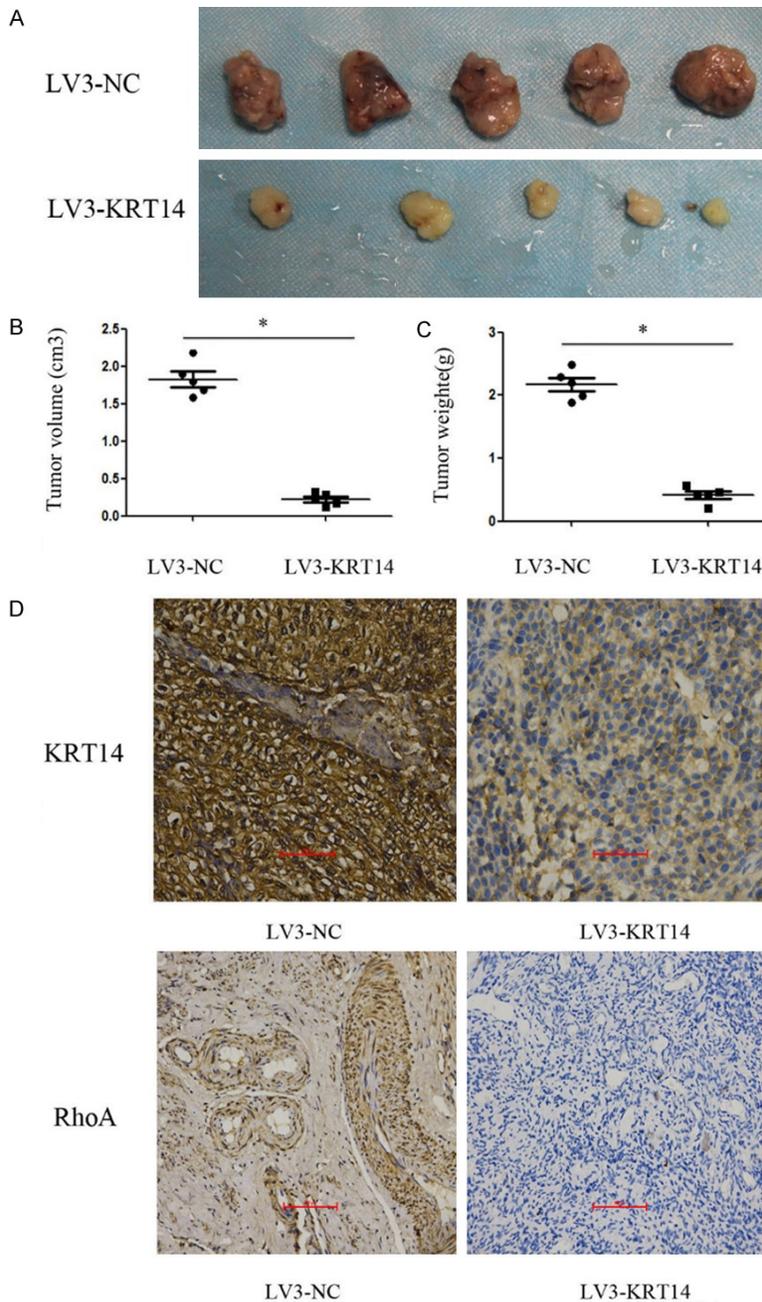


Figure 7. Effect of silencing KRT14 on tumor growth investigated in in vitro experiment. A. Comparison of tumor size in nude mice; B and C. Comparison of volume and weight of tumor in nude mice; D. The expression of KRT14 and RhoA in tumor tissues of the mice.

sion of KRT14 and RhoA decreased significantly in the LV3-KRT14 group.

Discussion

Cytokeratin is a complex intermediate filament protein with a molecular weight of 44 kDa-66

kDa. The keratin family is consisted of 20 members with various isoelectric points and molecular weights. Keratin plays a very important role in maintaining structural stability of epithelial cells, and is involved in a variety of stress response, signal transduction of epithelial cells, apoptosis, tumor cell apoptosis and proliferation [6-8]. The study by Nazarian [9] suggested that keratin also played an important part in tumor invasion and metastasis. The expression of various keratins is different in various tumor tissues, therefore this kind of proteins has great potential in diagnosis, typing and prognosis prediction of tumors.

As a member of the keratin family, KRT14 is a specifically differentiated protein and belongs to the acidic CK family [10]. It was demonstrated in a number of studies that KRT14 was expressed in squamous cell carcinoma of various origins and degrees of differentiation. The study by Cheung et al [11] suggested that KRT14 played a major role in metastasis of breast cancer, and the expression level of KRT14 was higher in the cell lines having stronger invasiveness. By IHC analysis with lung cancer tissues removed by surgery, Poovorawan et al [12] demonstrated that in lung cancer, the expression of KRT14 increased remarkably, mainly seen around the tumor tissues; and KRT14 was also expressed in metastatic lymph nodes, indicating that KRT14 played an important role in occurrence and development of tumors.

In this study, the invasion and migration abilities of lung cancer cells were measured by silencing KRT14, and it was revealed that the abilities were weakened, suggesting a major role of KRT14 in invasion and migration of the cells.

KRT14 promote lung cancer cells by ROCK-1 signaling pathway

ROCK is serine-threonine protein kinase, with two intracellular structures: ROCK-1 and ROCK-2 [13]. The Rho/ROCK signaling pathway plays an essential part in regulation of cell morphology, reconstruction of cytoskeleton and cell migration by regulating the movement of actin [14]. In invasion and migration of tumor cells, changes in cell adhesion ability, reconstruction of cytoskeleton and polarization of motion direction were all closely related to the Rho/ROCK signaling pathway [15]. It was observed that invasiveness of hepatic cancer cells strengthened after overexpression of ROCK and weakened after silencing ROCK; when Y-27632 (an inhibitor of ROCK) was used, it also decreased significantly, suggesting an important role of ROCK in invasion of hepatic cancer cells [16]. Similar studies on tumors like ovary cancer and urinary bladder cancer also demonstrated that Rho/ROCK had effects in tumor invasion and migration [17, 18]. In this study, the invasion and migration abilities of lung cancer cells were weakened after silencing KRT14, thus the Rho/ROCK-1 signaling pathway might be involved; and in detection of the ROCK signaling pathway-related proteins by Western blotting, it was observed that after silencing KRT14, the expression of ROCK-1 and RhoA decreased remarkably. Direct interaction between KRT14 and RhoA was likely through prediction with bioinformatics, thus CoIP was designed for confirmation. Then, it was demonstrated *in vitro* that both volume and weight of tumor were reduced significantly in nude mice and it was reconfirmed by immunohistochemistry that the expression of KRT14 and RhoA was significantly lower in the LV3-KRT14 group than that in the LV3-NC group.

It has been revealed in this study that by silencing the expression KRT14 with lentivirus, the invasion and migration abilities of lung cancer cells can be inhibited via the RhoA/ROCK-1 signaling pathway, indicating that KRT14 is likely to be involved in invasion and migration of the cells, and it may become a marker for predicting progression and prognosis of lung cancer and for monitoring the treatment effects.

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Disclosure of conflict of interest

None.

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