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RESEARCH

Effect of shRNA Mediated Down-Regulation of Annexin A2 on Biological Behavior of Human Lung Adencarcinoma Cells A549

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Abstract In the previous study, we found that Annexin A2 was significantly up-regulated in lung cancer and could induce related-antigen in lung cancer patients' serum. To further study the function of Annexin A2, the short hairpin RNA plasmid targeting Annexin A2 was constructed in vitro and transfected into human lung adencarcinoma A549 cells. Knocking down Annexin A2 expression by shRNA, the mRNA level of Annexin A2 was investigated by semi-quantitative RT-PCR. The expression of Annexin A2 protein was examined by Western Blotting and Immuocytochemistry. MTT assay and Transwell chamber model were used to evaluate proliferation and invasion of A549 cells in vitro. The concentration of matrix metalloproteinase-2 (MMP-2) and cathepsin B (CB) in the supernatant was evaluated by ELISA. At 48 h after transfection, the expression of Annexin A2 mRNA and protein was down-regulated significantly, respectively ($p<0.05$).The proliferation and invasion capability of A549 cells also decreased significantly ($p<0.05$). The concentration of MMP-2 and CB was down-regulated obviously, respectively ($p<0.05$). This study implies that Annexin A2 might play an important role in the progression and invasion of human lung cancer cells, and could promote progression of lung cancer by regulating the expression of MMP-2 and CB.

Keywords Annexin A2 · Lung cancer · RNA interference · Proliferation · Invasion

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Introduction

The Annexins are a super-family of closely related calcium and membrane binding proteins which show cell type specific expression. The annexins are classified into five groups, A-E, and within each of these groups, individual annexins are identified numerically. As one of important members in Annexins family, Annexin A2 plays an important role in the biological behavior, such as DNA synthesis, cell proliferation, tumor invasion and metastasis [1–6]. Increased expression of annexin A2 has been described in several types of tumour, including gastric cancer [7], intestinal cancer [8], thyroid cancer [9], renal cell carcinoma [10], pancreatic cancer [11], breast cancer [12], high-grade gliomas [13] and vascular tumours [14]. In the previous studies [15–17], we found that Annexin A2 was one of 19 non-redundant differentially expressed proteins related to lung cancer. And it could induce auto- antibodies in lung cancer patients' serum. However, the mechanism that *Annexin A2* effects on lung carcinoma cell is not very clear. This study was to investigate the function of Annexin A2 in growth of lung adencarcinoma cells and provide experiment evidence for further molecule mechanism about malignant progression of lung cancer.

Materials and Methods

shRNA Plasmid Design and Synthesis

Short hairpin RNA (shRNA) plasmid against Annexin A2 was constructed as described [18] and synthesized in vitro. pGenesil-1.1 was used as the vector. 29-nucleotide DNA template oligonucleotides were designed to produce 21-nucleotide shRNAs and 8-nucleotide complementary to the human U6 Promoter Primer. Annexin A2 mRNA target sequence beginning with AA was 5-AA TGTCTACTGTTC ACGAAAT-3. Its antisense shRNA oligonucleotide template was 5-AATGTCTACTGTTCACGAAATCCTGTCTC-3, and the sense shRNA oligonucleotide template was 5-AAATTTCGTGAACAGTAGACACCTGTCTC-3. They were checked for sequence specificity by a BLAST search and did not show homology to any other known human gene. shRNA plasmid synthesis was completed by Wuhan Genesil Biotechnology Co. Ltd.

Cells and Cell Culture

The human lung adenocarcinoma cell line A549 was obtained from Center South University Cell Culture Collection. Cells were continuously cultured in DMEM supplemented with 10% fetal bovine serum, 100 $U \cdot ml^{-1}$ penicillin and 100 $U \cdot ml^{-1}$ streptomycin. Cells were routinely fed with fresh media, passaged as needed, and maintained at 37°C in a humidified atmosphere containing 5% of CO_2.

Transfection

A549 cells were sed into 6-well plates at a density of 400,000 cells /well and grown to 80% confluence prior to liposome-mediated transfection. The cells were transfected with shRNA plasmid in oligofectamine reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, for each well, 10 μl of oligofectamine reagent was incubated with 250 μl of serum-free medium for 5 min. Subsequently, 4 μg of shRNA plasmid was dealed with as above. Then the 500 μl transfection mixture was incubated for 20 min at room temperature and added to each well. The final volume was 2 ml /well. Except shRNA plasmid group, three control groups as follows: transfecting independence sequence plasmids (independence sequence control), transfecting blank plasmids (keno-carrier control), no transfecting group (blank control). After 4–6 h, 2 ml of DMEM supplemented with 10% fetal bovine serum was added. After 48 h cells were harvested for further analysis.

Detection of Transfection Efficiency with Fluorescence Microscope

At 48 h posttransfection, three fields of vision were selected randomly and transfection efficiency was observed and evaluated under fluorescence microscope.

Semi-Quantitative Reverse Transcription (RT)-Polymerase Chain Reaction (PCR)

Total RNA was extracted from A549 cells using the Trizol reagent (Invitrogen) and quantified by UV absorbance spectroscopy. The reverse transcription reaction was performed with 3 μg total RNA and carried out at 65°C for 5 min followed by at 42°C for 1 h and at 70°C for 5 min according to protocol of RevertAidTM First Strand cDNA Synthesis Kit (Fermentas, life sciences). The newly synthesized cDNA was stored at −20°C until use. In the following experiment, 4 μl of cDNA was amplified in a 25 μl PCR reaction volume. Both of the *Annexin A2* primer 5′-CAGAACCAACCAGGAGCT-3′ (sense), 5′-TTCACTG CGGGAGACCAT-3′ (antisense) and *β-actin* primer 5′-TAACTGGAACGGTGAAGGTG-3′ (sense), 5′-AGGGC ACGAAGGGGCTCATCAT-3′ (antisense) were synthesized by Biologic Project Company (Shanghai). The *β-actin* expression was used as a control to normalize data of *Annexin A2* level. Amplification cycles were 94°C for 5 min, the 30 cycles at 94°C for 30 s, 53°Cfor 30 s, 72°C for 30 s, followed by 72°C for 10 min. After prepared by routine procedures, PCR products were visualized by electrophoresis on 1.5% agarose gel stained with ethidium bromide and analysized with UVP gel shaping instrument. Images of RT-PCR were scanned. The experiments were repeated three times.

Western Blotting

Western blotting was used to examine Annexin A2 protein expression. Confluent cells were washed three times with ice-cold PBS and then lysed in buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1%SDS, 10 mM PMSF, 0.5 mM DTT for 30 min on ice. After removal of cell debris by centrifugation at 12,000 g for 5 min, the supernatant was boiled for 3 min with the sample buffer. 30 μg of proteins were separated by 12% SDS-PAGE and then transferred onto nitrocellulose membrane. After unspecific reactivity was blocked by 5% fat-free milk in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature, the membrane was incubated in turn with mouse monoclonal antibody against human Annexin A2 (Santa Cruz) diluted in TBST (1:400) overnight at 4°C and horseradish peroxidase-conjugated

goat antimouse IgG diluted in TBST (1:40,000) for 1 h at room temperature. As internal control for Annexin A2, β-actin protein expression was carried out at the same time. The protein complexes were finally visualized by using ECL chemiluminescence analysis system (ECL system, KPL). Images of Western Blotting (WB) were scanned. The experiments were repeated three times.

Immuocytochemistry

Immuocytochemistry was performed according to standard procedures as described. We used mouse monoclonal antibodies from Santa Cruz for detection of Annexin A2. Slides covered with transfected cells were fixed with acetone at 4°C for 10 min and washed three times with cold PBS. To eliminate unspecific dyeing, the slides were dripped with 5% goat serum and incubated at 37°C for 30 min in wet case. Primary antibody was diluted 1:500 for Annexin A2 in PBS and incubated at 37°C for 60 min or at 4°C overnight. After washing with PBS, a biotinylated secondary antibody IgG followed by antibiotiny-horseradish peroxidase was applied. Brown color in cytoplasm or cell membrane was developed by DAB reagent kit (Boaosen, Beijing).

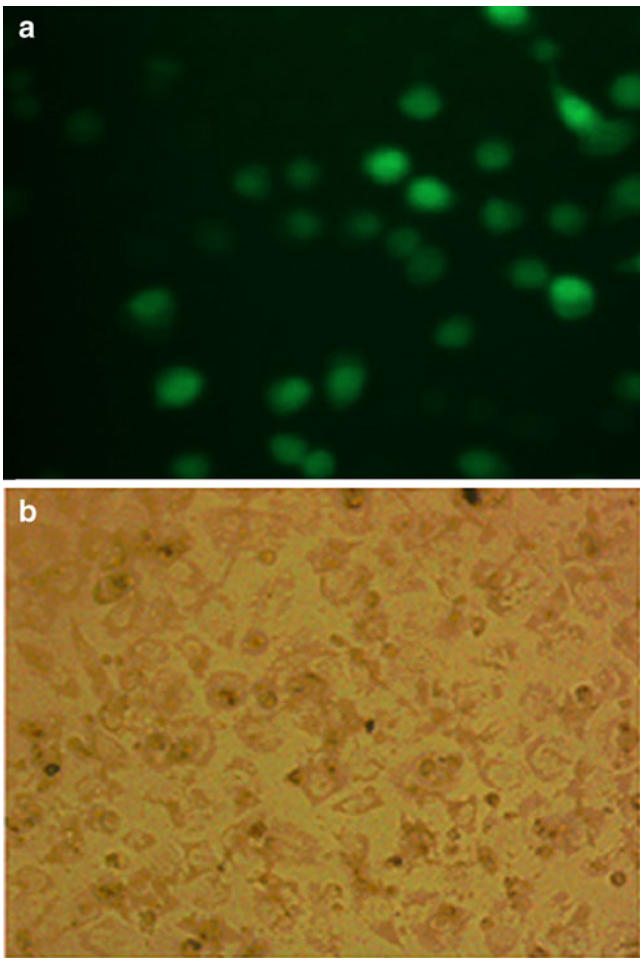

Fig. 1 Detection of transfection efficiency by fluorescence microscope. **a**: human lung adencarcinoma cell line A549 with Annexin A2 shRNA plasmid at 48 h post-transfection. The positive cells emitted green fluorescence. **b**: the same cells from A under phase-contrast microscope. Original magnification, 200×

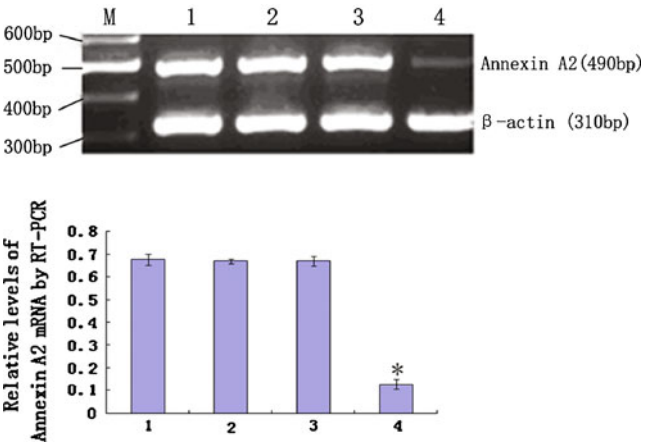

Fig. 2 The result of RT-PCR for Annexin A2 mRNA expression in lung adencarcinoma cell line A549. Lane M: DL 1000 marker; Lanes 1–4: blank control, keno-carrier control, independence sequence control, Annexin A2 shRNA plasmid group, respectively. β-actin expression was used as internal controls. The below is the corresponding plots (* $P<0.05$)

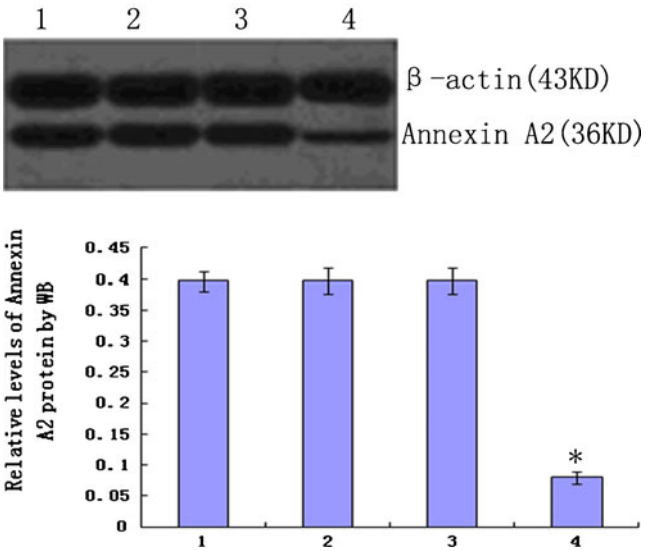

Fig. 3 Representative result of Western blotting for Annexin A2 protein expression in lung adencarcinoma cell line A549. Lane 1: blank control; Lane 2: keno-carrier control; Lane 3: independence sequence control; Lane 4: Annexin A2 shRNA plasmid group. The below is the corresponding plots (*$P<0.05$)

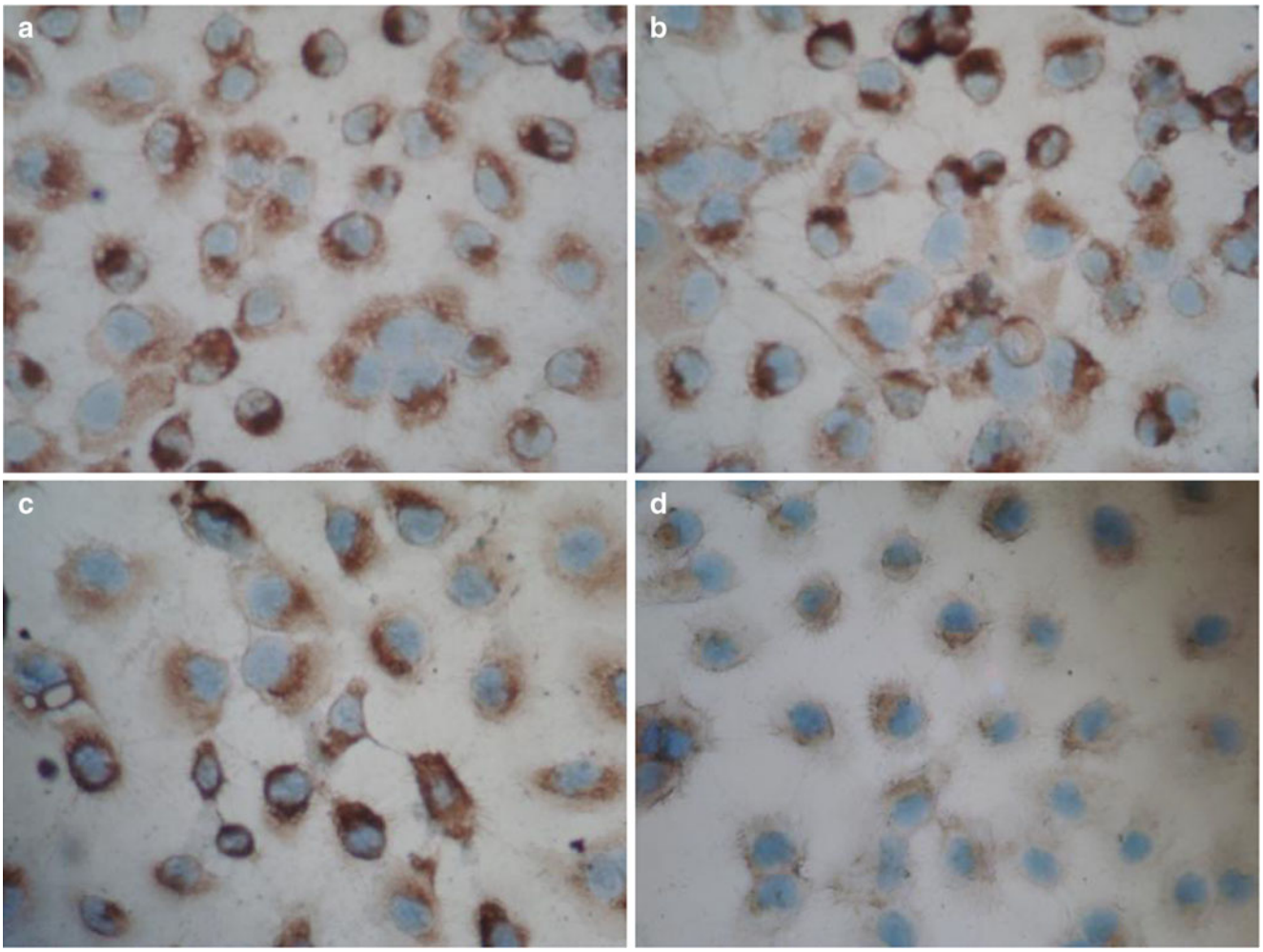

Fig. 4 Immuocytochemistry staining of Annexin A2 in lung adencarcinoma cell line A549. Strong cytoplasmic staining of Annexin A2 was observed in blank control (**a**), keno-carrier control (**b**) and independence sequence control (**c**). There was a little weak staining in shRNA plasmid group (**d**). Original magnification, 200×

MTT Assay

Parallel samples of transfected cells were plated in 96 well flat-bottomed micro-plate (4,000 cells/well) and cultured for 24, 48, 72 h in 200 μl DMEM supplemented with 10% FBS, respectively. The number of living cells was determined using the MTT assay as previously described. 20 μl of MTT {3–(4, 5)-dimethylthiahiazo (-z-y1)-3, 5-diphenytetrazoliumromide, Sigma} dye was added to each well 4 h before the end of the incubation. The wells were decanted and 150 μl of DMSO (dimethy sulfoxide) was added to solubilize the reactive formazan crystals. An automatic micro-plate reader (Model 550; Bio-Tek, CA) was used to measure absorbency at 490 nm. Data was collected for drawing proliferation curve and counting proliferation inhibition ratio. For statistical comparison with controls, Student *t*-test was used with significance $p<0.05$ from three independent experiments.

Matrigel Invasion Assay

The invasion of tumor cells in vitro was assessed by transwell chamber usually. Transwell chambers with poly-

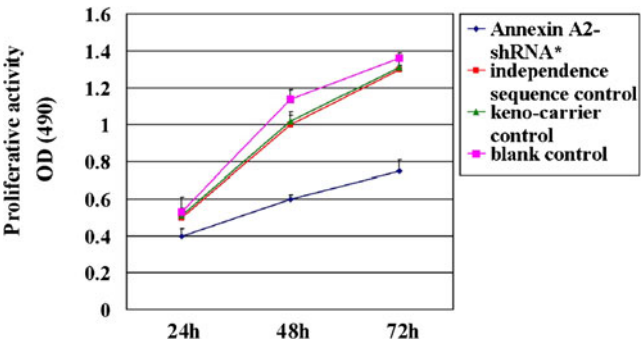

Fig. 5 Results of MTT assay. The growth curve revealed that knowing-out Annexin A2 could decrease the proliferation of A549 cells. The cell proliferative velocity of A549 in shRNA plasmid group greatly decreased compared with three control groups at 24, 48, 72 h; respectively ($P<0.05$)

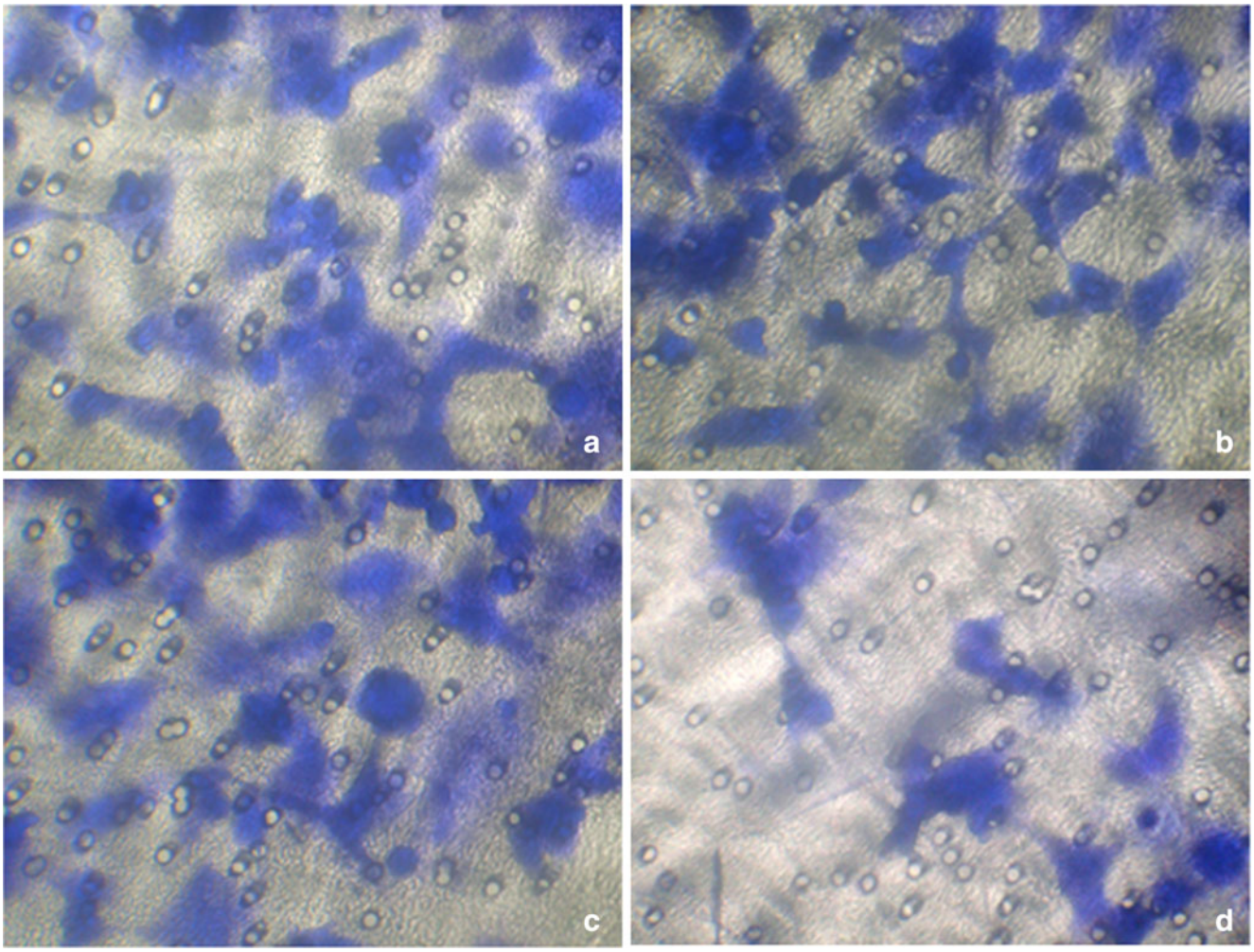

Fig. 6 Transwell invasion experiments.The number of cells penetrating the Matrigel membrane in shRNA plasmid group (**d**) displayed a striking decrease contrast to three groups: blank control (**a**), keno-carrier control (**b**) and independence sequence control (**c**) ($P<0.05$) Original magnification, 200×

carbonate membranes (8 μm pores, Costar, CA) in 24-well culture plates were coated with matrigel (Collaborative Rsearch, CA) diluted in serum-free DMEM, and incubated at 37°C for 30 min. A 200 μl suspension of 1×10^5 transfected cells was layered to the upper compartment of the transwell chambers and cultured for a further 24 h at 37°C with serum-free DMEM. Then the cells were motile and invaded through the matrigel membrane into the lower chamber. In the end, the matrigel was scraped away and the remaining polycarbonate membranes were stained with gentian violet (hexamethylpararosanlline, Dingguo, Beijing) to calculate cells that had penetrated the matrigel membrane. The data was analysed according to the number of cells. All incubations were done in triplicate.

Enzyme-linked Immunoadsorbent Assay (ELISA)

The concentration of MMP-2 and CB in the culture supernatant was quantified using commercially available ELISA kits (RD, USA). Methods were as described in the manufacturers' instructions. Briefly, the supernatant was collected by centrifugation at 2000 rpm for 20 min. The data need to be compared according to time gradient and the concentration of MMP-2 and CB in the culture supernatant was corrected for cell number. Each sample was assayed in duplicate and the values were within the linear portion of the standard curve. The experiments were repeated three times.

Statistical Analysis

All experiments were performed at least three times. For all statistical procedures, SPSS for Windows version 11.0 software (SPSS Inc., USA) was used for statistical analyses. Normality was checked for continuous variables. Data were expressed as mean±SD (standard deviation), median minimum-maximum, n (number of cases) and percent (%). Results were compared from two groups using the

Student *t*-test and from many groups using single factor mean square test. Generally, *p* value less than 0.05 was considered as statistically significant in difference.

Results

Detection of Transfection Efficiency

Under fluorescence microscope, three visions of transfected cells were selected randomly and about 60% of cells emitted green fluorescence at 48 h after transfection (Fig. 1).

Expression of Annexin A2 mRNA

The Annexin A2 mRNA expression level of shRNA plasmid group was drastically decreased compared with three control groups, respectively ($P<0.05$). In contrast, there was no difference about the Annexin A2 mRNA level among blank control, independence sequence control and keno-carrier control ($P>0.05$) (Fig. 2).

Expression of Annexin A2 Protein

The level of Annexin A2 protein of shRNA plasmid group had greatly decreased contrast to control groups ($P<0.05$), while no change of the Annexin A2 protein level was seen among blank control, independence sequence control and keno-carrier control ($P>0.05$). Compared to Annexin A2, internal control β-actin did not vary (Fig. 3).

Immuocytochemistry Staining

A549 cells from blank control, independence sequence control and keno-carrier control revealed a high level of brown staining for Annexin A2 protein in cytoplasm or cell membrane (Fig. 4a, b, c), while a relatively weaker brown staining was observed in shRNA plasmid group (Fig. 4d).

Effect of shRNA Plasmid on Proliferation of A549 Cells

Results of MTT assay showed cell proliferative velocity of blank control was slightly quicker than that of independence sequence control and keno-carrier control, but among these three controls there was no discrepancy ($P>0.05$). However, cell proliferation velocity of A549 in shRNA plasmid group greatly decreased compared with three control groups at 24, 48, 72 h; respectively ($P<0.05$). The proliferation inhibition ratio of A549 cells of shRNA plasmid group was 21.60%±0.11, 60.04%±0. 20 and 43.22%±0.17 at 24 h , 48 h and 72 h, respectively (Fig. 5).

Effect of shRNA Plasmid on Invasion of A549 Cells In Vitro

The number of cells penetrating the Matrigel membrane among shRNA plasmid group, blank control, independence sequence control and keno-carrier control were 23.0±7.5, 68.0±9.3, 80.0±7.1, 72.0±8.6, respectively. According to above data, there was no diference among three control groups ($P>0.05$). But A549 cells of shRNA plasmid group displayed a striking decrease in the number of cells penetrating membrane contrast to three groups ($P<0.05$) (Fig. 6).

Measurement of MMP-2 and CB in Culture Supernatant

Results of ELISA showed concentration of MMP-2 in the supernatant of shRNA plasmid group decreased extremely compared with three control groups at 8 h, 12 h, 24 h, 48 h posttransfection, respectively ($P<0.05$) (Fig. 7a), and concentration of CB in the supernatant of A549 cells of shRNA plasmid group also showed the same change with MMP-2 ($P<0.05$) (Fig. 7b)

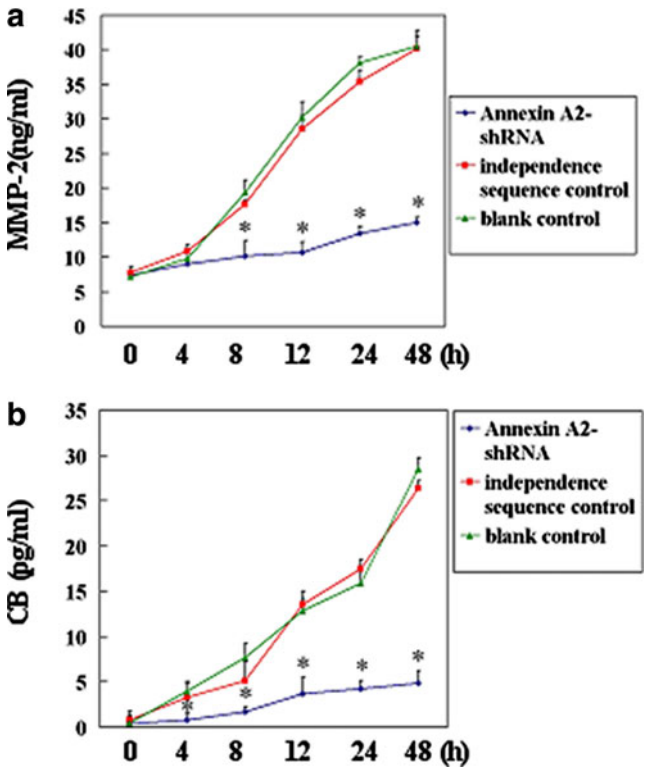

Fig. 7 The levels of MMP-2 and CB in the culture supernatant of A549 cells by ELISA. **a**: The concentration of MMP-2 in the supernatant of shRNA plasmid group decreased significantly compared with independence sequence control and blank control at 8, 12, 24, 48 h post-transfection, respectively($P<0.05$). **b**: The concentration of CB showed the same change with MMP-2 ($P<0.05$)

Discussion

Annexin A2 is strongly expressed in most tumors. The expression level of Annexin A2 has a close relation with development, invasion and metastasis of tumors [19–21]. Frohlich [22] reported increasing expression of Annexin A2 correlated to generation of hepatoma firstly in 1990. Immunohistochemical analysis revealed that Annexin A2 was strongly expressed in follicular thyroid carcinoma, papillary carcinoma, anaplastic carcinoma and medullary carcinoma, while in normal thyroid gland follicular epithelium and follicular adenoma the expression of Annexin A2 showed negative staining [9]. Two-dimensional PAGE and Western blotting techniques were applied to detect protein expression in normal cervix and squamous carcinoma of the cervix, and Annexin A2 expression of the latter was predominantly up-regulation [23].

Although Annexin A2 had a close connection with multiple tumors, the report about function of Annexin A2 in lung cancer cells is still very little. shRNA plasmid mediated RNA interference is a kind of gene silence technique of post-transcriptional level, and shRNA possesses highly efficient function and feature of knocking-down gene expression. We designed shRNA sequence and constructed the shRNA plasmid targeting Annexin A2 in vitro followed by transfecting the specific plasmid into human lung adencarcinoma cell line A549 via lipofectamine 2000 mediation. This study proved both mRNA and protein expression levels of Annexin A2 in A549 cells post-transcription were significantly down-regulation compared to control groups. The results of MTT revealed that reproductive activity of A549 cells in shRNA plasmid group were decreased obviously post-transcription contrast to three control groups. The research of Transwell invasion experiments also presented the number of cells penetrating membrane of shRNA plasmid group reduced apparently compared with three control groups post-transcription. This suggested that knocking down Annexin A2 expression by shRNA could restrain reproductive activity and invasion competence of lung cancer cell A549 in vitro.

Lung cancer is a kind of malignant tumor which possesses high level of incidence and low therapeutic efficacy in the world, and has become the major cause of malignancy-related deaths in most countries. The high mortality of this disease is attributable to difficulties in early diagnosis. In many cases, local invasion and/or metastasis to distant organs have already occurred by the time of the diagnosis. Malignant proliferation, invasion and metastasis are the essence for malignant phenotype of lung cancer, and the main factor impacting on survival rate and case fatality. Degradation of the extracellular matrix (ECM) and components of the basement membrane caused by a concerted action of proteinases play a crucial role in tumor invasion, metastasis and angiogenesis [24, 25]. Among these enzymes, matrix metalloproteinase-2 (MMP-2) and cathepsin B (CB) could degrade and destroy most components of the ECM directly [26–33]. Matrix metalloproteinases are a closely related multigene family of zinc-dependent proteolytic enzymes. As one member of the MMP family, MMP-2 has unique ability to degrade type IV collagen, one of the main constituents of the basement membrane. Similar to MMP-2, the lysosomal cysteine proteinase CB also could facilitate ECM degradation such as type IV collagen, fibronectin and laminin hydrolization. Annexin A2 is a coreceptor of plasminogen, tissue-type plasminogen activator and procathepsin B, which form active plasmin and CB after combining with Annexin A2 monomer in cell surface. Plasmin then activates matrix metalloproteinases including MMP-2. To confirm the signal pathway among Annexin A2 and downstream factors, the concentration of MMP-2 and CB was measured by ELISA in the supernatant of A549 cells. As shown in the current study, the concentration of MMP-2 and CB in the supernatant of A549 cells with specific shRNA plasmid decreased obviously compared with control groups at 8, 12, 24, 48 h post-transfection. It implied that down-regulated expression levels of Annexin A2 could suppress the secretion of MMP-2 and CB, consequently MMP-2 and CB are downstream target genes of Annexin A2, which could promote progression of lung cancer by regulating the expression of MMP-2 and CB.

In general, in this study the expression of Annexin A2 had been effectively inhibited and significantly decrease proliferation and invasion capability of A549 cells in vitro by synthesizing the specific Annexin A2-shRNA plasmid, implying that Annexin A2 might play an important role in the progression and invasion of human lung cancer cells and could promote progression of lung cancer by regulating the expression of MMP-2 and CB.

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