

• Basic Research •

Inhibitory effect of novel internalized fully human phage antibody fragments on proliferation of lung adenocarcinoma cell line overexpressing peroxiredoxin I

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[Abstract] Background and Objective: Previous researches have implicated the close relationship between peroxiredoxin I (Prx I) and cancer progression. A lung adenocarcinoma-related human phage antibody library has been constructed by using phage display techniques. This study was to screen out the single chain variable fragment (scFv) antibodies from the library against a lung adenocarcinoma cell line overexpressing Prx I and to analyze their anti-proliferation ability. Methods: The insertion ratio of scFv gene was identified by polymerase chain reaction (PCR). The products were digested by *Sfi* I and *Not* I, and analyzed on 1% agarose gel. Three rounds of panning against lung adenocarcinoma cell line A549 and Prx I were performed separately, and the positive clones were chosen for soluble expression. The internalization of radiolabeled scFv fragments was then quantified. The proliferation and apoptosis of A549 cells were detected by MTT assay and flow cytometry (FCM). The protein expression of Prx I in A549 cells was analyzed by Western blot. Results: The insertion ratio of scFv gene was 77% (23/30) and enzyme digestion showed the target products. The sixth phage harvest yielded 180 times as much as that of the first one. Positive reactions with A549 cells were detected in six (60%) of ten random clones. The human scFv fragments against Prx I of lung adenocarcinoma were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and enzyme-linked immunosorbent assay (ELISA). The internalized scFvs mediated cell apoptosis and Prx I expression down-regulation. Conclusions: The scFv fragments against Prx I of lung adenocarcinoma are acquired by screening the phage antibody library. The soluble antibodies have specific avidity and inhibitory effect on proliferation of human lung adenocarcinoma cells.

Key words: lung neoplasm, phage antibody library, single chain variable fragment, peroxiredoxin I, proliferation inhibition

Cellular redox status is closely associated with tumorigenesis, and the proteions participating in cellular redox reaction can be applied to the studies on tumorigenesis and progression. Peroxiredoxin is a kind of newly identified peroxidase which play an important role in the clearance of reactive oxygen species, and protect cells from membrane damage caused by peroxidation.¹ Peroxiredoxin I (Prx I), a member of Peroxiredoxin protein family, is located in cytoplasm and has a high expression level in lung carcinoma cells.² Prx I can reduce the generation of reactive oxygen radicals, and the upregulation of Prx I expression can

enhance antioxidant capacity of tumors, which is an important reason for anti-apoptosis and chemotherapy resistance.³ It has been confirmed that, through the Prx I target, inhibition of gene expression and protein translation of Prx I can induce proliferation inhibition and reverse chemotherapy resistance of tumor cells.^{4,5}

Lung carcinoma is a severe disease threatening the human health. In recent years, the incidence of lung carcinoma showed an increasing trend. Also, studies on the treatments of lung carcinoma are attracting more and more attention. Antibody-targeted therapy plays unique and important roles in clinical practice. In tumor treatments, antibody technology has also gradually become an unneglectable therapeutic means. Currently, most of tumor antibodies are heterogeneous, and have slow clearance rates in vivo.^{6,7} Also, they can induce anti-antibody response in human body in clinical practices, which enhances the therapeutic risk of patients and reduces the therapeutic efficacy. Therefore, tumor antibodies are not still really effectively and extensively applied in clinical practices. However, the technology of human phage antibody library can effectively solve the above problem, and bring the new hope for the antibody therapy of tumors.

In view of this, we screened out the human scFv antibody against Prx I of lung carcinoma by the phage antibody library. Moreover, we detected the inhibitory effect on the proliferation of lung adenocarcinoma cells, which provided a basis for further applications of this phage antibody library.

Materials and Methods

Materials and reagents. Plasmid extraction kit was purchased from Shanghai Watson Biotech Company; T4 DNA ligase, *Sfi* I and *Not* I restriction enzymes were the products of TaKaRa Biotechnology (Dalian) Company; Helper phage M13KO7, *E. coli* TG1 and *E. coli* HB2151 strains, horseradish peroxidase labeled anti-M13 monoclonal antibody (HRP/Anti-M13 Monoclonal Antibody), mouse anti-E-tag monoclonal antibody were purchased from Amersham Company; Peroxiredoxin I antigen was from BPS Company; Prx I rabbit anti-human polyclonal antibody was the product of ABCAM Company; Human lung adenocarcinoma cell line A549 was purchased from Nanjing KeyGen Biotech

Company; Human bronchial epithelial cell line HBE16 was preserved at our lab; Breast carcinoma cell line MDA-MB-435 was a gift from Dr. Hong Duan in the Department of Pathophysiology in Chongqing Medical University.

Determination of insertion ratio of antibody gene by PCR. Thirty single colonies were randomly picked from the lung adenocarcinoma related phage antibody library constructed by ourselves,⁸ and were respectively cultured in 2-YT culture medium with vigorous shaking overnight. Then, plasmids were extracted by plasmid extraction kit according to manufacturers instruction. Finally, the plasmids were respectively identified by PCR, and the insertion ratio of antibody gene was calculated.

Identification by *Sfi* I/*Not* I double restriction digestion. Positive PCR products of plasmids were randomly selected and subjected to double restriction digestion through *Sfi* I and *Not* I enzymes. Then, the target genes were verified by 1% agarose gel electrophoresis.

Screening of specific antibody library of lung carcinoma against Prx I. Whole cell screening based on lung adenocarcinoma A549. Firstly, normal bronchial epithelial cells HBE16 were used to adsorb 1.5 mL solution of phage antibody library so as to block non-specific binding sites of antibodies. Then, three rounds of screening of antibody library were performed through the "absorption – elution – amplification" procedure using 1×10^7 A549 cells.

Screening of antibody library against Prx I antigen. The antibody library solution, which has been previously screened against A549 cells, was further screened against Prx I purified antigen. After Prx I antigen coated an immune tube at 4°C overnight, blocking was carried out at 37°C for 2 h by addition of 2% BSA/PBS. Then, the solid-phase screening was performed through the addition of the antibody library solution previously screened against A549 cells and subsequent incubation at 37°C for 1 h. Free and weakly bound phages were removed by washing with PBST, and the washing times were increased with the round numbers of screening. Total three rounds of screening were carried out.

Identification of positive clones. After being infected by the enriched phages, *E. coli* TG1 were spread on the SOBAG plate and then cultured at 30 overnight. Then, single colonies were picked and inoculated into a 96-well plate to culture for preparing single chain phage antibody. After Prx I

antigen coated the 96-well plate at 4°C overnight, blocking was performed at 37°C for 2 h with 2% BSA/PBS. Subsequently, 100 L of phage antibody was added into each well and incubated at 4°C overnight. In control group, the scFv antibody was replaced with PBS. After each well was washed with PBS for three times, HRP/Anti-M13 Monoclonal Antibody (1:5000 dilution) was added into each well and incubated at room temperature for 1 h. Finally, color development was performed in dark for 20 min using ABTS. The absorbance values at 405 nm were determined by an enzyme-linked measuring instrument, and P/N > 4 was considered positive.

Expression of soluble scFv antibody. After being infected by the strongly positive phage antibody with the highest measurement reading, *E.coli* HB2151 were spread on the SOBAG plate and then incubated at 30°C overnight. Then, single colonies were picked and inoculated into 5 mL 2-YT medium to culture at 26°C and 200×g overnight. Subsequently, 100 mL of 2-YTAG culture medium was added, and further culture was carried out at 26 and 200 rpm until the absorbance value at 600 nm (A_{600}) reached 0.5. After IPTG was added at a final concentration of 1 mmol/L, induction culture was performed at 26°C and 200 xg for 4 h. Then, the cultured bacterial cells were collected by centrifugation at 8,000×g and 4°C for 10 min. Then, bacterial cells were resuspended with 2 mL of HEPES extraction buffer (20 mmol/L HEPES, 0.5 mol/L NaCl, pH was adjusted by NaOH). After ultrasonic lysis of bacterial cells, centrifugation was carried out at 12,000×g and 4°C for 30 min, and then the supernatant containing soluble scFv antibody⁹ was collected. Finally, the scFv antibody was purified through the affinity chromatography column of HisTrap Anti-E Tag (GE Company).

Identification of soluble antibody. SDS-PAGE detection. Raw soluble antibody sample (10 µL) and purified soluble antibody (10 µL) were respectively mixed with equal volume of loading buffer, and then were denatured by boiling for 3 min. Finally, SDS-PAGE was carried out.

Determination of immune activity of soluble antibody by ELISA. A549, MDA-MB-435 and HBE16 cells were respectively seeded into a 96-well culture plate at a density of 1×10^4 cells/well, and then were fixed with 4% paraformaldehyde. Immune activity of the scFv antibody was determined by ELISA. Mouse anti-E-tag monoclonal antibody (1:2,500 dilution) and HRP/goat anti mouse antibody (1:5,000 dilution)

were respectively used as primary and secondary antibodies. Finally, color development was performed in dark for 20 min using ABTS. The absorbance values at 405 nm were determined by an enzyme-linked measuring instrument.

Determination of antibody affinity by competitive ELISA. A549 cells were seeded into a 96-well culture plate at a density of 1×10^4 cells/well. 50 µL of IgG (1 mg/mL) was added into each well. Subsequently, 50 µL of soluble antibodies with various dilutions (1:1, 1:5, 1:25, 1:125, 1:250) were respectively added, and then were incubated at 4°C overnight. In control group, no soluble antibody was added. After washing, HRP labeled anti-E-tag monoclonal antibody was respectively added into each well. After color development using ABTS, the absorbance values at 405 nm were determined by an enzyme-linked measuring instrument, and the inhibition rate of binding was calculated.

Determination of specificity of soluble antibody by immunocytochemistry method. A549, MDA-MB-435 and HBE16 cells were respectively seeded into a 96-well culture plate at a density of 1×10^4 cells/well. Then, the specificity of soluble antibody was determined through kit by immunocytochemistry method, and the color development was performed using DAB.

Determination of internalization amount of soluble antibody. The internalization amount of soluble antibody was determined by the chloramine T method, and the radionuclide Na¹²⁵I was used to label the antibody (3.7×10^6 Bq/mg of protein). The labeled antibody was purified on sephadex G50. The lung adenocarcinoma A549 cells were incubated with the ¹²⁵I-scFv (1.5×10^6 cpm/ 1×10^4 cells) at 37°C for 30 and 120 min, respectively. The incubation at 4°C was used as control. Each group was in triplicate. After incubation, cells were washed with PBS for three times, and further washed with ice-precooling glycine buffer (0.1 mol/L glycine-HCl, 2 mol/L urea, pH 2.2) for 2 min for twice in order to elute the ¹²⁵I-scFv bound on cell surface. And, the eluent was collected. In addition, cells were lysed with 2% SDS, and cells lysates were collected. The radioactive count of the eluent and cells lysates were determined by a γ-ray counter, respectively.

Detection of cells proliferation inhibition. A549 cells were seeded into a 96-well culture plate at a density of 3×10^4 cells/mL, and the volume of culture medium in each well was 100 L. Then, 50 µL of

scFv containing 1% fetal bovine serum at various concentrations were respectively added, and the anti-iASPP scFv prepared by our lab was used as control. Each group included triplicate wells. After 72-hour cell culture, MTT assay at 490 nm was performed to detect the proliferation of cells.

A549 cells were seeded into a culture flask at a density of 1×10^6 cells/mL, and the volume of culture medium was 1 mL. Then, 1 mL of Prx I scFv ($0.5 \mu\text{mol/L}$) was added. After 72-hour cell culture, cells were collected through trypsinization. Finally, the Annexin V-FITC/PI double-staining method was used to detect the cells apoptosis by flow cytometry, and the anti-iASPP scFv was used as control.

Expression of Prx I protein in A549 cells. A549 cells were seeded into a culture flask at a density of 5×10^6 cells/mL, and the volume of culture medium was 1 mL. Then, 1 mL of Prx I scFv ($0.5 \mu\text{mol/L}$) was added, and the anti-iASPP scFv was used as control. After 72-hour cell culture, total cellular protein was extracted, and the protein concentration was assayed by Bradford method. Subsequently, the expression of Prx I protein was detected by Western blot assay in which rabbit anti human Prx I polyclonal antibody (ABCAM) and HRP/goat anti rabbit IgG (SBA) were respectively used as primary and secondary antibodies. The β -actin was used as the internal control. Finally, the ratio of optical density of Prx I protein bands to that of β -actin internal control bands was calculated and used to express the relative strength of Prx I protein expression.

Statistical analysis. The *t* test and correlation analysis were performed by SPSS 13.0 software. $P < 0.05$ was considered significant.

Results

Identification of insertion ratio of antibody gene by PCR. Thirty single colonies were used to determine the insertion ratio of antibody gene by PCR. Therein, the scFv fragments were amplified from twenty-three clones, respectively. Therefore, the insertion ratio of antibody gene was 77%.

Identification of positive clones by double restriction digestion. Six positive clones were randomly selected and subjected to double restriction digestion using *Sfi* I and *Not* I enzymes. The results of 1% agarose gel electrophoresis have shown that the target fragments were released from all of six positive clones, and 750 bp length bands could be

clearly observed (Fig. 1).

Screening enrichment of phage scFv of human lung adenocarcinoma against Prx I and identification of positive clones

Three rounds of screening of phage antibody library were performed through the "absorption-elution-amplification" procedure using lung adenocarcinoma cell line A549. Further, another three rounds of screening was carried out using Prx I antigen. With the increase of washing times, the harvesting rate of phage antibody showed an increasing trend. After six rounds of screening, the harvesting rate was increased about 180-fold, which suggested that the phage antibody library was effectively enriched. After being infected by the enriched phages, *E. coli* cells were spread on a plate and then cultured. Ten single colonies were picked and inoculated into a 96-well plate to culture for preparing scFv antibodies. ELISA assay was performed to identify the prepared scFv antibodies. The results, which were determined at 405 nm by an enzyme-linked measuring instrument, showed that six antibody reactions were positive, and the positive rate of antibodies was 60% (Fig. 2).

Detection of soluble antibody by SDS-PAGE. The purified soluble antibody and bacterial lysates supernatant containing soluble antibody were assayed by SDS-PAGE. After staining with coomassie brilliant blue R250, a protein band at the 30 ku site was observed, which suggested that the soluble expression of the scFv antibody fragment in *E. coli* HB2151 was carried out (Fig. 3).

Determination of immune activity of soluble antibody by ELISA. The soluble antibody had high immune activity. Moreover, it specifically bound to A549 cells ($A_{405} = 0.63 \pm 0.08$), but not bound to MDA-MB-435 cells ($A_{405} = 0.36 \pm 0.05$) and HBE16 cells ($A_{405} = 0.37 \pm 0.06$) ($P < 0.05$).

Determination of antibody affinity. The affinity between the soluble antibody and A549 cells was evaluated by competitive ELISA. The results have shown that the binding inhibition rate of IgG was 4.4% with the 1:250 dilution of antibody, and was increased to 72.1% with the 1:1 dilution.

Determination of specificity of soluble antibody by immunocytochemistry method. Compared with MDA-MB-435 and HBE16 cells, A549 cells were significantly deeply stained (Fig. 4), indicating that the scFv antibody specifically bound to A549 cells.

Internalization of soluble antibody by A549 cells.

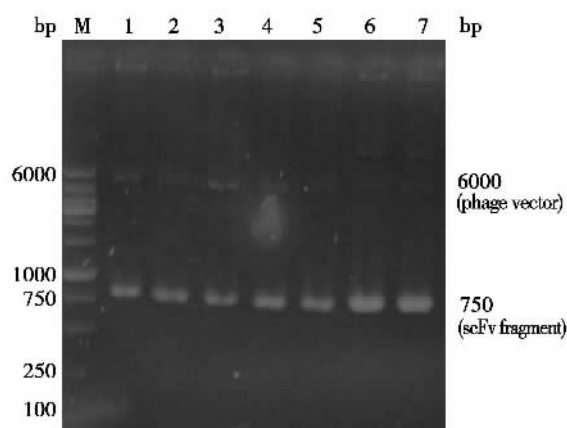


Figure 1 Identification of positive clones with scFv fragments by enzyme digest
Lane M: DNA marker; lanes 1–7: positive clones.

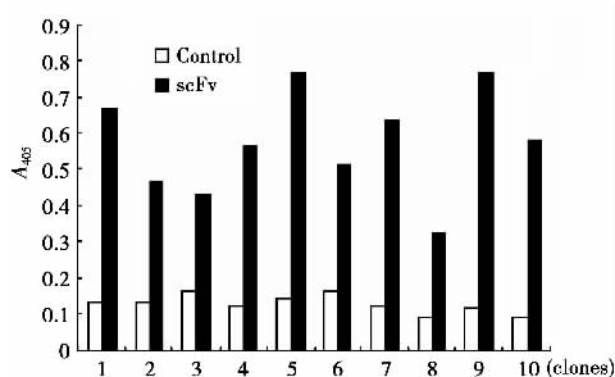


Figure 2 Positive clones with scFv fragments detected by ELISA

The amount of ¹²⁵I–scFv internalized into cells were evaluated by the radioactive count of cells lysates, and the total amount of ¹²⁵I–scFv bound to cells were evaluated by the sum of the radioactive count of the glycine buffer eluent and cells lysates. The results

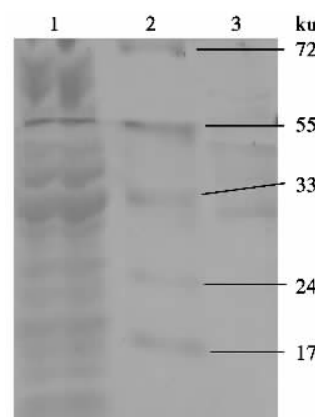


Figure 3 SDS-PAGE analysis of soluble antibodies
Lane 1: scFv fragments induced with IPTG; lane 2: molecular mass marker; lane 3: purified soluble antibodies.

showed that the total binding amount was (1,924.3 ± 457.3) cpm and the internalization amount was (857.4 ± 101.3) cpm at the time point of 120 min, which all were two times of those at the 30 min point [total amount: (1037.1 ± 177.5) cpm, internalization amount: (421.1 ± 69.9) cpm]. The ratios of the amount of internalized antibody to that of antibody bound to cells were relatively constant at the above two time points, and were respectively 40.3% (30 min) and 44.7% (120 min). In control group, the labeled antibodies were only detected on cell surface.

Detection of cell proliferation and apoptosis by MTT and flow cytometry. After A549 cells were treated with the scFv antibody for 72 h, MTT assay was performed. It was found that the antibody had a dose –dependent anti –proliferation effect on cells (Fig. 5). The apoptosis rate was significantly higher in antibody–treated group than in control group (Fig. 6). Determination of expression level of Prx I protein

After A549 cells were treated with the scFv antibody for 72 h, total cellular protein was extracted

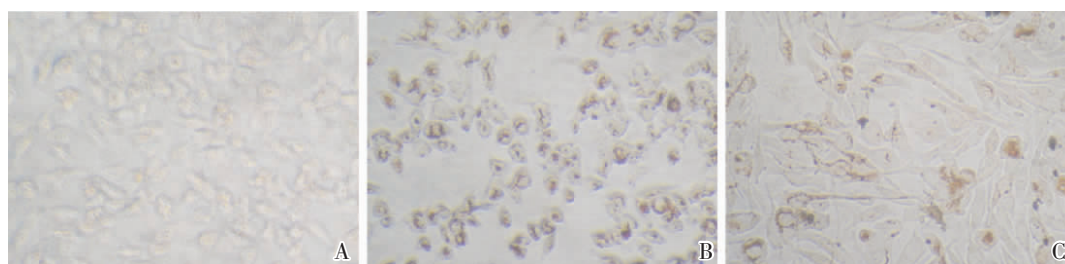


Figure 4 Specificity determination of scFv fragments by immunocytochemical staining (×200)
A: normal human bronchial epithelial HBE16 cells; B: human lung adenocarcinoma A549 cells; C: breast cancer MDA-MB-435 cells. With horseradish peroxidase-diaminobenzidine staining, the phage antibodies are observed in A549 cells, whereas few are observed in HBE16 and MDA-MB-435 cells.

and assayed by Western blot. The expression of Prx I protein in antibody-treated group was significantly lower than that in control group ($P < 0.05$) (Fig. 7).

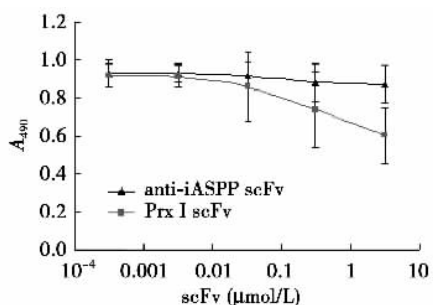


Figure 5 Proliferation of A549 cells measured by MTT assay

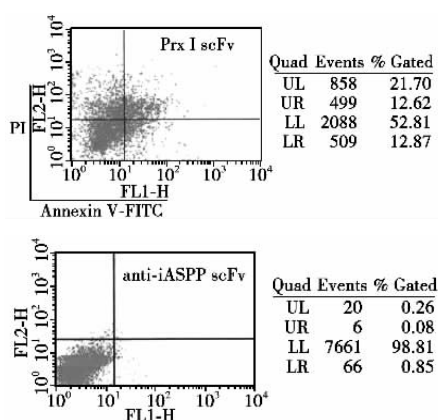


Figure 6 Apoptosis of A549 cells measured by flow cytometry (FCM) with Annexin V/PI staining

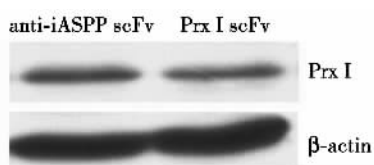


Figure 7 Expression of Prx I in A549 cells detected by Western blot

Discussion

Hypoxia is a common phenomenon in the progression of solid tumors, and transitory microenvironment hypoxia plays an important effect on tumor proliferation and treatment results. Hypoxia can upregulate expression levels of antioxidant proteins in tumor cells and enhance antioxidant capacity of tumor cells, which lead to resist drug-induced apoptosis.¹⁰ By dynamic observation, Kim et al.²

found that the expression of Prx I gene was upregulated in anoxic A549 cells, and the Prx I expression level in lung carcinoma tissue was also significantly higher than that in normal lung tissue. They concluded that the changes of redox in the microenvironment of lung tumors triggered the upregulation of Prx I expression, which promoted tumors proliferation and chemotherapy resistance. Compared with the other members of Peroxiredoxin family, Prx I exerts unique function and regulation mechanism in non-small cell lung carcinoma (NSCLC), and can be used as the therapeutic target and biomarker for estimation of NSCLC prognosis.¹¹

Currently, the genes being involved in human antibody library were mainly originated from peripheral blood lymphocytes. Moreover, large amount of peripheral blood and inconvenient operations were required for antibody preparation. In our previous construction of antibody library, lymph node tissues adjacent to carcinoma in patients with lung cancer were selected as the source of antibody genes, and more B cells could be collected. It is the best selection for constructing the antibody library.¹²

The performances of human single chain variable fragments (scFv) antibody are mainly determined by the enrichment of antibody library by screening. The antibody library can exert maximally biological function through choosing correct screening strategy. Most of cell surface membrane proteins were modified after translation, and so have specific space conformation. By whole cell screening, the prepared antibodies can specifically and high efficiently bind to targets.^{13, 14} However, solid-phase screening with purified antigen can provide definite screening targets, and so improve specificity and efficiency of screening.¹⁵ In present study, the negative screening against antibody library was firstly performed using normal bronchial epithelial cells HBE16. Then, three rounds of positive screening were carried out through Prx I overexpressing cells A549. Finally, three rounds of screening were done by Prx I antigen. The results have shown that, through the combination of whole cell screening and purified antigen solid-phase screening, the prepared antibodies have good specificity, which provide support for preparing high affinity scFv antibody against Prx I of lung adenocarcinoma.

Phage display technique has advantages of simplicity, high efficiency, large library capacity of screening and low production cost. Moreover,

immunizing animals is not required for antibody preparation by phage display technique. This technique solves the problems of antibody antigenicity and low production efficiency in previous preparation of antibody, and its application is very extensive.¹⁶⁻¹⁸ Therein, antibody therapy, in which specific tumor antigens are used as targets, has the advantages of high efficiency and low toxicity, and provide new choice and supplement for tumors cytotoxicity therapy in clinical practice.^{19,20}

It has been reported that scFv could bind to cell surface molecules and then be internalized into cells, and further mediate important physiological processes in tumor cells, which suggested that scFv had intrinsic anti-proliferation activity.²¹ In present study, the prepared scFv antibody could target to A549 cells and Prx I antigen. After binding to A549 cell surface, the prepared scFv antibody could be effectively internalized into A549 cells. Thereafter, the internalized scFv antibody could mediate dose-related anti-proliferation effect on tumor cells, and promote the apoptosis of target cells. Moreover, it was found that the expression level of Prx I protein in tumor cells was decreased. Based on the above results, it is speculated that antibody anti-proliferation effect could be attributed to limitation of cell receptor numbers through the internalized antibody. Moreover, after binding to Prx I epitope, the antibody reduced antioxidant capacity of tumor cells, and so promoted proliferation inhibition and apoptosis of tumor cells. With respect to the mechanism that the antibody in present study inhibited the proliferation of lung adenocarcinoma cells, further study will be required.

In summary, through screening lung adenocarcinoma related phage antibody library, we have prepared the human scFv antibody targeting to Prx I antigen and lung adenocarcinoma A549 cells with high expression of Prx I antigen. The prepared antibody could specifically bind A549 cells. After internalization into cells, the antibody could effectively inhibit the tumor cells proliferation and promote the apoptosis of tumor cells, which suggested that the antibody had potential application in immunotherapy of lung adenocarcinoma.

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