Screening and identification of a peptide specifically targeted to NCI-H1299 cells from a phage display peptide library

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Abstract. Ligands that are capable of binding to tumor cell surface biomarkers specifically used in the early diagnosis of cancer and targeted drug delivery in cancer chemotherapy have been extensively investigated. Phage display technology has been demonstrated to be a powerful tool in this field. In this study, the non-small cell lung cancer NCI-H1299 and the normal lung small airway epithelial cell lines were used for subtractive screening *in vitro* with a phage display 12-peptide library. After three rounds of panning, there was an obvious enrichment in the phages specifically binding to the NCI-H1299 cells, and the output/input ratio of phages increased approximately 875-fold (from $0.4x10^4$ to $3.5x10^6$). A group of peptides capable of binding specifically to the NCI-H1299 cells was obtained, and the affinity of these peptides to bind to the targeted cells and tissues was studied. Through cell-based ELISA, immunocytochemical staining, immunohistochemical staining and immunofluorescence, an M13 phage was isolated and identified from the above screenings, and a synthetic peptide, ZT-1 (sequence QQMHLMSYAPGP), corresponding to the sequence of the surface protein of the M13 phage, was demonstrated to be capable of binding to the tumor cell surfaces of NCI-H1299 and A549 cells and biopsy specimens, but not to normal lung tissue samples, other cancer cells, or non-tumor adjacent lung tissues. In conclusion, the peptide ZT-1 may be a potential candidate biomarker ligand that can be used for targeted drug delivery in lung cancer therapy.

Introduction

Genetic and proteomic changes occur during carcinogenesis, resulting in the alteration of cell surface features (1). This difference in the cell surface profile between cancerous cells and their non-malignant counterparts could serve as a molecular address for targeting reagents to deliver a molecule of choice to desired cell types. Identification of biomarkers for cancerous cells is a critical step in terms of developing new targeting reagents for cell-specific delivery of chemotherapeutics or imaging reagents. Mass spectrometric-based proteomic approaches have been used to identify biomarkers useful for disease diagnosis and prognosis, or drug targets (2,3). However, it is difficult to detect proteins at low levels using conventional methods, and such classes of proteins are underrepresented, especially membrane proteins, which are usually used as biomarkers. These biomarkers could be used for targeted drug delivery. Phage display biopanning on viable cells has proven to be a powerful approach to identifying cell-specific membrane proteins that are capable of binding to a targeted tumor type, even in the absence of knowledge of the targeted cellular receptors (4-7). Thus, peptides recognize distinct cell surface receptors, and this may have clinical value. Targeting reagents can be identified using biopanning, and the corresponded peptides can be used for the discovery of unique cell surface biomarkers. These peptides are capable of delivering a chemotherapeutic agent, resulting in the death of the target cell (8). Our results suggest that such peptides may have value as a diagnostic standard and as cell-targeting reagents.

In the present study, we identified a specific novel peptide that was able to bind to the cell surface of NCI-H1299 cells generated in our laboratory using *in vitro* phage-displayed random peptide libraries. Our results demonstrated that this biopanning strategy can be used to identify tumor-specific targeting peptides. One of our selected peptides was particularly effective in targeting cells and tissues, indicating its potential for use in the early diagnosis or therapy of lung cancer.

Materials and methods

Materials. The NCI-H1299 and A549 cell lines, a normal lung small airway epithelial (SAE) cell line and a human cervical cancer cell line (HeLa), as well as nude mice with a body weight of 15-25 g, were all obtained from the Medical Academy of China. Fetal calf serum (FCS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco (USA). Phage DNA sequencing was performed by Shanghai Sangon Corp.

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(Shanghai, China). Peptide ZT-1 (QQMHLMSYAPGP) and non-specific control peptide (EAFSILQWPFAH) were synthesized and labeled with fluorescein isothiocyanate (FITC) by Shanghai Bioengineering Ltd. Mass analysis of the peptides was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, and all peptides were >90% pure as determined by a reverse-phase HPLC. Stock solutions of the peptides were prepared in phosphate-buffered saline (PBS) (pH 7.4). Horseradish peroxidase-conjugated sheep anti-rabbit antibody and rabbit anti-M13 bacteriophage antibody were purchased from Pharmacia (Peapack, NJ, USA). Trizol reagent was purchased from Gibco BRL (Gaithersburg, MD, USA), and a reverse transcriptase polymerase chain reaction (RT-PCR) system kit was purchased from Promega (Madison, WI, USA). All experiments were repeated a minimum of three times in duplicate.

The Ph.D.-12 Phage Display Peptide Library Kit (New England Biolabs, Beverly, MA, USA) was used to screen specific peptides binding to NCI-H1299 cells. The phage display library contains random peptides constructed at the N-terminus of the minor coat protein (cpIII) of the M13 phage. The titer of the library is $2.3x10^{13}$ pfu (plaque-forming units). The library contains a mixture of $3.1x10^{9}$ individual clones, representing an entire obtainable repertoire of 12-mer peptide sequences that expresses random 12-amino acid sequences. Extensively sequencing the naive library revealed a wide diversity of sequences with no obvious positional biases.

The *E. coli* host strain ER2738 (a robust F^+ strain with a rapid growth rate; New England Biolabs) was used for M13 phage propagation. The NCI-H1299 and small airway epithelial (SAE) cell lines were cultured in DMEM supplemented with penicillin, streptomycin and 10% fetal bovine serum. Cells were harvested at subconfluence, and the total number of cells was counted using a hemocytometer.

In vitro panning. NCI-H1299 cells were selected as the target cells, and the normal lung SAE cell line as the absorber cells for a whole-cell subtractive screening from a phage display 12-peptide library. Cells were cultured in DMEM with 10% FCS at 37˚C in a humidified atmosphere containing 5% CO_2. SAE cells were washed with PBS and kept in serum-free DMEM for 1 h before blocking with 3 ml blocking buffer (BF, PBS + 5% BSA) for 10 min at 37˚C. Approximately $2x10^{11}$ pfu phages was added and mixed gently with the blocked SAE cells for 1 h at 37˚C. Cells were then pelleted by centrifuging at 1000 rpm (80 x g) for 5 min. SAE cells and bound phages were removed by centrifugation. These phages in the supernatant were incubated with the BF-blocked NCI-H1299 cells for 1 h at 37˚C before cells were pelleted again. After that, the pelleted cells were washed twice with 0.1% TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) to remove unbound phage particles. NCI-H1299 cells and bound phages were both incubated with *E. coli* host strain ER2738. Then, the phages were rescued by infection with bacteria while the cells died. The phage titer was subsequently evaluated by a blue plaque-forming assay on agar plates containing tetracycline. Finally, a portion of purified phage preparation was used as the input phage for the next round of *in vitro* selection.

For each round of selection, $2x10^{10}$ pfu of collected phages was used. The panning intensity was increased by prolonging the phage incubation period with SAE cells for 1.25 or 1.5 h, shortening the phage incubation with NCI-H1299 cells by 45, 30 or 15 min in the second and third rounds individually and increasing washing with TBST by 4, 6 or 8 times in the second and third round individually.

Sequence analysis of selected phages and peptide synthesis. After three rounds of *in vitro* panning, 60 blue plaques were randomly selected, and their sequences were analyzed with an ABI Automatic DNA Analyzer (Shanghai Sangon Corp.). The primer used for sequencing was 5'-CCC TCA TAG TTA GCG TAA CG-3' (-96 gIII sequencing primer, provided in the Ph.D.-12 Phage Display Peptide Library Kit). Homologous analysis and multiple sequence alignment were conducted using the BLAST and Clustal W programs to determine the groups of related peptides.

Cell-based ELISA with phageS. NCI-H1299 and SAE cells were cultured in DMEM with 10% FCS at 37˚C in a humidified atmosphere containing 5% CO_2, and the cells were seeded into 96-well plates ($1x10^{5}$ cells/well) overnight. Cells were then fixed on 96-well plates by 4% paraformaldehyde for 15 min at room temperature until they were attached to the plates. Subsequently, the cells were washed three times with PBS. The cells and the amplified phage clones were then chosen randomly and blocked individually with 1% BSA at 37˚C for 30 min. These phage clones were added to the wells ($1x10^{10}$ pfu/well) and incubated with the cells at 37˚C for 2 h. The plates were washed three times with PBS containing TBST before being reacted with a mouse anti-M13 phage antibody. The mouse anti-M13 phage antibody was diluted 1:5000 and added to the wells at 37˚C for 1 h. The plates were washed three times with TBST, and then 100 μl horseradish peroxidase-conjugated sheep anti-mouse Ig (diluted to 1:500 in the blocking buffer) was added. Subsequently, color development was carried out by adding 100 μl/well of freshly prepared TMB solution and incubating the plates for 5 min at 37˚C. The plates were read on an automated ELISA plate reader at an absorbence of 450 nm. As negative controls, PBS and unrelated phages with the same titers were added to the wells instead of the selected phage clones. Triplicate determinations were carried out at each data point. Selectivity was determined using the formula (9): Selectivity = OD_{M13} - OD_{C1}/OD_{S2} - OD_{C2}, where OD_{M13} and OD_{C1} represent the OD values from the selected and control phages binding to NCI-H1299 cells, respectively, and OD_{S2} and OD_{C2} represent the OD values from the selected and control phages binding the control SAE cell line, respectively.

Immunocytochemical staining and immunohistochemical staining of phage M13. Before the staining of phage M13 (10), the cells in the different groups (NCI-H1299, A549, SAE and HeLa) were cultured on coverslips and fixed with acetone at 4˚C for 20 min. Then, ~$1x10^{11}$ pfu of phage M13 diluted in PBS was added to the coverslips and incubated at 4˚C overnight. Subsequently, the coverslips were washed five times with TBST, then blocked by H_2O_2 (3% in PBS) at room temperature for 5-10 min. After being washed with PBS for 5 min at 37˚C, the coverslips were incubated with normal sheep serum for 20 min at 37˚C. Subsequently, the coverslips were incubated overnight at 4˚C with a mouse anti-M13 phage antibody with a working

dilution of 1:5000. The next day, the coverslips were rinsed three times (10 min for each rinse) in PBS and incubated with a secondary antibody for 1 h at room temperature. Afterwards, the coverslips were rinsed three times (5 min for each rinse) in PBS. The bound antibody was visualized using TMB. The coverslips were rinsed three times (5 min for each rinse) using running tap water before staining with hematoxylin and eosin. Finally, the coverslips were rinsed for 10 min with running tap water before dehydration and mounting.

Frozen sections of human lung tissues with and without tumors (provided by the Pathology Department of Sun Yat-sen University Cancer Center) were also prepared. The steps involved in immunohistochemical staining were similar to those of the immunocytochemical staining described above.

Instead of the selected phage clone M13, PBS and non-specific control phage with the same titers were used as negative controls in this study.

Peptide synthesis and labeling. The ZT-1 peptide (QQMHLMSYAPGP; translated from the selected M13 phage DNA sequence) and non-specific control peptide (EAFSILQWPFAH) were synthesized and purified by Shanghai Bioengineering Ltd. FITC-conjugated peptides were produced by the same company.

Peptide competitive inhibition assay for characterization of specific phage clones. The *in vitro* blue-plaque forming assay was performed to observe the competitive inhibition effect of ZT-1 peptide with its phage counterparts (M13). NCI-H1299 cells were cultured in a 12-well plate overnight, and then pre-incubated with blocking buffer to block non-specific binding at 4˚C for 30 min. The synthetic peptide (0, 0.0001, 0.001, 0.01, 0.1, 1 or 10 μM) was diluted in PBS and incubated with cells at 4˚C for 1 h, and then incubated with $1x10^{11}$ pfu of phage M13 at 4˚C for 1 h. The bound phages were recovered and titered in ER2738 culture. The phages binding to NCI-H1299 cells were evaluated by the blue plaque-forming assay, and the rate of inhibition was calculated by the formula: rate of inhibition = (number of blue plaques in NCI-H1299 cells incubated with PBS - number of blue plaques in NCI-H1299 cells with ZT-1 peptide)/number of blue plaques in NCI-H1299 cells incubated with PBS x 100%. Non-specific control phages (a synthetic peptide corresponding to an unrelated phage picked randomly from the original phage peptide library) were used as negative controls.

Immunofluorescence microscopy and image analysis. Immunofluorescence microscopy was used to study the affinity of synthetic peptide (ZT-1) binding to NCI-H1299 cells. NCI-H1299 and SAE cells were digested with 0.25% trypsin and respectively plated on coverslips overnight. Cells were washed three times with PBS and fixed with acetone at 4˚C for 20 min before analysis. ZT-1 peptide labeled with FITC was incubated with the cells. PBS and control peptides labeled with FITC were used as negative controls. After being washed three times with PBS, the slips were observed using a fluorescence microscope.

Analysis of the tissue distribution of the M13 phage in an animal model. A NCI-H1299 model in nude mice was used to analyze the binding activity of the M13 phage. We injected $1x10^7$ NCI-H1299 cells subcutaneously into the flank of mice. Solid tumors were formed from the inoculated human lung cancer cells (NCI-H1299) with sizes of ~1 cm x 2 cm x 1 cm 15 days after the mice were inoculated. Then, $2x10^{11}$ pfu of the M13 phage was blocked with 500 μl BF for 30 min at 37˚C. The blocked phages were injected intravenously in 1 ml PBS. Mice were sacrificed 10 min later. Before being sacrificed, the mice were perfused with PBS to facilitate phage elimination from the vasculature. Organs such as the lungs, heart and brain along with non-tumor lung tissue and tumor samples were removed, weighed, and washed using plain DMEM with protease inhibitors (DMEM-PI) (1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin and 1 μg/ml leupeptin). The organ and tumor samples were homogenized with a homogenate apparatus (IKA, Gaungzhou Germany). The phages were titered on agar plates with *E. coli* in the presence of 1 mg/ lisopropyl-1-thio-β-D-galactopyranoside/5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

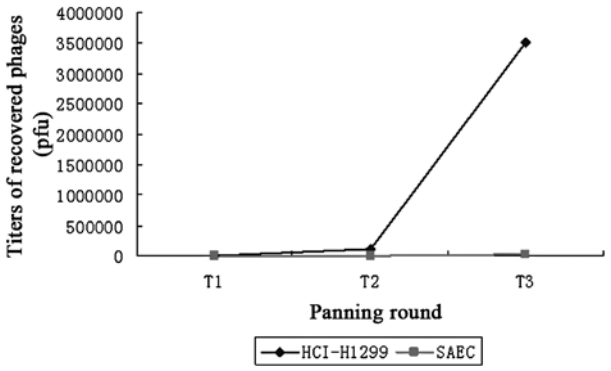

Figure 1. Specific enrichment of NCI-H1299 cell-bound phages as noted when the third round of selection was finished. The titers of the recovered phages from each round were evaluated using a blue plaque-forming assay on agar plates containing tetracycline. The alteration of the titers on the different cells in three rounds is shown.

Results

Specific enrichment of NCI-H1299 cell-bound phages. Phages specifically able to bind to human NCI-H1299 cells were identified through three rounds of *in vitro* panning. In each round, the bound phages were rescued and amplified in *E. coli* for the following round of panning, while the unbound phages were removed by washing with TBST. After the third round of *in vitro* selection, the number of phages recovered from NCI-H1299 cells was increased 875-fold (from $0.4x10^4$ to $3.5x10^6$), as shown in Fig. 1. However, there was a decrease in the number of phages recovered from SAE control cells. The output/input ratio of phages recovered after each round of panning was used to determine the phage recovery efficiency. These results indicated an obvious enrichment of phages specifically binding to NCI-H1299 cells in this study.

Homology analysis of exogenous sequences of the selected phage clones. After *in vitro* selection, 60 individual clones were chosen, amplified and sequenced. Each of the clones and

Table I. Amino acid sequences of the seven peptides and a multiple sequence alignment.

Phage clones	Phage no.	Peptide no.	Sequence (NC)	Frequency
P1-5/P8-10/P15/P21/P27-28/P30/P32-33/P51/P53/P55	P1	ZT-1	QQMHLMSYAPGP	19
P6-7/P12/P17-19/P40-41/P45-46/P56-57	P4	ZT-2	AHRHPISFLSTL	12
P13/P16/P34-39/P59-60	P13	ZT-3	VEAPLHRAQPHY	10
P22-26/P48-50	P22	ZT-4	KMDRHDPSPALL	8
P14/P20	P14	ZT-5	AYYPQNHKSKAE	2
P58	P50	ZT-6	APNHIPRPPGLT	1
P47	P20	ZT-7	YPHYSLPGSSTL	1

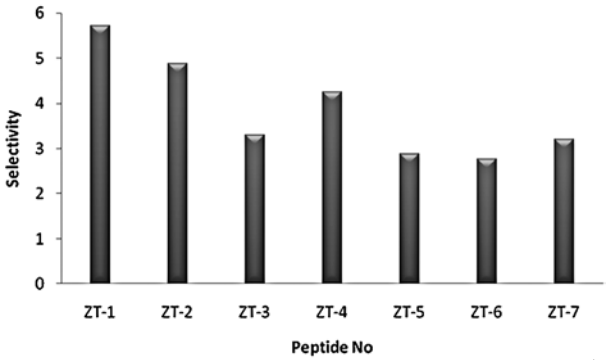

Figure 2. Evaluation of the binding selectivity for the seven peptides using a cell-ELISA. Cells ($1x10^5$/well) were seeded and grown in 96-well plates overnight. Approximately $1x10^{10}$ pfu phages was added to each well at 37˚C for 1 h. A mouse anti-M13 bacteriophage antibody and a HRP-conjugated sheep anti-mouse Ig were added in turn. An OD_{450} was obtained after the reaction blocking. The selectivity values for these phage clones calculated by the formula mentioned in the text (Selectivity = $OD_{M13} - OD_{C1}/OD_{S2} - OD_{C2}$). were 5.71, 4.87, 3.29, 4.25, 2.88, 2.76 and 3.21, respectively. Therefore, clone ZT-1 appeared to bind most effectively.

the corresponding sequence was given a sequential name from P1 to P60. Seven of these clones (P29, P31, P42, P43, P44, P52, P54) were found to be lacking exogenous sequences. The remaining clones were identified correctly by DNA sequencing. After the analysis of the peptide sequences was deduced from P1-5/P8-10/P15/P21/P27-28/P30/P32-33/P51/P53/P55, P6-7/P12/P17-19/P40-41/P45-46/P56-57, P13/P16/P34-39/P59-60, P22-26/P48-50, P14/P20, P58 and P47; seven different phage clones or peptide sequences were obtained (Table I). Sixteen of the 60 clones were shown to contain the peptide sequence of ZT-1.

Verification of in vitro specific binding by immunocytochemistry. A cellular ELISA was used to identify the affinities for the seven selected phages binding to NCI-H1299 cells. To ascertain selectivity, the affinity of each phage binding to NCI-H1299 and to the control normal lung SAE cells was compared. The results indicated that these phage clones were effectively binded to NCI-H1299 cells as compared with the PBS and SAE cell control groups. Furthermore, clone ZT-1 appeared to bind most effectively to NCI-H1299 cells in this study (Fig. 2). Therefore, we further analyzed phage M13 and its displaying peptide, ZT-1.

Affinity of phage M13 to NCI-H1299 cells and lung cancer tissues. To confirm the binding ability of the selected phage toward target NCI-H1299 cells and lung cancer tissues, the phage clone M13 (clone ZT-1) was isolated, amplified and purified for immunochemical assay. The SAE cell line and human non-tumor lung tissues were also tested for clone ZT-1 as negative controls. The interaction of the M13 phage and target cells (NCI-H1299) was evaluated using immunocytochemical staining as shown in Fig. 3. The surfaces of cells were stained a brown color, indicating that the phage M13 was able to bind to NCI-H1299 cells. In contrast, no positive staining was observed in control SAE cells. Negative results were also obtained when NCI-H1299 cells were bound to an unrelated phage clone. Subsequently, immunohistochemical staining was performed to observe the specific binding of the phage clone ZT-1 to human lung cancer tissues, as shown in Fig. 4. The cells in the NCI-H1299 tumor tissue sections when bound to the phage clone ZT-1 were distinctively stained brown. When NCI-H1299 tumor tissue sections were bound to the unrelated phage clone, or when the non-tumor lung tissue sections were bound with the phage clone ZT-1, they showed negative staining. It was clearly evident that the phage clone ZT-1 was able to bind specifically to the NCI-H1299 cells.

Competitive inhibition assay. A peptide-competitive inhibition assay was performed to discover whether the synthetic peptide ZT-1 and the corresponding phage clone competed for the same binding site. The results showed that, when the synthetic peptide ZT-1 was pre-incubated with NCI-H1299 cells, the ability of the phage ZT-1 to bind to NCI-H1299 cells was decreased in a dose-dependent manner (Fig. 5). With an increase in the concentration of the peptide ZT-1, the titer of phages recovered from NCI-H1299 cells was decreased and the inhibition was gradually increased. When the concentration of peptide ZT-1 was increased above 5 μM, the inhibition reached a flat phase. The control peptide (EAFSILQWPFAH) had no effect on the binding of the phage ZT-1 to NCI-H1299 cells.

In vivo binding assay. Nude mice bearing NCI-H1299 xenografts were injected with $1.8x10^{10}$ pfu of M13 wild-type (control) and ZT-1 clone phages through the tail vein. The distribution and specificity of the phages binding to different tissues were further verified by the titers of bound phages in

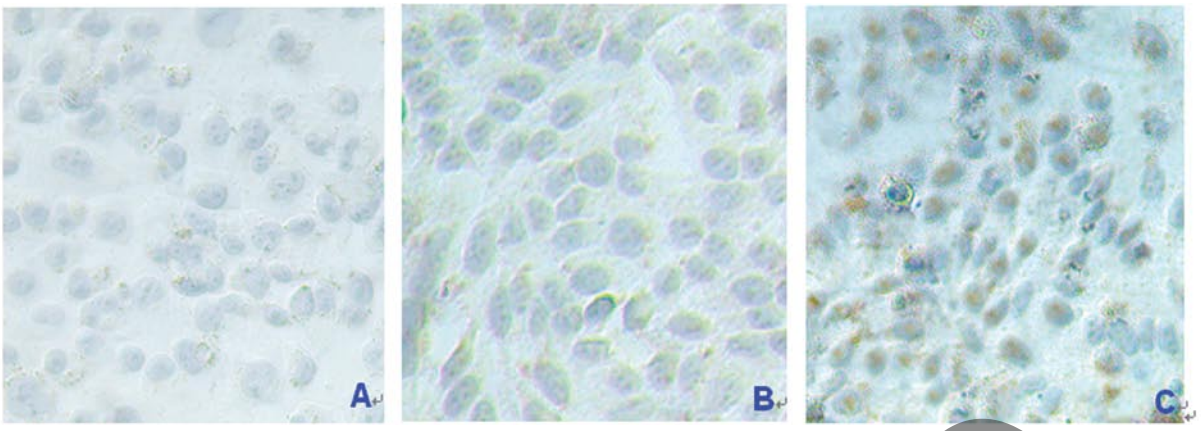

Figure 3. Immunocytochemical staining of NCI-H1299 and control cells when bound with phage ZT-1 (ABC method, x200). Cell-bound phages were detected using anti-M13 phage monoclonal antibody, secondary antibody and ABC complex. The cells were stained with diaminobenzidine (DAB). (A) Immunocytochemical staining of NCI-H1299 cells when bound with phages without exogenous sequences (wild-type phage); (B) immunocytochemical staining of NCI-H1299 cells when bound with an unrelated phage; and (C) immunocytochemical staining of NCI-H1299 cells when bound with phage ZT-1.

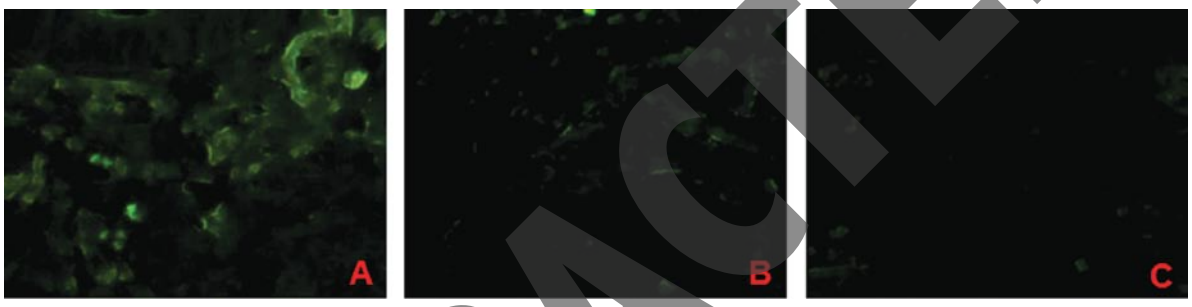

Figure 4. Immunohistochemical staining of lung cancer and non-tumorous lung tissue sections when bound with ZT-1 peptide-FITC (x200). To investigate whether the free ZT-1 peptide maintained its binding affinity to lung cancer cells, we constucted a synthetic peptide ZT-1 (EHMALTYPFRPP) labeled with FITC. (A) Immunohistochemical staining of lung cancer tissues bound with phage ZT-1-FITC. The specific binding sites on tumor cells were distinctly seen as green fluorescence. (B) Immunohistochemical staining of non-tumorous lung tissues bound with phage ZT-1; (C) a negative control section with immunohistochemical staining with random peptide-FITC in lung cancer tissues.

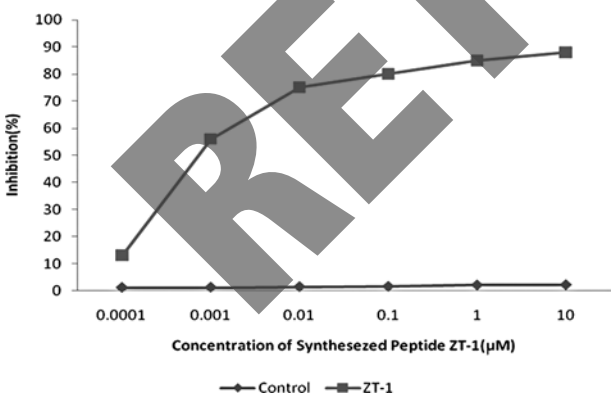

Figure 5. Competitive inhibition of the binding of the phage ZT-1 to NCI-H1299 cells by the synthetic peptide ZT-1 (EHMALTYPFRPP). The average inhibition rates at different concentrations of the peptide are shown. When the concentration of the peptide ZT-1 reached >0.001 μM, a significant inhibition occurred. The independent experiments were repeated three times.

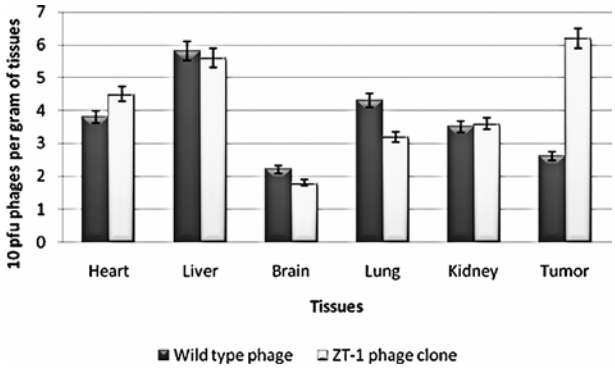

Figure 6. Distribution and specificity of ZT-1 phage clone in tissues from the animal model. Nude mice bearing NCI-H1299 xenografts received intravenous injections of ZT-1 phage clones. Xenografts and the heart, liver, brain, kidney and lung were removed for titer phages 30 min later. The level of ZT-1 phages identified in the tumor tissue was 1.66- to 5.4-fold higher than that in other tissues, such as the heart, liver, brain, kidney and lung, and in non-tumor lung tissue. However, there were fewer phages distributed in the tumor tissue of the mice treated with the non-specific control phages.

the tumor compared to the non-tumor tissues. The ZT-1 clone phages were found in tumor tissues at concentrations 1.66- to 5.42-fold higher than in non-tumor organs, such as the heart, liver, brain, kidney and lung, and in the non-tumor lung tissue. Meanwhile, the wild-type phage did not show any targeted binding to tumor tissues (Fig. 6).

Discussion

Since 1985, phage display has been widely used as an important tool for drug discovery and the research of biological molecules as ligands (11,12). Unbiased phage panning on intact cells allows the isolation of highly specific cell targeting peptides, even for cells for which the cell surface is undefined. Once identified, these peptides can be used as 'fishing hooks' to identify unique cellular receptors. Thus, in addition to yielding cell-targeting reagents for drug delivery, this approach is useful for identifying features present on the cell surface. Phage display technology permits peptides, antibodies and other protein libraries to be displayed on the surface of filamentous phages, and allows for the selection of specific peptides, antibodies and proteins with high affinity (13-16). Panning of phage-displayed peptide libraries on molecular targets such as receptors, enzymes, cells and tissues of living animals, which is often called biopanning, has proven successful for isolating peptides that show high cell- and tissue-binding specificities. This approach may be used to explore and discern cancer-specific antigens from normal cells (17,18).

To target specific ligands that bind to specific tumor antigens is an efficient way to increase the selectivity of therapeutic targets in clinical oncology, and is helpful for the early detection and therapy of cancer. Tumor cells often display certain cell surface antigens, which are different from the antigens on normal tissues, in high quantities. These include tumor-associated or tumor-specific antigens. To develop more biomarkers for the diagnosis of lung cancer, we used peptide phage display technology to identify potential molecular biomarkers of NCI-H1299 cancer cells (11,12). After panning for three rounds, 60 clones were selected for further characterization. A cell-based ELISA assay was used to confirm the specific binding of the phage clones to NCI-H1299 cells *in vitro*. As a result, ZT-1 was demonstrated to be the best candidate phage clone with the highest specificity. Immunocytochemical and immunohistochemical staining were performed to confirm the selectivity of the phage ZT-1 to bind to NCI-H1299 cells and the lung tumor tissues. The results of the competitive inhibitory assays suggest that the peptide displayed by the phage M13-ZT-1, not other parts of this phage, can bind to the NCI-H1299 cell surface. Under the same conditions, the normal lung SAE cell line did not show significant fluorescence when stained with ZT-1 peptide-FITC, a finding that further confirmed the target of ZT-1 to be NCI-H1299 cells. Our analysis of the tissue distribution of the M13 phage in an animal model indicated that the M13-ZT-1 phage was distributed mainly in the lung tumor masses and livers, but not in the brain, heart or non-tumor lung tissues. These results also support our conclusion that the M13-ZT-1 phage can specifically bind to xenograft tumor cells, but not to normal lung tissue and cells. The above findings strongly suggest that the ZT-1 peptide may be specific to NCI-H1299 cells, and would therefore be useful in the diagnosis of lung cancer or as an antitumor therapy agent delivery tool. However, further research is required.

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