

RESEARCH COMMUNICATION

Screening Peptides Binding Specifically to Colorectal Cancer Cells from a Phage Random Peptide Library

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Abstract

The aim of this study was to screen for polypeptides binding specifically to LoVo human colorectal cancer cells using a phage-displayed peptide library as a targeting vector for colorectal cancer therapy. Human normal colorectal mucous epithelial cells were applied as absorber cells for subtraction biopanning with a c7c phage display peptide library. Positive phage clones were identified by enzyme-linked immunosorbent assay and immunofluorescence detection; amino acid sequences were deduced by DNA sequencing. After 3 rounds of screening, 5 of 20 phage clones screened positive, showing specific binding to LoVo cells and a conserved RPM motif. Specific peptides against colorectal cancer cells could be obtained from a phage display peptide library and may be used as potential vectors for targeting therapy for colorectal cancer.

Keywords: Phage peptide library - LoVo cells - subtraction biopanning - colorectal cancer - M13

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Introduction

Colorectal cancer is one of the most common malignant tumors of the alimentary tract, which is strongly invasive in the progressive stage, hard to achieve radical cure, and has a high post-operative recurrence rate (Xu et al., 2006). The elimination of residual cancer cells is a primary goal of chemotherapy (Shadidi and Sioud, 2003). But the traditional chemotherapeutic agent for the disease has weak tissue selectivity for colorectal tissue, which makes it hard to achieve an effective killing concentration at the site of the tumor. Targeting therapy against the tumor provides a new solution for this problem (Oh et al., 2004; Shukla and Krag, 2005; Lo et al., 2008). Phage display peptide library technology is gradually becoming a hot topic for research in tumor targeting therapy (Landon et al., 2004; Samoylova et al., 2006). In this study, LoVo cells were used as the target cells and normal human colorectal mucous epithelial (NHCME) cells as the absorber cells for subtraction biopanning from a c7c phage display peptide library for screening polypeptides binding specifically to colorectal cancer cells and thus using them as vectors for colorectal cancer targeting therapy. The study also provides a new experimental basis for clinical research in the mechanism underlying the genesis and development of colorectal cancer, and for the development of new targeting agents.

Materials and Methods

Materials

Human colorectal cancer cells LoVo were provided by the Institute of Digestion, Nanfang Hospital of Southern Medical University, Guangzhou, China. NHCME cells were primarily cultured by the Chinese medicine symptom library, Department of Chinese medicine, Southern Medical University. The kits of c7c phage display peptide library (including c7c phage display peptide library with a concentration of 2.0×10^{13} pfu/mL of host bacterium *E. coli* ER2738 and the sequencing primer 5'-CCC TCA TAG TTA GCG TAA CG-3') were purchased from New England Biolabs. Horseradish peroxidase (HRP)/anti-M13 monoclonal conjugate antibody was purchased from Amersham Pharmacia Biotech. Dispase, type I collagenase, epidermal growth factor (EGF) and fetal calf serum (FCS) were all purchased from Gibco. Fluorescein isothiocyanate (FITC)-labeled goat anti-mouse secondary antibody was provided by the Key Laboratory of Functional Proteomics of Guangdong Province. Poly-L-lysine was purchased from Guangzhou Weijia Technology Co. Limited.

Primary culture of NHCME cells

Following informed consent of patients diagnosed with colorectal cancer by pathological examination at the

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Department of General Surgery of Nanfang Hospital, 5 g of normal colorectal mucosa tissue from a site at least 10 cm away from the tumor was obtained during surgery. After the contaminated surface of the mucosa was cleaned with Hank's Balanced Salt Solution (HBBS) containing penicillin (200 U/ml) and streptomycin (200 mg/ml), the mucosa was sheared into pieces of about 3 mm³, and then dispase (0.5 mg/mL) and collagenase type I (0.5 mg/mL) were used in combination to obtain NHCME cells. Based on the culture method provided by Dorina Rusu and Johannes Grossmann (Grossmann et al., 2003; Rusu et al., 2005), these cells were seeded into 25-cm² plastic culture flasks that had been precoated with poly-L-lysine with high-glucose Dulbecco's Modified Eagle's Medium (DMEM) culture medium (containing 20 mmol/L HEPES, 200 U/L insulin, 100 mg/L heparin sodium, 100 mg/L FCS, and 0.011 µg/mL EGF), and placed in a tissue culture incubator at 37 °C in an atmosphere of 5% CO₂. The culture medium was changed 12–24 hours after observing the adherence of mucous epithelial cells under an inverted microscope, and then every 48 hours. Cells were used for experiments when they achieved about 60% confluence in about 7 days.

Serial subcultivation of LoVo cells

LoVo cells were thawed out from liquid nitrogen and cultured in RPMI-1640 culture medium containing 10% FCS. Following the first passage, LoVo cells were subcultured in plastic 25-cm² culture flasks and incubated at 37 °C in 5% CO₂ atmosphere. Cells were passaged once they reached about 60% confluence. After the third passage, cells in the logarithmic growth phase were used for experiments.

Subtraction biopanning and enrichment effect analysis of c7c phage display peptide library

LoVo and NHCME cells were both digested with 0.25% pancreatin (containing 0.02% EDTA), and then transferred into 96-well Nunc microtiter plates that had been precoated with poly-L-lysine. Screening occurred after cell adherence and growth, which was carried out according to the kit instructions of the c7c phage random peptide library (Ph.D.-C7C™ Phage Display Peptide Library Kit). The LoVo cells were blocked with RPMI-1640 containing 1% bovine serum albumin (BSA) at 37°C for 1 hr and then washed 5 times with 0.1% TBS-Tween 20 (TBST) for 5 min each time. After 10 µl of c7c phage-display peptide library (2.0×10¹¹ pfu/mL titer) and 90 µl of TBS were added, the cells were kept at 37°C for 1 hr, washed 5 times to remove any unconjugated phage or phage of low affinity, and then they were rocked gently at 100 rpm for 10 min together with 100 µl 0.2 mol/L Gly-HCl (pH 2.2). After the wells were tapped gently, the phage eluant at the surface of LoVo cells was decanted and then neutralized with 15 µl 1 mol/L Tris-HCl (pH 9.1). The neutralized eluant was then added to the blocked NHCME cells, which were placed at 37°C for 1 h to absorb the phages, which can combine with NHCME cells; the remaining phages were screened out for infection of host *E. coli* ER2738 for amplifications. The phage peptide library was purified and amplified according to the

operating instruction protocol, resulting in a yield of 10 µl, which was used to identify the phage titre; the remaining eluant was used for the next screen. The procedures above were repeated for the 2nd and 3rd rounds of screening. For the 1st round, the original phage library was used for amplification, whereas for the 2nd and 3rd rounds, phages from the previous round were used. The conditions for 2nd and 3rd rounds were the same as those for 1st round, expect that the elution buffer was changed to 0.3% TBST, 0.5% TBST, respectively. The phages screened or amplified from each round were cultured on LB/IPTG/X-gal plates, and the phage titers were determined. The recovery rates were then calculated (i.e. the ratio of output to input; input is phage titer before screen and output is phage titre after screen).

At the end of 3 rounds of screening, the binding peptide libraries of suitable titers were obtained and cultured on LB/IPTG/X-gal plates at 37°C overnight. 20 negative (blue) colonies were randomly chosen and added to host *E. coli* ER273, previously prepared for amplification, each of which was cloned to a volume of 3 mL. Other procedures were carried out according to the phage amplification and purification protocol; the final phage sediments were dissolved in 500 µl TBST for ELISA.

Primary identification of positive phage clones with ELISA

LoVo cells were cultured in enzyme-linked 96-well Nunc microtiter plates at 2×10⁵ cells per well until the cells adhered as a monolayer. The supernatant was aspirated, and the cells were fixed with 10% formalin. 100 µl of 3% hydrogen dioxide was added to each well; the plates were placed in a 37 °C incubator for 30 min to inhibit the activity of endogenous peroxidase and then washed with PBS 3 times, for 5 min each time. The plates were blocked with 5% BSA, and then 100 µl of phage clones was added to each well containing the LoVo cells (experimental group); for the control or blank group, the wells were coated with PBS at 37°C for 1 hr. HRP/anti-M13 monoclonal conjugate antibody (dilution 1:5,000) was added and the plates were kept at 37°C for 30 min. The plates were washed 3 times with PBS, for 5 min each time, and stained with OPD. The reaction was stopped with 2 mol/L sulfuric acid. OD490 nm was measured using a spectrophotometer (Model MQX200R; BIO-TEK Instruments, Inc., Winooski, VT, USA). Positive clones were defined as those with an OD490 nm that was 3 times higher than that of the control (i.e., negative control). Using this method, positive clones were identified in plates with colorectal cancer cells LoVo or NHCME cells (negative control).

Identifying the targeting character of positive clones with immunofluorescence

Adherent LoVo and NHCME cells were made into cell suspensions and transferred to sterile slides. The cells were cultured until a monolayer of cells covered the slide. The slides were fixed with 4% paraformaldehyde and washed with PBS (pH 7) 3 times, for 5 min each time. 3% hydrogen dioxide was then added to the slides, and the slides were placed in an incubator at 37 °C for 30 min to block the activity of endogenous peroxidase. After 3 5-min washes with PBS, the slides were blocked,

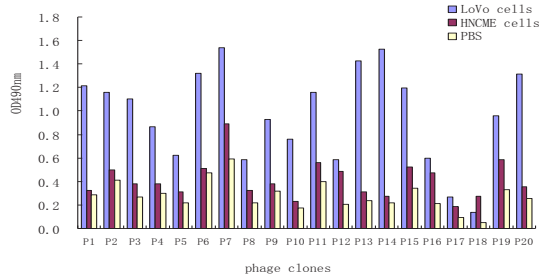


Figure 1. ELISA for Phage Clones Binding to both Colorectal Cancer LoVo Cells and Human Normal Colorectal Mucous Epithelial Cells; 5 positive phage clones: P1, P10, P13, P14, P20

Table 1. Enrichment of Positive Phage Clones from c7c Phage Library by Subtraction Biopanning

| Round | Input phage of screening (pfu/ml) | Output phage (pfu/ml) | Recovery (%) |
|-------|-----------------------------------|-----------------------|----------------------|
| 1 | 2.0×10^{11} | 5.6×10^6 | 2.8×10^{-5} |
| 2 | 2.0×10^{11} | 8.4×10^8 | 4.2×10^{-3} |
| 3 | 2.0×10^{11} | 2.2×10^9 | 1.1×10^{-2} |

Table 2. Amino Acid Sequence Analysis of 5 Phage Positive Clones

| Phage positive clones | DNA sequence | Peptide sequence |
|-----------------------|-----------------------|------------------|
| P1 | CCTCATATGACGGCTCCGTTT | PHMTAPF |
| P10 | GCTCCTTATTCGCGGTTTATG | APYSRFQ |
| P13 | TAGCCTCCTGATCGGCCGATG | QPPDRPM |
| P14 | CCTATTCATGAGCGGCCGATG | PDHERPM |
| P20 | CCTCTGCGGGAGCATCCGATG | PLREHPM |

this time with 5% BSA, and placed in a 37 °C incubator for 30 min. They were then covered with purified positive clone P14 and placed in a 37 °C incubator for 1 hr. The slides were incubated at 37 °C for another 30 min, and HRP/anti-M13 monoclonal conjugate antibody (dilution 1:5,000) was added to them. The slides were then washed with PBS, 3 times, for 5 min each time, and then 30 µl of FITC-labeled goat anti-mouse secondary antibody (dilution 1:1,000) was added, and the slides were stored in the dark at 37 °C for 30 min. The slides were washed with PBS for 10 min in the dark and then embedded in glycerine and placed under a fluorescence microscope for observation and photo shooting.

DNA sequencing and analysis of positive phage clones

Positive phage clones were amplified with kits provided by New England Biolabs and purified with PEG/NaCl. Sodium iodide solution was used to extract single-stranded phage DNA, which was then sent to Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. for automatic sequencing and analysis with 96gIII sequencing primer: 5'-CCC TCA TAG TTA GCG TAA CG-3'.

Results

Subtraction biopanning and enrichment analysis of c7c phage display peptide library

LoVo cells were used as the target cells and NHCME cells were used as the absorber cells to achieve 3 rounds

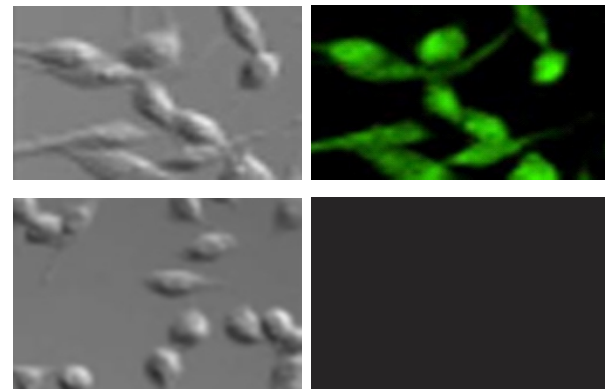


Figure 2. Immunofluorescence Detection of Phage Positive Clone Targeting to Colorectal Cancer Cells LoVo (×400); A and C: LoVo cells and human normal colorectal mucous epithelial cells under the light microscope; B and D: LoVo cells and human normal colorectal mucous epithelial cells under the fluorescent microscope

of subtraction biopanning of the c7c phage display peptide library. The phage recovery rate improved gradually, and positive phage clones were effectively enriched with phage input maintenance.

Primary identification of positive phage clones with ELISA

After 3 rounds of screening, 20 clones were randomly picked up for primary identification with ELISA. Clones with nonspecific binding (i.e., the blank control only coated by PBS) (Figure 1) were excluded based on the binding state between phage clones and LoVo cells. There were 7 clones with a ratio $OD_{490\text{ nm}}$ of the experimental group (LoVo cells) to the blank control group (PBS) that was higher than 3. They were P1, P3, P10, P13, P14, P15, and P20, which bound well with LoVo cells. The binding state of these 7 clones and NHCME cells was assessed with ELISA to determine the specificity of the clones. There were 5 clones with a ratio $OD_{490\text{ nm}}$ of the experimental group (LoVo cells) to the blank control group (PBS) that was higher than 3. They were P1, P10, P13, P14, and P20, and these clones were defined as positive clones (positive rate was 25%, 5/20). These clones could bind well to LoVo cells, but could not bind to NHCME cells, from which we concluded that what ultimately obtained was positive phage clones specifically binding to the colorectal cancer cells LoVo.

Assessing the targeting affinity of positive clones using immunofluorescence

The positive clone P14 was chosen and its targeting specificity for LoVo cells was primarily identified using immunofluorescence (Figure 2). P14 was found to specifically bind to LoVo cells, as seen by a fluorescent green color of the membrane of the LoVo cells (Figure 2B). There was no fluorescence with NHCME cells, indicating there was no binding of positive clone P14 to the primarily cultured NHCME cells.

DNA sequencing and homology analysis

According to Table 2, there are considerable pyrrolidinecarboxylic acid sequences in 5 peptide sequences, corresponding to 28.6% (10/35) of the

sequences, and there is a common RPM sequence in 3 peptide sequences. A search of the BLAST database did not reveal any similar sequences.

Discussion

The phage display technique was set up by Smith P in 1985 (Smith, 1985) and has become an important system for screening the interaction of molecules in the domain of a molecular organism (Cao et al., 2010; Luck et al., 2011; Shanmugam et al., 2011). The screening strategy most commonly used involves purifying a specified target, absorbing it to an affinity resin or ELISA plate, and then screening for binding by adding a phage peptide library. But the ligands obtained by this strategy only bind to the specified target. It is possible that other specific binding sites on the surface of cancer cells are not detected, however. Another important strategy for screening peptides that specifically bind to cancer cells is by making the whole cell as the target. The advantage of this approach is the ability to obtain specific binding peptides, without even predicting the specific molecules that are bound on the surface of cancer cells (Krag et al., 2006). The phage peptide library technique, with features of large capacity, high flux, and convenient operation, has become a powerful tool for screening peptides that specifically bind to cancer cells or tissues (Kehoe et al., 2005). We have successfully screened peptides that specifically bind to different cancer cells (i.e., neuroglioma, lung cancer, nasopharyngeal carcinoma, ovarian cancer, lymphoma) in vitro, which may have potential clinical value for diagnosis and targeted therapy of various cancers (Zhang et al., 2001; Oyama et al., 2003; Lee et al., 2004; Karjalainen et al., 2011; Zhang et al., 2011). In this study we randomly chose 20 clones from an eluate after the 3rd round of screening in subtraction biopanning for ELISA detection, 5 of which could bind specifically to the colorectal cancer cells LoVo. Therefore, by screening from a phage display peptide library, we could easily and rapidly obtain clones specifically binding to colorectal cancer cells, in this case, the LoVo cells as the target.

In this study, for the first time, the human colorectal cancer cells LoVo were used as the target cells and NHCME cells as the absorber cells to screen a c7c phage peptide library with space conformation, in an attempt to obtain phage clones that could specifically bind to the surface of LoVo cells. According to the literature, there is no similar report, either domestic or abroad, at present. After 3 rounds of subtraction biopanning, the phage recovery rate gradually increased, and positive phage clones are effectively enriched with maintenance of phage input. We primarily identified 20 clones that were randomly chosen after the 3rd round of screening with the use of ELISA, and obtained 5 positive phage clones that bound specifically to LoVo cells (i.e., positive phage clones). Of these clones, P14 had the highest OD_{490nm} for LoVo cells and was thus chosen for immunofluorescence. Using immunofluorescence, we were able to directly observe the binding of P14 and LoVo cells. This result showed specific binding of P14 to colorectal cancer cells (i.e., the combination of the two), which has not been

seen to date with primarily cultured NHCME cells as the target. Therefore, the peptide sequence of P14 may become a targeting vector for chemotherapeutic agent development, revealing a potential value of targeting therapy for colorectal cancer. We found some homology after sequencing the 5 positive phage clones obtained in this study, of which 3 clones contained the same RPM sequence. It is possible that a relationship exists between the RPM sequence of the clone and the binding peptide motif of the surface receptor on colorectal cancer cells, warranting further studies on the mechanism underlying this binding interaction. We also found a large proportion of pyrrolidinecarboxylic acid sequence in the 5 peptides, corresponding to 28.6% (10/35) of the total sequence, whereas the ratio of pyrrolidinecarboxylic acid to the general amino acid sequence was 4.6%. According to the literature, pyrrolidinecarboxylic acid can reduce the flexibility of free peptides, which play an important role in stabilizing the binding of ligand and peptide (De Bolle et al., 1999). This has provided a basis for the design of specific binding peptides as vectors cross-linked with agents.

The clones obtained in this study can combine specifically with colorectal cancer cells, but the targeting capability of the negative clones should also be analyzed to gain as much information as possible on candidate positive clones, which have important value for targeted therapy for colorectal cancer and new specific markers. However, this research has been performed only at cellular level in an in-vitro environment. An endosomatic screen should be performed on models with solid tumors to assist in the development of a targeting agent. Based on this research, the next step is to raise athymic mice with colorectal cancer, as the animal model, to imitate the environment of the colorectal cancer tissue in order to optimize an in-vivo screening strategy for vascular endothelial cell growth factors specifically binding to colorectal cancer tissue (Nicklin et al., 2000). This will provide a new way of studying the pathogenesis and biological behavior of colorectal cancer, as well as for developing targeted therapy against colorectal cancer.

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