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Screening of a specific peptide binding to esophageal squamous carcinoma cells from phage displayed peptide library

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ABSTRACT

To select a specifically binding peptide for imaging detection of human esophageal squamous cell carcinoma (ESCC), a phage-displayed 12-mer peptide library was used to screen the peptide that bind to ESCC cells specifically. After four rounds of bio-panning, the phage recovery rate gradually increased, and specific phage clones were effectively enriched. The 60 randomly selected phage clones were tested using cellular enzyme-linked immunosorbent assay (ELISA), and 41 phage clones were identified as positive clones with the over 2.10 ratio of absorbance higher than other clones, IRP and PBS controls. From the sequencing results of the positive clones, 14 peptide sequences were obtained and ESCP9 consensus sequence was identified as the peptide with best affinity to ESCC cells via competitive inhibition, fluorescence microscopy, and flow cytometry. The results indicate that the peptide ESCP9 can bind to ESCC cells specifically and sensitively, and it is a potential candidate to be developed as an useful molecule to the imaging detection and targeting therapy for ESCC.

1. Introduction

Esophageal cancer (EC) is the second most common cause of death from gastrointestinal cancers over the past decades [1]. Esophageal squamous cell carcinoma (ESCC) is the predominant histological type of EC in East Asian countries, and one of the most malignant tumors [2]. Despite of the development of various therapeutic strategies, the prognosis of ESCC remains poor. The 5-years survival rate of ESCC was 20–30% depending on the clinical stage at the time of diagnosis [3]. Thus, novel methods for the early detection of ESCC are urgently needed.

Molecular imaging is an emerging technology that enables minimally-invasive visualization of disease-specific functional tissue alterations, provide a wide field of view, and highlight abnormalities at the cellular even at molecular levels [4]. The early detection of cancers can be visualized with use of exogenous probes that target unique protein expression patterns [5,6]. Peptides have the advantages as detection probes because of their high clonal diversity, small size, rapid binding kinetics, and low immunogenicity [7,8]. Therefore, exploring novel peptides that bind to ESCC cells with high specificity and sensitivity is extremely important for the early detection and treatment in ESCC patients.

With the development of phage display technology, more and more peptide-based probes with high specificity and affinity have been identified for the imaging detection and targeting therapy of cancers. Phage display is a powerful technique that allows the presentation of multiple different peptides on the surface of filamentous phage particles for various applications, providing a means to improve peptide affinity and generate unique peptides that bind any given target [9]. Recently, the bio-panning of phage display peptide library on intact cells has proven successful for selecting peptides [10,11].

In this study, we have tried to find out novel peptides targeting ESCC cells from a 12-mer phage display peptide library. One of 14

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consensus sequences, ESCP9, was identified as the peptide with best affinity to ESCC cells via competitive inhibition, fluorescence microscopy, and flow cytometry. The results demonstrated that the peptide ESCP9 can bind to ESCC cells specifically and sensitively, and it is a potential candidate to be developed as an useful molecule to the imaging detection and targeting therapy for ESCC.

2. Materials and methods

2.1. Cell lines and cell culture

Human Eca-109 and TE-1 (esophageal squamous carcinoma cells), and HEK293 (human embryonic kidney cells) were cultured in either RPMI-1640 or DMEM media, respectively, supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (Amresco, USA). All cell lines were grown as adherent monolayer cultures at 37 °C with 5% CO_2 in a humidified incubator, and purchased from the Tissue Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China).

2.2. Subtractive bio-panning in vitro

The Ph.D.-12™ phage display peptide library kit consisting of M13 bacteriophage that expresses ~10^9 unique 12-amino acid sequences, *Escherichia coli* host strain ER2738, and a bio-panning protocol based on a subtractive whole cell approach was purchased from New England BioLabs (Beverly, MA, USA). Eca-109 and HEK293 cells were used as positive target cells and negative absorber cells, respectively. When Eca-109 and HEK293 cells were grown to log phase, the HEK293 cells were washed 3 times with phosphate-buffered saline (PBS) and blocked with 3% bovine serum albumin (BSA) for 2 h at 37 °C. Approximately 1–2 × 10^{11} pfu phages were incubated with HEK293 cells at 37 °C for 1 h with gentle agitation. After incubation, the supernatant containing unbound phages was incubated with the blocked Eca-109 cells at 37 °C for 2 h with gentle agitation. The Eca-109 cells were washed 3 times with 0.1% PBST (PBS/0.1% Tween-20, v/v) to remove the unbound phages. The cell membrane-bound phages were then eluted with 260 μl of 0.2 M glycine (pH 2.2) for 10 min on ice and immediately neutralized with 40 μl of 1 M Tris–HCL (pH 9.1). The eluted phages were amplified, purified, and tittered according to the manufacturer's instructions. Subsequently, 1–2 × 10^{11} pfu phages were subjected to the next round of bio-panning. After the fourth round of bio-panning, 60 phage clones were randomly picked out from titered phage plaques for enzyme-linked immunosorbent assay (ELISA).

2.3. ELISA assay

Eca-109 and HEK293 cells were seeded into 96-well plates (1 × 10^4 cells/well). The cells were washed twice and fixed with 4% paraformaldehyde for 30 min at room temperature. A solution of H_2O_2 (3%, 100 μl/well) was added, and the plates were placed at room temperature for 30 min to inhibit the activity of endogenous peroxidase. The cells were then blocked with 3% BSA at 37 °C for 2 h. The phages were added to Eca-109 and HEK293 cells (1 × 10^{10} pfu, 100 μl/well) and incubated at 37 °C for 1 h. After washing with 0.1% PBST 3 times, 100 μl of goat anti-M13 polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was incubated with the cells at 37 °C for 1 h. After washing 3 times with 0.1% PBST, 100 μl of horseradish peroxidase (HRP)-labeled rabbit anti-goat antibody IgG (Beijing Biosynthesis Biotechnology Co., Ltd., China) was incubated with the cells at 37 °C for 1 h. After washing 3 times with 0.1% PBST, 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma, Saint Louis, MO) was incubated with the cells at 37 °C for 10–30 min, and the reaction was then terminated by adding 2M H_2SO_4. The 96-well plates were then measured at 450 nm using an ELISA reader (Bio-Tek ELX800, USA). Irrelevant phage clone (IRP, an amplified phage randomly selected from the original phage peptide library) and PBS were used as control groups.

2.4. DNA sequencing of the positive phage clones

The selected positive phage clones were used to extract DNA for the sequencing analysis. An overnight culture of *E. coli* ER2738 was diluted to 1:100 in LB medium, and to a portion of 10 μl monoclonal phages were added. The mixture was shaken at 37 °C for 4.5 h, and the supernatant was harvested by centrifugation at 13,000 rpm for 10 min. Then, 200 μl of PEG/NaCl was added to the supernatant to precipitate the phages. The precipitate was suspended in iodide buffer (10 mM Tris–HCl (pH 8.0), 1 mM EDTA, and 4 M NaI), and followed by ethanol precipitation at room temperature for 10 min. The single-stranded DNA (ssDNA) was recovered and dissolved in TE buffer consisting of 10 mM Tris-HCL (pH 8.0), and 1 mM EDTA. DNA sequencing of the selected phages was carried out by GENEWIZ (Suzhou, China). The primer used for sequencing was -96gIII 5′-CCCTCATAGTTAGCG TAACG-3′. Homology analysis and multiple sequence alignment were performed according to the National Center of Biotechnology Information BLAST and Clustal W programs to determine groups of related peptides.

2.5. Cell immunofluorescence assay of positive phage clones

Eca-109 and HEK293 cells were cultured on coverslips overnight and then fixed with 4% paraformaldehyde for 30 min at room temperature. Blocking of non-specific binding was performed by the addition of 1% BSA at 37 °C for 30 min. Approximately 1 × 10^{10} pfu phages diluted in PBS were added and incubated with the cells at 37 °C for 1 h. IRP and PBS were used as control groups. After washing 3 times with 0.1% PBST, the cells were incubated with goat anti-M13 polyclonal antibody at 4 °C overnight. After washing 3 times with 0.1% PBST, the cells were then incubated with fluorescein isothiocyanate (FITC)-labeled rabbit anti-goat antibody IgG (Beijing Biosynthesis Biotechnology Co., Ltd., China) at 37 °C for 40 min. After washing 3 times with 0.1% PBST, 4′,6-diamidino-2-phenylindole (DAPI) (Sigma–Aldrich, St. Louis, MO, CA) was used to stain the nucleus. Fluorescence images were observed using laser scanning confocal microscope (LSCM, Leica, Germany).

2.6. Peptide synthesis

The candidate and control peptide were synthesized using standard solid-phase fluorenylmethoxycarbonyl chemistry. An Aminocaproic Acid linker was added to the N-terminus for FITC labeling. The compounds were purified to 95% by high performance liquid chromatography (HPLC), isolated by lyophilization, and stored at −20 °C. The sequence of each peptide was analyzed using mass spectrometry.

2.7. Competitive inhibition assay

Eca-109 cells were seeded into 96-well plates (1 × 10^4 cells/well). The cells were washed twice and fixed with 4% paraformaldehyde for 30 min at room temperature. Synthetic peptides at concentrations of 0, 100, 200, 300, 400, 500, and 600 μM were incubated with Eca-109 Cells at 37 °C for 1 h, and the random peptide was used as a control. Subsequently, the homologous positive phage clones (1–2 × 10^{11} pfu, 100 μl/well) was incubated with the cells at 37 °C for 1 h. The antibody was used as described in the section "ELISA assay". The rate of competitive inhibition was calculated according to the following