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In vitro panning of a targeting peptide to hepatocarcinoma from a phage display peptide library ☆

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Abstract

Phage display technology has been used as a powerful tool in the discovery of ligands specific to receptor(s) on the surface of a cancer cell and could also impact clinical issues including functional diagnosis and cell-specific drug delivery. After three rounds of in vitro panning and two rounds of reverse absorption, a group of phages capable of addressing BEL-7402 enormously were obtained for further analysis. Through a cell-based ELISA, immunofluorescence, FACS, and in vivo binding study, WP05 (sequence TACHQHVRMVRP) was demonstrated to be the most effective peptide in targeting four kinds of liver cancer cell lines (BEL-7402, BEL-7404, SMMC-7721, and HepG2), but not the normal liver cell line HL-7702. In conclusion, the peptide WP05 which was screened by in vitro phage display technology was proved to be a targeting peptide to several common hepatocellular carcinoma cell lines.

Keywords: Phage display library; In vitro; Hepatocellular carcinoma; Targeting peptides

Hepatocarcinoma is one of the most challenging malignancies with a very high mortality [12]. To date, no preoperative treatment has been proven useful [6]. The major drawback of current cytotoxics and gene therapy vectors is their lack of selectivity [14,16]. Thus, the targeting therapy has become a potential effective choice in the curing of hepatocarcinoma. However, a major complication of this approach is delivering sufficient amount of these therapeutics to the tumor tissues. Traditional targeting molecules mostly made up of monoclonal antibody or other ligands, so the specifically binding ability has become nothing the matter. But the immunogenicity to human body and the penetration to tumor tissues still cannot be treated ideally. In principle, the small size of peptides makes them attractive for tumor-targeting applications [11]. So, more and more scientists began to give their attention to low molecular weight protein and peptides [5].

Phage display was originally described in 1985 by Smith [15] who presented the use of the non-lytic filamentous bacteriophage fd for the display of specific binding peptides on the phage coat. Phage display is a molecular diversity technology that allows the presentation of a large number of peptides or proteins on the surface of filamentous phage for various applications. These libraries permit the selection of peptides or proteins with high affinity and specificity for almost any target. The most important feature of this technology is the direct link between the experimental phenotype and its encapsulated genotype [3]. Since 1985 [15], phage display has been an important tool for both basic

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research and drug discovery. It has provided a rich source of applications from the detection of protein–protein interactions to molecular evolution. Direct selection of markers inside a living organism (termed in vivo panning) is one of the most effective approaches available to date to investigate cellular and vascular heterogeneity. In the past few years, this technology has been used mainly for screening ligand to tissue-specific endothelial cell receptors [17].

In the present study, we used in vitro panning to identify peptides that can target the human hepatocellular carcinoma cell line BEL-7402. Our results have demonstrated that it is possible to identify tumor-specific targeting peptides by using in vitro panning strategy. In fact, one of our selected peptides is able to bind to the hepatocarcinoma cell lines BEL-7402, BEL-7404, SMMC-7721, and HepG2 in vitro, pointing to its great potential in novel diagnostics and therapeutics of liver cancer.

Experimental procedures

Materials. The hepatocellular carcinoma cell lines BEL-7402, BEL-7404, SMMC-7721, HepG2, and the normal liver cell line HL-7702 were all purchased from the cell bank of Shanghai Institute for Biological Sciences (Shanghai, China). SCID mice were purchased from Shanghai Laboratory Animal Center (Shanghai, China). The 12-mer and C7C PhD Phage-displayed Peptide Library were purchased from New England BioLabs (Beverly, MA, USA). Fetal calf serums (FCS) and RPMI 1640 medium were purchased from Invitrogen (Carlsbad, CA, USA). Peptides WP05 (TACHQHVRMVRP) and C10 (HSYHSHSLLRMF, a non-specific peptide control) were synthesized and labeled with FAM (6-carboxyfluorescein; Ex-490 nm, Em-520 nm) by HD Biosciences Ltd (Shanghai, China). Phage DNA isolation and sequencing was performed by shDNA Biosciences Ltd (Shanghai, China). Horseradish peroxidase-conjugated sheep anti-rabbit antibody was purchased from Sigma Chemicals (St. Louis, Missouri, USA). Fluorescein isothiocyanate (FITC)-conjugated sheep anti-rabbit antibody was purchased from SINO-American Biotechnology Company (Shanghai, China). Rabbit anti-M13 bacteriophage antibody was kindly provided by Dr. Lin-Fa Wang at CSIRO Australian Animal Health Laboratory.

In vitro panning. BEL-7402 and control normal liver cell line HL-7702 were cultured in RPMI 1640 10% FCS at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were washed with PBST-05 (PBS with 0.05 % Tween 20) and kept in serum-free RPMI 1640 for 1 h before use. Then the solution (both 12-mer and 7-mer PhD Phage-displayed Peptide Library are equally mixed) which contains 10^{11} colony-forming units (CFU) and 5% BSA was added into cells at 37 °C for 1 h. Next, unbound phages were wiped off by washing with PBST-05 for about eight times. And the bound phages were collected together with target cells by digestion with 0.25% trypsin. Two microliter of collected phages was picked out for phage tittering. For the amplification of selected phage clones to be used in the next round of panning, the remaining cells and phages were mixed with 20 ml of *Escherichia coli* ER2738 culture (at early-log stage) and incubated at 37 °C with vigorous shaking for 4.5 h. After the first round of panning, the phages were treated with normal liver cell line HL-7702 in the same way for reverse absorption, and the unbound phages were collected to eliminate the phages which can bind to liver cells. The residual phages were amplified and tittered for next round panning. After three rounds of panning and two rounds of reverse absorption the peptide sequences of randomly picked phage clones were analyzed by DNA sequencing.

Phage binding assay using cell-ELISA. BEL-7402 and control cell line HL-7702 were cultured in RPMI 1640/10% FCS at 37 °C in a humidified atmosphere containing 5% CO_2 and plated into 96-well plate (1×10^4 cells/well) the day before use. Cells were washed, incubated in serum free RPMI 1640 at 37 °C for 1 h, and fixed in 4% of paraformaldehyde (PFA) in PBS for 20 min. Cells were washed three times with PBST-05 (PBS with 0.05 % Tween 20) and blocked with blocking buffer (PBST-05 contained 3% BSA) at 37 °C for 1 h. Selected phage clones were each added into the cells at 10^{10} cfu per well and the plate was incubated at 37 °C for 1 h. Subsequently, unbounded phages were removed by washing the plate three times with PBST-05. To detect phages bound to the cells, wells were incubated for 1 h with 100 μl/well of rabbit anti-M13 antibody (diluted 1:5000 in the blocking buffer), washed three times with PBST-05, and subsequently incubated with 100 μl HRP-conjugated sheep anti-rabbit Ig (diluted 1:2000 in the blocking buffer). Subsequently, color development was carried out by adding 100 μl (per well) of freshly prepared substrate solution (1 mg/ml *o*-phenylenediamine (OPD) (Amresco Inc, Ohio, USA) in 0.1 M citrate buffer, pH 4.2, containing 0.03% H_2O_2) and incubating the plate for 20 min at room temperature. The absorbance (A_{490}) was then determined on a PowerWave™ XS Microplate spectrophotometer (Bio-Tek, Winooski, VT, USA) [19]. Selectivity is determined using the formula:

$$\text{selectivity} = \frac{\text{OD}_{\text{S1}} - \text{OD}_{\text{C1}}}{\text{OD}_{\text{S2}} - \text{OD}_{\text{C2}}}.$$

Here, OD_{S1} and OD_{C1} represent the OD values from the binding to BEL-7402 cells by the selected phage and control phage, respectively; OD_{S2} and OD_{C2} represent the OD values from the binding to control cell line HL-7702 by the selected phage and control phage, respectively.

Fluorescence microscopy and image analysis. BEL-7402, BEL-7404, SMMC-7721, HepG2, and HL-7702 cells were all digested with 0.25% trypsin, resuspended in serum containing medium to 10^4 cells/ml, and grown on a 24-well chamber slide overnight. Cells were washed three times with PBS to clear the cellular receptors and fixed with 4% paraformaldehyde before analysis. Cells were incubated with the selected phage clones or FAM-labeled synthetic peptides and washed, following the same protocol as that used for antibodies, as described above. Fluorescence microscopy was performed with an Olympus BX51TF microscope with a cooled frame CCD camera for FAM with excitation and emission wavelengths of 490 and 520 nm, respectively, and Image-pro Plus 5.0 image analysis software.

Fluorescence-activated cell sorting. Cells were processed the same way as for fluorescence microscopy described above. FACS analysis was conducted using a FACScalibur flow cytometer (BD Biosciences, San Diego, CA 92121, USA) equipped with an argon laser set to excite samples at 488 nm and detect light emission at 530/35 nm. Mean fluorescence intensities were calculated using Cell Quest software (BD Biosciences, San Diego, CA 92121, USA) [13].

In vivo binding assay. BEL-7402 hepatocellular carcinoma cells were inoculated subcutaneously on back of SCID mice. When tumors reached the size of ~1 cm in diameter, selected targeting phage clone (10^{11} cfu) was injected into the tail veins of tumor-bearing mice. After 15 min, mice were sacrificed (4 mice were used for panning or negative control) and perfused through heart with 100 ml of D-Hank's buffer. Phage titer per gram of tissue was then determined. For immunohistochemistry analysis, tumors were recovered (three mice were sacrificed for the analysis), sectioned (5 μm thick) in a cryostat (LEICA CM 1850, Germany) at −20 °C, mounted on poly-L-lysine-coated glass microslides, and fixed in ice-cold acetone for 10 min for hematoxylin–eosin (HE) staining and immunohistochemistry. After washing the slides in PBST-05, endogenous peroxidase was blocked by incubating the slides in methanol containing 0.75% (v/v) H_2O_2. The slides were washed three times (5 min for each wash) in PBST-05, incubated with blocking buffer (3% BSA in PBST-05) for 15 min at 37 °C, and then for 1 h at room temperature in a moist chamber. Subsequently, slides were incubated overnight at 4 °C with rabbit anti-M13 antibody (diluted 1:750 in blocking buffer). On the following day slides were rinsed three times (10 min for each rinse) in PBST-05 and incubated with HRP-conjugated sheep anti-rabbit antibody (diluted 1:500 in blocking buffer) for 1 h at room temperature. Afterwards, the slides were rinsed three times (5 min for each rinse) in PBST-05. The bound antibody was visualized using DAB (3,3-diaminobenzidine tetrachloride) (Sigma Chemicals, St. Louis, Missouri, USA) in PBS (pH 7.6) containing