Screening and Identification of a Targeting Peptide to Hepatocarcinoma from a Phage Display Peptide Library

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Ligands specific to cell surface receptors have been heavily investigated in cancer research. Phage display technology is a powerful tool in this field and may impact clinical issues including functional diagnosis and targeted drug delivery. In this study, a hepatocellular carcinoma cell line (HepG2) and a normal hepatocyte line (L-02) were used to carry out subtractive screening in vitro with a phage display-7 peptide library. After four rounds of panning, there was an obvious enrichment for the phages specifically binding to the HepG2 cells, and the output/input ratio of phages increased about 976-fold (from 0.3×10^{-7} to 292.8×10^{-7}). A group of peptides capable of binding specifically to the hepatoma cells were obtained, and the affinity of these peptides to the targeting cells and tissues was studied. Through a cell-based ELISA, immunocytochemical staining, immunohistochemical staining, and immunofluorescence, the S1 phage and synthetic peptide HCBP1 (sequence FQHPSFI) were shown to bind to the tumor cell surfaces of two hepatoma cell lines and biopsy specimens, but not to normal hepatocytes, other different cancer cells, or nontumor liver tissues. In conclusion, the peptide HCBP1 may be a potential candidate for targeted drug delivery in therapy of hepatoma cancer.

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INTRODUCTION

Hepatocellular carcinoma, a form of cancer originating in liver cells, is a challenging malignancy with high patient mortality rates (1). Although therapies are available, drawbacks such as cytotoxicity have prompted researchers to seek more effective means of treatment. The identification of high-affinity ligand biomarkers that can specifically discriminate between normal and cancerous cells as well as differentiate between specific types of cancer cells is key to the development of early cancer detection methods and preoperative treatment strategies. As such, the development of ligand identification methods is an important approach to the development of an effective therapeutic method that may overcome the major drawbacks of cytotoxicity and gene therapy.

Since 1985 (6,7), phage display has been an important tool for both basic research and drug discovery. Phage display technology allows small peptides and protein libraries to be presented on the surface of filamentous phages and permits selection of specific peptides and proteins with high affinity (2–5). This technology has been used to identify peptides for species-specific recognition of *Bacillus anthracis* spores and peptides that bind specifically to the H7 flagellin of *Escherichia coli*. Panning of phage-displayed peptide libraries on intact cells in culture and on the tissues of living animals, often called biopanning, has proven successful for isolating peptides that show high cell and tissue specificities. This approach may be used to distinguish cancerous from normal cells as well as to enable selective binding to different tumor types, even those with similar classifications (8,9).

In the present study, we established human hepatoma cell lines in our laboratory and used in vitro phage-displayed random peptide libraries to pan these cell lines to identify a specific novel peptide that could bind the cell surface. Our results demonstrate that this biopanning strategy can be used to identify tumor-specific targeting peptides. One of our selected peptides is effective in targeting

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cells and tissues, underscoring its potential for use in early diagnosis and therapy of hepatoma.

MATERIALS AND METHODS

Materials

The hepatocellular carcinoma cell lines HepG2 and BEL-7402, the normal liver cell line L-02, and the passage cell line of human cervical cancer Hela were all obtained from National Key Laboratory of Nanobiological Technology, PR China; Balb-c mice were purchased from the Animal Center of Central South University (Changsha, PR China) ranging in weight from 15 to 25g; fetal calf serum (FCS) and RPMI 1640 medium were purchased from Hyclone (Logan, UT, USA); phage DNA sequencing was performed by Shanghai Bioengineering Ltd (Shanghai, PR China); unrelated phages were the amplified phages from original phage peptide library; peptide HCBP1 (FQHPSFI) and nonspecific control peptide (AFSIKQW) were synthesized and labeled with fluorescein isothiocyanate (FITC) by Shanghai Bioengineering Ltd; horseradish peroxidase-conjugated sheep anti-rabbit antibody was purchased from Sigma Chemicals (St Louis, MO, USA); rabbit anti-M13 bacteriophage antibody was purchased from Pharmacia (Peapack, NJ, USA); Trizol reagent from Gibco BRL; (Gaithersburg, MD, USA) and the reverse transcriptase polymerase chain reaction (RT-PCR) system kit from Promega (Madison, WI, USA).

RT-PCR primer pairs for the cDNA sequences of human albumin were designed according to the primer 5.0 software and synthesized by Sangon Co. (Shanghai, China). The primers for β-actin were purchased from Promega USA. The albumin upstream primer was 5′-TCG ACA ACG GCT CCG GCA T-3′, and the albumin downstream primer was 5′-AAG GTG TGG TGC CAG ATT TTC-3′. β-actin upstream was 5′-GTG GGG CGC CCC AGG CAC CA-3′, and β-actin downstream primer was 5′-CTC CTT AAT GTC ACG CAC GA-3′. The sizes of the primers were 241 and 491 bp, respectively.

The Ph.D.-7 phage display peptide library kit (New England Biolabs, Berverly, MA, USA) was used to screen the specific peptide binding to hepatoma cells. The phage display library contains random heptapeptides constrained at the N terminus of the minor coat protein (cpIII) of M13 phage. The titer of the library is 2×10^{13} pfu (plaque-forming units). The library contains a complexity of 2.7×10^{9} individual clones, representing the entire obtainable repertoire of 7-mer peptide sequences, which expresses a random seven-amino-acid sequence. Extensive sequencing of the naive library has 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.

Quantitative Comparison of Albumin mRNA Levels in HepG2 Cells and L-02 Cells

To confirm that the L-02 cells were the normal liver cells and absorber cells in the panning procedure, the albumin levels in HepG2 cells and L-02 cells were compared quantitatively.

The HepG2 and L-02 cell lines were cultivated in RPMI 1640 medium supplemented with penicillin, streptomycin, and 10% fetal bovine serum. Cells were harvested when the growth was subconfluent, and the total number of cells was counted in a hemocytometer. After centrifugation, RNA was extracted from cell pellets (1×10^{7} cells) with Trizol reagent (Gibco BRL) according to the protocol.

The level of albumin in cells was analyzed by semiquantitative RT-PCR, and β-actin mRNA served as an internal control. With the access RT-PCR system kit (Promega, USA), cDNA synthesis and amplification were done in one tube according to the manufacturer's instructions. The PCR profile for each primer set consisted of an initial melting step of 2 min at 94 °C, followed by 40 cycles of 40 sec at 94 °C, 90 sec at 60 °C, and 2 min at 68 °C, and a final elongation step of 7 min at 68 °C and a soak at 4°C. Gene expression was calculated as the ratio of analyzed RT-PCR product to the internal standard (β-actin). Equal volumes of amplified products were loaded in each lane of 2.5% TAE (Tris, acetic acid, and EDTA)-buffered agarose gel (Promega, USA) and electrophoresed. Finally, the levels of albumin expressed in cells were quantified by scoring the intensity of bands obtained from semiquantitative RT-PCR using computerized image analysis with Gel-pro Analyzer 4.5 software (Media Cybernetics, Silver Spring, MD, USA).

In Vitro Panning

HepG2 cells were taken as the target cells, as well as the normal liver cell line L-02 as the absorber cells for whole-cell subtractive screening from a phage display 7-peptide library. Cells were cultured in RPMI 1640 and 10% FCS at 37 °C in a humidified atmosphere containing 5% CO_2. L-02 cells were washed with PBS and kept in serum-free RPMI 1640 for 1 h before blocking with 3 mL blocking buffer (BF, PBS + 5% BSA) for 10 min at 37 °C. Phage amounts of approximately 2×1011 pfu were added to the blocked L-02 cells and mixed gently for 1 h at 37 °C. Cells were then pelleted at this and subsequent panning steps, by centrifugation at 800 rpm (59*g*) for 5 min. L-02 cells and the phages bound to them were removed by centrifugation. The supernatant-containing phage was incubated with the BF-blocked HepG2 cells for 1 h at 37 °C before cells were pelleted again. The 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. HepG2 cells and bound phages were both incubated with *E. coli* host strain ER2738 and 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 tetracy-

cline. 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, 2×10^{11} pfu of collected phages were used, and the panning intensity was increased, prolonging the phages incubation process with L-02 cells to 1.25 h, 1.5 h, and 2 h and shortening that with HepG2 cells to 45 min, 30 min, and 15 min in the second, third, and fourth rounds individually and increasing TBST washing times to 4 times, 6 times, and 8 times in the second, third, and fourth round individually.

Sequence Analysis of Selected Phages and Peptide Synthesis

After four rounds of in vitro panning, 50 blue plaques were randomly selected and their sequences analyzed with an ABI Automatic DNA Analyzer (Shanghai Bioengineering Ltd.). The primer used for sequencing was 5′-^{HO}CCC TCA TAG TTA GCG TAA CG-3′ (–96 gIII sequencing primer, provided in the Ph.D.-7 Phage display peptide library kit, New England Biolabs). Homologous analysis and multiple sequence alignment were done using BLAST and Clustal W programs to determine the groups of related peptides.

Cell-Based ELISA with Phage

HepG2 and L-02 cells were cultured in RPMI 1640 10% FCS at 37 °C in a humidified atmosphere containing 5% CO_2, and seeded into 96-well plates (1×10^5 cells/well) 1 d before use. Cells were then fixed on 96-multiwell plates by 4% paraform for 15 min at room temperature until they were attached to the plates, then washed three times with PBS. The cells and the amplified phage clones were then picked out randomly and blocked individually with 1% BSA at 37 °C for 30 min. These phage clones were added into the wells (1×10^{10} pfu/well) and incubated with cells at 37 °C for 2 h. The plates were washed three times with PBS containing TBST prior to work with mouse anti-M13 phage antibody, a dilution of 1:1000 being added to the wells at 37 °C for 1 h. The plate was washed three times with TBST, and 100 µL horseradish peroxidase-conjugated sheep anti-mouse Ig (diluted 1:500 in the blocking buffer) was added. Subsequently, color development was carried out by adding 100 µL/well of freshly prepared DAB solution and incubating the plate for 5 min at 37 °C. The plates were read on an automated ELISA plate reader at the absorbency (A) of 450 nm. As negative controls, PBS and unrelated phages with the same titer were added into wells instead of the selected phage clones. Triplicate determinations were done at each data point. Selectivity was determined using the formula (10): Selectivity = $OD_{S1} - OD_{C1}/OD_{S2} - OD_{C2}$. Here, OD_{s1} and OD_{c1} represent the OD values from the binding to HepG2 cells by the selected phage and control phage, respectively, and OD_{s2} and OD_{c2} represent the OD values from the binding to the control cell line L-02 by the selected phage and control phage, respectively.

Immunocytochemical Staining and Immunohistochemical Staining with Phage S1

Before staining with phage S1 (11) the cells in the different groups (HepG2, BEL-7402 and L-02, Hela) were cultured on coverslips and fixed with acetone at 4 °C for 20 min. Then, about 1×1011 pfu of phage S1 diluted in PBS were added onto the coverslips and incubated at 4 °C overnight. Coverslips were then washed five times with TBST . The coverslips were blocked by H_2O_2 (3% in PBS) at room temperature for 510 min. After being washed by PBS for 5 min at 37 °C, the coverslips were incubated with normal sheep serum for 20 min at 37 °C. Subsequently, coverslips were incubated overnight at 4 °C with mouse anti-M13 phage antibody with a work dilution of 1:1000. The next day, the coverslips were rinsed three times (10 min for each rinse) in PBS and incubated with secondary antibody for 1 h at room temperature. Afterward, the coverslips were rinsed three times (5 min for each rinse) in PBS. The bound antibody was visualized using DAB. Coverslips were rinsed three times (5 min for each rinse) in running tap water before staining by hematoxylin-eosin. Finally, coverslips were rinsed for 10 min in running tap water before dehydration and mounting.

Frozen sections of human liver tissues with and without tumors (provided by the pathology department of Xiangya Hospital of Central South University) were also prepared. The steps of immunohistochemical staining were similar to those of immunocytochemical staining described above.

Instead of the selected phage clone S1, PBS and nonspecific control phage with the same titer were used as negative controls in this experiment .

Peptide Synthesis and Labeling

The HCBP1 peptide (FQHPSFI; translated from the selected S1 phage DNA sequence) and nonspecific control peptide (AFSILQW) were synthesized and purified by Shanghai Bioengineering Ltd. Fluorescein isothiocyanate (FITC)-conjugated peptides were also 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 the synthetic heptapeptide (HCBP1) with its phage counterparts (S1). HepG2 cells were cultured in a 12-well plate overnight and then preincubated with blocking buffer to block nonspecific binding at 4 °C for 30 min. Synthetic peptides (0, 0.0005, 0.005, 0.05, 0.5, 5 and 50 µM) were diluted in PBS and incubated with cells at 4 °C for 1 h, then incubated with 1×10^{11} pfu of phage S1 at 4 °C for 1 h. The bound phages were recovered and titered in ER2738 culture. The phages binding to HepG2 cells were evaluated by blue plaque-forming assay, and the rate of inhibition was calculated by the formula: Rate of inhibition = (number of blue plaques in HepG2 incubated with PBS –

number of blue plaques in HepG2 with the synthetic peptide)/number of blue plaques in HepG2 incubated with PBS × 100%. Nonspecific control phages (synthetic peptides 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 (HCBP1) binding to hepatoma cell lines. HepG2, BEL-7402, L-02, and Hela cells were all digested with 0.25% trypsin and plated on coverslips overnight. Cells were washed three times with PBS to clear the cellular receptors and fixed with acetone at 4 °C for 20 min before analysis. The synthetic peptides labeled with FITC were incubated with cells. PBS and control peptides labeled with FITC were used as negative controls. After being washed three times with PBS, the slips were observed in the fluorescence microscope.

Analysis of the Tissue Distribution of S1 Phage in an Animal Model

The hepatoma models of Balb-c mice were used to analyze the binding activity of S1 phage. We injected 1×10^7 HepG2 cells subcutaneously at the oxter of mice. After 15 d, establishment of the transplanted hepatic cancer models was confirmed when the volume of solid tumors had grown growing to 1 cm x 2 cm x 1 cm, and 2×10^{11} pfu of S1 phage were blocked with 500 µL BF for 30 min at 37 °C. The blocked phage was injected intravenously in 1 mL PBS. Mice were killed 10 min later, and perfused with PBS to facilitate phage elimination from the vasculature. Organs such as lung, heart, and brain and nontumor liver tissue and tumor nodules were removed, weighed, and washed with DMEM with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 20 µg/mL aprotinin, and 1 µg/mL leupeptin, DMEM-PI). The organ and tumor samples were homogenized with a Homogenate Apparatus (IKA, Wilmington, NC, USA). The phages were titered on agar plates in the presence of 1 mg/L isopropyl-1-thio-β-D-galactopyranoside/5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

Data obtained were expressed as mean ± standard deviation and analyzed by one-way ANOVA with the post-hoc Tukey's test applied for paired comparisons. A difference between means was considered significant if the *P* value was less than 0.05.

RESULTS

Quantitative Comparison of Albumin mRNA Levels in HepG2 and L-02 Cells

By semiquantitative RT-PCR, we prospectively compared the expression level of albumin mRNA in tumor cells (HepG2) and normal liver cells (L-02). We found that expression was heterogeneous between 2 cell lines, and the albumin-mRNA levels in L-02 cells were 5.6-fold higher than in HepG2 cells (Figure 1). Similar results have also been reported (12–13), so it was reasonable to consider L-02 cells as normal liver cells and absorber cells in the panning process.

Specific Enrichment of Hepatoma Cell–Bound Phages

Phages specifically binding to human hepatoma cells were identified through four rounds 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 cleared via washing with TBST. After four rounds of in vitro selection, the number of phages recovered from hepatoma cells increased 976-fold (from 0.6×10^4 pfu to 5.856×10^6 pfu) compared with that of recovered phages in the first round, as shown in Figure 2. On the other hand, there was a decrease in the number of phages recovered from L-02 control cells. The output/input ratio of phages after each round of panning was used to determine the phage recovery efficiency, which increased from 0.3×10^{-7} to 292.8×10^{-7}. These results indicated an obvious enrichment of phages specifically binding to HepG2.

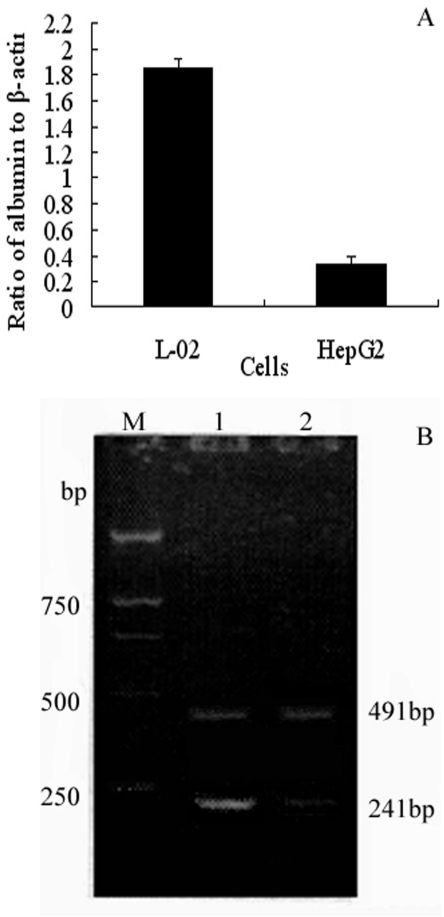

Figure 1. RT-PCR products of albumin (241 bp) and _-actin (491 bp) analyzed using RNA isolated from L-02 and HepG2 cells. _-Actin was used as an internal standard. (A) The expression levels of albumin in L-02 and HepG2 cells. The values expressed as ratios to β-actin in semiquantitative RT-PCR. (B) The expression of albumin in cells, M = marker; 1 = albumin level in L-02 cells; 2 = albumin level in HepG2 cells. The results show that the expression of albumin was heterogeneous between 2 cell lines and the albumin-mRNA levels in L-02 cells were 5.6-fold higher than in HepG2 cells.

Homology Analysis of Exogenous Sequences of Selected Phage Clones

After in vitro selection, 50 individual clones were picked out and sequenced. Each of the clones and corresponding ex-

ogenous sequence was given a sequential name from S1 to S50. Eight clones (S10, S11, S16, and S22–26) lacked an exogenous sequence; all the other clones were identified correctly by DNA sequencing. The deduced peptide sequences from S1-3/S5-6/S15/S21/S27–33, S4/S7/S12/S17–19/S40–46, S13/S16/S34–39, S8/S48–49, S9/S47, S14/S50, and S20 were accordant, and in the final stage of this process, seven different phage clones or peptide sequences were obtained. These peptides were designated hepatoma cell binding peptides (HCBPs) (Table 1). The peptide sequence of HCBP1 appeared 14 times in 50 clones. Multiple sequence alignment analysis did not reveal any strong homology among all the peptides (Table 2).

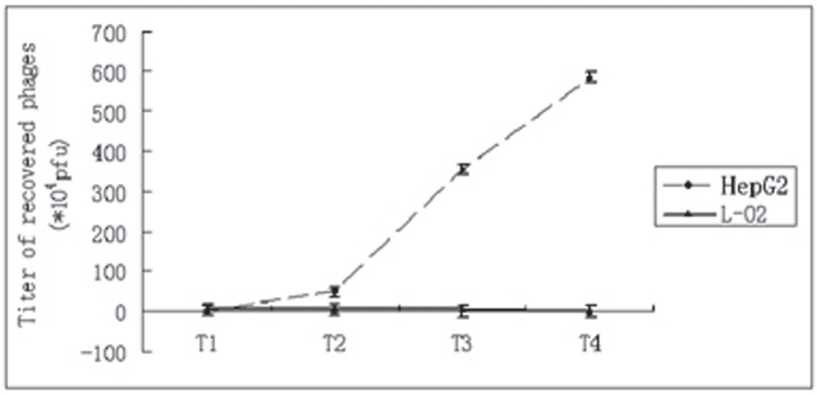

Figure 2. Specific enrichment of HepG2 cells and bound phages. Four rounds of selection were finished. The titers of recovered phages from each round were evaluated by blue plaque-forming assay on agar plates containing tetracycline. The changes of the titers on different cells in four rounds are shown above. T1-T4 refer to first round to fourth round in vitro panning.

Confirmation of In Vitro Binding by Cell-Based ELISA

To identify the affinity of the seven selected phages with HepG2, cellular ELISA was performed to exclude false-positive colonies and those binding with equal affinity to HepG2 and controls. For calculating selectivity, the binding of each phage to HepG2 cells was compared with the control normal liver cell line L-02. Results indicated that these phage clones could bind effectively to HepG2 compared with PBS and L-02 control groups. Furthermore, clone S1 appeared to bind more effectively than the other clones (see Figure 3) and was the most remarkable clone. Therefore we further analyzed the phage S1 and its displaying peptide HCBP1 (FQHPSFI).

Binding Ability of Phage S1 to HepG2 Cells and Hepatoma Tissues

To confirm the binding ability of the selected phage to target hepatoma cells and tissues, phage clone S1 was picked out for immunochemical assay. L-02 cell line, Hela cell line, and human nontumor liver tissues were also tested as negative controls. The interaction of S1 and target hepatoma cells (HepG2 and BEL-7402 cells) was evaluated by immunocytochemical staining as shown in Figure 4. The area that stained dark

Table 1. The Amino Acid Sequences of Seven Peptides and Multiple Sequence Alignment

Phage clones	Phage no.	Peptide no.	Sequence (N⊂C)	Frequency
S1-3/S5-6/S15/S21/S27–33	S1	HCBP1	FQHPSFI	14
S4/S7/S12/S17-19/S40–46	S4	HCBP4	PLPTLPL	13
S13/S16/S34–39	S13	HCBP13	LPPQSFH	8
S8/S48–49	S8	HCBP8	ATYQHAT	3
S9/S47	S9	HCBP9	WAESKTF	2
S14/S50	S14	HCBP14	HSALPKW	2
S20	S20	HCBP20	VSFPFGF	1

Table 2. Alignment of Selective Peptide Sequences

Name	Len(aa[a])	Name	Len(aa)	Score[b] (%)
HCPB1	7	HCPB4	7	14
HCPB1	7	HCPB13	7	28
HCPB1	7	HCPB9	7	14
HCPB1	7	HCPB14	7	14
HCPB1	7	HCPB20	7	14
HCPB4	7	HCPB13	7	28
HCPB4	7	HCPB8	7	14
HCPB4	7	HCPB9	7	14
HCPB4	7	HCPB14	7	14
HCPB4	7	HCPB20	7	14
HCPB13	7	HCPB9	7	14
HCPB13	7	HCPB14	7	28
HCPB13	7	HCPB20	7	28
HCPB8	7	HCPB9	7	14
HCPB8	7	HCPB14	7	14
HCPB9	7	HCPB14	7	14
HCPB9	7	HCPB20	7	14
HCPB14	7	HCPB20	7	14

[a]Length of peptide sequence; [b]homology degree of different sequences.

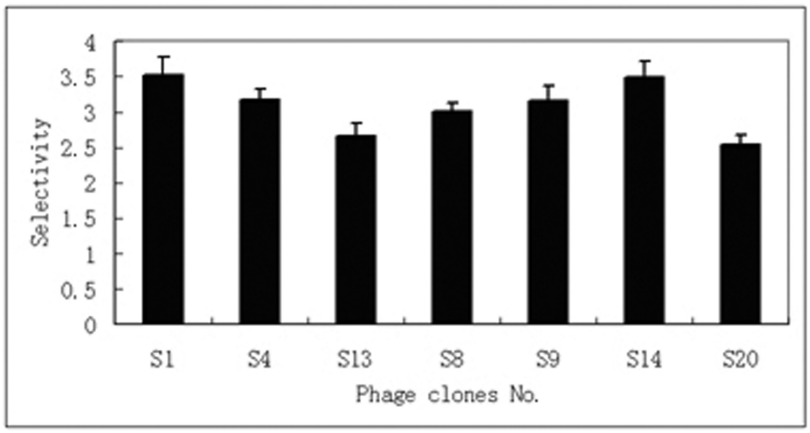

Figure 3. Evaluation by cell-ELISA of the binding selectivity of seven phage clones.. Cells were grown on 96-well plates overnight at the same concentration (1×10^5 cells/well). About 1×10^{10} pfu phages were added to each well at 37 °C for 2 h. Mouse anti-M13 bacteriophage antibody and HRP-conjugated sheep anti-mouse Ig were added in turn. OD_{490} was obtained after blocking of the reaction. The selectivity values of each phage clone (S1S20), calculated by the formula mentioned in the text, were 3.52, 3.18, 2.66, 3.01, 3.15, 3.49, and 2.55, respectively. Therefore clone S1 appeared to bind more effectively than the other clones.

brown, was located at the surfaces, and in the perinuclear cytoplasm of cells, indicated the positive binding region of the phage S1 to HepG2 and BEL-7402 cells. In contrast, no positive staining was observed in control L-02 cells or Hela cells. The negative results were also obtained when HepG2 cells and BEL-7402 cells were stained with unrelated phage clone or PBS. Subsequently, immunohistochemical staining was performed to observe the specific binding of phage S1 to human hepatoma tissues as shown in Figure 5. The cells in hepatoma tissue sections were stained brown distinctly. The hepatoma tissue sections stained with unrelated phage clone or the nontumor liver tissue sections showed negative staining. It is thus clear that the phage S1 was able to bind specifically to hepatoma cells.

Competitive Inhibition Assay

A peptide-competitive inhibition assay was performed to discover whether the synthetic peptide and the selected phage clone competed for the same binding site. The results showed that if the synthetic peptide HCBP1 (FQHPSFI) was preincubated with HepG2 cells, the binding of phage S1 to HepG2 cells was inhibited in a dose-dependent manner (Figure 6). With an increase in concentration of HCBP1, the titer of phages recovered from HepG2 cells decreased and the rate of inhibition increased gradually. When peptide concentration increased above 5 μM, the inhibition rate came to a flat phase. The control peptide (AFSIKQW) had no effect on the binding of phage S1.

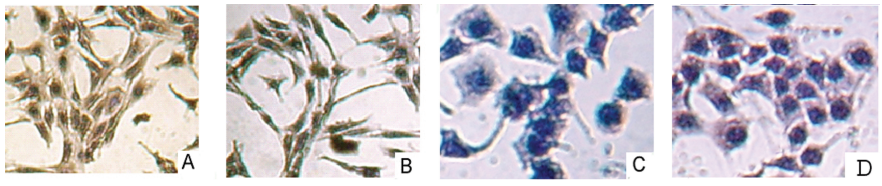

Figure 4. Immunocytochemical staining of HepG2 and control cells with phage S1 (by ABC means, x400). Cell-bound phages were detected using anti-M13 phage monoclonal antibody, secondary antibody, and ABC complex. Then the cells were stained with diaminobenzidine (DAB) followed by hematine crystal. Panel (A) shows immunocytochemical staining with phage S1 on HepG2 cells; (B) shows immunocytochemical staining with phage S1 on BEL-7402 cells; (C) shows immunocytochemical staining with phage S1 on L-02; (D) shows immunocytochemical staining with phage S1 on Hela. L-02 (c) and Hela (d) were treated as negative controls.

Binding Ability of Peptide HCBP1 to Hepatoma Cells

Results of experiments described above suggest that the phage S1 has specificity to hepatoma cells. To investigate whether the free peptide maintained this binding affinity after removal from its phage protein framework, we made a synthetic peptide HCBP1 (FQHPSFI) with an FITC label. After the HepG2, BEL7402, L-02, and Hela cells were incubated with FITC-HCBP1, green fluorescence was sent out on the membrane and in the perinuclear cytoplasm of HepG2 and BEL7402 cells and observed under a fluorescence microscope. In contrast, negative results were obtained on L-02 cells, Hela cells, and FITC-labeled nonspecific control peptide (Figure 7). These results suggest that HCBP1 may bind specifically to hepatoma cells.

In Vivo Binding Assay

We injected 2×10^{11} pfu of S1 phage through the tail vein into Balb-c mice bearing HepG2 xenografts. The specificity of the phage clone was further confirmed by the titer of bound phage in the tumor tissue compared to nontumor tissues. S1 phages were found in tumor masses at concentrations 2.2- to 10.5-fold higher than in nontumor organs such as brain, lung, and heart and nontumor liver tissue; in contrast, the nonspecific control phage did not show any targeting to tumor tissues (Figure 8).

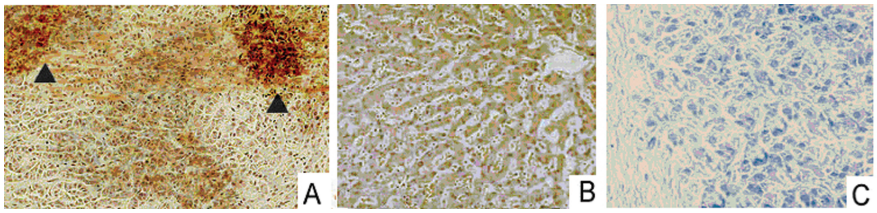

Figure 5. Immunohistochemical staining of hepatoma and nontumorous hepatic tissue sections with phage S1 (by ABC means, x400). The bound phages were detected using anti-M13 phage monoclonal antibody, secondary antibody, and ABC complex. Then the cells were stained with diaminobenzidine (DAB) followed by hematine crystal. Panel (A) shows immunohistochemical staining with phage S1 in hepatoma tissues, and the specific binding sites on tumor cells are shown as brown distinctly; (B) shows immunohistochemical staining with phage S1 in nontumorous hepatic tissues with no specific binding; (C) shows a negative control section with immunohistochemical staining with unrelated phages in hepatoma tissues.

DISCUSSION

Targeting specific binding ligands on the tumor site is an efficient way to improve the selectivity of therapeutic molecules in clinical oncology, and this process is critical to the development of cancer early detection and therapy. It has been reported that cancer cells often display high numbers of certain cell surface molecules such as tumor-associated antigens or tumor-specific antigens that are sparse on normal tissues and represent potential sites for delivery of toxic agents (5). Therefore, improvement of targeting accuracy and efficiency to tumor tissues is a major challenge in clinical trials. For this purpose, we used peptide phage display technology, introduced by Smith and Parmley (6,7), to identify potential molecular markers of hepatoma cancer.

Our phage-panning procedure for selection of peptides with tumor cellular binding specificity uses an M13 random peptide phage display library and thus differs from other previously reported phage display library panning strategies (8-10). Prior to the panning process, semiquantitative RT-PCR was performed to ensure that L-02 cells could be used as absorber cells for subtractive screening, and our results show that the albumin-mRNA levels in L-02 cells were much higher than in HepG2 cells, a finding similar to other reports (12-13). Therefore we used L-02 cells as negative controls in the following experiments. We performed four rounds of in vitro panning on hepatoma cells in culture and some optimizing procedures (eg, several rounds of whole-cell subtractive screening and enhanced gradually selection pressures) to improve the probability of obtaining specific phages with high affinity. After panning, approximately 50 clones were chosen for further characterization. First, cell-based ELISA was performed to confirm specific binding of HepG2 cells in vitro. As a result, S1, the best candidate clone with the highest specificity, was selected for a phage-binding assay. Next, immunocytochemical and immunohistochemical staining were used to confirm the selectivity of the phage S1 to hepatoma cells and tissues compared with that of normal liver cells, nontumor liver tissues, and other types of cancer cells. Third, the results of our competitive inhibitory assay suggest that the peptide displayed by the S1 phage, and not other parts of this phage, bound to the hepatoma cell surface. Fourth, we demonstrated that the displaying peptide of the phage S1, HCBP1, was also able to maintain binding specificity to hepatoma cells rather than normal liver cells in vitro, also proving that its binding ability was determined by the peptide sequence alone without the involvement of flanking phage-coat protein sequences. Under the same conditions, the normal liver cell line L-02 and Hela cells did not show any significant fluorescence, a finding that further confirmed the targeting of HCBP1 to hepatoma cells. Finally, our analysis of the tis-

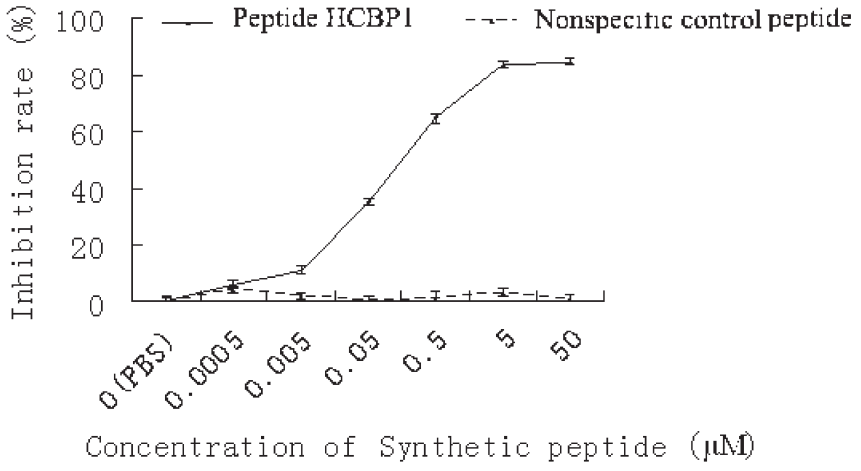

Figure 6. The competitive and inhibitory results between the synthetic peptide HCBP1 and the phage S1 on HepG2 cells. Comparable data were obtained in three independent experiments, and the average inhibition rates at different peptide concentration are shown. When the concentration of peptide HCBP1 reached more than 0.005 μM, significant inhibition occurred.

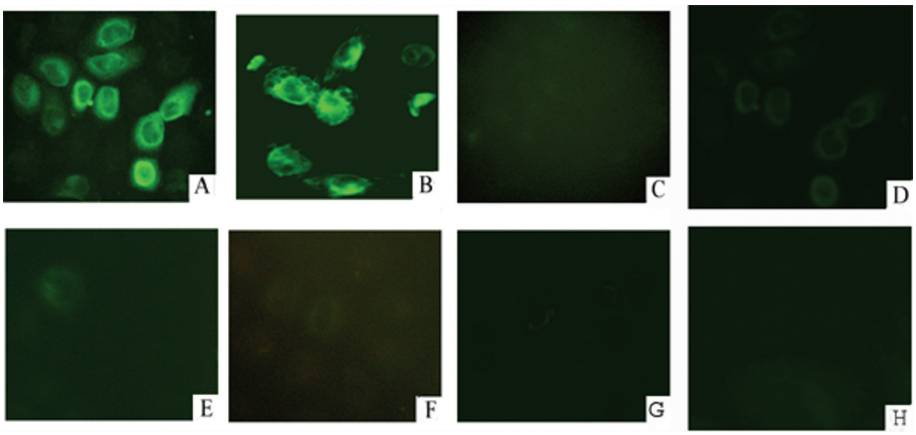

Figure 7. Binding specificity of synthetic peptide HCBP1 to HepG2 and BEL-7402 cells (x200). The FITC-labeled synthetic peptide HCBP1 was incubated with HepG2 (A), BEL-7402 (B), L-02 (C), and Hela cells (D). At the same time, the control FITC-labeled peptide (AFSIKQW) was incubated with HepG2 (E), BEL-7402 (F), L-02 (G), and Hela cells (H) as negative control. The cells were observed in the fluorescence microscope. As the figure shows, the peptide HCBP1 bound preferentially to hepatoma cells rather than control cells.

sue distribution of the S1 phage in vivo in an animal model indicated that S1 phage was localized only in tumor masses but not in brain, lung, heart, or nontumor liver tissues, results that also support our conclusion that S1 phage can specifically bind to xenograft tumor cells but not normal tissue and cells. These results also suggest that the HCBP1 peptide may be specific to hepatoma and therefore useful for diagnosis, antitumor therapy, and further research.

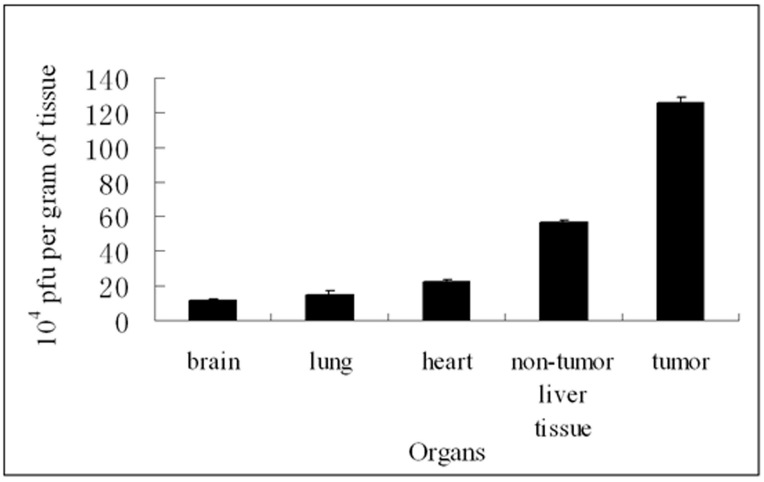

Figure 8. Distribution of S1 phage in organs of animal models. Balb-c mice bearing HepG2 xenografts received intravenous injections of S1 phage clones. After 10 min, the free phages were washed out. Xenografts and some organs were removed for determination of phage titers. The level of S1 phage identified in the tumor mass is 2.2–10.5-fold higher than that in some organs such as the brain, lung, and heart and in nontumor liver tissue. However, there is no phage observed in the tumor mass in the mice treated with the nonspecific control phage.

Through encapsulation of drugs in a macromolecular carrier such as chitosan nanocarriers, which our group has investigated (14), the concentration of drug in the tumor is increased, resulting in a decrease in the amount of nonspecific toxicities and an increase in the amount of drug that can be effectively delivered to the tumor. A tumor-targeting ligand is needed, however, to deliver the carriers to the tumor site. Tumor cells may express a number of molecular markers at very low levels, but the structures and characteristics of these markers are unknown, making it difficult to identify them with conventional methods. In some cases, markers are undetectable (15–16). The use of a phage display library provides a powerful tool to obtain the specific binding peptides through the selection of desired binding properties (17). Strategies for panning cells in vitro or tissues in vivo with complex phage libraries have been reported to yield phages with organ- or tumor-binding specificity (18–19). Compared with in vivo phage display technology, cell-based panning is simple and effective. Capillary vessels of the vascular system may act as barriers for phage passage so that with the majority of recovered phages in vivo panning is actually binding to the vascular endothelium cells and not tumor cells. The use of in vivo phage display technology has led to reports of many specifically vascular endothelium cell-binding peptides (20). Therefore, in this study we chose intact tumor cells as targets for panning peptides specific to hepatoma cells.

In conclusion, we report the use of phage display technology to identify peptides specific to hepatoma cells. We have developed an improved panning system, and the peptides generated by this method preferably bind to hepatoma cells (HepG2 and BEL-7402) rather than to normal liver cells (L-02) or other types of cancer cells (Hela), and bind to hepatoma tissues rather than nontumor liver tissue. The targeting peptide HCBP1 may

have significant implications for the targeting of liver cancer treatment. Further studies are needed to investigate the binding specificity of HCBP1 to human hepatoma tissues and the application of HCBP1 to clinical oncology.

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