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Identification of an Oligopeptide Binding to Hepatocellular Carcinoma

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Key Words
Biopanning · Hepatocellular carcinoma · Peptide · Phage display · Tumor targeting

Abstract
Objectives: We carried out identification of a small peptide binding to human hepatocellular carcinoma (HCC) cells with the aim of applying the peptide for future HCC-targeted therapy or imaging. ***Methods:*** The biopanning technique using phage peptide display libraries was performed on HCC cells in vitro, and a phage clone expressing the HCC-binding peptide motif was selected. The binding activity of the selected phage was evaluated by plaque infection assay and immunofluorescence on cell lines. In addition, the binding activity of the peptide-expressing phage was investigated using HCC specimens derived from patients who had undergone hepatectomy for HCC. ***Results:*** A heptapetide, Thr-Thr-Pro-Arg-Asp-Ala-Tyr (TTPRDAY), was identified as a motif binding to HCC. TTPRDAY bound specifically to HCC cells in comparison with other cancer cells, and the binding to HCC cells was also confirmed by immunofluorescence. In addition, the synthesized TTPRDAY peptide showed binding activity and a non-mitogenic effect on HCC cells in vitro. TTPRDAY-presenting phage showed more significant binding to HCC cells derived from specimens obtained from actual patients than to non-cancerous liver tissue. ***Conclusion:*** The motif TTPRDAY, identified by the biopanning technique, shows significant binding to HCC cells.

Introduction

Hepatocellular carcinoma (HCC) is one of the commonest malignancies worldwide [1], and the prognosis of affected patients remains poor [2–4]. In many patients with HCC, obtaining curativity by surgery or radiofrequency ablation of the cancer is difficult due to the high frequency of intrahepatic metastases or multicentric occurrence. Other therapeutic options for intrahepatic localized HCC are percutaneous ethanol injection, microwave therapy, cryotherapy and transarterial chemoembolization. However, the therapeutic efficacy of these treatments is limited. For example, the 5-year survival rate after transarterial chemoembolization for HCC is only 12.5% [5]. Patients with distant metastatic HCC are treated with systemic chemotherapy, but its reported

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therapeutic efficacy is dismal; Chung et al. [6] reported that intra-arterial cisplatin infusion and systemic interferon-α yielded a response rate of 33% and a median survival time of 19 weeks for HCC patients with major portal vein thrombosis or distant metastases.

The chief obstacles to establishing effective therapies for HCC are the multiplicity of the lesions (multicentric occurrence of the tumor and intrahepatic or distant metastases) and severe liver dysfunction due to underlying liver cirrhosis. Therefore, establishment of a methodology that could target HCC efficiently would bring about a dramatic improvement in the efficacy of treatments for HCC. For example, HCC-selective ligands would enable targeted delivery of a variety of therapeutic agents, such as genes or cytotoxic drugs, and would also facilitate better imaging of multiple HCC lesions. Such agents are clearly appropriate for HCC patients with liver cirrhosis, because they could minimize the degree of drug entry into non-HCC cells and the resulting damage to liver function.

Candidate targeting agents have been studied by several groups in attempts to achieve tumor tropism. The ligands that have been evaluated include some antibodies, as well as fragments and single-chain Fv molecules and growth factors. Recently, however, this empirical approach to the identification of targeting ligands has been largely superseded by the use of library-based screening systems, which have been designed to allow iterative selection of high-affinity ligands by repeated screening and enrichment of living libraries.

Barry et al. [7] have introduced peptide-presenting phage libraries to enable selection of peptides binding to several different cell types. Since then, the biopanning technique using phage peptide display libraries has been shown to be a powerful tool for identifying specific ligands on target organs and tumors [8–10]. The peptide-presenting phage library used is based on a combinatorial library of random peptide heptamers fused to a minor coat protein (pIII) of phage M13 and contains about 2.8×10^9 different sequences. With successive rounds of biopanning, different peptides selective for the target cells can be identified.

In the present study, we used the phage panning technique to identify a peptide that would have the specific ability to bind to human HCC cells. In addition, in order to confirm the feasibility of applying the identified peptide to clinical practice, its HCC-binding activity was assessed on HCC samples derived from patients who had undergone hepatectomy for operable HCC.

Materials and Methods

Cell Lines

The cell line Huh-7 (human HCC) was obtained from the Health Science Research Resources Bank (HSRRB, Osaka, Japan), and the cell lines Hep3B (human HCC), HepG2 (human hepatoblastoma), DLD-1 (human colorectal carcinoma), AZ521 (human gastric cancer) and Saos2 (human osteosarcoma) were obtained from the Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). Cells were maintained in Dulbecco's modified Eagle medium (DMEM, Sigma Chemical, Poole, UK) or RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in 5% CO_2. The cells were passaged and expanded by trypsinization of the cell monolayers followed by replating every 4 days.

Selection of Cell-Targeting Peptides

The Ph.C7C M13 heptapeptide phage display library (New England Biolabs, Beverly, Mass., USA) was used for biopanning. Huh-7 cells were grown to confluence in a 6-well plate. They were acclimated to 4°C for 30 min and washed twice in phosphate-buffered saline (PBS, Dainippon, Osaka, Japan) containing 1% (w/v) bovine serum albumin (BSA, Sigma). The phage library of 2×10^{11} plaque-forming units (pfu)/well diluted in 1 ml DMEM containing 1% BSA was added to each well at 4°C. After 1 h of incubation with gentle agitation, medium containing unbound phage was removed, and the cells were washed 4 times in PBS containing 1% BSA. This was followed by addition of 1 ml/well 0.2 M glycine-HCl (pH 2.2) as a general buffer for nonspecific disruption of binding interactions for 5 min, and fluids were neutralized with 150 μl 1 M Tris-HCl (pH 9.1).

The cells were scraped from the 6-well plate. Numbers of eluted phages were established by titering on X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Wako, Osaka, Japan) and IPTG (isopropyl-β-D-thiogalactopyranoside; Wako) agar plates containing *Escherichia coli* strain ER2738. Remaining phages were amplified by addition to a 20-ml early log phase culture of ER2738 for 5 h at 37°C with vigorous shaking (150 rpm). Amplified phages were isolated from the resulting culture using the manufacturer's recommended protocol, concentrated, titered and used for subsequent rounds of biopanning. In total, four rounds of biopanning were performed in triplicate.

Isolation and Sequencing of Phage DNA

After each round of biopanning, individual phage clones were isolated from each replicate and their total DNA was isolated according to the recommended protocol of the sequencing kit manufacturer (Biosystems, Perklin Elmer, Foster City, Calif., USA). The resulting DNA was used for sequencing analysis using the –96 primer together with a BigDye terminator cycle sequencing kit (Amersham Biosciences, Amersham, UK). The DNA sequences were determined using an automated ABI PRISM sequencer 3100 Genetic Analyzer (Applied Biosystems). Next, searches for human proteins mimicked by the selected peptide motifs were carried out using online databases through the National Center for Biotechnology Information (http:www.ncbi.nlm.nih.gov/BLAST/).

Evaluation of Selected Phage Clones for Binding to Human Cancer Cells

The cells were grown to confluency in 6-well plates, acclimated to 4°C for 30 min and washed briefly in PBS containing 1% BSA two times. Then, each selected phage clone diluted in 1 ml of DMEM containing 1% BSA at a concentration of 5×10^7–10^9 pfu was added to each well at 4°C. After 1 h of incubation with gentle agitation, medium containing unbound phages was discarded, and the cells were washed four times in PBS containing 1% BSA. Then, the phage binding to cells was evaluated as described in 'Selection of Cell-Targeting Peptides'. In addition, the ability of selected phage clones to bind to other human cancer cell lines (Hep3B, HepG2, AZ521, DLD-1 and Saos2) was determined in 6-well plates as described above.

Immunofluorescence Analysis of Binding of Selected Phage Clones to Huh-7 Cells in vitro

Huh-7 cells were grown to confluence on an 8-well chamber slide in DMEM containing 10% FBS. The cells were washed with PBS containing 1% BSA, and incubated for 1 h at 4°C in DMEM (containing 1% BSA) containing 2×10^{11} pfu of either a selected phage clone or a control phage. A phage clone displaying no oligopeptide insert (insertless) was used as a negative control. The medium was discarded and the cells were washed four times with PBS containing 1% BSA, fixed with methanol/acetone (–20°C), and blocked with 10% goat serum for 20 min at room temperature. An anti-M13 monoclonal antibody diluted 1:600 in 2% goat serum was added and incubated for 1 h at room temperature. The cells were washed in PBS three times, and then incubated with FITC-conjugated goat anti-mouse immunoglobulin for 1 h at room temperature. The cells were washed in PBS three times, 4′-6-diamino-2-phenylindole was used for nuclear counterstain, and visualized using an Olympus BX60 fluorescence microscope (Olympus, Tokyo, Japan).

The motif Thr-Thr-Pro-Arg-Asp-Ala-Thr (TTPRDAY) was selected as the most promising consensus sequence binding to HCC and studied in more detail.

Competitive Inhibition of the Synthesized Peptide on Phage Accumulation in vitro

To confirm the capacity of the synthesized peptide (TTPRDAY) to bind to HCC, its inhibitory effects on phage accumulation were examined. Huh-7 cells were pre-incubated with the TTPRDAY peptide (synthesized by Sigma Genosys Japan, Ishikari, Japan) or control peptide at 1 or 10 μM for 30 min at 4°C, and then 5×10^8 pfu of the selected phage diluted in 1 ml DMEM containing 1% BSA were added. The phages were allowed to bind to the cells for 1 h at 4°C with gentle agitation. Medium containing unbound phages was discarded, and the cells were then washed four times for 5 min each time in PBS containing 1% BSA, before the cell-associated phages were recovered by lysing the cells in 1 ml/well of 30 mM Tris-HCl (pH 8.0) containing 10 mM EDTA on ice for 1 h. The number of phages recovered was determined by titering multiple dilutions of the eluted phages as described above. The same experiment was repeated using insertless phages and TTPRDAY peptide.

Effect of the TTPRDAY Peptide on Cell Viability

Huh-7 cells were plated in 96-well plates at 5×10^3 cells/well and incubated at 37°C in DMEM containing 10% FCS in either the presence or absence of the TTPRDAY peptide at 0.1, 1 or 10 μM. After 24, 48, 72 and 96 h, the viability of the Huh-7 cells was assessed using the MTS assay, as described previously [9]. Media were replaced with 120 μl of FCS-free DMEM containing 20 μl of CellTiter®96 AQueous One solution reagent (Promega, Madison, Wisc., USA), and the culture plates were incubated at 37°C for 2 h. Next, 100 μl of the medium were transferred to a new 96-well plate and the quantity of the formazan product present was determined by measuring the absorbance at 490 nm using a microplate autoreader (Molecular Devices, Sunnyvale, Calif., USA).

Mutagenicity Study

The Ames test [11, 12] was carried out using histidine-deficient (*his*$^-$) *Salmonella typhimurium* tester strains, TA100 and TA98. The tester strains 100 are responsive to base-pair substitutions, whereas the 98 detect deletions or additions of base pairs (frameshifts). Strains TA100 and TA98 were cultured at 37°C in the presence of different concentrations (0.1, 1, 10, 100 and 1,000 μM) of the selected peptide and revertant *his*$^+$ colonies were counted after a 48-hour incubation period. The presence of revertant colonies after plating on histidine-poor growth media indicates the presence of a mutagen. Each experimental condition was run with duplicate samples. Additionally, the Ames test was performed in the absence or presence of a S9 fraction (mix of metabolizing enzymes from rat liver) to allow not only detection of a direct mutagenic effect, but also of an indirect mutagenic effect brought about by possible metabolites of the applied compound.

Binding of the Selected Phage Clone to Surgically Resected HCC Specimens

Samples of tumor and non-cancerous liver tissue were obtained at the time of hepatectomy from patients with operable HCC. Samples were weighed and homogenized using a motor-driven Teflon-glass homogenizer. The homogenized samples of both the tumor and non-cancerous liver tissue were each divided into two portions, one being used to assess the binding activity of the selected phage clone and the other to assess that of the control phage. Either the selected or the control phage was added to the homogenates of tumor and non-cancerous liver tissue at 5×10^{10} pfu per 100 mg tissue, and allowed to bind to the samples for 20 min at 37°C with agitation (150 rpm). The samples were washed with PBS twice, and then 2 ml of 0.2 M glycine-HCl were added as a general buffer for nonspecific disruption of binding interactions for 3 min, and fluids were neutralized with 300 μl of 1 M Tris-HCl. After washing with PBS, 5 ml of PBS containing 0.5% Tween 20 were added to the cells. Recovery was quantified by titering multiple dilutions of the homogenates, as described above. The experiment was performed on 6 patients with HCC, and in each case, the results were presented as a ratio relative to the recovery of the insertless phage from the non-cancerous liver tissue. In addition, the binding activity of the selected phage clone on surgically resected specimens was evaluated by immunofluorescence analysis. Sample from the tumor tissue was mounted in OCT compound and frozen, and sectioned at 6 μm in a cryostat at –20°C. After washing with PBS twice, the sectioned sample was incubated with 5×10^{10} pfu of the selected phage clone or control phage (insertless) for 30 min. The sample was then washed four times in PBS. This was followed by addition of 100 μl 0.2 M glycine-HCl (pH 2.2) as a general buffer for nonspecific disruption of binding interac-

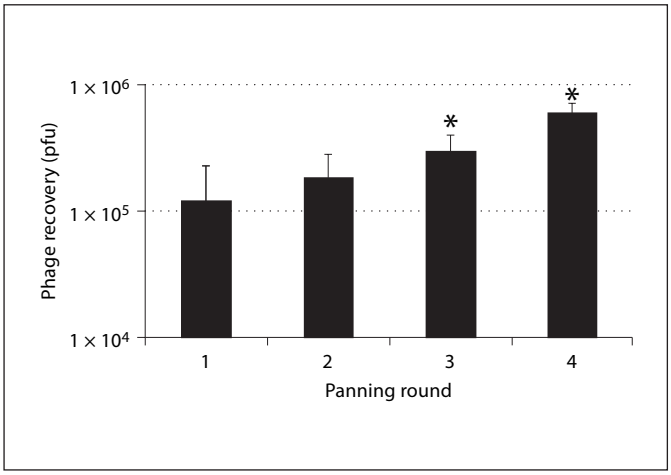


Fig. 1. Phage recoveries from Huh-7 cells in each round of biopanning. Four consecutive rounds of biopanning were performed on the human HCC cell line Huh-7. The phage recovery from each round increased with the number of biopanning passages, being approximately 6-fold higher in the final round than in the first. * $p < 0.05$ compared to the recovery in round 1.

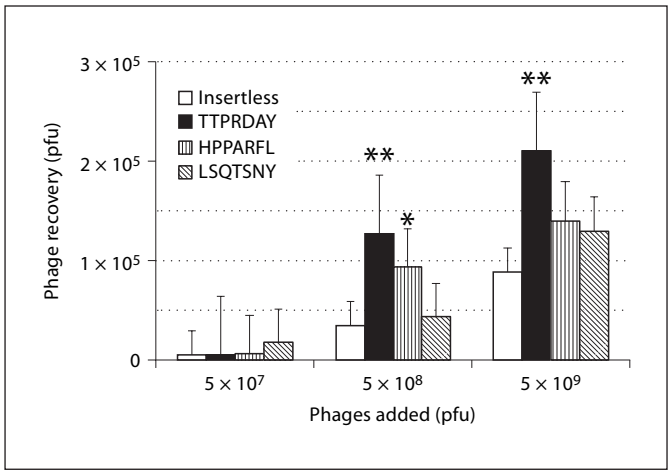


Fig. 2. Assessment of binding activities of selected phage clones expressing TTPRDAY, HPPARFL and LSQTSNY on Huh-7 cells in vitro. Huh-7 cells were cultured in 6-well plates and incubated with phage clones expressing TTPRDAY, HPPARFL or LSQTSNY or control phage (insertless) at a concentration of 5×10^7–10^9 pfu. Cells were then scraped off and phage recoveries were titered by plaque infection assay. The phage clone expressing TTPRDAY showed the highest binding activity at plural concentrations of added phage. ** $p < 0.01$ and * $p < 0.05$ compared to the recovery of insertless phage.

tions for 5 min, and fluids were neutralized with 15 μl 1 M Tris-HCl (pH 9.1). Then, the sample was washed with PBS and immunostained as described in 'Immunofluorescence Analysis of Binding of Selected Phage Clones to Huh-7 Cells in vitro'.

Statistics

Results are presented as the mean and standard deviation of data from three independent experiments, with significance of differences evaluated using Student's t test. In the experiment, to examine the binding of phages to surgically resected human tissue specimens, the results are presented as the mean and standard deviation of the data from six patients.

Approval from the Shinshu University Ethics Committee was obtained before the study, and written informed consent was obtained from all patients for the use of their tissue specimens.

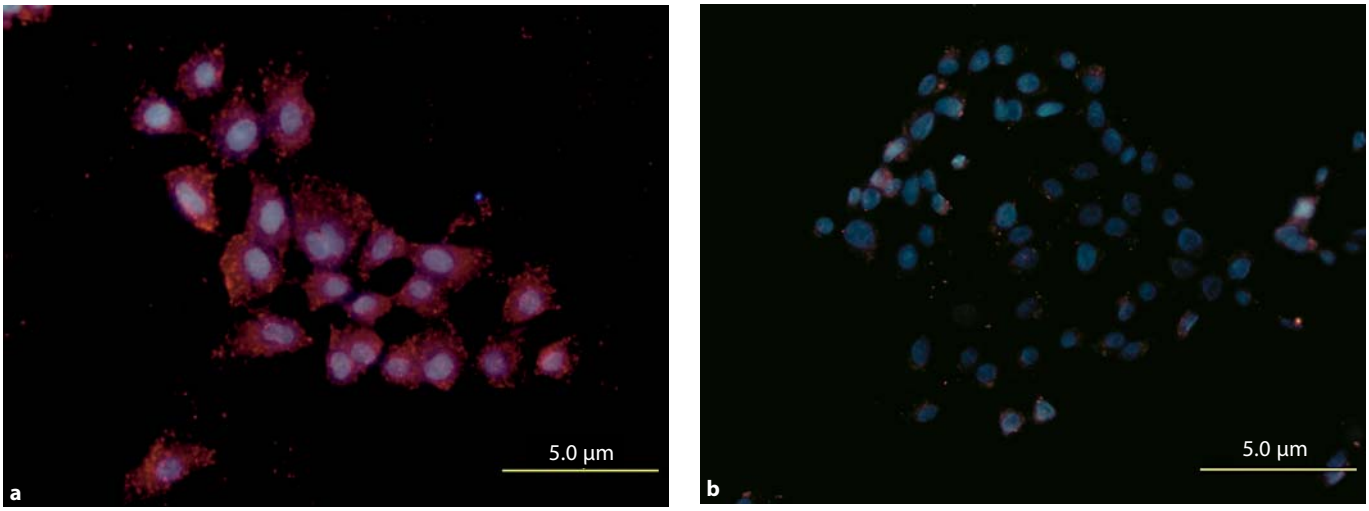
Results

Isolation of the Specific Peptide Binding to Huh-7 Cells

Four consecutive rounds of in vitro biopanning were performed on human HCC. The phage recovery from each round increased with the number of biopanning passages, indicating selection of phage binding to HCC cells (fig. 1).

After each round of biopanning, individual phage plaques were picked up. Their DNA was isolated and sequenced, and the corresponding amino acid sequences of the inserts were deduced. After the first and second rounds of biopanning, the HCC-derived sequences displayed no distinguishable homology (data not shown). However, the HCC-derived sequences from the third and fourth rounds displayed some consensus motifs, and these were selected as candidate peptides that could bind to HCC. After the third and fourth rounds of biopanning, 24 phage plaques were picked up from each replicate, and their DNA was sequenced. We then compared the relative frequencies of every tripeptide motif in each replicate. The motif frequencies were calculated as the prevalence of each motif-containing peptide divided by the total number of isolated peptides. The Thr-Thr-Pro-Arg-Asp-Ala-Tyr (TTPRDAY) motif was the most frequently encountered (5.6%), followed by His-Pro-Pro-Ala-Arg-Phe-Leu (HPPARFL; 4.2%) and Leu-Ser-Gln-Thr-Ser-Asn-Thr (LSQTSNY; 2.8%).

To determine which motif was the best binding peptide, the binding activities of selected phage clones expressing the candidate oligopeptides were assessed in vitro as described above. The phage clone expressing the TTPRDAY motif showed the highest binding activity at plural concentrations of added phage clones (fig. 2), and therefore this motif was selected as the most promising for binding to HCC.



TTPRDAY phage

Insertless phage

Fig. 3. Immunolocalization of phages on Huh-7 cells. Cells were incubated with phage clones expressing TTPRDAY (**a**) or with control phage (insertless, **b**). Phage-incubated cells were fixed and permeabilized. Phages were visualized by fluorescence using mouse anti-M13 phage antibody followed by an FITC-conjugated goat anti-mouse antibody. 4′-6-Diamino-2-phenylindole was used for nuclear counterstain. ×400.

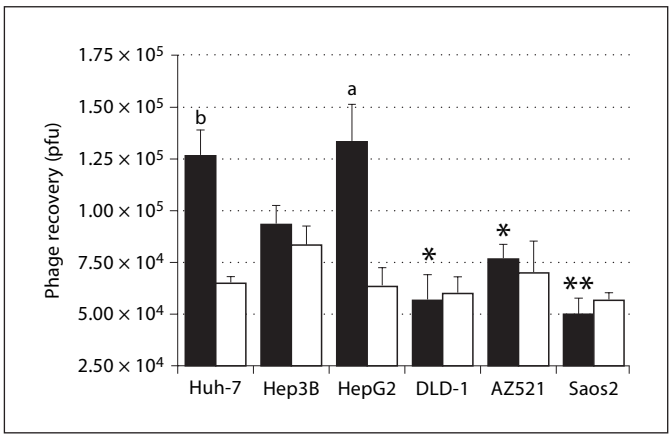


Fig. 4. Evaluation of binding of phage clones expressing TTPRDAY to cultures of Hep3B, HepG2, DLD-1, AZ521 and Saos2 cells in comparison with Huh-7 cells. Each cell line was cultured in 6-well plates, and incubated with phage clones expressing TTPRDAY or control phage (insertless). Cells were scraped off, and phage recoveries from each cell line were titered by plaque infection assay. Black bar = Recovery of TTPRDAY phage; white bar = recovery of insertless phage; ** $p < 0.01$ and * $p < 0.05$ compared to the recovery of TTPRDAY phage from Huh-7 cells; [b] $p < 0.01$ and [a] $p < 0.05$ compared to the recovery of insertless phage from the same cell line.

Heptapeptides containing the consensus motif were analyzed using BLAST (National Center for Biotechnology Information) to search for similarity to known human peptides. Interestingly, TTPRDAY showed 6/7 homology with colonic and hepatic tumor-overexpressed gene protein (ch-TOGp, TTPRDxY).

Immunofluorescence Analysis of Phage Clone Binding to Huh-7 Cells

The ability of the phage clone expressing TTPRDAY to bind to Huh-7 cells was assessed using immunofluorescence (fig. 3). The TTPRDAY phage showed apparently stronger binding activity to Huh-7 than the control phage.

Binding of TTPRDAY-Conjugated Phage Clone to Human Cancer Cell Lines

The binding of the phage clone expressing the TTPRDAY motif to confluent cultures of Hep3B, HepG2, DLD-1, AZ521 and Saos2 cells was evaluated in comparison with its binding to Huh-7 cells (fig. 4). The recoveries of the TTPRDAY phage from HepG2 and Hep3B cells were similar to that from Huh-7. The recoveries of the phage from non-HCC cell lines (DLD-1, AZ521 and Saos2) were significantly lower than that from Huh-7. In addition, the recovery of TTPRDAY phage from Huh-7 and HepG2 was significantly higher than that of the insertless phage

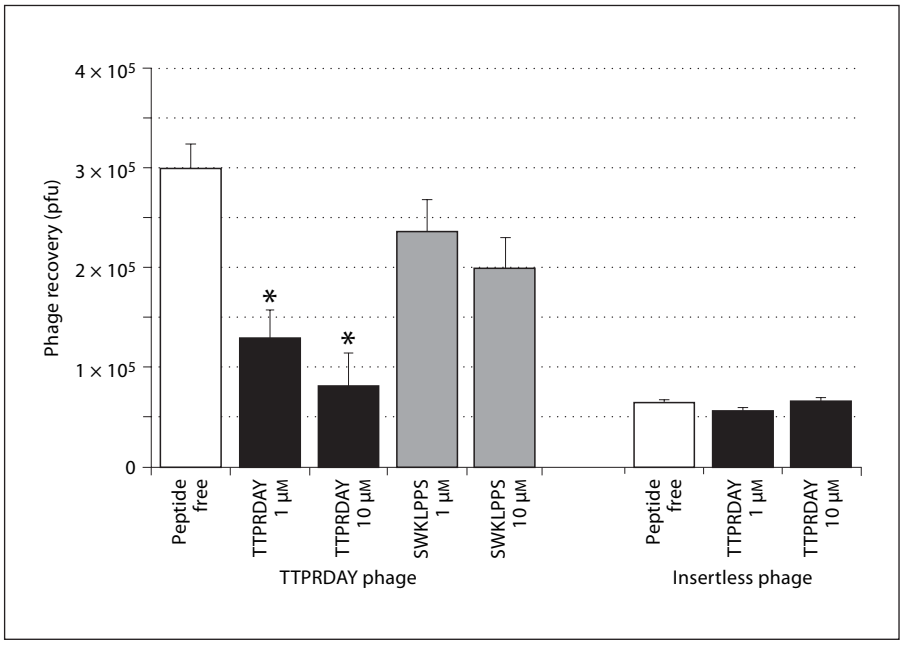


Fig. 5. Competitive inhibition of the synthesized peptides on phage accumulation. Huh-7 cells were cultured on a 6-well plate and pre-incubated with the synthesized TTPRDAY peptide or control peptide (SWKLPPS) at 1 and 10 μM before incubation with phage expressing TTPRDAY. Cells were scraped off and phage recoveries were titered. The same experiment was repeated using insertless phage and TTPRDAY peptide. * $p < 0.01$ compared to the recovery of peptide-free condition.

from the same cell lines. On the other hand, there was no significant difference between the recoveries of TTPRDAY phage and the insertless phage from the other cell lines.

Competitive Inhibition of the Synthesized Peptides on Phage Accumulation in vitro

To confirm the capacity of the synthesized peptides to bind to HCC, cells were pre-incubated with 1 or 10 μM TTPRDAY peptide or control peptide before addition of 5×10^8 pfu of the selected phage. The inhibitory effects of the synthesized peptides on phage accumulation were examined by titering the phages bound to cancer cells. It was found that pre-incubation of cells with the TTPRDAY peptide caused 73% inhibition of the binding activity of the TTPRDAY phage to HCC (fig. 5), while this inhibitory effect of TTPRDAY peptide was not observed when the experiment was performed with insertless phage.

Effect of the Selected Peptide on Cell Viability and Mutagenicity

The possibility that the TTPRDAY peptide might influence cancer cell growth (promotion or inhibition) was evaluated using the MTS assay. The presence of the TTPRDAY peptide had no discernible effect on cell growth (fig. 6).

In addition, the mutagenic potential of TTPRDAY peptide was evaluated using the Ames test. Figure 7 shows the number of revertant colonies obtained with different concentrations of TTPRDAY peptide: positive control: 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide [S9mix(–)], and 2-aminoanthracene [S9mix(+)]. The incubation of

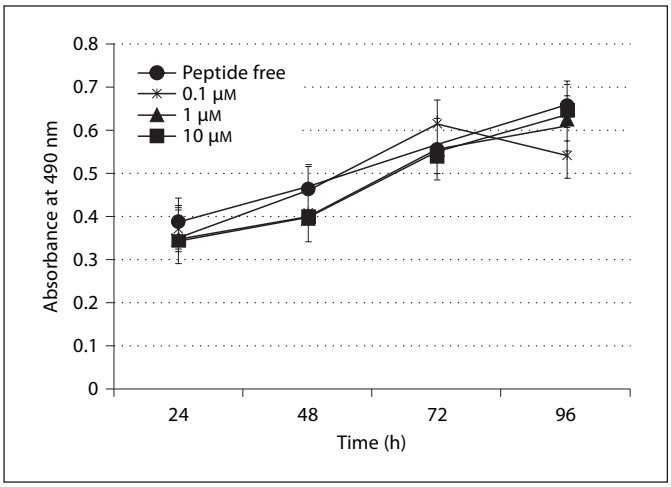


Fig. 6. Effect of the TTPRDAY peptide on cell viability. Huh-7 cells were plated at a density of 5×10^3 per well in a 96-well plate and incubated in DMEM containing 10% FCS, in either the presence or absence of the TTPRDAY peptide at 0.1, 1 or 10 μM. After 24, 48, 72 and 96 h, the viability of the Huh-7 cells was assessed using the MTS assay. Presence or absence of the TTPRDAY peptide made no significant difference to cell growth.

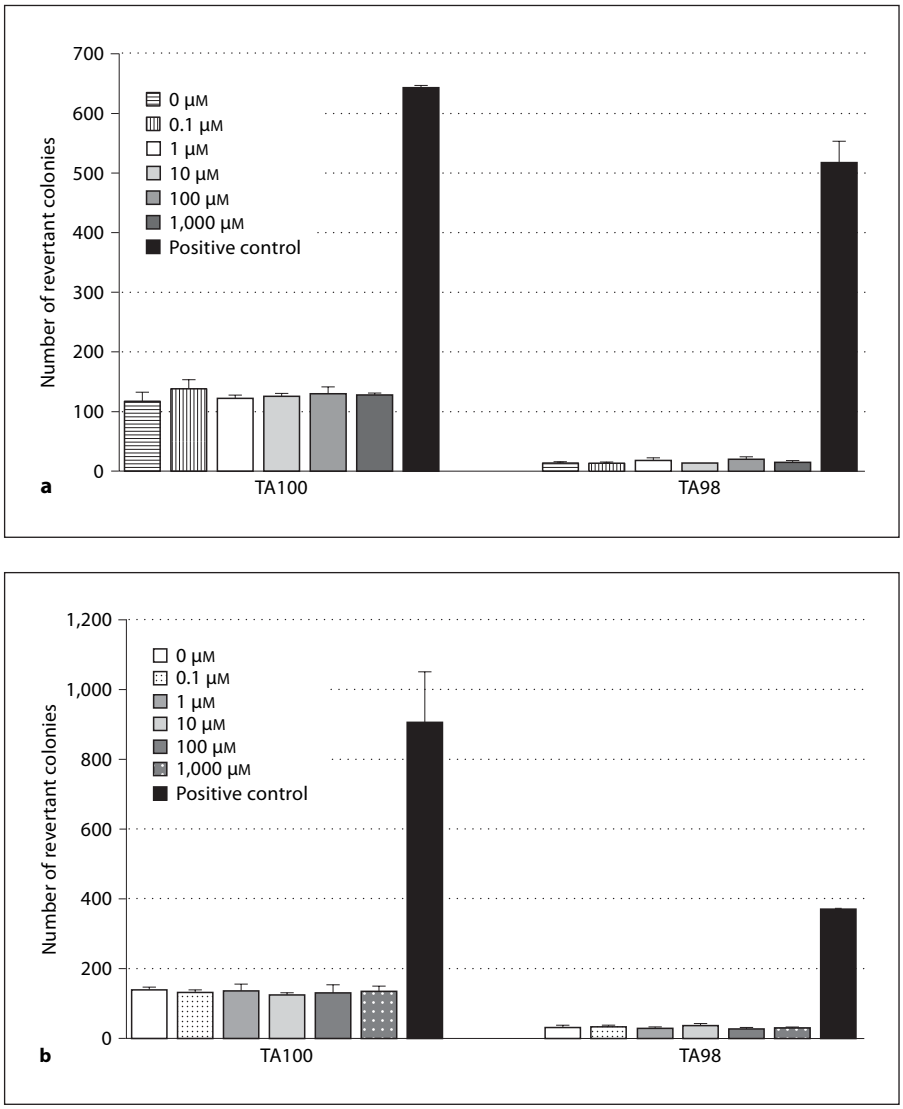


Fig. 7. Evaluation of mutagenicity of TTPRDAY peptide. The Ames test was performed using *S. typhimurium* tester strains, TA100 and TA98, to evaluate the mutagenic potential of TTPRDAY peptide. TA100 and TA98 were cultured in the presence of different concentrations of TTPRDAY peptide (0.1, 1, 10, 100 and 1,000 μM) in the absence (**a**) or presence (**b**) of a S9 fraction isolated from rat hepatocytes. After a 48-hour incubation period, revertant *his*$^+$ colonies were counted. Any concentrations of TTPRDAY peptide cause no increase in the number of revertant colonies.

TTPRDAY peptide with liver enzymes allowed evaluation of the pro-mutagenic potential of TTPRDAY peptide. As seen in figure 7, TTPRDAY peptide did not cause any increase in the number of revertant colonies in the absence (fig. 7a) or presence (fig. 7b) of S9 fraction compared to the control group. The result of this study suggests that TTPRDAY peptide has no mutagenic or promutagenic potential.

Evaluation of Binding of the Selected Phage Clone to Surgically Resected Human Tissue Specimens

Samples of tumor and non-cancerous liver tissue were collected at the time of hepatectomy from 6 patients with operable HCC. The median age of the patients was 70 years (range 59–73 years), and all 6 patients were male. The histology of the tumor was well-differentiated HCC in 4 patients and moderately differentiated HCC in 2. Five patients had liver cirrhosis or chronic hepatitis (hepatitis B virus-related hepatitis in 1; hepatitis C virus-related cirrhosis in 1; hepatitis virus-unrelated cirrhosis or hepatitis in 3, including 1 case of alcoholic hepatitis). TTPRDAY phage showed significantly higher binding to tumor tissue than the control phage (fig. 8a) and also significantly higher accumulation in tumor tissue than in the corresponding non-cancerous liver tissue (fig. 8b). Immunofluorescence analysis was used to characterize the distribution of phage clones expressing the TTPRDAY peptide in the tumor from a patient who had under-

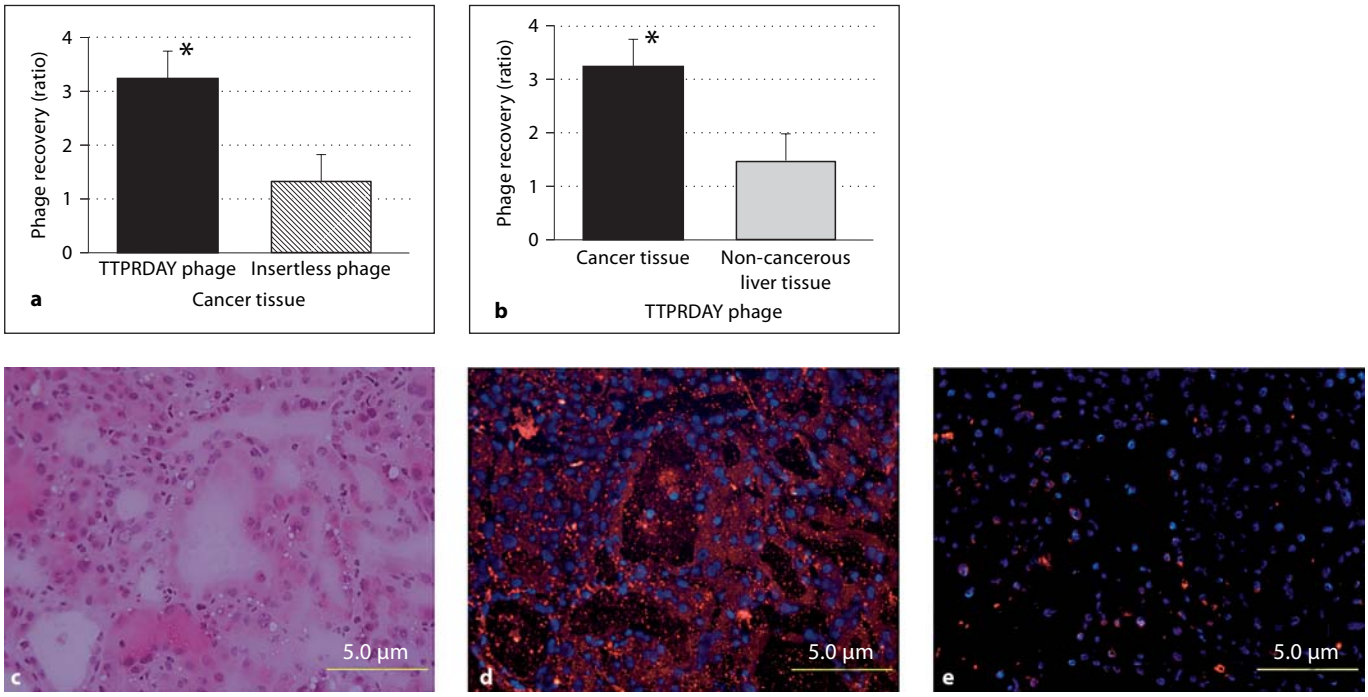


Fig. 8. Evaluation of TTPRDAY phage binding to surgically resected HCC tissue. Samples of cancer and non-cancerous liver tissue were obtained at the time of hepatectomy from 6 patients with HCC. The samples were homogenized and incubated with TTPRDAY phage or control phage (insertless) for 20 min. Phage binding activities were evaluated by plaque infection assay. Recovery of each phage was expressed as a ratio relative to that of the control phage from background liver tissues. In addition, binding of TTPRDAY phage to surgically resected HCC tissue was assessed by immunofluorescence analysis. Sample of cancer tissue was incubated with TTPRDAY phage or insertless phage for 30 min. Phages were visualized by fluorescence using mouse anti-M13 phage antibody followed by an FITC-conjugated goat anti-mouse antibody. 4′-6-Diamino-2-phenylindole was used for nuclear counterstain. **a** Comparison of binding to cancer tissue between TTPRDAY phage and insertless phage. **b** Comparison of TTPRDAY phage binding between cancer tissue and non-cancerous liver tissue. * $p < 0.05$ compared to the recovery of insertless phage or non-cancerous liver tissue. **c** HE staining of surgically resected HCC. **d** Immunostaining for TTPRDAY phage. **e** Immunostaining for insertless phage HCC. ×400.

gone hepatectomy for HCC. The results revealed that TTPRDAY phage particles bound to HCC cells, but the insertless phage showed only low signals (fig. 8d, e).

Discussion

In the present study, we used a phage display library to identify peptide sequences capable of binding to HCC cells. After four rounds of selection, the consensus sequence TTPRDAY was identified and examined in more detail. TTPRDAY showed apparent binding to receptors that are most commonly and specifically upregulated in HCC, since TTPRDAY bound to three different types of HCC cell lines, whereas it showed lower binding to non-HCC cells (fig. 4). In addition, TTPRDAY bound to HCC derived from actual specimens resected from human patients, whereas it showed lower binding to non-cancerous liver tissue from the same patient (fig. 8).

A search for similarity to known human peptides revealed that TTPRDAY has 6/7 homology (TTPRDxY) with colonic and hepatic tumor-overexpressed gene protein (ch-TOGp), which has been reported by Charrasse et al. [13] to be a new human cDNA overexpressed in human HCCs and colonic adenocarcinomas. Strong expression of the ch-TOG gene was also found in the colonic Caco-2 cell line at the proliferative stage, whereas the transcript became undetectable when the cells were in a quiescent enterocyte-like differentiated state [13]. ch-TOGp is reported to be structurally homologous to a high-molecular-weight microtubule-associated protein in *Xenopus* oocytes (XMAP215) [14], but in humans the precise function of ch-TOGp is still unknown [15]. In the present study, TTPRDAY was shown to bind efficiently

to HCC cells but less so to colon cancer cells (fig. 4), despite the fact that ch-TOGp is reportedly related to both HCC and colon cancer. The significance of the homology between TTPRDAY and ch-TOGp is currently unclear, and warrants further investigation.

For the treatment of HCC, tumor-specific targeting is necessary, since HCC has a tendency to develop intrahepatic metastases and multicentric occurrence. Furthermore, an important requirement is that drugs directed against HCC should have no effect on the non-cancerous liver parenchyma, because HCC patients usually have deteriorated liver function. For this reason, research aimed at finding gene therapy vectors that could be targeted to HCC cells using specific monoclonal antibodies is being actively conducted [16]. Because monoclonal antibodies such as AF-20 [17] and Hep27 [18] bind to the antigen uniformly expressed in HCC-derived cell lines and human tumors, including those with distant metastasis, it is likely that they would have high specificity and efficiency as HCC-targeting gene transfer vectors. In addition, single-chain antibody fragments may have potential application for tumor targeting [19]. In fact, Bing et al. [19] have reported HCC-specific single-chain antibody fragments. However, these are relatively large proteins and have some pharmacological limitations, notably a short plasma half-life, unwanted interactions with serum components and a high cost of manufacture. In contrast, TTPRDAY is a simple peptide with excellent stability and a low manufacturing cost. Furthermore, although it consists of only seven amino-acid residues, TTPRDAY is expected to work sufficiently well as a targeting ligand, since peptides containing three amino-acid residues, such as RGD, have been reported to provide the minimal framework for structural formation and protein-protein interactions [20]. In fact, the competitive inhibition of TTPRDAY-presenting phage binding to HCC by the synthesized TTPRDAY peptide implies that the peptide itself has HCC-binding activity.

This study confirmed the ability of TTPRDAY to bind to HCC using surgical specimens derived from patients with operable HCC. Such confirmation is important, because sometimes a peptide that works well in vitro does not work in actual patients. TTPRDAY phage bound to non-cancerous liver tissue approximately 1.4 times more than control phage (fig. 8b), although the difference was not statistically significant. Five of 6 patients contributing to this study had liver cirrhosis or chronic hepatitis, therefore the non-cancerous liver tissue in these cases was not completely normal, and there was a possibility that they had already included micro-dysplasia lesions in the liver. This may be one reason why TTPRDAY phage accumulates in non-cancerous liver tissue slightly higher than control phage. Nevertheless, TTPRDAY phage bound to HCC significantly more than surrounding non-cancerous liver tissue (fig. 8b), and this difference between HCC and non-cancerous tissue could be important for the application of TTPRDAY to HCC-targeted therapy.

In addition, TTPRDAY showed no significant ability to mediate cell proliferation in vitro after binding to HCC cells. This is an important consideration when applying a novel peptide to actual cancer treatment, since it is undesirable to administer potent mitogens to cancer patients. The TTPRDAY peptide has potential therapeutic applications to well-established treatments such as transarterial chemoembolization or systemic chemotherapy for HCC patients. Furthermore, it could be used as a targeting molecule to direct imaging agents to primary HCC and its metastases by using well-developed strategies for chelation to radiolabels. Finally, a peptide-presenting phage display library is a powerful method for identifying ligands that can bind to specific targets [21]. Our findings suggest that the TTPRDAY peptide has considerable potential as a therapeutic and diagnostic reagent for HCC.

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