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# Targeting of hepatocellular carcinoma with glypican-3-targeting peptide ligand

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**Hepatocellular carcinoma is a common malignancy. The carcinoma cells express glypican-3 (GPC-3) on the cell membrane. GPC-3 is also expressed in melanoma cells. Therefore, GPC-3 might be a potential target for tumor imaging or therapy. Here, proteomic mass spectrometry was used to identify peptides that target GPC-3-expressing tumors. A mammalian expression vector expressing a FLAG-GPC-3 fusion protein was cloned for immunoprecipitation. With the use of liposomes, the vector was transfected into HepG2 (HepG2/FLAG-GPC-3) and HEK 293 cells, and the transfected cell lines were selected with geneticin. HepG2/FLAG-GPC-3 cells were used for immunoprecipitation of FLAG-GPC-3 fusion protein. Seven peptide candidates (L1–L7) were selected for GPC-3-targeting ligands by mass spectrometric analysis. The L5 peptide with 14 amino acids (Arg-Leu-Asn-Val-Gly-Gly-Thr-Tyr-Phe-Leu-Thr-Thr-Arg-Gln) showed selective binding to the GPC-3-expressing tumor cells, as did a shortened L5 peptide (L5-2) with seven amino acids (Tyr-Phe-Leu-Thr-Thr-Arg-Gln). These peptide ligands have potential as targeting moieties to GPC-3-expressing tumors for diagnostic and/or therapeutic purposes. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.**

**Keywords:** glypican-3; proteomics; peptide ligand; tumor targeting; hepatocellular carcinoma

## Introduction

Hepatocellular carcinoma (HCC), which accounts for 75% of liver cancer cases, is the fifth-most common malignancy, causing approximately 600,000 annual deaths worldwide, with mortality increasing in developed countries [1,2]. Surgical resection with or without transplantation requires evidence of localized intrahepatic lesions and no extrahepatic metastasis. All HCC lesions must be imaged before deciding on a therapeutic plan. However, imaging of HCC is challenging because the tumor has a variable radiological appearance and frequently coexists with regenerative and dysplastic nodules in patients with underlying liver cirrhosis [3].

Active targeting of HCC with targeting moieties that have radioisotope or magnetic resonance contrast could improve imaging outcomes and may even have therapeutic applications with cytotoxic agents or beta-emitting radioisotopes [4,5].

Glypican-3 (GPC-3) is a cell surface protein that is exuberantly expressed on the membrane of HCC cells and other human cancer cells including melanoma and germ cell tumor [2–4,6,7]. Although GPC-3 can be secreted into the blood stream, it is mainly expressed on the cell membrane [2]; therefore, it has potential as a promising target for tumor-specific diagnosis and treatment [2,6,8]. Diagnostic or therapeutic materials, such as imaging agents, nanomaterials, or drugs, can be targeted to sites of tumors expressing GPC-3 with a number of different GPC-3 affinity ligands such as antibody, peptides, and aptamers that specifically target GPC-3 [9].

As a GPC-3-targeting ligand, anti-GPC-3 antibody was generated, as well as a humanized version of the antibody; both targeting were shown to be toxic to GPC-3-positive HCC cells [10,11]. Although the antibody exhibits exceedingly high target selectivity and binding affinity, drawbacks include difficulty of the laboratory procedures, immunogenicity, and poor *in vivo* kinetics [9,12].

As compared with antibody, peptide-based targeting ligands offer several advantages such as lack of immunogenicity, ease of performance, and low-cost production. The peptide ligand can be identified by mass spectrometry (MS) followed by separation or phage display methods [13,14]. However, as far as we know, there have been no previous reports about a peptide ligand targeting GPC-3.

The present study identified novel GPC-3-targeting peptides by MS and tested the binding capability of the peptides to GPC-3-expressing tumors *in vitro*.

## Materials and Methods

### Cell Culture

HepG2 and Huh-7 human hepatoma cells (Korean Cell Line Bank, Seoul, Korea), HEK 293 human embryonic kidney cells (Korean Cell Line Bank), and SkMel28 human melanoma cells were routinely grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and antibiotic–antimycotic liquid (100×, GIBCO, Grand Island, NY, USA) at 37 °C in a 5% $CO_2$ incubator.

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**Abbreviations used:** *GPC-3, glypican-3; HCC, hepatocellular carcinoma.*

### GPC-3 Gene Amplification

The human GPC-3 gene was inserted in a pDNRLIB vector (Korea Unigene Information, Seoul, Korea). The GPC-3 gene construct was amplified by PCR using primers with *Xba*I or *Eco*RI sites (forward primer: 5′-AATTAGAATTCCAGGATGGCCGGGACCG TC-3′; reverse primer: 5′-TTACTCTAGAGTAGCACATGAGCTGGGC A-3′).

### Construction and Transfection of FLAG-GPC-3 Vector into Cell Lines

The amplified GPC-3 gene with the aforementioned restriction sites was inserted into a p3 × FLAGCMV vector (Sigma-Aldrich, St Louis, MO, USA). The mammalian expression vector (FLAG-GPC-3 vector) was transfected into HepG2 and HEK 293 cell lines using Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA, USA) as recommended by the manufacturer. Stably transfected cells were selected with 600 μg/ml geneticin (Invitrogen). HepG2 and HEK 293 cells stably expressing FLAG-GPC-3 were designated as HepG2/FLAG-GPC-3 and HEK/FLAG-GPC-3, respectively.

### Identification of FLAG-GPC-3 mRNA Expression with Reverse Transcription PCR

The total RNA from transfected cells was isolated using TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions. Reverse transcription (RT) was performed with M-MuLV reverse transcriptase (Fermentas, St Leon-Rot, Germany) to produce cDNA. PCR amplification of the RT products was performed with FLAG primers (420 bp product; forward primer: 5′-ATGGGTGAAACTCTGGGA-3′, reverse primer: 5′-TCGGCACT-CAATGGCCAT-3′), and GAPDH (560 bp product; forward: 5′-ATCTTCTTGTGCAGTGCCAGCC-3′, reverse primer: 5′-GGTCAT-GAGCCCTTCCACAATG-3′) was used as the internal control. Following amplification, the PCR products were separated by 0.9% agarose gel electrophoresis, stained with ethidium bromide, and visualized under ultraviolet light.

### Identification of GPC-3 Protein Expression with Western Blotting

Cells were washed twice in phosphate-buffered saline (PBS) and lysed with lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 100 μM phenylmethanesulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM dithiothreitol). Total protein in cell lysates was quantified using bicinchoninic acid. Forty micrograms of the protein was separated by 9% acrylamide electrophoresis, and the resolved proteins were transferred to a nitrocellulose membrane. Each membrane was blocked using 5% skim milk and exposed to a 1:1000 dilution of primary antibody cocktail containing GPC-3 antibody (BioMosaics, Burlington, VT, USA) or a 1:1000 dilution of anti-FLAG monoclonal antibody (Sigma-Aldrich). Membranes were washed with Tris-buffered saline Tween and then exposed to secondary antibody conjugated to horseradish peroxidase for 2 h at room temperature. After three washes, immunoblots were developed with ECL Plus (Amersham Bioscience, Piscataway, NJ, USA). Western blotting of GPC-3 was performed in a melanoma cell line.

### Immunoprecipitation with Anti-FLAG Antibody

Cells were washed twice with PBS and lysed with lysis buffer [150 mM NaCl, 1 mM EDTA, 20 mM Tris pH 8.0, 1% Triton X-100, 10% glycerol, 1:30 diluted protease inhibitor mixture (Roche Applied Science, Basel, Switzerland), and a 1:100 dilution of phosphatase inhibitor cocktail (Sigma-Aldrich)]. The insoluble cell debris was removed by centrifugation at 14,000 *g* for 20 min at 47 °C, and the supernatants were transferred to new tubes. Total cell lysates were diluted at a 4 : 6 ratio with immunoprecipitation dilution buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and a 1:30 dilution of protease inhibitor mixture). Twenty microliters of anti-FLAG monoclonal-antibody-conjugated M2-agarose beads (Sigma-Aldrich) was added to the cell lysates, and immunoprecipitation was performed overnight at 47 °C with slow rotation on a shaker. Immunoprecipitated complexes were washed five times with washing buffer (25 mM, Tris, 2.7 mM KCl, and 137 mM NaCl, pH 7.4). After the final wash, the bound proteins were eluted in non-reducing SDS sample buffer (63 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, and 0.005% bromophenol blue) or with 36 FLAG peptides (150 ng/ml final concentration; Sigma-Aldrich). The samples were boiled for 3 min in SDS sample buffer and loaded onto a 15% acrylamide gel.

### Colloidal Coomassie Brilliant Blue Staining and In-Gel Trypsin Digestion

Gels were washed three times with distilled water for 5 min each and stained with colloidal Coomassie Brilliant Blue dye solution for 1 h with gentle agitation at room temperature. The gels were destained by washing in distilled water for 30 min and changing the water every 10 min. The isolated bands from SDS–PAGE were digested with trypsin. The dye was removed by destaining, and the SDS in the gel was removed by washing the gel pieces twice in 0.4 ml of a washing buffer (0.1 M ammonium bicarbonate) for 10 min and once again with a washing buffer [50% acetonitrile (ACN), 0.1 M $NH_4HCO_3$] for 1 h on a shaker at room temperature. The gel pieces were dehydrated by adding 50 ml 100% ACN to each tube. After incubation for 15 min, the ACN was removed; the gel pieces were dried in a SpeedVac for 15 min; and 5–10 ml of freshly diluted, modified sequencing-grade trypsin (Roche) in 25 mM $NH_4HCO_3$, pH 8.0, was added to a final concentration of 0.02 mg/ml (a total of 10–20 mg trypsin) to each tube containing dried gel pieces. After incubating at room temperature for 15 min, an additional 5–25 ml of 25 mM $NH_4HCO_3$, pH 8.0, was added to cover the gel pieces if necessary. Trypsin digestion was performed overnight at 37 °C, and the supernatants were transferred to fresh Protein LoBind Eppendorf (Hamburg, Germany) tubes.

### MS Analysis

For MALDI-TOF MS analysis, trypsin-digested samples (0.5 ml) were mixed in a MALDI target plate with 0.5 ml of 5 mM diammonium citrate, and 1 ml CHCA (10 mg/ml in 60% ACN containing 0.1% TFA) was added. The sample was dried, and MALDI-TOF spectra were measured in a Voyager DE-STR Biospectrometry Workstation (Applied Biosystems, Foster City, CA, USA) in the reflectron and positive ion modes. A database search of the peptide masses was performed using PROFOUND software (The Rockefeller University Edition). A Web-based software probability of 100% and a *Z*-score of 1.65 were considered as positive for protein identification.

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### *In Vitro* Imaging of GPC-3-Expressing Cells with FITC-Labeled Peptides

Cells cultured on eight-chamber slides were blocked with 1% BSA for 60 min, incubated at 4 °C for 1 h with 10 μmol/l solution of a fluorescein isothiocyanate (FITC)-labeled peptide ligand or scrambled control peptide (green) in PBS/1% BSA, and then stained with 4′,6-diamidino-2-phenylindole (DAPI) for nuclear counterstaining (blue). Slides were visualized under a fluorescent microscope (Zeiss, Oberkochen, Germany). To assess the degree of GPC-3 specificity of the ligands, we performed a blocking assay. Ten micrograms of L5 peptide was co-incubated with FITC-labeled L5 peptide, and then *in vitro* imaging was obtained.

## Results

### Stable Expression of FLAG-GPC-3 in HepG2 and HEK 293 Cells

HepG2/FLAG-GPC-3 and HEK/FLAG-GPC-3 (HepG2 and HEK 293 cells stably expressing FLAG-GPC-3, respectively) were successfully established with parent HepG2 and HEK 293 cells (Figures 1 and 2). GPC-3 expression was detected in HCC cell lines (parent HepG2, Huh-7 cells), melanoma cell line (SkMel28), and HEK/FLAG-GPC-3, but not in HEK 293 and COS-7 cells, by RT–PCR and Western blot analyses (Figure 1). GPC-3 expression was detected in HepG2 and HepG2/FLAG-GPC-3 cells. However, FLAG expression was detected only in HepG2/FLAG-GPC-3 cells (Figure 2).

### Identification of Peptide Ligands Targeting GPC-3

HepG2/FLAG-GPC-3 cells were used for identification of peptide ligands targeting GPC-3. After immunoprecipitation with anti-FLAG M2 agarose beads, the bound proteins from the cell lysates were resolved by SDS–PAGE, and the gel was excised. With MALDI-TOF MS analysis after in-gel trypsin digestion, seven peptide candidates were selected as GPC-3-targeting ligands (Table 1).

### *In Vitro* Overlay of FITC-Labeled Peptide Candidates on GPC-3-Expressing Cells

Selective binding of the peptide candidates to GPC-3-expressing cells was tested after FITC conjugation to the candidates. The 14-amino-acid peptide ligand L5 bound to the HepG2, Huh-7, and SkMel28 cells (cells with GPC-3 expression) but not to

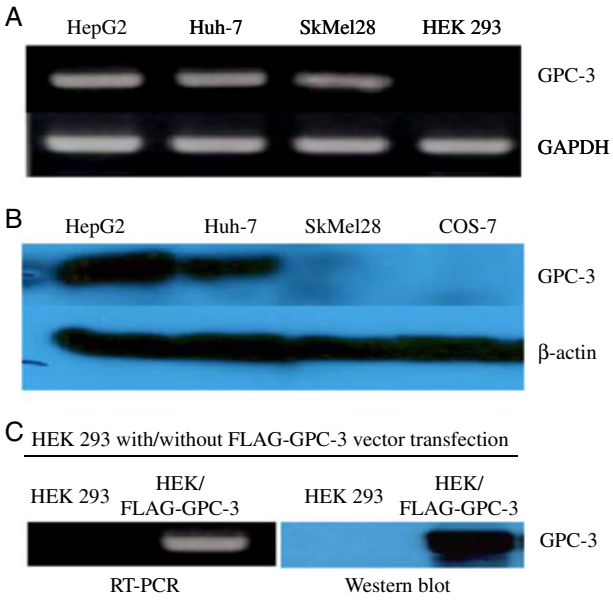


**Figure 1**. The expressions of GPC-3 mRNA (A) and protein (B) in HepG2, Huh-7, SkMel28, HEK 293, and COS7 cells. GAPDH and β-actin served as a loading control. The expression of GPC-3 in HEK 293 cells transduced GPC-3 expression vector (C).

HEK293 and COS-7 cells (cells without GPC-3 expression). But the ligand bound to HEK 293 cells after stable transfection of GPC-3 (HEK/FLAG-GPC-3) (Figure 3). Other peptide ligands do not bind to the GPC-3-expressing cells.

### *In Vitro* Overlay of FITC-Labeled Short Peptide Candidates on GPC-3-Expressing Cells

Five short variants from the L5 peptide were designed to find shorter peptide ligands having specificity for GPC-3 (Table 2). The FITC-labeled L5-2 peptide bound to HepG2, SkMel28, and HEK/FLAG-GPC-3 cells (cells with GPC-3 expression), but not to COS-7 (cells without GPC-3 expression) (Figure 4). But affinity of the L5-2 peptide to GPC-3-expressing cells was weaker than that of L5 peptide (Figure 5).

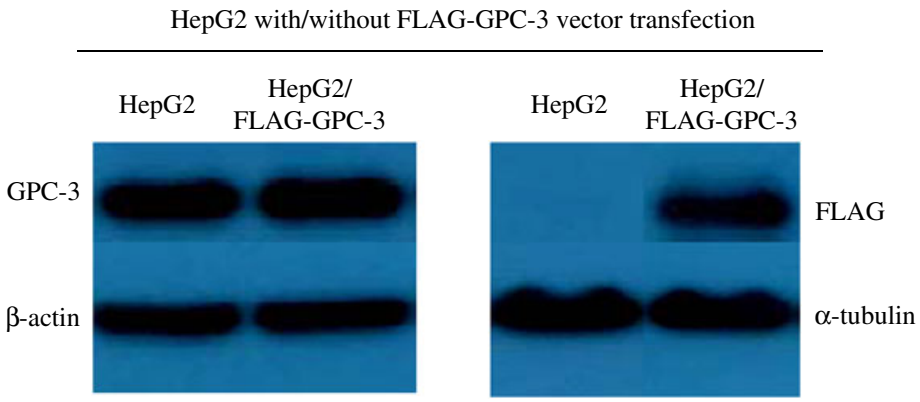


**Figure 2**. Western blottings for GPC-3 and FLAG in HepG2 cells with or without transfection with p3XFLAG-GPC-3 vector. β-Actin and α-tubulin served as a loading control. GPC-3 expression was visualized in HepG2 cells with or without the transfection; however, FLAG expression was visualized only in HepG2 cells with the transfection.



  

**Table 1.** Sequences and GPC-3-targeting capability of GPC-3-targeting peptide ligand candidates

| Peptide candidates | Sequence | GPC-3 targeting capability |
|---|---|---|
| L1 | Lys-Glu-Leu-His-Asn-Thr-Pro-Tyr-Gly-Thr-Ala-Ser-Glu-Pro-Ser-Glu-Lys-Ala-Lys-Ile | No |
| L2 | Lys-Glu-Leu-His-Asn-Thr-Pro-Tyr-Gly-Thr-Ala-Ser-Glu-Pro-Ser-Glu-Lys-Ala | No |
| L3 | Arg-Cys-Ser-Ala-Gly-Leu-Gly-Ala-Leu-Ala-Gln-Arg-Pro-Gly-Ser-Val-Asp-Ser-Lys-Trp | No |
| L4 | Arg-Gly-Gly-Ile-Gly-Ala-Gly-Leu-Gly-Gly-Gly-Leu-Cys-Arg-Arg | No |
| L5 | Arg-Leu-Asn-Val-Gly-Gly-Thr-Tyr-Phe-Leu-Thr-Thr-Arg-Gln | Strong targeting |
| L6 | Lys-Gln-Phe-Ile-Glu-Asn-Arg-Ser-Glu-Phe-Ala-Gln-Lys-Leu | No |
| L7 | Lys-Ser-Ile-Leu-Leu-Thr-Glu-Gln-Ala-Leu-Ala-Lys-Ala | No |

### Blocking Study with L5 Peptide Without FITC Labeling

Binding of FITC-labeled L5 peptide to GPC-3-expressing HepG2 cells was markedly decreased with 10 μM of blocking L5 peptide (non-FITC labeled) (Figure 6).

## Discussion

In this study, we found peptide ligands specifically targeting GPC-3 by proteomic technique using MALDI-TOF MS analysis and compared their affinity with GPC-3 in several GPC-3-expressing cell lines. L5 peptide ligand (Arg-Leu-Asn-Val-Gly-Gly-Thr-Tyr-Phe-Leu-Thr-Thr-Arg-Gln) and its short variants (Tyr-Phe-Leu-Thr-Thr-Arg-Gln) showed specific and strong affinity to the GPC-3; therefore, they might be used as a homing moiety to cancers expressing the GPC-3 for diagnostic and therapeutic purposes.

Cancer-targeting moieties having high specificity for cancer surface marker can improve diagnostic and therapeutic aspects of cancer management by carrying imaging probes or therapeutic agents to cancer lesions [15]. Specific antibodies can target malignant tumors, but the high molecular weight of antibody precludes rapid pharmacokinetics and results in slow blood clearance and poor tissue penetration [9,12]. These *in vivo* kinetic characteristics of antibody are manifested as poor tumor targeting [16]. Although the poor pharmacokinetics of antibody can be improved by engineering and modifications [17], peptides still have several advantages over antibodies [16]. Peptides are smaller than antibodies; therefore, they can distribute rapidly into target tissue by diffusion and are also cleared rapidly from the blood. These kinetic characteristics of peptide lead to high tumor levels and/or tumor-to-background ratio soon after intravenous administration [16]. Other advantages of peptide ligands are a

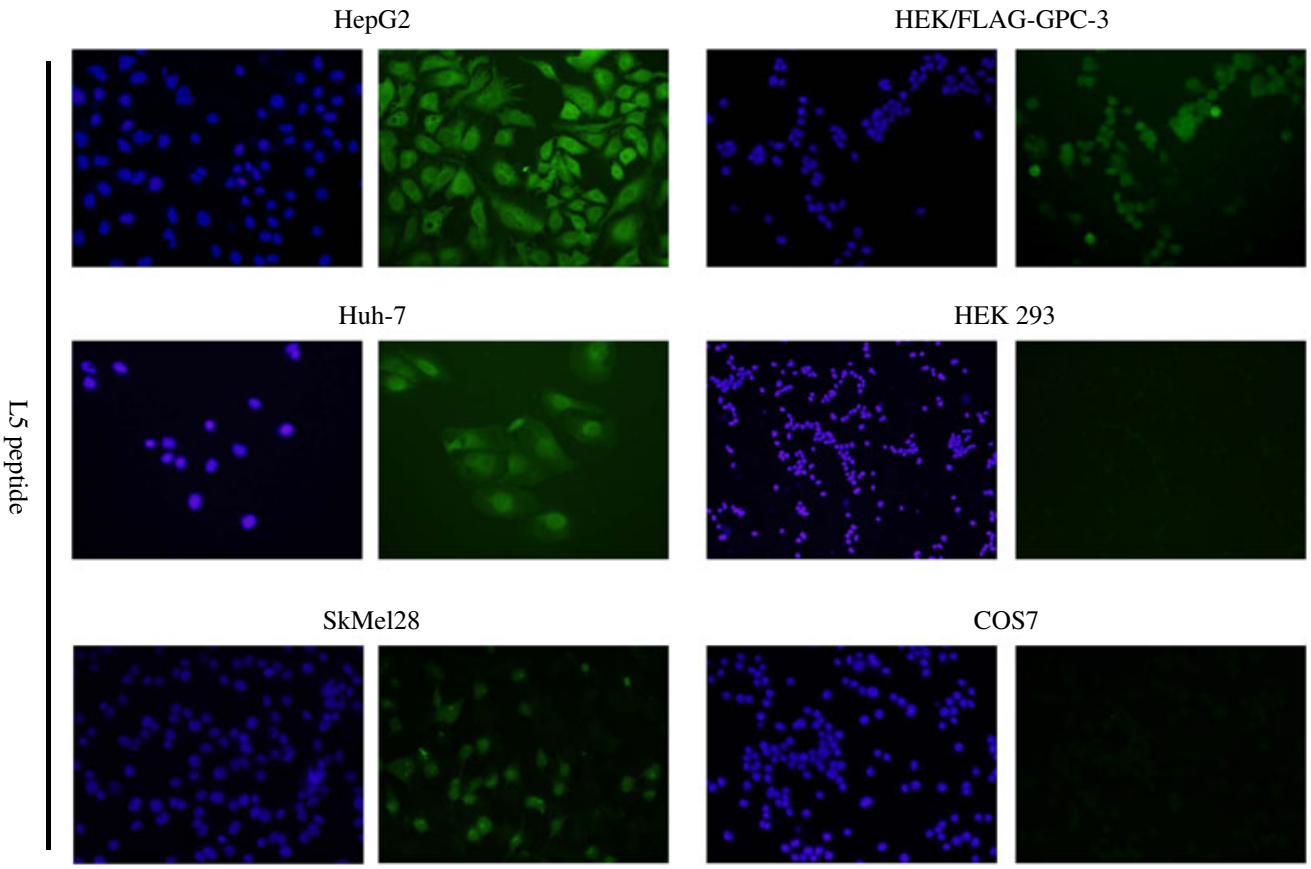


**Figure 3**. L5 peptide binding assays. HEK 293 cells with or without GPC-3 transfection, HepG2, Huh-7, SkMel28, and COS7 cells were treated with L5 peptide labeled with FITC and mounted with DAPI-containing media. Fluorescence microscopy was performed with filters for DAPI and FITC. L5 peptide showed significant binding affinity to cells with GPC-3 expression but not to cells without GPC-3 expression.



  

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**Table 2.** Sequences and GPC-3-targeting capability of five short variants from the L5 peptide

| Variants of L5 | Sequence | GPC-3-targeting capability among the variants |
|---|---|---|
| L5-1 | Arg-Leu-Asn-Val-Gly-Gly-Thr | No |
| L5-2 | Tyr-Phe-Leu-Thr-Thr-Arg-Gln | Strong |
| L5-3 | Thr-Tyr-Phe-Leu-Thr-Thr-Arg | No |
| L5-4 | Thr-Thr-Arg-Gln | Weak |
| Gly-Gly-Thr-Tyr-Phe | Gly-Gly-Thr-Tyr-Phe | Weak |

lack of immunogenicity, low cost, and ease of synthesis compared with antibody .In addition, the peptide sequences can be used to modify adenoviral tropism from hepatocytes to specific tumors by changing the native fiber shaft of the virus into targeting peptide ligands [18]. Therefore, peptides that can bind to tumor receptors are also being actively investigated for diagnostic imaging and therapeutic studies [16,19,20]. Some cancer-targeting peptides such as octreotide, which is used to target somatostatin receptors expressed on neuroendocrine tumors, are used for clinical diagnosis and treatment [16].

Here, we established HEK 293 and HepG2 cell lines that expressed GPC-3 with a FLAG epitope to facilitate immunoprecipitation with FLAG antibody. Proteins that bound GPC-3 were analyzed after protease treatment. With this method, we identified seven candidates that may have binding affinity to GPC-3 and found a novel GPC-3-targeting peptide motif (L5 peptide). L5 peptide sequence belongs to the protein–protein interaction domain called the bric-a-brac, tramtrack, and broad complex (BTB)/poxvirus zinc finger domain containing potassium channel tetramerization domain 5. The BTB/poxvirus zinc finger-domain-containing proteins constitute a diverse group of proteins participating in cell function, but the function of potassium channel tetramerization domain protein from a group of proteins containing a BTB domain is largely unknown [21].

The L5 peptide showed binding affinity to GPC-3 on HepG2, Huh-7, SkMel28, and FLAG-GPC-3-transfected HEK 293 cells, but not to COS-7 and parent HEK cells that did not express GPC-3. An unlabeled L5 peptide blocked binding of the FITC-labeled L5 peptide to GPC-3-expressing cells, indicating the specific binding of L5 peptide for GPC-3. We then designed five shorter peptide sequences based on the L5 peptide sequence and found a shorter peptide ligand with specific binding affinity to GPC-3, but weaker binding affinity compared with the L5 peptide. These results are consistent with an important role of the amino acid sequence of L5-2 in the binding to GPC-3; in addition, amino acid sequences included in the L5 peptide, but not in the L5-2 peptide, also contributed to the binding affinity for GPC-3.

Protein analysis with MS can determine the precise mass and charge of a protein, which are crucial in identification [22]. MS, such as the MALDI-TOF that was presently used, deduces the peptide sequences of the samples [23].

*In vivo* assays in animal models are also needed to confirm the usefulness of the L5 and L5-2 peptides for diagnostic and therapeutic applications for various GPC-3-expressing tumors. However, on the basis of the results of *in vitro* assays, the peptides might be a suitable carrier for specific delivery of diagnostic or therapeutic agents to the GPC-3-expressing tumors. For example, the GPC-3-targeting L5 peptide labeled with a gamma-emitting radioisotope could be used to detect HCC or melanoma lesions, and the L5 peptide labeled with a beta-emitting radioisotope or chemotherapeutic drug could be used to treat the cancers. In addition, for transductional therapeutic targeting of adenoviral

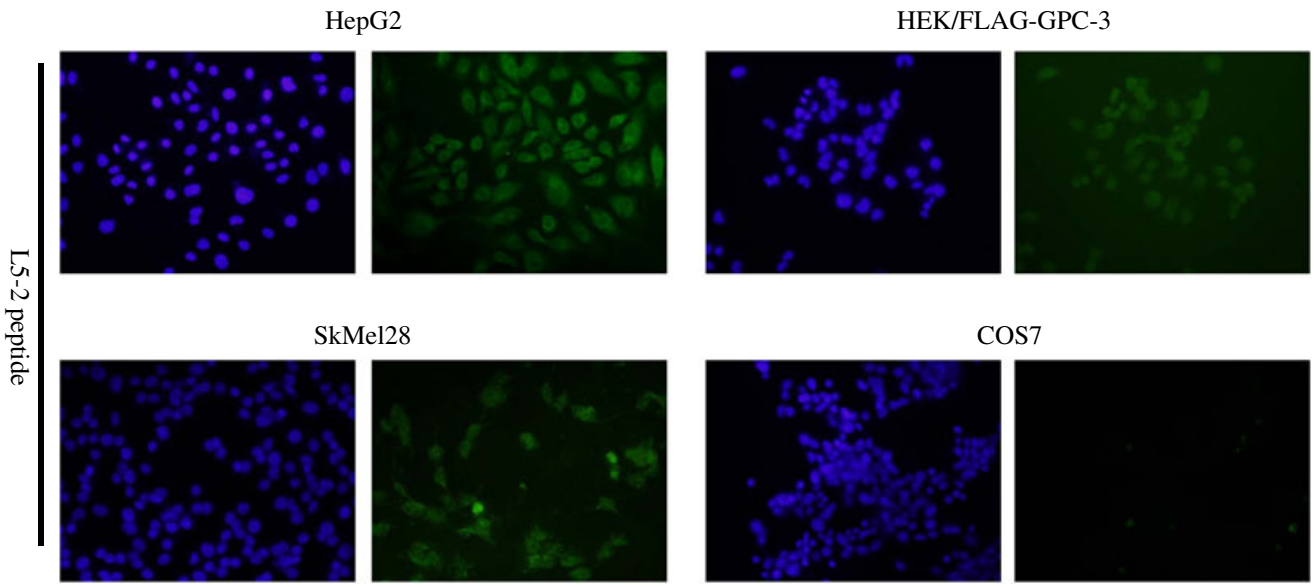


**Figure 4**. L5-2 peptide binding assays. HEK 293 cells with GPC-3 transfection, HepG2, SkMel28, and COS7 cells were treated with an L5-2 peptide labeled with FITC and mounted with DAPI-containing media. Fluorescence microscopy was performed with filters for DAPI and FITC. L5 peptide showed significant binding affinity to cells with GPC-3 expression but not to cells without GPC-3 expression.



  

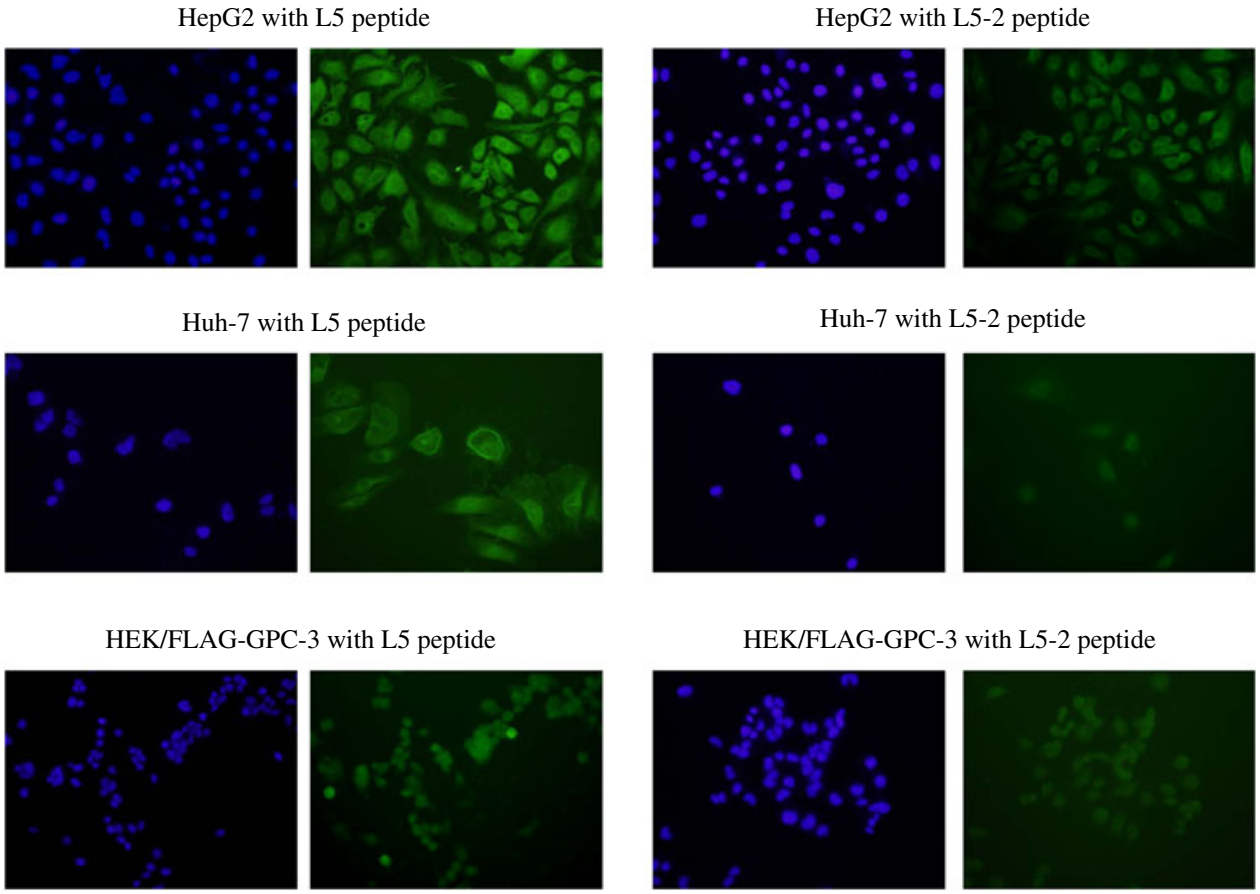


**Figure 5**. The comparison of peptide binding affinity between L5 and L5-2 peptides for GPC-3. HEK 293 cells with GPC-3 transfection, HepG2, and Huh-7 cells were treated with L5 labeled with FITC or L5-2 peptide labeled with FITC and mounted with DAPI-containing media. Fluorescence microscopy was performed with filters for DAPI and FITC. L5 peptide showed higher binding affinity to cells with GPC-3 expression than L5-2 peptide.

delivered gene(s) into GPC-3-expressing tumors, the L5 or L5-2 peptide sequence might be used for establishing an internal ligand incorporated into a fiber shaft of the therapeutic virus.

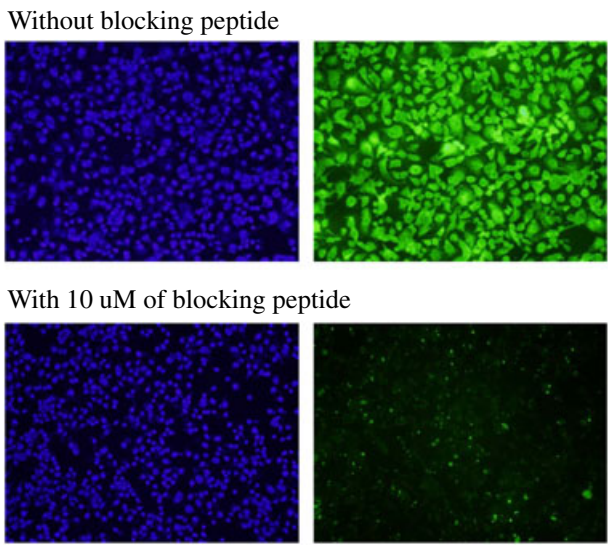


**Figure 6**. Blocking study with L5 peptide without FITC labeling. Unlabeled L5 peptide blocked the binding of L5 labeled with FITC and mounted with DAPI-containing media. Fluorescence microscopy was performed with filters for DAPI and FITC.

In conclusion, peptide sequences targeting GPC-3 were found by MS following immunoprecipitation of FLAG-GPC-3, and their specific affinity to tumor cells expressing GPC-3 was verified. These sequences may be useful as a suitable homing moiety of various carriers targeting the tumors expressing GPC-3 for diagnostic and therapeutic purposes.

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