A Novel Peptide Specifically Binding to Nasopharyngeal Carcinoma For Targeted Drug Delivery

Tong-Young Lee,1 Han-Chung Wu,1,2 Yun-Long Tseng,1 and Chin-Tarng Lin1,3

1Institute of Pathology and 2Graduate Institute of Oral Biology, College of Medicine, National Taiwan University; and 3Department of Pathology, National Taiwan University Hospital, Taipei, Taiwan

ABSTRACT

Nasopharyngeal carcinoma (NPC) is a common cancer among Chinese living in southern China, Taiwan, and Singapore. The 5-year survival rate in the early stage of NPC has been reported as high as 90 to 95% with the use of radiotherapy, but in the advanced cases, even with the use of both chemotherapy and radiotherapy, the survival rate is still <50%. To improve the survival rate, we identify a 12-mer peptide (L-peptide) specifically binding to NPC cells with a phage displayed random peptide library. The L-phase and synthetic L-peptide bound to the tumor cell surfaces of most NPC cell lines and biopsy specimens, but not normal nasal mucosal cells, and the L-peptide-linked liposomes containing fluorescent substance (L-peptide-Lipo-HPTS) were capable of binding to and translocating across plasma membranes. L-Phase-linked liposomes that carried doxorubicin (L-peptide-Lipo-Dox) caused marked cytotoxicity in NPC cells. In SCID mice bearing NPC xenografts, the L-phage specifically bound to the tumor mass, an effect that was inhibited by competition with synthetic L-peptide. In addition, the L-peptide-Lipo-Dox suppressed tumor growth better than Lipo-Dox. These results indicate that the novel L-peptide specifically binds NPC cells and is a good candidate for targeted drug delivery to NPC solid tumors.

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is one of the most common cancers among Chinese living in southern China, Taiwan, and Singapore. Although etiological factors have not yet been clearly identified, heredity and environmental factors such as the consumption of salted fish and Chinese herbs and long-term exposure to sulfuric acid vapor have been suspected to be related to NPC induction (1–3). EBV has also been associated with NPC (4). Radiotherapy, surgical removal, and chemotherapy have been used for decades with varying degrees of success. The 5-year survival rate has been improved for localized NPC cases to >90% in some hospitals, but for advanced cases, the survival rate remains below the 50% margin. A combination of radiotherapy, chemotherapy, bone marrow stem cell transplantation, and methods that use target therapy and so on are needed to better control this malady.

Most small-molecule chemotherapeutic regimes have a large volume of distribution when given i.v. (5). The result of this treatment is often a narrow therapeutic index because of a high level of toxicity in normal tissues. Through encapsulation of drugs in a macromolecular carrier such as liposomes, the volume of distribution is significantly reduced and the concentration of drug in the tumor is increased (6), resulting in a decrease in the amount and types of nonspecific toxicities and an increase in the amount of drug that can be effectively delivered to the tumor (7, 8). Liposomes containing various lipid derivatives of polyethylene glycol (PEG) have resulted in extension of half-life (9). However, they need a tumor targeting ligand to carry the liposomes to the tumor site. For solid malignancies, which comprise >90% of human cancers, antibodies recognizing tumor-specific antigens have provided only some utility for drug delivery because the immunocongenates cannot easily penetrate tumor tissue (10, 11). Therefore, the development of additional targeted technologies is highly desirable. Recently, phage-displayed peptide libraries have been used to select peptides that bind to specific receptors (12, 13) or antibodies (14, 15). Strategies for phasing cells in vitro (16, 17) or tissues in vivo (18–22) with complex phage libraries have been described to yield phages with organ- or tumor-binding specificity. Screening phage-displayed peptide libraries against specific target tissues would, consequently, seem a direct and fast method of identifying novel peptide sequences to be used for targeting of gene delivery vectors. Therefore, in this experiment, to identify a specific novel peptide (L-peptide) that could bind NPC cell surface, we used in vitro phage-displayed random peptide libraries to screen NPC cell lines that were established in our laboratory (23, 24). We then linked the L-peptide with the liposomes containing anticancer drugs for targeting NPC cells both in vitro and in vivo.

MATERIALS AND METHODS

Cell Lines and Cell Culture. We used 12 NPC cell lines containing NPC-TW 01-08, NPC-CGBM-1, HOME-1, CNE-1, and CNE-2 for this experiment. NPC-TW 01, 02, 05, 08 were derived from keratinizing squamous cancer line (WHO type I) and NPC-TW 03, 04, 06, 07 from undifferentiated carcinoma (WHO type II). All lines were established in our laboratory (23, 24). For comparison, we received four cell lines established from other laboratories, NPC-CGBM-1 was established by Dr. Shuen-Kuei Liao of Chang-Gung University (Linkou, Taiwan) from bone marrow metastatic NPC tumor tissue. NPC-HONE-1, CNE-1, and CNE-2 were established in mainland China. All cell lines were grown in DMEM containing 5% FCS (Life Technologies, Inc., Carlsbad, CA). In addition, we also used three normal nasal mucosal (NNM) epithelia, which were cultured from the nasal polyposis that were obtained from polypectomy, and a fibroblast primary culture cell, which was developed from the human abdominal dermis during cesarean section. NNM cells and fibroblasts were grown in 20% FCS. For comparison, we also included five other cancer cell lines: three oral cancer cell lines (Cal-27, Cal 9–22, and SAS); one laryngeal carcinoma (HEp-2); and one uterine cervical cancer line (CaSki). All of these lines were obtained from American Type Culture Collection and were cultured in DMEM plus 5% FCS, as was done to the NPC lines.

Phage-Display Biopanning Procedures. The Ph.D.-12 phage displayed random peptide library kit was purchased from New England Biolabs, Inc. (Beverly, MA). Biopanning procedures were done according to the manufacturer’s directions with some modifications. Briefly, NPC-TW 04 cells and NNM cells were plated in a Petri dish and incubated at 37°C for overnight. Before biopanning, the growth medium was removed and washed twice with the manufacturer’s directions with some modifications. Briefly, NPC-TW 04 cells and NNM cells were plated in a Petri dish and incubated at 37°C for overnight. Before biopanning, the growth medium was removed and washed twice with the manufacturer’s directions with some modifications. Briefly, NPC-TW 04 cells and NNM cells were plated in a Petri dish and incubated at 37°C for overnight. Before biopanning, the growth medium was removed and washed twice with serum-free DMEM and then blocked with blocking buffer. Then, 5 × 10^5 plaque-forming units (pfu) of UV-treated inactive control phage (insertless phage) were used to react with confluent cultures of NNM cells for blocking nonspecific binding. Then, the culture medium of NNM cells was added with 5 × 10^10 pfu of phage peptide library Ph.D.-12 and incubated for 1 hour at 37°C. The culture medium of NNM cells was added with 5 × 10^10 pfu of phage peptide library Ph.D.-12 and incubated for 1 hour at 37°C.
4°C. After being subtracted with NNM cells three times, the unbound phages were used to react with NPC-TW 04 cells. Bound phages were recovered by lysis buffer on ice. The bound phages were amplified and titered in Escherichia coli ER2738 culture (New England BioLabs, Inc.). Recovered phages were subjected to four more rounds of biopanning with NPC-TW 04 cells. The fifth round phage eluate was subjected on LB/isopropyl-1-thio-β-D-galactopyranoside/5-bromo-4-chloro-3-indoly1-β-D-galactopyranoside plates and amplified.

Identification of Phage Clones by ELISA. The 96-well ELISA plates (Falcon, Oxnard, CA) were seeded with NPC-TW 04 cells and NNM cells separately. Cells were washed with serum-free DMEM and then blocked with blocking buffer for 30 minutes at 4°C. Then, 10⁴ individual phage particles were added to the cell-coated plates and incubated at 4°C for 2 hours, followed by incubation with 1:1000-diluted horseradish peroxidase-conjugated mouse anti-M13 monoclonal antibody (Amersham Biosciences, Uppsala, Sweden) and then with the peroxidase substrate O-phenylenediamine dihydrochloride (OPD; Sigma, St. Louis, MO). The reaction was read with a microplate reader at 490 nm.

The selected phage clones were additionally analyzed by DNA sequencing. Phage DNA was isolated according to manufacturer’s directions. The DNA sequences were determined according to the dideoxynucleotide chain termination method with an automated DNA sequencer (ABI PRISM 377; Perkin-Elmer, CA). The phage-displayed peptide sequences were translated and aligned with the Genetics Computer Group program.

Identification of the Selected Phage Clones’ Specific Binding to NPC Cells by Immunohistochemistry. All cancer cell lines, NNM, and fibroblasts were plated and grown to ~80% confluence on coverslips. The coverslips were incubated in the blocking buffer, treated with 1% hydrogen peroxide plus 0.1% Na₂O₂ to block endogenous peroxidase activity, and then incubated with 10⁵ pfu of each selected phage at 4°C for 1 hour. The coverslips were then incubated with horseradish peroxidase-labeled mouse anti-M13 monoclonal antibody at 4°C for 1 hour and fixed with 3% formaldehyde for 10 minutes and subjected to peroxidase substrate incubation and mounted with 50% glycerol in PBS as described previously (24).

Peptide Synthesis and Labeling. The 1-peptide (RLLDTNRPPLPY; translated from the selected I-phage DNA sequence) and nonspecific control peptide (SHRLHTMPSIE) were synthesized and purified by Advanced ChemTech (Louisville, KY). Conjugation of biotin–1-peptide and biotin-control peptide were also produced by the same company.

Peptide Competitive Inhibition Assay for Characterization of Specific Phage Clones. NPC cells were cultured in a 12-well plate overnight and then preincubated with 10⁶ pfu of UV-treated inactive control phage in blocking solution to block nonspecific binding at 4°C for 30 minutes. Synthetic peptides (2 to ~20,000 ng/mL) were diluted in PBS and incubated with cells at 4°C for 1 hour, then incubated with 10⁵ pfu of selected phage clones at 4°C for 1 hour. The bound phages were recovered by 0.5 mL of lysis buffer on ice and titrated in ER2738 culture.

Analysis of the Binding Activity of Biotin-labeled Peptide with NPC Cells. Phage Clones. The NPC and other cell lines grown on coverslips were prepared as above. The coverslips were incubated with biotin-1-peptide or biotin-control-peptide at 4°C for 1 hour. Then, they were incubated with goat anti-biotin antibody, biotinylated horse antigen antibody, and avidin-biotin-peroxidase complex reagent (ABC kit; Vector, Burlingame, CA), fixed, and then incubated with peroxidase substrate, as mentioned above.

Preparation of 1-PEPTIDE–LIPOSOME CONTAINING DOXORUBICIN OR 8-HYDROXYPYRINE-1,3,6-TRISULFONIC ACID (HPTS). The procedures for preparation of 1-PEPTIDE–liposome containing doxorubicin or HPTS were adopted from the methods published in one of our previous reports (25). Briefly, 1-PEPTIDE was coupled to NHS-PEG-DSP (N-hydroxysuccinimidem-carboxylPEG (Mr, 3400)-derived diestearoylphosphatidylethanolamine (NOF Corporation, Tokyo, Japan)) at a 1:1.5 molar ratio. This coupling was done with the free unique amine group in the NH₂ terminus of the peptide to produce peptide-PEG-DSP. The reaction was completed and confirmed by quantitation of the remaining amino groups. The amino groups were measured with trinitrobenzenesulfonate reagent (26).

Liposomes composed of diestearoylphosphatidylcholine, cholesterol, and PEG-DSP were hydrated at 55°C in ammonium sulfate solution (250 mmol/L (NH₄)₂SO₄ ph 5.0) and 530 mOs] and extruded through polycarbonate membrane filters (Costar, Cambridge, MA) of 0.1- and 0.05-μm pore size with high-pressure extrusion equipment (Lipex Biomembranes, Vancouver, British Columbia, Canada) at 60°C, and doxorubicin was encapsulated in the liposomes by a remote loading method at a concentration of 1 mg of doxorubicin per 10 μmol phospholipid. The final concentration of liposomes was determined by phosphate assay. After adding 1 mL of acidic isopropanol (81 mmol/L HCl) to 0.2 mL of diluted drug-loaded liposomes, the amount of doxorubicin trapped inside the liposomes was determined with a spectrofluorometer (Hitachi F-4500; Hitachi, Ltd., Tokyo, Japan) with 470 nm as excitation wavelength and 582 nm as emission wavelength. Vesicle sizes were measured by dynamic laser scattering with a submicron particle analyzer (model N4 plus; Coulter Electronics, Hialeah, FL). After preparation, the liposomes contained 110 to 130 μg doxorubicin per μmol phospholipid and had a particle size ranging from 65 to 75 nm in diameter. For encapsulation of the fluorescent substance HPTS (trisodium salt), small unilamellar vesicles were prepared by reverse-phase evaporation. At a molar ratio of 2:1, EPC (Egg phosphatidylcholine) and cholesterol were extruded repeatedly through poly-carbonate membrane filters of pore sizes of 0.1 and 0.05 μm sequentially. A solution of liposomes encapsulating 30 mmol/L HPTS was prepared in distilled water. The same method was used to prepare a control peptide to replace the 1-PEPTIDE and couple to NHS-PEG-DSP for comparison. Peptidyl-PEG-DSP was transferred to pre-formed liposomes after co-incubation at temperature above the transition temperature of lipid bilayer (27). There were 300 to 500 peptide molecules per liposome, as computed as described previously (28).

Verification of Binding Specificity of 1-PEPTIDE–conjugated Liposome Containing HPTS (1-PEPTIDE–Lipo-HPTS) to NPC Cells. NPC cells were incubated at 4°C or 37°C for 90 minutes with HPTS-encapsulated 1-PEPTIDE liposomes (1-PEPTIDE–Lipo-HPTS), control peptide liposome (control peptide–Lipo-HPTS), or liposomes (Lipo-HPTS) in growth medium respectively (25). After treatment, cells were counterstained with Hoechst 33258 (Molecular Probes, OR). Unrelated liposomes were removed by washing with cold PBS and mounted with mounting solution (Vector). The cells were then examined under a Leica Universal Microscope.

Animal Model for Targeting Study. SCID mice were obtained from the Animal Center, College of Medicine, National Taiwan University. At the time of the experiments, the mice were between 4 and 6 weeks old. The mice, ranging in weight from 18 to 22 g, received s.c. injections at the flank with 1 × 10⁶ NPC-TW 01 cells. After 3 weeks, they were injected with 10⁹ pfu of 1-PEPTIDE or control phage through the tail vein (19). Eight minutes after injection, the mice were anesthetized with diethyl ether. The mice were perfused with 50 mL of PBS, and the organs such as lung, heart, brain, and tumor nodules were removed, weighed, and washed with PBS buffer (protease inhibitor mixture tablet; Roche, Penzberg, Germany) on ice. The organ and tumor samples were homogenized, and the phage particles were rescued by RE2738 bacterial. The phages were titrated 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. In peptide competitive inhibition experiments, 10⁴ pfu of 1-PEPTIDE were co-injected with 100 μg of 1-PEPTIDE. We also used unrelated individual phage clone R3-17 (TLATTASPDSDAQ) as another control.

Distribution of Phage Particle Binding in Oranss and Xenografts. The SCID mice bearing NPC-TW 01-derived tumor received injections of 10⁹ pfu of 1-PEPTIDE or 1-PEPTIDE plus 1-PEPTIDE or control phage through the tail vein. Eight minutes after injection, the anesthetized mice were then perfused through the heart with 50 mL of PBS to wash unbound phage. The organs and tumor nodules were fixed in Bouin’s solution ~2 hours (19). After fixation, the samples were embedded in paraffin blocks. The paraffin sections were deparaffinized, rehydrated, and subjected to immunostaining using M13 monoclonal antibody, as mentioned above.

Animal Model for Study of Targeted NPC Therapy. After transplantation of NPC cells s.c. for 10 days (tumor sizes ranging 50 to 100 mm³), 72 SCID mice bearing NPC xenografts were randomly assigned into four different experimental groups. Each group contained 18 mice and was divided into three subgroups for different treatments (group A, 1-PEPTIDE–Lipo-Dox; group B, Lipo-Dox; and group C, PBS). Each of the six mice in each subgroup was administered the drug through the tail vein. In group 1, the mice were treated with doxorubicin three times (5 mg/kg each time per week; total doxorubicin, 15 mg/kg). In group 2, the mice were treated with the same drug three times (2 mg/kg each time per week; total doxorubicin, 6 mg/kg). In group 3, the mice were treated with the drug five times (1 mg/kg each time per week; total doxorubicin, 5 mg/kg). In group 4, the mice were treated with the drug five times (0.2 mg/kg each time per week; total doxorubicin, 1 mg/kg). The mouse...
body weights and the tumor sizes were measured twice a week by pair of calipers. The tumor volumes were calculated using the equation: \( \text{volume} = \text{length} \times (\text{width})^2 \times 0.52 \). After 48 days, all mice were killed, and the tumor masses were removed and weighed. The differences in mean tumor volume were evaluated by ANOVA. In addition, the tumor nodules from each mouse and their visceral organs such as heart, liver, lung, brain, and kidney were removed and fixed for histopathological examination.

RESULTS

Identification of Specific Phage Clones Binding to Target Cell Surface and Their Binding Motifs. We screened a phage-displayed random peptide library against the NPC-TW 04 line and found that the fifth biopanning recovery rate was 40-fold above the first one. Of 44 phage clones reacted with NPC and NNM cells, 16 NPC-bound phage clones were selected by ELISA assay (data not shown). We additionally sequenced nine phage clones with higher NPC-binding activity, including clone 1-8, 1-11, 1-18, 1-19, 1-29, 1-37, 1-39, 1-41, and 1-44. Using GCG software, these nine had consistent residue Pro, whereas five clones (1-8, 1-11, 1-18, 1-37, and 1-39) were found to have consensus amino acid residues, Leu and Pro; two clones [1-29 (\( \text{L-phage} \)) and 1-44] were found to have a consensus motif of Leu-Pro-Tyr (Table 1). Then, we used immunohistochemistry to verify whether these nine candidate phage clones could specifically bind to NPC cell lines but not NNM cells or fibroblasts. The results showed that the cellular binding of those nine phage clones varied widely (data not shown). The 1-29 (\( \text{L-phage} \)) phage clone bound specifically to the tested NPC tumor cell lines, including NPC-TW 01 cells (Fig. 1A-a, arrowhead), NPC-CGBM-1 cells (Fig. 1A-b, arrowhead), other NPC lines such as NPC-TW 03, 04 (data not shown) and NPC biopsy section (Fig. 1A-d, arrowhead) but not to other cancer cell lines such as oral cancer line (Ca9-22; Fig. 1A-e), uterine cervical cancer line (CaSki; Fig. 1A-f), or the NNM cells (Fig. 1A-g). Other phage clones bound to different cell types. For example, the 1-39 phage clone also bound to oral cancer cells and laryngeal cancer cells, and the 1-41 phage clone also bound to laryngeal cancer cells and normal nasal mucosal cells. The control phage could not bind to NPC-TW 01 line (Fig. 1A-c) or biopsy specimen (Fig. 1A-h).

Verification of the Target-binding Activity of Phage-displayed Peptides Derived from \( \text{L-phage} \). When 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 the binding activity of NPC cells with the \( \text{L-phage} \) was inhibited by synthetic peptide (\( \text{l-peptide} \)) in a dose-dependent manner. Twenty micrograms per milliliter \( \text{l-peptide} \) were able to inhibit 50% of the binding activity, whereas the arbitrary control peptide had no such effect (data not shown). Furthermore, to verify that the peptide sequences displayed on the \( \text{L-phage} \)indeed interacted with the NPC-TW 04 cells, a synthetic peptide-binding assay was performed by ELISA assay, too. The data showed that the biotin-labeled \( \text{l-peptide} \) bound the NPC-TW 04 cells in a dose-dependent manner, yet the control peptide revealed no such specific binding activity (data not shown).

NPC cell lines, including NPC-TW 01–08, NPC-CGBM-1, and CNE-1 were shown by immunohistochemical staining to exhibit specific binding reaction products, indicating they were bound by biotin-\( \text{l-peptide} \) (Fig. 1B-a, NPC-TW04; Fig. 1B-b, NPC-TW07; Fig. 1B-c, NPC-CGBM-1; arrowheads), and the oral cancer cell line SAS (Fig. 1B-d), normal epithelial cells (Fig. 1B-e), and fibroblasts (Fig. 1B-f) were not bound by the peptide (Table 2). The biotin-labeled control peptide had no binding activity (data not shown).

Binding and Endocytosis Assay of \( \text{l-peptide} \)-Lipo-HPTS on NPC Cells. The binding and uptake of \( \text{l-peptide} \)-Lipo-HPTS to NPC cells were studied by immunofluorescent localization. When

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Table 1

<table>
<thead>
<tr>
<th>Phage clone</th>
<th>Phage-displayed peptide sequence *</th>
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<tr>
<td>1–19</td>
<td>PFKCTGAPVFS</td>
</tr>
<tr>
<td>1–41</td>
<td>NNSQKAPVSPF</td>
</tr>
<tr>
<td>1–37</td>
<td>QLGLFLARNHIS</td>
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<td>1–11</td>
<td>TRGMLLTKDPF</td>
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<td>1–8</td>
<td>SMSLLPFAPTTT</td>
</tr>
<tr>
<td>1–39</td>
<td>PRGWTTSMSMPS</td>
</tr>
<tr>
<td>1–18</td>
<td>LGVLSLMPGLH</td>
</tr>
<tr>
<td>1–44</td>
<td>SVLRPSANLATH</td>
</tr>
<tr>
<td>1–29 (( \text{L-phage} ))</td>
<td>ELLEVQNLQELPX</td>
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* Phage-displayed consensus amino acids are shown in boldface.
Table 2 The binding activity of biotin-labeled 1-peptide to different human cell type *

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Biotin-labeled 1-peptide</th>
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<tbody>
<tr>
<td>NPC-TW 01</td>
<td>+</td>
</tr>
<tr>
<td>NPC-TW 02</td>
<td>+</td>
</tr>
<tr>
<td>NPC-TW 03</td>
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</tr>
<tr>
<td>NPC-CNE-2</td>
<td>+</td>
</tr>
<tr>
<td>CaSkI</td>
<td>+</td>
</tr>
<tr>
<td>Hep-2</td>
<td>-</td>
</tr>
<tr>
<td>SAS</td>
<td>-</td>
</tr>
<tr>
<td>Ca 9–22</td>
<td>-</td>
</tr>
<tr>
<td>NNM-11</td>
<td>-</td>
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<td>NNM-13</td>
<td>-</td>
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<tr>
<td>NNM-14</td>
<td>-</td>
</tr>
<tr>
<td>Fibroblast</td>
<td>-</td>
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</tbody>
</table>

* (+ +), a value of reaction 40–60%; (+ +), a value of reaction 20–40%; (+), a value of reaction 5–20%; (–), a value of reaction < 5%.

NPC-TW 04 cells were incubated at 4°C with 1-peptide-Lipo-HPTS for 90 minutes, the fluorescence was localized on the cell surface (Fig. 2A). If the cells were moved to 37°C and incubated for 90 minutes, brighter punctuated fluorescent granules were seen randomly distributed in the cytoplasm surrounding the nuclei (Fig. 2C, arrowheads). When Lipo-HPTS was used to replace 1-peptide–Lipo-HPTS at 4°C, no specific fluorescence could be detected either inside or on NPC cell surfaces (Fig. 2B). However, if the cells were moved to 37°C and incubated for 90 minutes, a few weaker punctuated fluorescent granules were also seen in some NPC cells (Fig. 2D, arrowhead). Likewise, if control peptide-Lipo-HPTS was used, the results were similar to the application of Lipo-HPTS (data not shown). The regular light microscopic pictures corresponding to the fluorescent microscopic pictures in Fig. 2A–D were shown in Fig. 2E–H.

Animal Model for Study of 1-Peptide–targeted Therapy. When 10^8 pfu of 1-phages were injected through the tail vein into SCID mice bearing NPC xenografts, the 1-phage were found in tumor masses (Fig. 3A, column 4) at concentration 4.3 to 11.7-fold higher than nontumor organs, including the brain (Fig. 3A, column 1), lung (Fig. 3A, column 2; Fig. 3B, column 2), and heart (Fig. 3A, column 3); in addition, another unrelated phage (R3-17; data not shown) and control phage did not show any specific targeting to tumor tissues (Fig. 3B, columns 5, 6). The specificity of 1-phage binding to the tumor was confirmed by the ligand inhibition experiment, in which co-injection of a synthetic-free 1-peptide with the 1-phage markedly inhibited 1-phage recovery from tumor mass (Fig. 3B, columns 3, 4). One hundred micrograms of 1-peptide inhibited 87.3% of 1-phage binding to NPC tumor mass.

1-Phage homing to tumors was also studied by immunostaining. SCID mice bearing NPC xenografts received injections of 1-phage, and subsequently, xenografts and several organs were removed and fixed for localization of the phage-binding site. The 1-phage was localized in the tumor nests (Fig. 3C-b, arrows) but not in the stroma. At a higher magnification, the anti-phage immunoreactivity was seen on the plasma membrane (Fig. 3C-c, short arrow) with some diffusion in the surrounding cytoplasm of tumor cells (Fig. 3C-c, long arrow), whereas the central region of the tumor nests revealed less immunoreactivity (Fig. 3, C-b and C-c, arrowheads). There was no reaction product on normal organs, such as lung tissue (Fig. 3C-d), or on control phage-treated NPC xenografts (Fig. 3C-f). The specific binding of 1-phage with NPC cells was also inhibited by the synthetic 1-peptide (Fig. 3C-e). The H&E staining of xenograft (Fig. 3C-a) showed a keratinizing squamous carcinoma.

Animal Model for Study of 1-Peptide–targeted Therapy. When 1-peptide-Lipo-Dox conjugates were used to treat mice bearing NPC xenografts, the group of tumor-bearing mice that received the L-peptide-Lipo-Dox (1 mg/kg/dose: group 3A) were found to have significantly smaller-sized tumors than the respective Lipo-Dox and PBS groups and normal body weights (group 3B and 3C; P < 0.001; Fig. 4). Treated mice in group 3A were found to have markedly suppressed tumor sizes before day 30. By days 33 to 40, the tumor sizes had slightly increased, but they remained approximately the
same sizes until day 48. During the first 26 days, the treated mice in group 3B were found to have slightly increased tumor sizes, which were larger than those in group 3A, but from days 30 to 48, tumor sizes in group 3B gradually increased to 3.1-fold the size of those in group 3A. Treated mice in the control PBS group (group 3C) were found to have tumors that gradually increased in size from day 10 to 48, and by day 48, their group 3C’s tumors had become 8.4-fold the size of those in group 3A (Fig. 4A). The xenografts also showed more apoptotic cells in group 3A (data not shown). In groups 4A and 4B, both treated with 0.2 mg/kg/dose, the tumor sizes gradually increased from day 10 to 48, showing a similar growth rate as the control PBS-treated mice (group 4C; data not shown). Group 2A, those treated with 2 mg/kg/dose/week, had similar results as those in group 3A (1 mg/kg/dose/week), although group 2 mice were generally found...
to have slightly decreased body weights. When comparing group 2A with 2B, 2A was found to have slightly smaller tumor sizes than 2B, although the body weight of the mice in 2B remained lower than those in group 2A. If we applied 5 mg/kg/dose L-1-1-peptide-Lipo-Dox (group 1A) and Lipo-Dox (group 1B), most of the treated mice died after the third dose treatment (data not shown).

**DISCUSSION**

In this study, we used the phage displayed technique to identify the phage clones that could specifically bind to the cell surface of the NPC-TW 04 cell line. Taken together, our results suggest that the L-phage displayed peptide, not other parts of this phage, bound to the NPC cell surface and that the binding motif for the L-phage may be L-P-Y.

Although L-phage could specifically bind to NPC cells, phage particles have been found to have some limitations as diagnostic agents. So, we synthesized the same sequence peptide (L-peptide) to mimic phage-binding activity. The binding activity of L-peptide to the unfixed tumor cells was confirmed by biotin-labeled L-peptide in vitro using modified immunohistochemistry, indicating that L-peptide could bind to NPC cell lines (Fig. 1B; Table 2). However, if a NPC cell line was first fixed with formaldehyde and then incubated with biotin-labeled L-peptides, no L-peptide–binding activity was shown, probably due to the conformation of L-peptide–binding motif on the tumor cells, which has been altered by formaldehyde fixation. Similarly, biotin-labeled L-peptide could not bind to the tumor cells in the formaldehyde-fixed, paraffin-embedded NPC biopsy specimen sections (data not shown). These results suggest that the phage-displayed L-peptide is a good candidate as a target guider for NPC chemotherapy. To prove that liposome targeting is caused by the L-peptide, in one of our unpublished data, we have used an excess of L-peptide to compete with L-peptide-Lipo-HPTS binding with NPC cells at 37°C for 90 minutes. Results showed no specific fluorescent staining in the cells (a finding similar to Fig. 2B), indicating that the L-peptide is an important guider for liposome binding.

Several articles, with findings similar to ours (Fig. 3, A and B), have also suggested that a very small fraction of phage particles could in animal models bind nonspecifically to certain normal tissues (18, 29). When we used a low dosage of L-peptide to inhibit the L-phage binding, the results of blocking effect were clearly better than those found in a previous report in which a large quantity of competing peptide was needed to block their phage binding (20), suggesting that our L-phage is capable of binding specifically to its target. In addition, the modified immunohistochemical localization of L-phage done in our study also demonstrated that L-phage was only localized in tumor masses but not in brain, lung, and heart (Fig. 3C), additionally supporting our conclusion that L-phage can specifically bind to xenograft tumor cells but not normal tissue and cells. It is well known that most tumor vessels have an irregular diameter and an abnormal branching pattern and have thin, leaky walls (30); some reports even suggest that tumor vessels lack endothelial cells, pericytes, or basement membrane (31, 32). This phenomenon could explain how the L-phages could penetrate the vessel walls after i.v. injection and bind to the marginal zone of tumor cells in each tumor nest, whereas the central regions of the tumor nests revealed less L-phage binding.

Previously, several methods had been developed to make liposomes capable of targeting specific cells, the most common approach was to use immunoliposomes. Most of these liposomes have good targeting ability in vitro or in vivo, but data on their therapeutic efficacy have been either lacking or negative (9, 33). Two major factors may affect the systemic therapeutic effect: first, liposomes are removed nonspecifically, primarily by the reticuloendothelial system; and second, the leakage of doxorubicin from liposomes, which are composed of phospholipid with low phase-transition temperature, increases in biological fluids and results in loss of drug targeting (33). In the present study, we used neutral and sterically stabilized liposomes, also known as PEGylated liposomes. This formulation has been shown previously to have a much longer circulation time in blood than non-PEGylated liposomes (9). The use of PEG as a protector of immunoliposomes could result in steric hindrance for ligand-receptor interaction (28, 34). To solve this problem, we immobilized the L-peptide on the liposome surface using a long spacer (NH2-PEG3400-DSPE) directly on the termini of some PEG molecules (28). We also loaded liposomes with doxorubicin using an ammonium sulfate gradient (35). The loading efficiency was >95%, which was quite suitable for additional pharmaceutical industrial production because no additional separation step was needed to separate the liposomal drug from the free drug (25).

However, long-time exposure of Lipo-HPTS to NPC cells also revealed binding and entrapment into the cytoplasm in very low quantities, possibly explaining the fact that the Lipo-Dox, itself, could also inhibit tumor growth to some extent. A different study in our unpublished data, we had also performed cytotoxic 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide assay with doxorubicin (500 ng/mL) to replace HPTS and found that L-peptide-Lipo-Dox was more toxic to NPC cells than Lipo-Dox, with NPC cell survival rates being 54 and 67%, respectively, whereas the free doxorubicin treatment resulted in only a 42% survival rate after 48 hours of treatment. These survival rates suggest that L-peptide can guide the Lipo-Dox binding to NPC cells, whereas Lipo-Dox, by itself, cannot bind specifically to the tumor cell, although it can attach nonspecifically to the tumor cell surface, but the free doxorubicin can diffuse directly into the tumor and other normal cells, resulting in higher cytotoxic effect to both tumor and normal cells.

In the present experiment, when we used L-peptide-Lipo-Dox as the therapeutic drug, L-peptide-Lipo-Dox showed an improvement of therapeutic efficacy in SCID mice bearing NPC xenograft models in group 3A with no specific side effect to the animals (Fig. 4C). Comparing the effect of L-peptide-Lipo-Dox and Lipo-Dox on the tumor growth, we found a marked difference in tumor size and tumor weight, especially in treatment on group 3A after 48 days (P < 0.001), although the tumor growth was also partially inhibited by Lipo-Dox treatment (group 3B; Fig. 4). This partial inhibition may be due to the accumulation of nonspecific attachment of liposomes in tumor tissue through a leaky microvasculature and impaired lymphatic supporting the tumor area (36–38). The principal pathway for the movement of liposomes into the tumor interstitium is via extravasation through the discontinuous endothelium of the tumor microvasculature. Transcytosis is thought to be a relatively minor pathway. Once in the tumors, nontargeted liposomes are localized in the interstitium surrounding the tumor cells (37).

Our experiments also identified the range of doses that are effective without causing noticeable side effects. Our medium dose (1 mg/kg/week) produced clear benefits without any reductions in body weight compared with untreated controls. When we doubled this dose, to 2 mg/kg/week, the tumor size was moderately reduced and smaller than at the medium dose, but there were more side effects, particularly a decrease in body weight. When the mice were treated with an even higher dose (group 1), they died after three doses. A much lower dose of 0.2 mg/kg/dose treatment resulted in larger tumor sizes than all other doses and a time course of tumor growth that resembled untreated animals.

During the whole course of experiments, the unchanged or slightly increased body weight of mice treated with L-peptide–guided Lipo-Dox (1 mg/kg/dose; group 3A) indicated that they experienced min-
inal side effect. In fact, all of these mice maintained normal body weight (Fig. 4C), and their organs have normal weight and histopathological configuration (data not shown). In our unpublished data, we have also observed the mice treated with 1 mg/kg/week for 68 days. At the end of 68 days, the tumor size was decreased, even smaller than the original size. However, all of our control mice treated with PBS were expired in between day 55 and 70. The potential of L-peptide as a shuttle is strengthened by the fact that it is nontoxic, has no histologic evidence of organotoxicity (data not shown), is nonimmunogenic in mice, and can concentrate the liposomes in tumor cells. Other potential uses of L-peptide may include tumor diagnosis, imaging, or radiolabeling.

In conclusion, using phage-displayed random peptide libraries to screen NPC cell surface, we have identified a novel L-peptide, which could specifically bind to NPC cell surface both in vitro and in vivo. The peptides could be linked to the liposomes containing doxorubicin, which could bind specifically to the NPC tumor cell surfaces, resulting in the killing of NPC tumor cells in vivo without systemic side effects. L-Peptide appears to be an excellent guide for drug delivery to NPC cells, and it allows us to reduce the drug dose to one fifth of previously used (5 mg/kg) drug treatment. L-Peptide clearly has strong clinical potential as a drug delivery guider in the treatment of NPC.

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A Novel Peptide Specifically Binding to Nasopharyngeal Carcinoma For Targeted Drug Delivery

Tong-Young Lee, Han-Chung Wu, Yun-Long Tseng, et al.


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