Targeting Bladder Tumor Cells In vivo and in the Urine with a Peptide Identified by Phage Display

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Abstract
Bladder cancer is one of the most common tumors of the genitourinary tract. Here, we use phage display to identify a peptide that targets bladder tumor cells. A phage library containing random peptides was screened for binding to cells from human bladder tumor xenografts. Phage clones were further selected for binding to a bladder tumor cell line in culture. Six clones displaying the consensus sequence CXNXDXR3-C showed selective binding to cells from primary human bladder cancer tissue. Of these, the CSNRDARRC sequence was selected for further study as a synthetic peptide. Fluorescein-conjugated CSNRDARRC peptide selectively bound to frozen sections of human bladder tumor tissue, whereas only negligible binding to normal bladder tissue was observed. When the fluorescent peptide was introduced into the bladder lumen, in a carcinogen-induced rat tumor model, it selectively bound to tumor epithelium. Moreover, when the peptide was intravenously injected into the tail vein, it homed to the bladder tumor but was not detectable in normal bladder and control organs. Next, we examined whether the peptide can detect tumor cells in urine. The fluorescent peptide bound to cultured bladder tumor cells but not to other types of tumor cell lines. Moreover, it bound to urinary cells of patients with bladder cancer, while showing little binding to urinary cells of patients with inflammation or healthy individuals. The CSNRDARRC peptide may be useful as a targeting moiety for selective delivery of therapeutics and as a diagnostic probe for the detection of bladder cancer. (Mol Cancer Res 2007;5(1):11–19)

Received 3/14/06; revised 9/15/06; accepted 10/25/06.

Grant support: National Cancer Institute grants CA82271 and CA115010 and Center Support grant CA 30199 (E. Ruoslahti), Kyungpook National University Fund 2003, Regional Technology Innovation Program of the Ministry of Commerce, Industry and Energy, Korea grant RTB04-01-01, and Brain Korea 21 Project in 2006. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://molcanresearch.aacrjournals.org/).

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Introduction
Bladder cancer is one of the most common malignancies in developed countries, ranking as the sixth most frequent neoplasm (1). Although ~70% of bladder tumor patients have a superficial, noninvasive cancer, they are at high risk for recurrence, and a small number of patients will have their tumors progress and become invasive (2). Transurethral resection is a sufficient therapy for most superficial bladder tumors. Sometimes, intravesical chemotherapy and immunotherapy are added. Bacillus Calmette-Guerin still remains as an effective intravesical treatment but has local toxicity (e.g., cystitis; ref. 3). For advanced and metastatic tumors, combination chemotherapy of various drugs produce symptom palliation and median survival in the range of 13 to 18 months (4). In this regard, patients with superficial bladder cancer are regularly monitored for tumor recurrence and progression. Although cystoscopy is the most efficient method, it causes significant patient discomfort. Cytology is a standard noninvasive method but has poor sensitivity (5, 6). The limitations of current diagnosis and chemotherapy call for the development of more efficient noninvasive tests for the early detection of cancer and a targeted drug delivery in the management of bladder cancer.

Targeted delivery of drugs to tumor is the central challenge for improving existing cancer therapies. Directing therapeutic agents, such as chemotherapeutic or radiotherapeutic drugs or viruses for gene therapy, to tumors is likely to enhance the drug efficacy and decrease side effects (7). In this purpose, phage-displayed peptide libraries have been used to find peptides that target specific organs, tumors, or proteins (8-11). Phage libraries can have as many as 1010 different peptides (12). Peptides have several advantages over antibodies as a targeting moiety and include better organ penetration and less chance of unintended immune reaction (13). A prominent example of a targeting peptide is the three-amino-acid sequence RGD motif that homes to tumor vascular endothelial cells through binding to the α5β3 integrin, which is exclusively expressed in the angiogenic endothelial cells (14, 15). Inhibiting tumor angiogenesis using the RGD peptide or peptidomimetics is currently undergoing clinical trials.

Tumor cells have many molecular markers distinct from normal cells. For example, tumor cells have up-regulated expression of growth factor receptors and may switch the types of integrins they express, favoring ones that transmits growth signal (16, 17). In bladder cancer, the expression of epithelial growth factor receptor, uroplakin, and the α6β4 integrin are
elevated, whereas that of E-cadherin is lost (18-21). These tumor cell markers may provide potential molecular targets for tumor cell-selective peptides.

Here, we identified a peptide that targets bladder tumor cells using phage-displayed peptide libraries. The peptide homes in vivo to bladder tumor cells in rats bearing carcinogen-induced bladder cancers and selectively labels cells in the urine of bladder cancer patients.

Results
Selection of Phage that Bind to Bladder Tumor Cells
A T7 phage library containing CX7C random peptides was screened to enrich phage that selectively bind to bladder tumor cells. The tumor cells were isolated from s.c. tumor xenografts in nude mice prepared using the HT-1376 human bladder tumor cell line. Phage that bound to normal bladder cells isolated from mouse bladder tissues, normal rat kidney cells, or human umbilical vascular endothelial cells were subtracted during the biopanning procedure. After the second round of screening, the numbers of phage were enriched approximately by 1,000-fold and did not increase in further steps (Fig. 1A).

Ninety-six phage clones were picked from the enriched phage library after the second and third rounds of screening. They were evaluated for binding to cultured tumor cells over normal cells using the phage cell binding ELISA. Twenty of the 96 clones showed selective binding to HT-1376 cells compared with normal rat kidney cells and were picked up (data not shown). The DNA inserts of the phage clones were amplified by PCR, sequenced, and then translated into corresponding peptide sequences electronically. Alignment analysis of the peptide sequences using Clustal W program revealed a predominant motif, CXNXDXRX/RC, in six of those 20 clones (Table 1). Table 1 also lists a selected sample of human proteins shown by database search to contain motifs homologous to the peptides. The phage-displayed peptides may mimic one or more of these proteins in cancer cell binding.

To validate the selective binding of these six phage clones to human bladder tumor cells, we used primary tissue samples from three patients with bladder cancer (transitional cell carcinoma) and two samples of normal bladder tissue. Cell suspension was prepared from tissue samples and incubated with each phage clone. Epithelial cells from tumor or normal cell suspension were isolated using BerEP4 magnetic beads, and then cell-bound phages were eluted. Fold ratio of binding relative to nonrecombinant control phage was measured. Columns, mean of measurements with three cancer samples and two normal samples; bars, SD. Ps for each group were calculated using ANOVA test. *, P < 0.05; **, P < 0.01.

Selective Binding of the CSNRDARRC Peptide to Primary Human Bladder Tumor Tissues
We wanted to confirm that the selective binding of the CSNRDARRC phage to tumor cells was mediated by the peptide displayed on the phage. Synthetic fluorescein-conjugated CSNRDARRC peptide selectively bound to primary bladder cancer tissue in peptide overlay (Fig. 2A and B). A scrambled control peptide, CDASRRNRC, did not bind significantly to tumor tissue (Fig. 2C). In contrast, a little binding of the CSNRDARRC peptide to the adjacent normal bladder tissue was observed (Fig. 2D and E). The quantification of the fluorescence intensities in three primary bladder tumor and two normal bladder tissue specimens showed that the CSNRDARRC peptide binds much more to tumor tissue than to normal tissue (Fig. 2G). In addition, the CSNRDARRC peptide did not bind to a section of primary lung cancer (Fig. 2F), suggesting the tumor-type specificity of the CSNRDARRC peptide.

In vivo Targeting of Bladder Cancer by the CSNRDARRC Peptide in a Carcinogen-Induced Rat Tumor Model
We examined in vivo targeting of bladder cancer by the CSNRDARRC peptide in two ways: the instillation of the

![FIGURE 1.](image-url)
peptide into the bladder lumen and the injection of the peptide into the tail vein of tumor-bearing rats. Rat bladder cancer was induced by oral uptake of N-(4-hydroxybutyl) nitrosamine (BBN) for 8 weeks. Histologic analysis of rat bladders showed tumors characterized by epithelium of multiple layers and cellular proliferation (Fig. 3A and Fig. 4A). When the fluorescein-conjugated CSNRDARRC peptide was introduced into the bladder, it labeled the luminal epithelium of the tumor region but not that of normal regions in the same bladder (Fig. 3A-D) or that of normal bladders (Fig. 3E and F). The peptide colocalized in tumor tissue with cytokeratin, a well-known marker of epithelial cells (Fig. 3C and D). The quantification of the fluorescence intensities in tissue sections of three tumor-bearing and control rats showed similar results (Fig. 3G). The scrambled control peptide did not bind to the epithelium of either tumor or normal bladder (data not shown).

Next, we examined in vivo tumor homing of the CSNRDARRC peptide by injecting the fluorescein peptide through the tail vein. In previous studies, peptides that target the vascular endothelium of tumors were allowed to circulate for a short period of time (e.g., 5-10 min; refs. 8-10). We reasoned that homing of a peptide to tumor epithelial cells would require longer circulation time than that to vascular endothelium because it should diffuse and penetrate into tumor tissues. To test this expectation, tissues were collected at 2 h after injection of the peptide. Immunofluorescence analysis showed that the fluorescein-conjugated CSNRDARRC peptide homed to bladder tumor tissue and colocalized in tumor with cytokeratin (Fig. 4A-D) but not to other organs, such as lung (Fig. 4G and H), of tumor-bearing rats. It did not home to normal bladder tissue of control rats (Fig. 4E and F). The scrambled control peptide was not detected in tumor tissue (data not shown). Furthermore, the CSNRDARRC peptide did not home to a B16/F10 mouse melanoma xenograft (Fig. 4I and J), suggesting the tumor-type specificity of the peptide. In vivo homing examinations and the quantification of the fluorescence intensities in tissue sections of three tumor-bearing and control rats confirm that the CSNRDARRC peptide specifically homes to bladder tumor tissue (Fig. 4K).

Detection of Exfoliated Cells in the Urine of Bladder Cancer Patients

We first tested the binding of the fluorescein-conjugated CSNRDARRC peptide to cultured cells. The CSNRDARRC peptide bound to HT-1376 bladder tumor cells (Fig. 5A), whereas control peptide did not (Fig. 5B). It also bound to 5637 bladder tumor cells (Fig. 5C) and to a portion of T24 bladder tumor cells (Fig. 5D). The peptide did not bind to other type of tumor cells, including A549 lung cancer cells, HT-29 colon tumor cells, and MDA-MB231 breast tumor cells (Fig. 5E-G, respectively), or to HEK293 embryonic kidney epithelial cells (Fig. 5H).

Next, we examined whether the targeting peptide could bind to cells in the urine of bladder cancer patients. Urine samples of 16 patients with bladder cancer scheduled to undergo transurethral resection were tested in this study. As controls, patients of similar age with noncancerous diseases (two patients with benign tumors and four patients with cystitis or urinary tract infection) and 14 healthy individuals were included. The fluorescent CSNRDARRC peptide bound to exfoliated cells from urine sample of a cancer patient, whereas the scrambled control peptide did not (Fig. 6A and B). Little fluorescence by the peptide binding was detected with exfoliated cells from control urine samples of a cystitis patient and a healthy individual (Fig. 6C and D, respectively). The percentage of positive (peptide bound) cells was significant in all cancer patient urine samples: 37% to 78% (mean, 58%) in T1, 17% to 60% (mean, 43%) in T2, and 15% to 28% (mean, 23%) in T2 stage tumors (Fig. 6E). In contrast, it was low in urine samples of benign tumor (0-7%; mean, 4%) or inflammation (3-6%; mean, 4%) and almost none (0-3%; mean, 0%) in urine samples of healthy individuals (Fig. 6E). There were no significant differences in the values depending on sex or age (Supplementary Table S1). Of importance, the peptide could label cells in urine samples of two cancer patients, in which the urine cytology findings were negative (Supplementary Table S1). These findings suggest that the CSNRDARRC peptide is capable of distinguishing malignant cells from cells that exfoliate from benign tumors or inflammatory lesions as well as normal tissue.

Discussion

In this study, we took advantage of phage-displayed peptide libraries to screen for peptides specific to bladder tumor cells. Several promising peptide sequences with a consensus motif, CXNXDXRX^2/C, were obtained. Of these, we identified the CSNRDARRC peptide as a targeting peptide for bladder cancer cells. This peptide may be useful as a targeting moiety for selective delivery of therapeutics and as a diagnostic probe for the detection of bladder cancer.

Several lines of evidence document the specificity of the CSNRDARRC peptide. First, the CSNRDARRC phage bound

Table 1. Selected Peptide Sequences and Example of Human Proteins Containing Homologous Motifs

<table>
<thead>
<tr>
<th>Peptide sequence</th>
<th>Homologous motif</th>
<th>Example of human proteins containing the homologous motif</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSNRDARRC</td>
<td>SIRDARR</td>
<td>Siglec-6</td>
<td>O43699</td>
</tr>
<tr>
<td>CANKDBVRC</td>
<td>NKKVRR</td>
<td>B-lymphocyte cell adhesion molecule</td>
<td>P20273</td>
</tr>
<tr>
<td>CANLDTRRC</td>
<td>LDTRTR</td>
<td>Multiple epidermal growth factor-like domains 1</td>
<td>Q9NYQ9</td>
</tr>
<tr>
<td>CPNQDSRC</td>
<td>QDSRR</td>
<td>Podocadherin beta 15 precursor</td>
<td>Q9Y5E8</td>
</tr>
<tr>
<td>CPNQDERNC</td>
<td>DERN</td>
<td>Tumor necrosis factor ligand superfamily member 5 (CD40 ligand)</td>
<td>P29965</td>
</tr>
<tr>
<td>CVNNDGRC</td>
<td>VGNQDR</td>
<td>Nitogen-2 precursor</td>
<td>Q14112</td>
</tr>
</tbody>
</table>

NOTE: Peptides were analyzed using the National Center for Biotechnology Information BLAST search against the SWISSPROT database, using the option for short nearly exact matches, to identify human proteins with homologous sequences.
to cultured HT-1376 human bladder cancer cells used in the screening and to cell suspensions prepared from primary human tumors. Second, fluorescent peptide overlay of tissue sections showed that the CSNRDARRC peptide bound to primary human bladder cancer sections but not to sections from adjacent normal bladder tissue or from other tissues. Third, the peptide selectively bound to bladder tumor epithelium when instilled into the bladder lumen of rats bearing carcinogen-induced bladder cancers. Fourth, intravenously injected CSNRDARRC peptide specifically accumulated in the tumors of rats. Finally, the peptide labeled bladder-exfoliated cells in urine samples from bladder cancer patients. These results indicate that the CSNRDARRC peptide distinguishes between bladder cancer cells and normal cells, including those from normal bladder. In addition, limited analysis of tumor cells and tumors of other lineages suggests tumor type specificity of the peptide.

Although we did not study premalignant stages of tumor development, it is significant that exfoliated cells in the urine of

![Figure 2](https://example.com/fig2.png)

**FIGURE 2.** Selective binding of the CSNRDARRC peptide to primary human bladder tumor tissues in peptide overlay. Frozen sections of primary bladder cancer (A-C), normal bladder (D-E), and lung cancer (F) tissues were incubated with 10 μmol/L solutions of fluorescein-conjugated peptides at 4°C for 1 h and then costained with 4',6-diamidino-2-phenylindole (DAPI) for observation under a fluorescence microscope. Localization of the CSNRDARRC or scrambled control peptide and 4',6-diamidino-2-phenylindole staining for nucleus (B, C, E, and F). H&E staining (A and D). Magnification, ×400 (A-C) and ×200 (D-F). G. Quantification of fluorescence intensity. The average intensities (integrated intensity per unit area) of fluorescence in tumor (n = 3) and normal tissues (n = 2) were calculated on the MetaMorph Imaging System. *, P < 0.05, ANOVA test.

![Figure 3](https://example.com/fig3.png)

**FIGURE 3.** Selective binding of the CSNRDARRC peptide to rat bladder tumors upon instillation into the bladder. A 50 μmol/L solution of fluorescein-labeled CSNRDARRC peptide was introduced into the bladder of tumor-bearing (A-D) or normal (E and F) rats and incubated for 30 min with changing body position every 10 min. After incubation, the bladder was instilled with PBS buffer to wash out unbound peptides, removed, and fixed. The CSNRDARRC peptide localization in malignant epithelium (B). Costaining for cytokeratin 18 and the merge (C and D). Note that the peptide mainly distributed at the malignant epithelium (arrows) with little binding to the normal epithelium (arrowheads). The peptide does not bind to the epithelium of normal bladder, which is outlined with cytokeratin 18 staining in (F). H&E staining (A and E). Magnification, ×200. G. Quantification of fluorescence intensity. The average intensities (integrated intensity per unit area) of fluorescence in tumor and normal tissues (n = 3) were calculated. **, P < 0.01, ANOVA test.
patients with stage T1 (early) disease were positive for CSNRDARRC binding, and that our rat studies in the BBN model indicated availability of tumor cells to CSNRDARRC peptide introduced into the lumen of the bladder. The BBN-induced tumors are very similar to human transitional bladder carcinomas and are regarded as an excellent model for clinical bladder cancer (22). In the bladder, a specialized layer consisting of glycosaminoglycan and mucin protects the urothelium against concentrated urine chemicals and microbial adhesion (23). This protective layer may become an obstacle to effective delivery of therapeutic or diagnostic agents to the bladder by intravesical application. Our study showed that when the CSNRDARRC peptide was introduced into the bladder, it specifically bound to malignantly transformed parts

FIGURE 4. In vivo homing of intravenously injected CSNRDARRC peptide to bladder tumor in rats. The fluorescein-labeled peptide (500 µL of a 1 mmol/L solution) was injected into the tail vein of bladder tumor–bearing (A-D and G-H) or normal (E and F) rats, or mice bearing a melanoma xenograft (I and J), and allowed to circulate for 2 h. The CSNRDARRC peptide localization (green) in a bladder tumor (B). Costaining for cytokeratin 18 (red) and the merge (C and D). The localization of the CSNRDARRC peptide together with cytokeratin 18 is shown for normal bladder (F), or with 4',6-diamidino-2-phenylindole in lung (H), and B16/F10 mouse melanoma (J). H&E staining (A, E, G, and I). Magnification, ×400 (A-D) and ×200 (E-J). K. Quantification of fluorescence intensity. The average intensities (integrated intensity per unit area) of fluorescence in tumor and normal tissues (n = 3) were calculated. *, P < 0.05, ANOVA test.
of the bladder epithelium. Thus, the targeting peptide identified in this study could bind to the epithelium despite the existence of the protective layer over the epithelium. A previous study showed the binding of a phage clone displaying an Ile-Ser-Gly-Leu peptide motif to the intact urothelial mucosa (24). In contrast to our tumor-specific peptide, the ISGL peptide motif did not discriminate between normal and malignant urothelial cells. Given the tumor cell selectivity of the CSNRDARRC peptide and its ability to bind to cancerous cells from the bladder lumen, this peptide may be useful in targeting intravesical treatments, such as bacillus Calmette-Guerin therapy or mitomycin C, that are effective but cause considerable toxicity (3).

Bladder tumor tissue was also accessible to the fluorescent CSNRDARRC peptide injected into the bloodstream. Within 2 h, we saw deposition of the peptide in the tumors, localizing to epithelial cells within the tumor. Similar results have been reported with other tumor cell–specific peptides, including colon cancer, head and neck cancers, and nasopharyngeal cancers (25-27). In the previous article (25), the homing of an intravenously injected fluorescent peptide to orthotopic colon tumors in mice was detectable by fluorescence colonoscopy. Our CSNRDARRC peptide produced strong fluorescence in tumors but not in the surrounding normal tissue in the BBN-induced rat bladder cancer model, suggesting that the homing of this peptide may similarly detectable in fluorescence cystoscopy. Furthermore, targeting tumor cells could complement the targeting of tumor vasculature, for which antiangiogenic agents have been extensively applied. Vascular targeting is unlikely to reach tumors that are smaller than 1 mm³ in size because small tumors can get nutrients by perfusion from neighboring blood vessels (28).

Urine cytology is the current standard method for noninvasive detection of bladder cancer. This procedure delivers high

![FIGURE 5. Binding of the CSNRDARRC peptide to cultured cells. Cultured cells, HT-1376 (A and B), 5637 (C), T24 (D), A549 (E), HT-29 (F), MDA-MB231 (G), and HEK293 (H), were incubated at 4°C for 1 h with 10 μmol/L solutions of the fluorescein-labeled CSNRDARRC (A and C-H) or scrambled control peptide (B), washed, and then stained with 4,6-diamidino-2-phenylindole for nuclear counterstaining. Images are the merge of fluorescence microscopy of peptide binding and 4,6-diamidino-2-phenylindole stain. Magnification, ×400.](image)

![FIGURE 6. Detection of bladder tumor cells in the urine with the CSNRDARRC peptide. Urinary cells were collected from urine samples of patients with bladder cancer (A and B) or cystitis (C), that are effective but cause considerable toxicity (3).](image)
specificity, but its sensitivity for low-grade carcinomas is poor, typically <20% in grade 1 tumors that are the most common type of transitional cell carcinoma of bladder (5, 6). Cystoscopy is more efficient in detecting bladder cancer than cytology, but it causes significant patient discomfort. Thus, there is a great need to improve the efficiency of urine cytology in bladder cancer diagnosis. A variety of methods, including the BTA test, NMP22 test, and ImmunoCyt/cuCyt test, have been developed to improve the overall sensitivity and specificity of detecting bladder cancer from urine samples, but represent relatively modest advances (6, 29). Our finding that the CSNRDARRC peptide labels exfoliated cells in the urine of bladder cancer patients even in early stage and low-grade tumor cases, suggests that it may be possible to detect tumor cells using this peptide, and that when done in parallel with cytology, the peptide detection may improve the sensitivity of urine cytology. Studies with larger numbers of patients will be needed to confirm this proposition.

Identification of the cancer cell molecule (receptor) that binds the CSNRDARRC peptide would allow further improvement of the peptide for potential clinical use. Homologies revealed by a protein database search between the selected peptide sequences and human proteins (Table 1) provided some initial clues regarding the possible nature of the receptor(s). For example, the CSNRDARRC sequence shows considerable homology with a SIRDARR motif in human sialic acid binding immunoglobulin-like lectin 6 (Siglec-6). Siglec-6 binds to Neu5Ac2-6GalNAc1 (sialosyl-Tn; refs. 30-32), which is a tumor-associated carbohydrate (30-32). The possibility that the CSNRDARRC peptide might mimic Siglec-6 in binding to sialosyl-Tn is being investigated.

Materials and Methods

Cells

HT-1376 human bladder transitional cell carcinoma cell line was purchased from the American Tissue Culture Collection (Manassas, VA). Cells were maintained in MEM supplemented with 10% fetal bovine serum, nonessential amino acids, and penicillin/streptomycin. Normal rat kidney epithelial cells and human umbilical vascular endothelial cells were maintained in DMEM containing 10% fetal bovine serum. A549 human lung cancer cells and HEK293 human embryonic kidney epithelial cells were maintained in RPMI 1640 containing 10% fetal bovine serum.

Animals

All animal experiments were conducted according to the guidelines of Kyungpook National University and Burnham Institute for Medical Research. To make s.c. tumor-bearing mice, 3-month-old BALB/c nu/nu female nude mice were s.c. injected with $1 \times 10^6$ HT-1376 cells in PBS; tumors usually developed within 1 month.

A carcinogen-induced rat bladder tumor model was prepared using BBN (Tokyo Kasei Kogyo Co., Tokyo, Japan). Seven-week-old female Fischer 344 rats were supplied ad libitum with tap water containing 0.95% BBN for 8 weeks and thereafter with tap water without BBN; tumors usually developed within 20 weeks after the start of BBN administration (22). BBN-induced tumors are very similar to human bladder transitional cell carcinomas and considered an excellent model of clinical bladder cancer (22).

Biopanning of a Phage Library

A phage peptide library based on T7 415-1b phage vector displaying CX-C (C, cysteine; X, any amino acid residue) were constructed in accordance to the manufacturer’s manual (Novagen, Madison, WI). The library had a diversity of $\sim 5 \times 10^8$ plaque-forming units (pfu). The DNA insert of selected phage clones were sequenced by Koma Biotech Co. (Daejeon, Korea).

Biopanning and validation of a phage library were done as previously described (33). A BALB/c nude mouse bearing a s.c. human bladder tumor was anesthetized by i.p. injection of 1.25% (w/v) avertin (15 μL/g). The tumor tissue was removed and minced with a sterile blade. Minced tissue was incubated with collagenase (0.5 mg/mL) by shaking at 37°C for 30 min. Cells were collected by centrifugation and then resuspended in DMEM containing 1% bovine serum albumin (BSA). Cell number was counted by hemocytometer. To label tumor epithelial cells, the tumor cell suspension containing 2 $\times 10^7$ cells in 1 mL of DMEM/1% BSA was incubated with 25 μL (1 $\times 10^7$ beads) of magnetic beads conjugated with BerEP4 anti-human epithelial cell adhesion molecule antibody (Dyna Biotech, Brown Deer, WI) at 4°C for 30 min. A normal cell suspension consisting of 5 $\times 10^5$ cells isolated from normal mouse bladder tissues, 1 $\times 10^7$ normal rat kidney cells, and 5 $\times 10^5$ human umbilical vascular endothelial cells was combined with the tumor cell suspension. Normal bladder cells and normal rat kidney cells were used for excluding phage that bind to normal epithelial cells. Human umbilical vascular endothelial cells were included to eliminate phages that nonspecifically bind to human cells. A total of 4 $\times 10^6$ cells were incubated with the phage library (10^12 pfu) at 4°C overnight. After incubation, BerEP4 magnetic bead–labeled tumor epithelial cells were isolated using a magnet and washed with DMEM/1% BSA. The cell-bound phages were eluted by lysing cells in 1% NP40 on ice for 5 min and adding BL21 bacteria to the lysates. The phage output in pfu was determined by counting the number of plaques. The eluted phages were propagated in bacterial cells and used for the next round of selection.

Phage Cell Binding ELISA

Cells (3 $\times 10^8$) were plated into a well of 96-well culture dishes and grown overnight. The cell layer was blocked with DMEM/1% BSA at 37°C for 1 h and then incubated with phage (1 $\times 10^8$ pfu) for 1 h at room temperature. The cell layer was washed with PBS to remove unbound phage and incubated with horseradish peroxidase–conjugated mouse monoclonal anti-T7 antibody (1:13,000; Novagen) for 1 h at room temperature. An enzyme reaction was done for 30 min by adding Turbo-TMB substrate (Pierce, Rockford, IL) and then stopped by adding 2 N H₂SO₄ solution. The absorbance was measured at 450 nm with a microplate reader.

Phage Binding to Primary Tissues

All primary human tissues used in this study were obtained according to the guidelines of Kyungpook National University.
Hospital. Tumor tissues were obtained by the transurethral resection of the transitional cell carcinoma of the bladder. Normal bladder tissues were taken from a noncancerous region of the bladder apart from the tumor margin. Single-cell suspension was prepared by homogenizing tissues using Medimachine (DAKO, Carpinteria, CA). Cell suspension was incubated with phage (5 \times 10^{10} \text{ pfu}) in 1 mL of DMEM/1% BSA at 4°C for 2 h and then washed with DMEM/1% BSA to remove unbound phage. Epithelial cells from tumor or normal cell suspension were isolated using BerEp4 antibody–conjugated magnetic beads as described above. Phages bound to cells were eluted, and the phage output in pfu was determined by plaque assays.

**Peptide Immunofluorescence on Frozen Sections (Peptide Overlay)**

Fluorescent-conjugated peptides were synthesized by Anigen Co. (Kwangju, Korea). Primary human bladder cancer tissues were fixed in 4% paraformaldehyde for 2 h, incubated overnight in 28% sucrose/PBS for cryoprotection, embedded in ornithine carbamyl transferase medium, and then frozen. Sections in 5-μm thickness were prepared on a cryostat. The fresh frozen sections were blocked with PBS/1% BSA at room temperature for 30 min, incubated with the 10 μmol/L solution of a fluorescein-labeled peptide in PBS/1% BSA at 4°C for 1 h, and then washed with PBS. After mounting with the media containing 4',6-diamidino-2-phenylindole for nuclear counterstaining (Vector Laboratories, Burlingame, CA), tissue slides were visualized under a fluorescent microscope (Zeiss, Oberkochen, Germany). Quantification of the fluorescence intensities was done on the MetaMorph Imaging System (Universal Imaging, Downingtown, PA). H&E staining for histologic grading was done on adjacent sections by standard methods.

**In vivo Homing of a Peptide Instilled or Intravenously Injected**

Rats bearing the BBN-induced bladder tumor were anesthetized by inhalation of enflurane. To examine the localization of a peptide instilled into the bladder lumen, aliquots of the fluorescent peptide at the 50 μmol/L concentration in 200 μL PBS were introduced into the bladder using a transurethral catheter. After incubation for 30 min with changing body position, the bladder lumen was washed out with PBS, and then the bladder was removed and prepared for frozen sections as above.

To examine in vivo tumor homing of a peptide intravenously injected, aliquots of the fluorescent peptide at the 1 mmol/L concentration in 500 μL PBS were injected into the tail vein of anesthetized rats. After 2 h of circulation, rats were perfused with PBS and then with 4% paraformaldehyde. Bladder and other organs were removed, fixed, and prepared for frozen sections.

To examine the distribution of the epithelium, adjacent frozen sections were stained with rabbit anti-cytokeratin 18 antibody (Molecular Probes, Carlsbad, CA) for 1 h at room temperature. Alexa Red–conjugated donkey anti-rabbit IgG (Molecular Probes) was used as a secondary antibody at 1:200 dilutions and incubated for 1 h at room temperature. After mounting with the media containing 4',6-diamidino-2-phenylindole, tissue slides were visualized under a fluorescent microscope.

**Binding of a Fluorescent Peptide to Cultured Cells and Exfoliated Cells in the Urine**

Cells cultured on eight-chamber slides were blocked with 1% BSA for 30 min, incubated at 4°C for 1 h with 10 μmol/L solution of a fluorescein-labeled CSNRDARCR or scrambled control peptide (green) in PBS/1% BSA, and then stained with 4',6-diamidino-2-phenylindole for nuclear counterstaining (blue). For urine samples, cells were collected by centrifugation of urine samples at 1,500 rpm for 5 min. Cells were washed with PBS, blocked with PBS/1% BSA at 4°C for 30 min, and incubated with the 10 μmol/L solution of a fluorescent peptide in 1 mL PBS/1% BSA at 4°C for 1 h. After incubation, cells were washed with PBS and then applied to the ThinPrep system (Cytyc Co., Boxborough, MA). Briefly, cells were placed overnight in a vial containing PreservCyt transport medium. The vial is then placed into the ThinPrep processor, which generates negative pressure and in turn draws fluid through a filter that collects a thin, even layer of cellular material. The cellular material is then transferred to a glass slide and fixed. Slides were incubated with the mounting media containing 4',6-diamidino-2-phenylindole for nuclear staining and examined under a fluorescence microscope. Parallel slides were prepared for conventional urinary cytology using Papanicolaou staining. The percentage of positive (peptide bound) cells was calculated by dividing the number of green fluorescent cells with the number of 4',6-diamidino-2-phenylindole–stained cells counted from five microscopic fields at the \times400 magnification.

**Acknowledgments**

We thank Youngeun Chae for technical assistance.

**References**

Targeting Bladder Tumor Cells \textit{In vivo} and in the Urine with a Peptide Identified by Phage Display


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