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ORIGINAL ARTICLE

Novel peptides functionally targeting in vivo human lung cancer discovered by in vivo peptide displayed phage screening

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Abstract Discovery of the cancer-specific peptidic ligands have been emphasized for active targeting drug delivery system and non-invasive imaging. For the discovery of useful and applicable peptidic ligands, in vivo peptide-displayed phage screening has been performed in this study using a xenograft mouse model as a mimic microenvironment to tumor. To seek human lung cancer-specific peptides, M13 phage library displaying 2.9×10^9 random peptides was intravenously injected into mouse model bearing A549-derived xenograft tumor through the tail vein. Then the phages emerged from a course of four rounds of biopanning in the xenograft tumor tissue. Novel peptides were categorized into four groups according to a sequence-homology phylogenicity, and in vivo tumor-targeting capacity of these peptides was validated by whole body imaging with Cy5.5-labeled phages in various cancer types. The result revealed that novel peptides accumulated only in adenocarcinoma lung cancer cell-derived xenograft tissue. For further confirmation of the specific targeting ability, in vitro cell-binding assay and immunohistochemistry in vivo tumor tissue were performed with a selected peptide. The peptide was found to bind intensely to lung cancer cells both in vitro and in vivo, which was efficiently compromised with unlabeled phages in an in vitro competition assay. In conclusion, the peptides specifically targeting human lung cancer were discovered in this study, which is warranted to provide substantive feasibilities for drug delivery and imaging in terms of a novel targeted therapeutics and diagnostics.

Keywords Peptide · In vivo screening · Human lung cancer · Phage display

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Introduction

Lung cancer is a major cause of cancer-related death and its metastatic feature results in a failure of treatment, leading to a low survival rate (Qi et al. 2014). There are two major types categorized in lung cancer: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), which are classified into adenocarcinoma, squamous cell carcinoma and large cell carcinoma (Collins et al. 2007). Lung adenocarcinoma is the most common type of lung cancer (de Bruin et al. 2014; Jemal et al. 2011; Wu et al. 2012) and its incidence has increased in recent years (Ko et al. 2014). Adenocarcinoma consists of heterogeneous peripheral masses with increasing early metastasis (Vincent et al. 1977). Modalities such as surgery, chemotherapy

and radiation therapy or combination treatment have been commonly used for the treatment of lung cancer including adenocarcinoma (Zhu et al. 2014). However, there are limitations caused by severe side effects in surgery and radiation therapy on the local and stage of cancer (Choi et al. 2012; Guckenberger et al. 2012; Palma et al. 2012). Thus, to overcome these limitations that frequently occur in common therapeutics of lung cancer, an early diagnosis system has been recently emphasized (Liu et al. 2014). Early diagnosis of lung cancer is a critical factor for the treatment decision, and targeted therapeutics and monitoring of treatment progression are required to treat lung cancer. For early diagnosis of lung cancer, a target-specific peptide can be applied by the diagnosis system, and peptide has the advantages of rapid clearance and tumor penetration (Larimer et al. 2014).

Phage display was first described in 1985 (Smith 1985), and M13 phage-based display of randomized peptides has become the standard tool for selecting peptides in pharmaceutical biotechnologies (Du et al. 2006; Molek et al. 2011). Peptides show rapid clearance and tumor penetration, and M13 phage-expressing peptide is an advanced technique with a high binding affinity. M13 phage-based peptides were constructed with random 7- or 12-amino acid peptide by a process of biopanning (Azzazy and Highsmith 2002; Ploss et al. 2014; Sidhu 2001). In spite of many targeting therapies for lung adenocarcinoma, results have not yet been significant. Early diagnosis and monitoring in lung adenocarcinoma require the development of novel targeted molecules such as peptides. In the context, we have tried in this study to find out novel peptides targeting human lung cancer from an M13 bacteriophage display library and validate the specific targeting activity both in vitro and in vivo models.

Materials and methods

Cell culture and animal model

A549, MDA-MB-231 and Hep3B (ATCC, Manassas, VA) were cultured in F12K nutrient mixture (Kaighn's modification), RPMI and MEM, respectively (Invitrogen Corporation, Carlsbad, CA) containing 10 % FBS (Invitrogen Corporation), 100 units/mL penicillin (Invitrogen Corporation) and 100 μg/mL streptomycin (Invitrogen Corporation) at 37 °C in a humidified incubator with 5 % CO_2. A549, MDA-MB-231 and Hep3B cancer cells were implanted into 5-week-old male Balb/c nude mouse (SLC, Shizuoka, Japan) to produce xenograft models. A suspension of 5×10^5 cells in a 50 μl volume was injected subcutaneously (s.c.) into the right hind limb of the mouse. Tumors were allowed to grow until the average tumor volume reached 200–250 mm^3.

In vivo biopanning of phage displayed library

The Ph.D.-C7C Phage Display Peptide Library (New England Biolabs, Beverly, MA) was used for peptide screening. The peptidic phage library contains random cyclic heptapeptides that fused to the minor coat protein (pIII) of the M13 phage. For in vivo biopanning, a mouse bearing A549-derived xenograft tumor was injected with 1.0×10^9 *pfu* of the random library in TBS buffer 200 μl via the tail vein. After 15 min of biocirculation, the mouse was anesthetized by *i.p.* injection of zoletil (40 mg/kg) plus rompun (10 mg/kg) mixture and perfused with 50 mL of Tris-buffered saline (TBS; 50 mM Tris–HCl (pH 7.6), 150 mM NaCl) to remove non-specific and unbound phages. In subsequent rounds of biopanning to fourth rounds, different concentrations of detergent in the TBS buffer were used to wash out effectively non-specific bound phages. The increasing concentrations of Tween-20 were as follows: 0.1 % for second round, 0.3 % for third round and 0.5 % for fourth round in the TBS buffer. Then tumor tissues were resected from the mouse and homogenized in 1 mL of cold TBS containing 1× protease inhibitor cocktail (Tech and Innovation Co., Ltd, Seoul, Korea) on ice. Bound phages were subsequently eluted from the homogenate by 0.2 M glycine–HCl (pH 2.2) and the mixture was neutralized with 1 M Tris–HCl (pH 9.1). Finally, eluted phages were titered by following the manufacturer's protocol and amplified using *E.coli* ER2738 strains for the next round of biopanning.

Analysis of DNA sequencing in the selected peptidic phages

After four rounds of in vivo biopanning, 96 phage clones were randomly picked out from titered phage plaques to extract DNA for the sequencing analysis. An overnight culture of *E. coli* ER2738 host strains was diluted to 1:100 in LB medium, and to a portion of 1 mL volume individual monoclonal phages were added. The mixture was shaken at 37 °C for 5 h and the supernatant was harvested by centrifugation. 170 μl of 20 % (w/v) polyethylene glycol-8000 and 2.5 M NaCl was added to the supernatant to precipitate the phages. The precipitate was suspended in iodide buffer consisting of 10 mM Tris–HCl, pH 8.0, 1 mM EDTA and 4 M NaI, followed by ethanol precipitation. The single-strand DNA (ssDNA) was recovered and dissolved in distilled water. The sequence of ssDNA was analyzed by an automatic sequencing services (Marcrogen Inc. Seoul, Korea) using the M13-96 gIII sequencing primer provided with the Ph.D.-C7C library kit.

Estimation of targeting efficacies by near-infrared fluorescence imaging

Phages were labeled using the NHS ester of Cy5.5 fluorescence dye (GE Healthcare, Buckinghamshire, UK). The 1.0×10^{12} *pfu* of phages were conjugated with 1 μg/μl fluorophore *N*-hydroxy succinimide ester by following the manufacturer's instructions. The phages were modified through the reaction with Cy5.5 fluorophore for 3 h at room temperature in the dark. After the reaction, the phages were purified by PEG precipitation to remove excessive Cy5.5 fluorophores. Cy5.5-labeled phages were titered in TBS buffer and determined by the fluorescence measurement system. The fluorescent phages were administered into the blood circulation of tumor-bearing mice via tail vein injection to validate the in vivo tumor-targeting efficacy. The fluorescent optical images of whole body of the mouse and ex vivo of tumors were taken at 11 days after injection by the IVIS spectrum (Perkin Elmer, Waltham, MA), and ROI of the interested region was measured with the radiance (photons/s/cm^2/sr) by the software program of IVIS.

In vitro cell-binding assay of the novel peptidic phage

The 1×10^4 cells of A549 were seeded on the four-well chamber glass slide (Thermo Fisher Scientific Inc., Waltham, MA) and incubated overnight. Cells were washed with phosphate-buffered saline (PBS; Invitrogen Corporation) and fixed with 4 % (w/v) paraformaldehyde (Sigma-Aldrich Co., St. Louis, MO) for 30 min at 4 °C. After washing three times with PBS, the cells were blocked with 2 % (w/v) bovine serum albumin (BSA; Sigma-Aldrich Co.) for 1 h at room temperature. The Cy5.5-labeled phages (1×10^{10} pfu) were added and incubated for 15 min at room temperature and then washed out to remove unbound phages. The cells were counterstained with DAPI (Vector Laboratories Inc., Burlingame, CA) and mounted.

Immunohistochemical staining

The Cy5.5-labeled peptide phage and empty phage were injected intravenously into mouse model bearing A549-derived xenograft tumor and perfused as described above. Tumor was isolated and embedded in the optical cutting temperature compound (OCT; Sakura Finetek Inc., Torrance, CA) for cryosection. For immunohistochemical staining, embedded tissues were sectioned in 7 μm thickness using cryostat at −25 °C and fixed in ice-cold acetone for 15 min. After washing with PBS, endogenous peroxidase was neutralized by methanol solution containing 3 % (v/v) H_2O_2. The section was washed and blocked with 10 % normal goat serum (Vector Laboratories, Inc.) for 1 h at room temperature. Subsequently, mouse IgG-anti-M13 (New England Biolabs) diluted to 1:200 in PBS containing 10 % normal goat serum was incubated overnight at 4 °C. On the following day, the section was washed in PBS and incubated with biotinylated antibody against mouse IgG (1:500 in PBS containing 10 % normal goat serum; Vector Laboratories, Inc.) for 30 min at room temperature. Immunoreactivity was

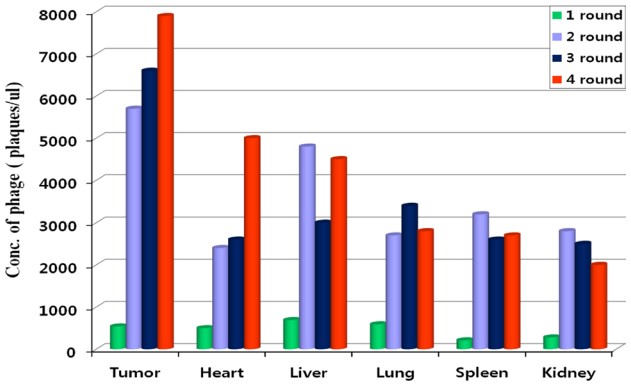

Fig. 1 Specific enrichment of tumor-targeting peptidic phages. Tumor homing peptide phages were isolated from the phage display library by in vivo biopanning. During four rounds of panning, titering of phages targeting tumor or other organs was performed. The concentration of amplified phages was calculated to follows: Phage concentration = (total amount of phages/6.02×10^{23})/suspension volume. (The total amount of phages = the number of plaques of phage by titering)

Table 1 Human lung adenocarcinoma-targeting peptides displayed on M13 phages

Name	Occurrences	Peptide sequences
G1	26	AKATCPA
G2	20	SWQIGGN
G3	12	TVRTSAD
G4	10	IGNSNTL
G5	2	IKVGKLQ
G6	2	NWGDRIL
G7	2	TNANHYF
G8	1	GWSGSLV
G9	1	LYANSPF
G10	1	SISSLTD
G11	1	NWGDRIR
G12	1	NWGDRIE
G13	1	RSANIFT
G14	1	FNMFSRF
G15	1	ISAWPTR
G16	1	SISSLTH
G17	1	FLNPTTT
G18	1	KNLTRLA
G19	1	MARYMSA
G20	1	IKVGKVQ

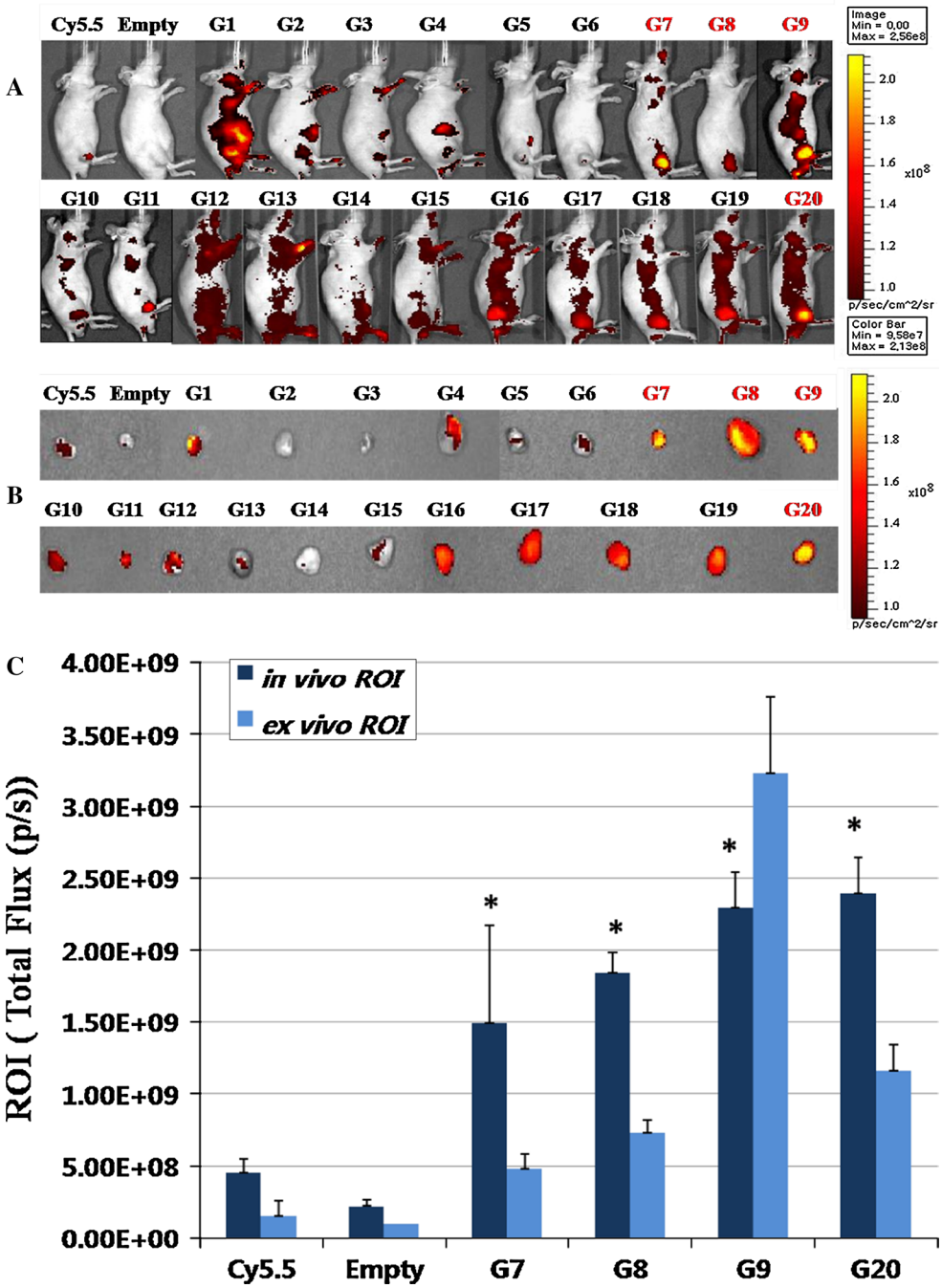

Fig. 2 In vivo tumor-targeting capabilities of discovered phages. Fluorescent optical images of tumor-targeting peptide phages. **a** whole body images, the representative image of triplicate **b** images of tumor tissues and the representative image of triplicate **c** calculated ROI values. Mice bearing A549 tumors were injected *i.v.* with Cy5.5, Cy5.5-labeled empty phage and Cy5.5-labeled-G7, G8, G9 and G20 through tail veins and the images were obtained on the 11th day. Cy5.5, Cy5.5 NHS ester dye; empty phage and Cy5.5-labeled wild-type phage without peptide insert; scale bar, p/s/cm2/sr; error bar, standard variation (SD); $*P < 0.001$ compared to in vivo ROI of empty phage ($n = 3$, Student's t test)

detected using the Elite ABC kit (Vector Laboratories, Inc.) by following the manufacturer's protocol.

Results

Discovery of tumor-specific targeting cyclic heptapeptide phages

To discover the novel peptide phages that specifically target in vivo human lung cancer, screening using the M13 phage display library pool was performed on the nude mouse bearing A549-derived xenograft tumor. After four repetitive cycles of biopanning, the number of phages specifically recovered from the tumor was increased to 13.9-fold compared with that in the first round (Fig. 1). This result suggested that tumor-targeting capability of selected peptide phages was significantly augmented through the cycles of biopanning. Although the other organs such as heart, lung, liver, spleen and kidney contained an increasing concentration of phages compared to the first round, the increasing ratio was much less than that of the tumor.

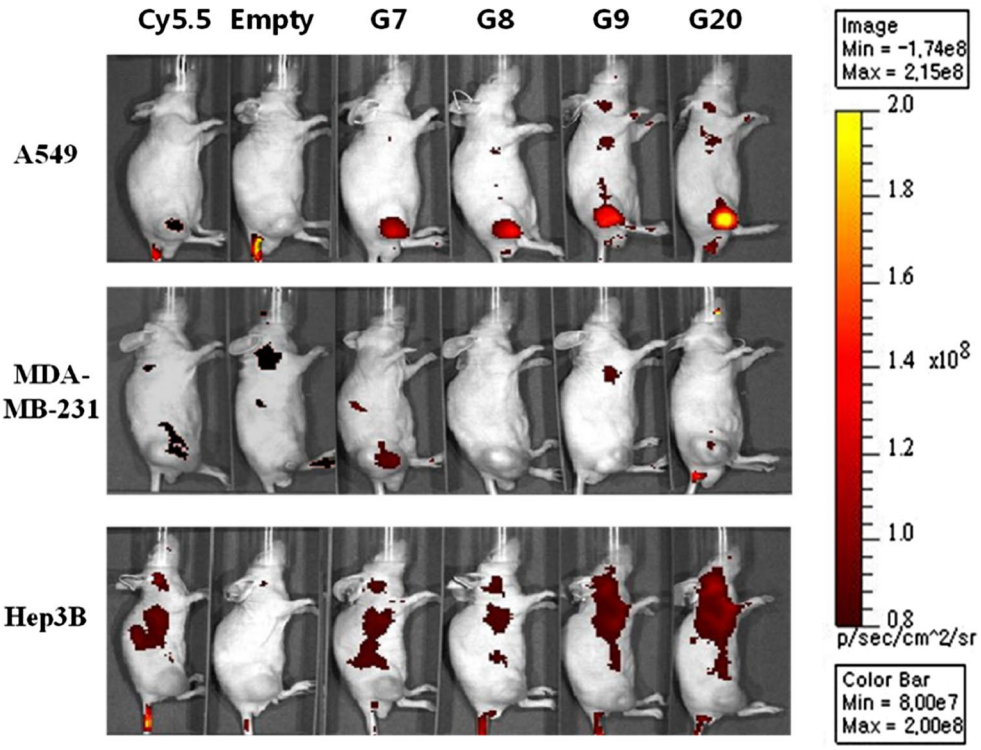

Fig. 3 Selective binding affinity of the peptide phages to human lung cancer. In vivo fluorescent optical images of peptide phages in mice bearing various types of xenograft tumor. Mouse bearing A549 tumor, MDA-MB-231 tumor and Hep3B tumor were *i.v.* injected with Cy5.5 or Cy5.5-labeled empty phages as a control, and Cy5.5 labeled-G7, G8, G9 and G20. Images were obtained on the 11th day

After four rounds of panning, 96 phage clones were randomly selected to analyze the peptide sequences. The 20 different peptide inserts were identified as shown in Table 1.

In vivo tumor-targeting capabilities of discovered peptide phages

To confirm the in vivo specific targeting of discovered phages, 20 peptide phages selected from discovered groups were conjugated with Cy5.5 NHS ester and subjected to non-invasive whole body imaging. The Cy5.5-labeled peptide phages were injected into the circulation of whole body through the tail vein of tumor-bearing mouse. Fluorescent optical imaging was obtained daily for 11 days using the IVIS spectrum (Perkin Elmer). On the 11th day, the Cy5.5-labeled wild-type phage without peptide insert (empty phage) was not detected in any place of the mouse, indicating that the peptide phages were cleared from the biocirculation. While all of the 20 peptide phages remained in the tumor on the same day, some of them were found to spread to other portions of the body. The fluorescence of four peptide phages (G7, TNANHYF; G8, GWSGLSV; G9, LYANSPF; G20, IKVGKVQ) particularly accumulated in the tumor and their ROI values were also highly increased (Fig. 2a, c). The in vivo fluorescence intensity of the selected peptide phages was significantly higher than in the Cy5.5-injected group. After obtaining the in vivo imaging, the tumor tissues were dissected and imaged for ex vivo fluorescence intensity. As shown in Fig. 2b, those four peptide phages have the most specific targeting ability compared with other phages. These results indicated that the selected peptide phages were specific to the in vivo human lung cancer.

Selective binding affinity of the peptide phages

To determine the targeting specificity of novel peptide phages to different tumor types, we investigated the peptide accumulation in xenograft tumor by in vivo imaging. The results showed that novel peptide phages bound only to the A549-derived xenograft tumor, but not to other tumor types such as breast adenocarcinoma MDA-MB-231 and hepatocellular carcinoma Hep3B-derived xenograft tumors (Fig. 3). This result indicated that our strategic approach of in vivo peptide screening was suitable for targeting the human lung cancer A549-derived xenograft tumor tissue.

In vitro specific binging ability of novel peptide phages

To confirm the specific binding ability against human lung cancer cells, we performed cellular binding and competitive assay in A549 cells with G9 peptide phage (G9) that was chosen as a representative peptide among them. Unlabeled empty phage and unlabeled G9 were

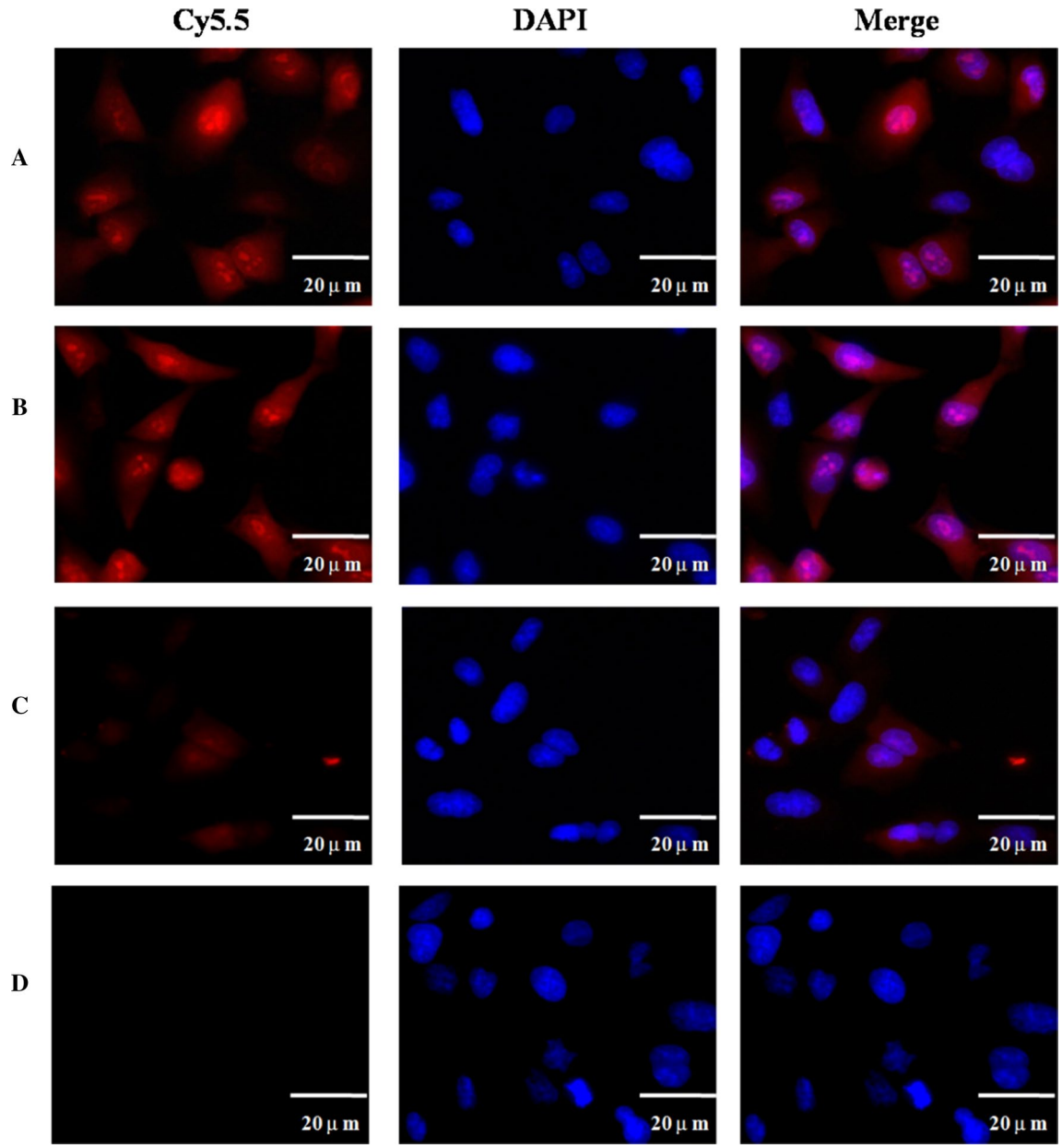

Fig. 4 In vitro specific binding ability of novel peptide phage to human lung cancer. Fluorescence images of A549 cells treated with G9 upon in vitro binding and competitive assay. Cells were incubated with Cy5.5-labeled G9 (10^{10} *pfu*) alone (**a**) or Cy5.5-labeled G9 (10^{10} *pfu*) in the presence of unlabeled empty phage (10^{12} *pfu*) (**b**) or unlabeled G9 (10^{12} *pfu*) for competition (**c**). **d** DAPI staining of A549 cells. The images were analyzed by immunofluorescence microscopy (scale bar, 20 μm)

used to compete with Cy5.5-labeled G9 in the A549 cell line. The fluorescence of Cy5.5-labeled G9 was definitely found in A549 cells (Fig. 4a) and its intensity was not decreased by the competition with the unlabeled empty phage (Fig. 4b). On the other hand, the fluorescence intensity of Cy5.5-labeled G9 that competed with unlabeled G9 was significantly decreased (Fig. 4c). Thus, these results indicated that G9 had a specific binding ability to A549 cells, which resulted from the peptide sequence-specific tendency.

In vivo functional targeting of the novel peptide phage

To address whether G9 functionally targets and specifically localizes in the tumor tissue, Cy5.5-labeled G9 was injected intravenously into A549-derived xenograft mice tumor and immunohistochemically examined in the tumor tissue. As shown in Fig. 5, M13 phages were intensely detected in the tumor tissue from the mouse treated with G9, in sharp contrast to no signal detected in other tumor tissues from mice treated with Cy5.5 or empty phage. Therefore, these results

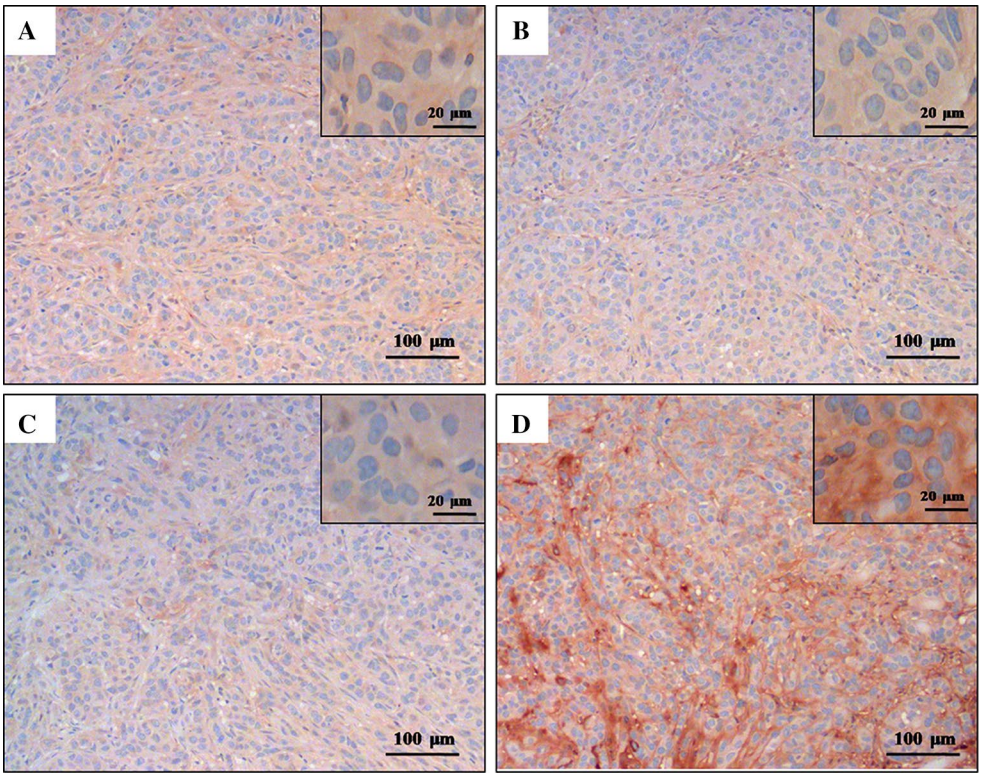

Fig. 5 In vivo functional targeting of the novel peptide phage. Immunohistochemical analysis for G9 targeting and localization in tumor tissue. Cy5.5-labeled G9 were *i.v.* injected into A549-derived xenograft tumor mice. The cryosectioned tumor tissues were incubated with mouse IgG-anti-M13, and then immunoreactivity was visualized using Elite ABC kit. Tumor tissues were from untreated mouse (**a**), empty phage-treated mouse (**b**), Cy5.5 dye-treated mouse (**c**) and Cy5.5-labeled G9-treated mouse

described that the novel peptide discovered through in vivo screening exhibited functional targeting ability toward in vivo human lung cancer tissue.

Discussion

Lung cancer is the leading cause of cancer-related deaths worldwide. Major modalities for cancer treatment such as chemotherapy, radiotherapy and surgical resection could be primarily applied for patients with lung cancer, while unfortunately the therapeutic influence is not at a satisfactory level yet. A strategic approach to open a new era of lung cancer treatment is required. Hence, the discovery of biomolecules functionally targeting in vivo lung cancer has received great attention to be applied to a potent drug delivery strategy to improve drug efficacy and introduce a new concept of imaging for more competent diagnostics.

Peptides as cancer-targeting ligands have been applied successfully in the fields of active targeting drug delivery and diagnostic imaging of cancer. Nonetheless, there have been unexpected failures with most discovered peptides during application with clinically available tools, because the peptides cannot display capability in a live body system. To increase the success rate of peptides from discovery to clinical application, screening process and validation should be done in a live animal model. Thus, we have designed and performed this study utilizing human lung cancer A549-derived xenograft model for in vivo phage-displayed peptide screening and validation of targeting activity with fluorescence dye-labeled phages.

In this study, phage display peptide library was intravenously injected into the circulation through the tail vein in a mouse bearing human lung cancer A549-derived xenograft tumor. The emerged phages were isolated from the tumor tissue in a course of four rounds of in vivo biopanning. By utilizing non-invasive in vivo imaging system for determining the distribution of fluorescence-labeling peptide phages in xenograft mouse model, the capability of the discovered peptides was successfully validated. The groups displaying higher fluorescence were taken as valuable groups for lung cancer-specific peptide phages. For further confirmation of specificity to human lung cancer, G9 was subjected to analysis in xenograft models bearing other types of tumors and in vitro and in vivo examination of binding and localization.

Screening technology using phage-displayed peptide has been considered as a powerful tool for the discovery of

clinically valuable ligands, which are able to bind specifically to surface biomarkers on particular tumor cells. The Ph.D.-C7C Phage Display Peptide Library has been used for the in vivo phage-displayed peptide screening. M13 phages of this library displayed random peptides which were expressed by the insertion of oligonucleotides coding a random sequence into phage DNA. This could be effectually used to discover tumor-specific peptides, since cancer cells or others cells in the tumor microenvironment possess unique biomarkers on the surface. Particularly, the cyclic 7-mer peptides, cyclized by two cysteines, show high binding affinity and stability, by which we were able to find potent 20 peptides targeting in vivo human lung cancer.

According to the previously reported findings, small molecule probes such as a novel peptide have been considered to be useful for a novel paradigm leading to multifunctional properties for both cancer treatment and imaging system (Park et al. 2011). Peptides could be utilized to provide a feasibility of therapeutic targets for the development of potential therapeutics (Fei et al. 2014). Moreover, it was shown that the peptides could be conjugated to apply as an imaging probe with various molecules such as iron oxide (Ghosh et al. 2012), radioisotope (Schumacher and Tsomides 2001) and fluorescent agent (Carrico et al. 2012). Furthermore, the peptides could be utilized to find and prove a diagnostic biomarker (Conraux et al. 2013) and tethered to nanoparticles including liposome through physical/chemical conjugation for the development of drug delivery systems (Stefanick et al. 2013). Therefore, peptides are versatile molecules that can be potentially applied to diverse creation of therapeutic and diagnostics (Bartlett et al. 2007; Daniels et al. 2006; Garanger et al. 2007). In conclusion, it is warranted that the peptides specifically targeting in vivo human lung cancer discovered in this study provide substantive feasibilities for drug delivery and imaging for improvement of human lung cancer treatment and diagnosis.

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Conflict of interest The authors declare that they have no conflict of interest.

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