

In Vivo Selection of Phage Sequences and Characterization of Peptide-specific Binding to Breast Cancer Cells

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This work was supported by a grant from the Hi-Tech Research and Development Program of China (863) (No. 2006AA02Z19C).

Received December 24, 2007; accepted March 24, 2008.

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OBJECTIVE To screen specific polypeptide target binding to breast cancer xenografts in vivo from a phage-displayed peptide library in order to provide peptide sequences for breast cancer tumor-targeting diagnosis and therapy.

METHODS A mouse model for carrying breast cancer xenografts was established using Tientsin Albino II mice (TA II). A 12-peptide library was biopanned through 4 rounds. Phages were recovered and titrated from tumor xenografts and control tissue (liver). The distribution of phages was detected by immunohistochemical staining.

RESULTS Phage homing to breast cancer was enriched through 4 rounds of biopanning, being 14-fold of that recovered from liver tissue. A peptide sequence, ASANPFPTKALL was characterized by randomly picked-up clones which appeared most frequently. Immunohistochemical staining revealed phage localization in cancer xenografts 40 min after injection of the enriched phages. When a specific phage was tested individually, the phage reclaimed from breast cancer xenografts was 14 times as those from control tissues.

CONCLUSION Tumor-specific homing peptides may provide an effective tool for breast cancer target therapy. The in vivo phage display selection technique employed in this study was feasible and applicable to screening peptides that home to breast cells.

KEY WORDS: phage display, breast cancer cells, in vivo selection.

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Introduction

Breast cancer, one of the most common primary malignant carcinomas in the world, has increased annually. Because breast cancer can remain undetected for a long period of time resulting in a poor prognosis, there is an urgent need to identify and treat this cancer as early as possible. At present, breast cancer detection is addressed by different diagnostic techniques, such as mammography, magnetic resonance imaging, spectroscopy, scintigraphy, or positron emission tomography. The main treatment for breast cancer includes surgical management, radiotherapy, chemotherapy, endocrine therapy and immunotherapy.

The major obstacles impeding current cancer therapies are side effects and low specificity. One way to improve the efficacy of therapeutic agents is to selectively target the tumor site, thereby sparing normal tissues. The development of targeted therapeutic methodologies relies, in most cases, on the availability of agents that specifically bind to tumor-associated markers^[1]. The display of polypeptides on the surface of filamentous phage, together with an efficient selective-amplification of the desired binding specificities using affinity cap-

ture, represents an efficient route towards the isolation of specific peptides and proteins that could act as vehicles for a tumor targeting application^[2]. We believe that in vivo-selected phage can serve as valuable first-line agents to determine if the phage and corresponding synthesized peptides would function as efficacious tumor-targeting and tumor-imaging agents.

In this study, we selected a small peptide from a 12-mer peptide library displayed on the surfaces of filamentous phages in TA II mice, which targets breast cancer cells. A single-phage clone was identified by immunostaining showing affinity for breast cancer tissues.

Materials and Methods

Materials

A Ph.D.-12 Phage Display Peptide Library Kit was purchased from New England BioLabs, Ipswich, MA, USA. The library consisted of 2.7×10^9 electroporated sequences with a titer of 1.5×10^{13} pfu. The host bacterium was *Escherichia coli* (*E.coli*) ER2738, and -96 gIII sequencing primer was 5'-CCC TCA TAG TTA GCG TAA CG-3'. The host bacterium and -96 gIII sequencing primer belonged to the kit. Aprotinin was purchased from Amresco, Solon, Ohio, USA. Leupeptin was obtained from Roche, Basel, Switzerland. Phenylmethanesulfonyl fluoride (PMSF) was bought from Hopebio, Tianjin, China. HRP-anti-M13mAb was obtained from Pharmacia Biotech, Uppsala, Sweden. Six-week-old TA II, female mice, (approximately 20 g) were obtained from the Animal Center of Tianjin Medical University.

Mouse model

A suspension of 0.1 ml of mouse spontaneous breast cancer cells (1×10^7) was injected into the left groin of the mice. Solid tumors were established in the TA II mice over several weeks, resulting in 1 cm-sized tumors for all the animals. The tumor-bearing mice were sacrificed by cervical dislocation, the tumor and organs (liver) were excised from the animal, fixed in 10% formalin, embed in paraffin, and examined under a microscope after H&E staining. After injecting the breast cancer cells, 1.0-cm-sized solid tumors were established in the TA II mice over a period of 4 weeks (Fig.1). Pathological examinations of the sections with H&E stain showed hyperplastic cancer cells irregularly arranged in a tubular form, which is consistent with the characteristics of breast cancer (Fig.2).

In vivo selection of tumor-targeting phage

About 10^{12} transducing units (TU) of a -12 phage display peptide library suspended in RPMI-1640 were injected into the tail vein of tumor-bearing TA II mice. Allowing 45 min for the binding to the breast cancer cells, the mice were then anesthetized by 10% chloral hydrate administration and the diaphragm incised to expose the entire thorax. The sternum was incised taking care to

avoid damage to the heart or large vessels. A vacutainer was inserted into the left ventricle while the right arterial chamber was incised to form an outlet for the blood and perfusate. Up to 50 ml of RPMI-1640 was injected into the heart to clear in vivo unbound phage. The tumor and control tissues were weighed and ground in 1 ml RPMI-1640-PI (1 mmol/L PMSF, 20 mg/L aprotinin, and 1 mg/L leupeptin). Tumor tissues were washed 5 times, and then centrifuged for 10 min and the supernatant discarded. At last, the washed tissue was mixed with 1 ml of host bacterium and 10 ml Luria-Bertani (LB) medium prewarmed to 37°C. After 45 min, the mixture titered the combining weight of tumor and then remained tissues were mixed in 200 ml LB. The mixture was shaken at 37°C to amplify the phages after which the phage was recovered in the mixture in preparation for the next procedure. The in vivo selection protocol was conducted 4 times.

Sequencing of the DNA phage insert

To know the sequence of the selected phages, we picked 10 phage monoclonal randomly and extracted ssDNA for sequencing. The amino acid sequences of displayed peptides were determined by the Sangon Co., Shanghai, China.

Identification of sequence by immunohistochemical staining

After the selected clone was amplified, 200 μ l (10^{12} TU) were injected into the tail vein of tumor-bearing TA II mice. After 40 min, the tumor tissue and control tissue were excised from the mice. Briefly, sections of routine formalin-fixed and paraffin-embedded material were deparaffinization with xylene and rehydrated in graded alcohols and distilled water. Antigen retrieval was carried out by high pressure treatment of the slides in sodium citrate buffer. To quench the endogenous peroxidase activity, the sections were treated with 3% H_2O_2 in methanol for 10 min. After blocking with 2% BSA, the sections were incubated with the selected phages overnight at 4°C and washed with PBS. After washing with PBS, the tissue sections were reacted with HRP-anti-M13mAb (1:5000) for 1 h. Color was developed with DAB, and all slides were counterstained with hematoxylin. For negative controls, the selected phages were replaced with control phages.

Results

Enrichment of the tissue-specific phages

After 4 rounds of successive in vivo selection, phage homing to the breast cancer in the fourth round was 4.1 times that of the first cycle. At the same time, phage homing to the breast cancer xenografts was enriched, 14-fold compared to that recovered from liver tissue, showing that by the fourth round binding of the phage to the breast cancer tissues was significantly increased

(Table1, Fig.3).

Identification of the selected phage

Four rounds of selection were performed, and foreign DNA inserts and encoded peptide sequences were determined for 10 random phage clones to gauge the selection process and absence of contamination. After analyzing the sequence of the inserted peptide, results showed that ASANPFPTKALL appeared 8 times and PSRNPFPKALL appeared 2 times. In conclusion, ASANPFPTKALL maybe the special peptide binding to breast cancer tissues.

Location of the selected phage

Immunohistochemical staining results indicated that the selected phage binding was found on cell membranes and in the cytoplasm of the tumor cells (Fig.4).

Discussion

A phage display is a selection technique in which a peptide or protein is expressed as a fusion with a coat protein of a bacteriophage, resulting in display of the fused protein on the surface of the virion, while the DNA encoding the fusion resides within the virion. Phage displays have been used to create a physical linkage between a vast library of random peptide sequences and the DNA encoding each sequence, so it is commonly used to select for peptides, antibodies, and recombinant proteins capable of binding to a target molecule in an in vitro or in vivo environment^[3]. In vitro phage displays have been used to select peptides that target cancer-associated antigens, as well as cultured carcinoma cell lines. The in vivo phage display selection procedure is a new technique in which peptide phage libraries are injected into animal models to screen the peptide which specially



Fig.1. Mouse bearing a breast cancer.

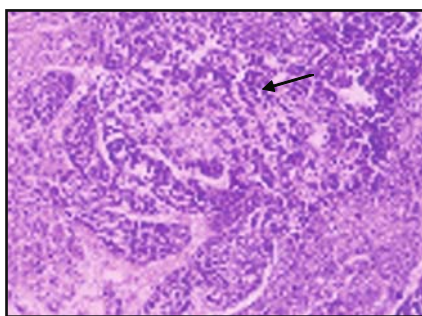


Fig.2. Histological examination of transplant (H&E stain, ×200).

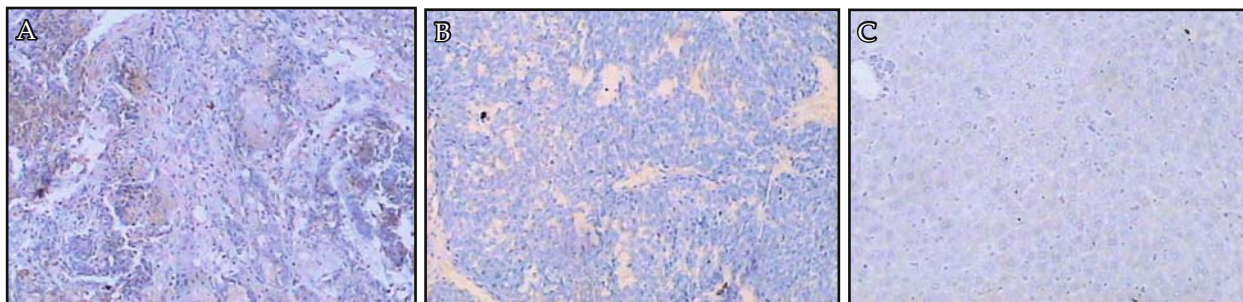


Fig.4. Detection of distribution of phage in transplanted tumor tissue by immunohistochemical staining (H&E stain, ×200). A, Positive phage in a tumor; B, Control phage in a tumor; C, Positive phage in liver tissue.

Table1. The result of the four selections in vivo.

| Round | 1st | 2nd | 3rd | 4th |
|---|--------------------|--------------------|--------------------|--------------------|
| Phage recovered from unit tumor (pfu/g tissue) | 8×10^4 | 1.3×10^5 | 2.34×10^5 | 3.29×10^5 |
| Phage recovered form unit liver tissue (pfu/g tissue) | 9.53×10^4 | 5.64×10^4 | 3.87×10^4 | 0.23×10^5 |

pfu, phage forming units.

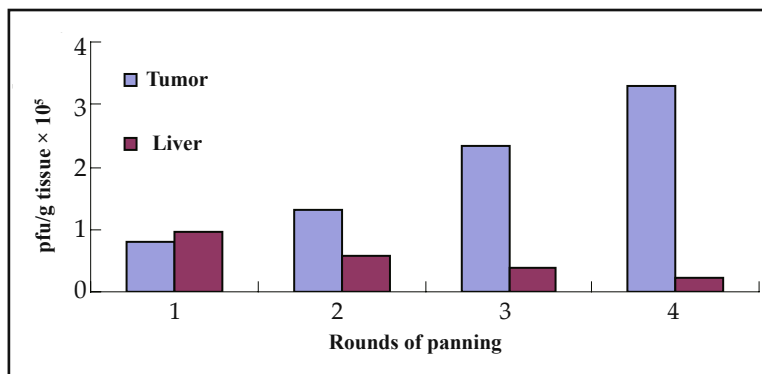


Fig.3. Titration of phage from tumor xenografts and liver tissue though 4 rounds of screening.

binds to an target organ or tissues. Compared with an in vitro screening protocol, in vivo phage displays can maintain the natural environment of the targeted receptor, and can obtain a stable, special small peptide. Furthermore, more complicated biological systems can be used as targets for biopanning. The selection of antibodies and peptides from libraries displayed on the surface of filamentous phage has proven significant for routine isolation of peptides and antibodies for diagnostic and therapeutic application^[4].

Peptides are thought to have clinically desirable benefits over currently used biomolecules, such as antibodies, because of their rapid blood clearance, increased diffusion and tissue penetration, non-immunogenic nature and ease of synthesis. Using a phage display, one can rapidly and simultaneously survey billion-clone peptide libraries, resulting in large numbers of “hits”. Since Pasqualini and Ruoslahti^[4] identified peptide binding to blood vessels of the brain or kidney, many peptides have been shown to bind to tumor vessels specificity, such as RGD-4C, CNGRC, GSL, SMSIARL and CPG-PEGAGC^[5-7]. However, there have been few reports that select a peptide which binds to cancer cells though an in vivo phage-display technology. Newton et al.^[8] selected phages that target human PC-3 prostate carcinomas in vivo, and fluorescently labeled versions of the phage resulting in increased signal intensity within PC-3 xenografted tumors in SCID mice.

We chose TA II mice which came from the Animal Center of Tianjin Medical University, and established a breast cancer model^[9]. The characteristics of the breast cancer were infiltrative growth and blood vessels that surrounded the tumor. Morphology, biochemistry and genetics in our breast cancer model were similar to human and biological characteristics of other stable tumor models^[10,11].

In our study, the breast cancer tumour cells were transplanted into TA II mice to establish the animal model. To acquire the peptide which bound to breast cancer, we injected phage into animals 40 min to bind tumor tissue completely^[8,12], and mice were perfused with RPMI-1640 medium to reduce the non-specific phage^[13]. The phage clones displaying the peptide sequence ASANPFPTKALL were found bound to breast cancer cells after four rounds in vivo. At the same time, the analysis of phage titration showed that the phage homing to the breast cancer xenografts were enriched 14-fold compared to that recovered from liver tissue. Furthermore, it was found that the selected phage bound to the cell membranes and cytoplasm by immunohistochemical staining. These results provide evidence that filamentous phage that specifically target cancer cells can be used to expand the applications in the field of cancer biology.

In summary, our findings demonstrate that phage, showing specific binding to breast cancer, were enriched and selected in our animal model. Those findings suggest that the peptide ASANPFPTKALL is specially bond to breast cancer cells. Future studies will be centered on detecting the selected peptide binding to breast cancer in humans^[14]. The peptide, ASANPFPTKALL, may have significant potential as a tumor-targeting and tumor-imaging agent^[15].

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