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SPECT and Near-Infrared Fluorescence Imaging of Breast Cancer with a Neuropilin-1-Targeting Peptide

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ABSTRACT

Breast cancer is the most common malignant cancer and is the leading cause of cancer death among females. Molecular imaging is a promising approach for the early detection and staging of breast cancer as well as for assessing therapeutic responses. Tumor-targeting peptides are effective targeting vehicles for molecular imaging. Here, we identified a breast cancer-targeting peptide CLKADKAKC (CK3) contains a cryptic C-end rule motif that may mediate its binding to neuropilin-1 (NRP-1), an attractive therapeutic target which expression was associated with poor outcome of the patients with breast cancer. Phage CK3 bound to NRP-1-positive breast cancer cells, which could be inhibited by peptide CK3 in a dose-dependent manner or by knock-down NRP-1 expression. Consistently, NRP-1 overexpression in cells increased the binding of phage CK3. Furthermore, peptide CK3 co-localized with NRP-1. Importantly, unlike previously reported NRP-1-targeting peptides with exposed C-end rule motifs, peptide CK3 did not penetrate into lungs and heart *in vivo*, which could make it more clinically applicable. Single-photon emission CT (SPECT) and near-infrared fluorescence (NIRF) imaging showed enrichment of peptide CK3 to the xenograft tumors in nude mice. In conclusion, as a novel NRP-1-targeting peptide, peptide CK3 could be used for breast cancer molecular imaging, which may represent a new avenue for breast cancer diagnostics, staging and assessments of therapeutic response.

1. Introduction

Breast cancer, the most common malignant cancer, is the leading cause of cancer death among females [1]. Early detection through mammography, a simple and effective x-ray imaging approach, increases treatment options and reduces mortality [1]. Other traditional imaging methods, such as x-ray computed tomography (CT), ultrasound and magnetic resonance imaging (MRI), have also been widely used for breast cancer diagnostics, staging and therapy response assessments [2]. These anatomic-based imaging methods are suitable for large masses or whole body imaging. However, they are limited in their ability to monitor breast cancer molecular processes in real-time. By contrast, molecular imaging methods rely on the regional biochemical, cellular, and molecular properties of tissues [2–4]; such methods include positron emission tomography (PET), single-photon emission CT (SPECT) and near-infrared fluorescence (NIRF). With these methods, one can image molecular biological processes specific to cancer in real-time and obtain information that is complementary to traditional imaging data. Molecular imaging has been used to measure breast cancer perfusion, metabolism, and proliferation and to study the breast cancer microenvironment [2]. However, few molecular imaging methods have been applied successfully to measure breast cancer-specific protein expression. These specific proteins are ideal biomarkers for the targeted therapy of breast cancer [5]. The current challenge is to identify more specifically expressed proteins for the molecular imaging and targeted therapy of breast cancer [6,7].

Phage display is a powerful and effective approach for identifying novel tumor-targeting peptides and their corresponding receptors [8–10]. Many tumor-targeting peptides and their receptors have been successfully used for tumor molecular imaging [10]. For example, many RGD-containing peptides have been successful used to visualize

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tumors, and certain of them have already been used at different stages of clinical trials [11–14].

This study aimed to identify breast cancer-targeting peptides for molecular imaging by phage display. A new identified breast cancer-targeting peptide CLKADKAKC (CK3), a cryptic C-end rule KXXK motif containing peptide, was selected and was validated as a new NRP-1-targeting peptide. Unlike previously reported NRP-1 targeting peptides with exposed C-end rule R/KXXR/K motifs, peptide CK3 did not accumulate into lung and heart. Both SPECT and NIRF imaging demonstrated that peptide CK3 homed to MDA-MB-231 tumors in mice. Our study suggested that this novel NRP-1-targeting peptide represents a new avenue for diagnosing, staging and assessing therapeutic responses in breast cancer.

2. Materials and Methods

2.1. Reagents

The Ph.D.-CX7C™ phage display peptide library kit was purchased from New England BioLabs (Ipswich, MA, USA). The T7 select packaging kit and T7 select 425-1b DNA were obtained from Merck-Millipore (Darmstadt, Germany). A horseradish peroxidase (HRP)-conjugated anti-M13 monoclonal antibody was purchased from GE Healthcare (Pittsburgh, PA, USA). The monoclonal rabbit anti-NRP-1 antibody was obtained from Merck-Millipore (Darmstadt, Germany). Tetramethylbenzidine (TMB), Streptavidin-Cy3 and DAPI were obtained from Sigma (St. Louis, MO, USA). The pEGFP-N1-NRP-1 and pMSCV-NRP-1 plasmids were constructed in our laboratory.

2.2. Cell lines

MDA-MB-231, 4 T-1, MDA-MB-435 and MCF-7 cells were maintained in DMEM supplemented with 10% fetal bovine serum, penicillin and streptomycin. HNE1 cells were cultivated in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin and streptomycin.

2.3. Nude Mice

All the animal experimentations were conducted according to the institutional guidelines of Guangdong Province and approved by the Use Committee for Animal Care. To create MDA-MB-231 tumors, 1×10^6 MDA-MB-231 cells were injected into the mammary fat pad of each BALB/c athymic nude mice.

2.4. Phage library screening and phage sequencing

The Ph.D.-CX7C™ phage display peptide library was used to select peptides that bound to MDA-MB-231 cells as previously described [15]. Briefly, 1×10^6 cells were suspended in DMEM containing 2% BSA and 1×10^9 transducing units (TU) of the phage display peptide library for 1 h on ice. Subsequently, the mixture was transferred to the top of a non-miscible organic lower phase [dibutyl phthalate: cyclohexane, 9:1 (vol:vol)] and centrifuged at 12,000 g for 10 min. Phage in the organic lower phase were recovered and infected with ER2738. After three rounds of screening, 30 clones from the third round were randomly selected, amplified and purified. ssDNA was extracted, and DNA sequencing was performed using the -96gIII primer (5'-CCCTCATAGT TAGCGTAACG-3').

2.5. Enzyme-linked immunosorbent assay and competitive inhibition assay

Enzyme-linked immunosorbent assays (ELISAs) were performed as previously described [16]. Briefly, cells were washed and fixed with 0.25% glutaral after plated in 96-well overnight. The cellular endogenous peroxidase activity was inhibited by hydrogen peroxide (3%). After blocked with 2% BSA, the phages were added to the cells for 2 h at 37 °C. The solutions were washed three times with 0.05% TBST, and then the horseradish peroxidase (HRP)-conjugated anti-M13 monoclonal antibody (1:5000) was added and incubated for 1 h at 37 °C. After washed three times with 0.05% TBST, tetramethylbenzidine (TMB) was added at room temperature for 30 min. Subsequently, 2 M H_2SO_4 was added, and absorbance of supernatant was determined against blank at 450 nm with an ELISA plate reader. The competitive inhibition assay was performed follow the same procedure, except the synthetic CLKADKAKC (CK3) peptide was added at various concentrations after the cells were blocked with 2% BSA.

2.6. Peptide synthesis and labeling

CLKADKAKC, CG7C, Biotin-CLKADKAKC, biotin-CG7C, D(KLAKLAK)2-CLKADKAKC, D(KLAKLAK)2-CG7C, Cy5-CLKADKAKC, Cy5-CG7C, HYNIC-CLKADKAKC and HYNIC-CG7C were synthesized by GL Biochem (Shanghai, China) and Chinese Peptide Company (Hangzhou, China) using standard solid-phase Fmoc chemistry. The peptides were purified to a minimum purity of 95% by high-performance liquid chromatography (HPLC) and were isolated by lyophilization.

2.7. Immunofluorescence

HNE1 cells were grown on coverslips in 24 well culture plates and transfected with pEGFP-NRP1. The cells were incubated with 50 mM biotin-CLKADKAKC (CK3) or biotin-CG7C, fixed with 4% paraformaldehyde, blocked with 2% BSA, and incubated with Streptavidin-Cy3. The nuclei were stained with DAPI. Images and fluorescence intensities were processed by using confocal laser-scanning system and FluoView application software FV10-ASW 3.0 (Olympus, Tokyo, Japan).

2.8. siRNA oligoribonucleotides

siRNA duplexes targeting human NRP1 (NM_003873.5) and negative control siRNA duplexes were synthesized by GenePharma (Shanghai, China). siRNA1: AACACCTAGTGGAGTGATAAA; siRNA2: AACAGCCTTG AATGCACTTAT.

2.9. SPECT imaging

^{99m}Tc-labeled peptides were constructed as previously described [17]. After labeling, peptides were injected intravenously mice bearing MDA-MB-231 tumors.

2.10. Near-infrared fluorescence imaging

Near-infrared fluorescence (NIRF) optical imaging was performed using the IVIS® Spectrum (Xenogen Corporation, Caliper Life Sciences, Hopkinton, MA, USA). The data were analyzed using Living Image® version 3.0 (Caliper Life Sciences, Hopkinton, MA, USA). The NIRF activity in different organs was quantified based on the following procedures: a region of interest (ROI) was defined, and the NIRF measurements were calculated and expressed as an efficiency using the Living Image® software.

2.11. Data analysis and statistics

Statistical analyses were performed using SPSS Statistics 13.0 (SPSS Inc.®). The data were analyzed using Student's *t* test, one-way analysis of variance (ANOVA) and Linear Regression. A p value less than 0.05 was considered statistically significant.