Near-infrared fluorescent labeled CGRRAGGSC peptides for optical imaging of IL-11Rα in athymic mice bearing tumor xenografts

Tiannv Li¹,△, Jin Sun¹,△, Yao Hu¹, Min Yang²,³,✉, Haibin Shi⁴,✉, Lijun Tang¹

¹Department of Nuclear Medicine, the First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu 210029, China; ²The First Clinical Medical College of Nanjing Medical University, Nanjing, Jiangsu 210029, China; ³Molecular Imaging Center, Key Laboratory of Nuclear Medicine, Ministry of Health, Jiangsu Key Laboratory of Molecular Nuclear Medicine, Jiangsu Institute of Nuclear Medicine, Wuxi, Jiangsu 214063, China; ⁴Department of Radiology, the First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu 210029, China.

Abstract

The interleukin-11 (IL-11) and the IL-11 receptor α-subunit (IL-11Rα) have been demonstrated to regulate the invasion and proliferation of tumor cells. Our study intends to evaluate a noninvasive imaging of IL-11Rα expression in breast tumors using near-infrared (NIR) fluorescent dye Cy7-labeled IL-11 mimic peptide CGRRAGGSC. This work evaluated the IL-11Rα expression of breast tumor cells and the binding status of this peptide to IL-11Rα in vitro and in vivo by using Western blotting, immunofluorescence staining and near-infrared fluorescence imaging. Our biochemical study showed that IL-11Rα was overexpressed in breast tumor cells (MCF-7). The cell-binding assay demonstrated specific binding of peptide CGRRAGGSC to MCF-7 cells in vitro. In vivo imaging results showed that NIR fluorescent signals of Cy7-CGRRAGGSC were selectively accumulated in tumor and metabolic organs. While in the blocking experiment, free CGRRAGGSC obviously blocked the concentration of the Cy7-CGRRAGGSC in the tumors. These results suggested that IL-11Rα may be used as a potential target for noninvasive imaging in IL-11Rα overexpressed tumors. Furthermore, the imaging agent of near-infrared fluorescent dye Cy7-labeled CGRRAGGSC is suitable for IL-11Rα expression imaging study in vivo.

Keywords: interleukin-11 receptor, molecular imaging, near-infrared, breast tumors

Introduction

Near-infrared (NIR) fluorescence imaging is a non-invasive molecular imaging alternative to more traditional imaging modalities such as radionuclide imaging[1–3]. NIR imaging does not require the use of radioactive materials and can be performed by using an appropriate NIR fluorescent dye to label peptides...
or antibodies at the molecular level in living subjects\textsuperscript{[4–5]}. Various molecular probes have been applied to detect tumors, such as antibody-based probes, peptide-based probes and activatable probes detecting tumor-associated proteases. NIR imaging can be performed using different NIR probes, which enables a diagnostic wavelength window of 650–900 nm that can increase tissue penetration and reduce background fluorescence\textsuperscript{[6]}

Interleukin-11 (IL-11), a member of the IL-6 family of pleiotropic cytokines\textsuperscript{[7–9]}, binds to its specific transmembrane receptor, IL-11R\textsubscript{α}\textsuperscript{[10–11]}. This dimeric complex interacts with gp130 as a tetrameric complex to regulate the occurrence and development of several tumors, including breast\textsuperscript{[12–14]}, gastric\textsuperscript{[15–16]} and colorectal tumors. The peptide c(Cys-Gly-Arg-Arg-Ala-Gly-Gly-Ser-Cys)NH\textsubscript{2} (denoted as CGRRAGGSC) is an artificially synthesized cyclic nonapeptide that binds to IL-11R\textsubscript{α}, as confirmed by phage display technology\textsuperscript{[17–19]}. Using CGRRAGGSC, imaging and therapeutic analysis based on IL-11R\textsubscript{α} expression level have been reported utilizing peptide conjugation with radioactive tracers such as \textsuperscript{111}I and \textsuperscript{153}Sm\textsuperscript{[19–20]}. In this study, we evaluated the expression level of IL-11R\textsubscript{α} in breast tumors and the possibility of using labelled CGRRAGGSC with Cy7, an NIR fluorescent dye, as an imaging probe for breast tumor study.

**Materials and methods**

**Cell culture**

The human breast tumor cell line MCF-7 and the breast normal cell line MCF-10A were established and reserved in our laboratory. The cells were cultured in DMEM medium (Hyclone, USA) with 10% fetal bovine serum (Hyclone, USA) at 37 °C with 5% CO\textsubscript{2}.

**Protein sample preparation**

Total protein was extracted from tumor cells using RIPA buffer with 0.5% sodium dodecyl sulfate (SDS) in the presence of a 3% proteinase inhibitor cocktail. After being lysed on ice for 30 minutes, the cell lysate was centrifuged at 11 000 g for 20 minutes, and the supernatant was collected for the experiment.

**Western blotting**

Western blotting was performed at room temperature with TBS buffer containing 0.1% Tween 20 and 5% non-fat milk powder. Proteins were separated by 10% SDS-PAGE gel and transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, USA). Membranes were blocked in TBST solution with 5% non-fat milk and then incubated with rabbit anti-IL-11R\textsubscript{α} antibody (1 : 1 000, Abcam, USA) in TBST 4 ºC overnight, followed by rinsing in TBST for 5 minutes three times and incubation with an anti-rabbit IgG in TBST for 1 hour at room temperature with shaking. After rinsing with TBST for 5 minutes three times, the bands were detected by chemiluminescence using ECL Western blotting Substrate. The band intensity was analyzed using the Quantity One analyzing system (Bio-Rad, USA). Equal protein loading was controlled using a GAPDH antibody.

**mRNA expression analysis**

Total RNA of 1×10\textsuperscript{7} MCF-7 cells was extracted using TRizol reagent (Invitrogen, USA) according to the protocol. Reverse transcription polymerase chain reaction (RT-PCR) was accomplished by the use of PrimeScript RT reagent Kit (Takara, Dalian, China). The expression level of genes was determined by quantitative real-time polymerase chain reactions and normalized against an endogenous control β-actin using SYBR Premix Ex Taq (Takara). The primer sequences were as follows: IL-11R\textsubscript{α} forward, 5′-CTGGGCTAGGGCATGAAGT-3′; reverse, 5′-CTGGGCTAGGGCATGAAGT-3′. Data were analyzed using a ΔΔCt approach and expressed as target gene/β-actin ratio \textsuperscript{[2]}.  

**Fluorescent microscope imaging**

The paraformaldehyde prefixed-cells were incubated with 10 nmol/L FITC-CGRRAGGSC and FITC-CGSPGWVRC (bankpeptide, HeFei, China) at 37 ºC for 1 hour. Cells were washed with PBS and then incubated with DAPI at room temperature for 3–5 minutes. The images of stained cells were recorded by an Olympus fluorescence microscope. DAPI was used to stain cell nuclei, which was pseudo-colored blue (emission at 416 nm). The FTIC-CGRRAGGSC, FITC-CGSPGWVRC were pseudo-colored green (emission at 525 nm). Binding specificity was tested by a blocking experiment \textit{in vitro}. MCF-7 cells were preincubated with unlabeled CGRRAGGSC or anti-IL-11R\textsubscript{α} antibody (1 hour, 37 ºC, 200 nmol/L).

**Tumor xenografts**

All female athymic nude mice (BALB/c-nu, 4–6 weeks old, 17–20 g) from Model Animal Research Center of Nanjing University were kept in the Laboratory Animal Environment of SPF and provided with sterilized pellet chow and sterilized water. MCF-7 cells were preincubated with unlabeled CGRRAGGSC or anti-IL-11R\textsubscript{α} antibody (1 hour, 37 ºC, 200 nmol/L). Tumor growth to
0.4–0.8 cm was achieved in the mice.

**Synthesis and purification of Cy7-CGRRAGGSC conjugates**

The protocol for the conjugation of Cy7-NHS and CGRRAGGSC was used with some modifications. Briefly, CGRRAGGSC (0.5 mg) in 100 μL of 0.1 mol/L sodium bicarbonate buffer (NaHCO₃, pH=8.2) was mixed with Cy7-NHS (1 mg) dissolved in 100 μL of DMSO. The reaction vessel was wrapped under aluminum foil to warm up to RT (25 °C) for 12 hours. Upon termination of the reaction, the reaction mixture was loaded on a PD-10 column (Disposable PD-10 Desalting Columns Improved) and then the synthesized product was collected after elution using PBS five times (0.01 mol/L, pH=7.4).

**In vivo NIR fluorescence imaging**

In vivo, NIR fluorescence imaging was performed with a Xenogen IVISTM 200 small animal imaging system (Caliper, Alameda, CA) and analyzed with the IVIS Living Imaging@4.5 software (PerkinElmer Inc., Alameda, USA). A Cy7 filter set (excitation: 680/30 nm; emission: 775/30 nm) was used to acquire the fluorescent signals of Cy7 in vivo. Identical illumination settings (lamp voltage, filters, f/stop, fields of view, binning) were used to acquire all images, and fluorescence emission was normalized to photons per second per centimeter squared per steradian (p/s/cm²/sr). Mice (n=3 for each group) were injected with Cy-7 or Cy7-CGRRAGGSC via tail vein, and in the blocking group (n=3) were injected with Cy7-CGRRAGGSC one hour after injection of CGRRAGGSC. Then, mice were imaged with an excitation wavelength of 640 nm and 675 nm and an emission wavelength of 680–760 nm separately for up to 24 hours with Caliper Life Sciences.

**Immunohistochemistry (IHC) and histopathology analysis**

Excised tumors were fixed in 10% formaldehyde solution overnight and then embedded in paraffin. Paraflin-embedded tissue samples were sectioned at 5 μm thickness, and stained for Haematoxylin and eosin (H&E) and IHC. The slides were incubated with anti-IL-11Rα antibody (EPR5446; 1:200 dilutions, Abcam, Cambridge, UK) at 4 °C. The following steps were performed as described.

**Statistical analysis**

All of the data were expressed as mean±SD (standard deviation). Statistical analysis was performed with a Student’s t-test. Statistical significance was assigned for P values <0.05. All statistical analyses were used by the SPSS 17. Every experiment was repeated at least three times.

**Results**

CGRRAGGSC binds specifically to IL-11Rα in vitro

The binding of CGRRAGGSC to IL-11Rα was detected in vitro using MCF-7 cells and MCF-10A cells, which differ in the expression levels of IL-11Rα (Fig. 1). The IL-11Rα messenger RNA and protein expression levels were determined by quantitative RT-PCR and immunoblotting, respectively. The results demonstrated that IL-11Rα expression was higher in MCF-7 cells than in MCF-10A cells. In accordance with the IL-11Rα expression data, only the FITC-labeled CGRRAGGSC agent bound to MCF-7 cells when incubated with FITC-labeled CGRRAGGSC or FITC-labeled CGSPGWVRC, whereas negligible signals were detected in MCF-10A cells (Fig. 2). These results confirmed the specific binding of CGRRAGGSC to IL-11Rα expressed on the tumor cell.

![Fig. 1 Characterization of the expression of IL-11Rα on human breast tumor MCF-7 cells and normal breast MCF-10A cells. A: IL-11Rα mRNA expression in MCF-7 and MCF-10A cell lines, quantified by qRT-PCR and normalized to –action mRNA expression (* indicates P<0.05, n=3). B: IL-11Rα protein expression in MCF-7 and MCF-10A cell lines, quantified by Western blotting and normalized to GAPDH protein expression.](image-url)
NIR imaging and biodistribution of Cy7-CGRRAGGSC in vivo

NIR imaging of Cy7-CGRRAGGSC in MCF-7 xenograft nude mice enables tumor localization. Fig. 3A shows that the uptake of Cy7-CGRRAGGSC in the blood was up to 24 hours. The Cy7-CGRRAGGSC probe was rapidly concentrated in the tumor as early as 1 hour after intravenous injection, and the best tumor-to-background contrast was observed at 3 hours post injection (p.i.), as determined by continuous monitoring of changes in fluorescence intensity. Fluorescence intensities in the MCF-7 tumor tissue and the muscle were plotted as the time-activity

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Fig. 2 Fluorescence microscopy results of treating FITC-labelled CGRRAGGSC and FITC-labelled CGSPGWVRC to MCF-7 and MCF-10A cells. FITC-labeled CGRRAGGSC and FITC-labelled CGSPGWVRC are shown in green, and counterstaining of nuclei by DAPI is shown in blue. To test the binding specificity, MCF-7 cells were pre-incubated with unlabeled CGRRAGGSC or anti-IL-11Rα antibody for blocking. A blocking experiment by unlabeled CGRRAGGSC or anti-IL-11Rα antibody leads to strong signal reduction, sc  

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Fig. 3 Time-course fluorescence imaging of MCF-7-tumour-bearing nude mice after injection of Cy7-CGRRAGGSC or Cy7. A: Time-course fluorescence imaging of subcutaneous MCF-7-tumor-bearing nude mice after intravenous injection of 1.5 nmol of Cy7-CGRRAGGSC and Cy7. The tumor can be clearly visualized as indicated by an arrow from 0.5 to 24 hours p.i.. B: Quantification and kinetics of in vivo targeting characteristics of Cy7-CGRRAGGSC in the MCF-7 tumor vs. muscle. The Cy7-CGRRAGGSC uptake in the MCF-7 tumor at various time points was significantly higher than that in the muscle. Error bar was calculated as the standard deviation (n=4).
curve (Fig. 3B). The maximum of the MCF-7 tumor tissue uptake was found at 3 hours and slowly degraded over time. Conversely, the muscle had little probe binding and quickly faded away. Therefore, the probe uptake in the MCF-7 tumor tissue was significantly higher than that in the muscle during the 24-hour imaging period.

A blocking experiment was performed to confirm the IL-11Rα-targeted specificity of the probe Cy7-CGRRAGGSC. Mice bearing subcutaneous MCF-7 tumors were intravenously injected with Cy7-CGRRAGGSC in the blocking group, while mice in the non-blocking group were not given the 1.5 mg CGRRAGGSC dose. Fig. 4A shows the NIR fluorescence imaging of MCF-7-tumour-bearing mice at 3 hours post-injection (p.i.) from the non-blocking group (right) and the blocking group (left). The fluorescent signal in the non-blocking group was stronger than that in the blocking group. As shown in Fig. 4B, the tumor-to-muscle ratio of the non-blocking group and the blocking group at 3 hours p.i. was decreased from 6.45±0.82 to 1.93±0.42 (P<0.05). The cold CGRRAGGSC significantly decreased the concentration of the Cy7-CGRRAGGSC probe in the tumor in the blocking group compared to that in the non-blocking group.

Ex vivo evaluation of excised organs at 3 hours p.i. demonstrated that the fluorescence signal during in vivo imaging predominantly originated from the Cy7-CGRRAGGSC uptake in the tumor and kidney (Fig. 5A). The fluorescence signal of the gastrointestinal tract decreased after taking out the solid mouse chow (Fig. 5A). In addition, the tumor-to-liver ratio, the tumor-to-kidney ratio and the tumor-to-muscle ratio for the blocking group at 3 hours p.i. were calculated as 3.92±0.57, 3.23±0.91 and 4.48±0.90, respectively, while those for the non-blocking group were 5.06±0.98, 4.74±0.97 and 8.74±1.43, respectively (Fig. 5B). The MCF-7 tumor was confirmed by H&E staining examination, and IL-11Rα immunohistochemistry revealed positive staining in the MCF-7 tumor cells (Fig. 5D-F).

Discussion

Optical imaging is a non-invasive imaging technique that utilizes the unique properties of light or photons to obtain images of organs, tissues, cells and molecules. This imaging technique has many advantages. In contrast to other radiological imaging techniques, this method does not employ ionizing radiation and thus reduces the exposure of patients to harmful radiation. Thus, research procedures can be safely repeated over time to monitor disease progression. Moreover, in optical imaging, different fluorescent dyes can be utilized in the same subject to simultaneously acquire multiple images [21–23].

In our study, we first used FITC-labelled peptide to evaluate the binding properties of CGRRAGGSC in vitro. FITC is a commonly used fluorescent dye in cell research. Following incubation of breast tumor cells (MCF-7) and normal breast cells (MCF-10A) with FITC-CGRRAGGSC, specific binding of FITC-CGRRAGGSC to MCF-7 cells was observed. In addition, weak fluorescence intensities were detected in MCF-7 cells that were pre-treated with CGRRAGGSC or anti-IL-11Rα antibody, indicating that the free cyclic peptide CGRRAGGSC mediated by IL-11Rα effectively blocked the binding of FITC-labelled CGRRAGGSC to culture cells. The peptide CGRRAGGSC displayed specific binding to MCF-7 cells, correlating with the IL-11Rα expression level in MCF-7 cells.

![Fig. 4 A blocking experiment of the IL-11Rα-targeted specificity of the probe Cy7-CGRRAGGSC. A: Representative optical imaging (at 3 hours p.i.) of mice bearing the MCF-7 tumor on the right shoulder demonstrating blocking of Cy7-CGRRAGGSC (1.5 nmol) uptake by pre-injection of CGRRAGGSC (15 mg/kg) in an hour. The tumors are indicated by arrows. B: Fluorescence intensity ratio of tumor-to-muscle based on the ROI analysis of Cy7-CGRRAGGSC uptake at 3 hours p.i. in MCF-7 tumors with (blocking) or without (non-blocking) injection of CGRRAGGSC (10 mg/kg) in an hour. Error bar was calculated as the standard deviation (n=4). *P<0.05.](image-url)
The Cy7 dye is a commonly used NIR fluorescent dye. Its NIR window is ideally suited for in vivo imaging because of the minimal light absorption by haemoglobin and water in this window. The decreased absorption of light in the NIR region results in deeper tissue penetration, up to the level of centimeters. The present study evaluated the possibility of using Cy7 to label the cyclic peptide CGRRAGGSC to create an imaging agent for tumors expressing IL-11Rα.

The specificity of Cy7-CGRRAGGSC in vivo was confirmed by a blocking experiment with unlabelled CGRRAGGSC and an excess of Cy7-CGRRAGGSC using a subcutaneous MCF-7 breast tumor xenograft (Fig. 4). Both in vitro and in vivo studies demonstrated that the CGRRAGGSC peptide can target MCF-7 breast tumor cells and tissues expressing IL-11Rα (Fig. 2 and 4). In addition, ex vivo imaging demonstrated Cy7-CGRRAGGSC uptake by the liver, gastrointestinal tract and kidney, suggesting that the Cy7-CGRRAGGSC probe was primarily excreted through the hepatic pathway and urinary system (Fig. 5). The fluorescence signals in the gastric region decreased significantly after the solid mouse chow was removed, although some fluorescence signals still remained. We speculate that the fluorescence signals of the gastrointestinal tract are attributed to some intrinsic IL-11Rα expression or to the solid mouse chow, its aqueous extract and organ-specific autofluorescence[24–25]. Therefore, further studies are required to optimize the pharmacokinetics of the Cy7-CGRRAGGSC probe to reduce uptake by important normal organs.

Despite a lack of safety and toxicology studies, the NIR fluorescence dye Cy7 has been used to label a variety of small molecular peptides and antibodies for in vivo optical imaging due to its high photostability, bright fluorescence and simple conjugation[26]. The Cy7-CGRRAGGSC probe can be used as a reliable tool for evaluating IL-11Rα expression levels in breast tumors. Some researchers have reported that IL-11 and IL-11Rα possibly play a role in bone metastasis in breast tumors, which stimulates osteoclasts[12,27]. Other studies have also confirmed that IL-11 and IL-11Rα expressions in patients with breast tumors induces bone metastasis[13,28]. Therefore, the Cy7-CGRRAGGSC probe may be used to assess the risk of bone metastasis in patients with primary breast tumors by detecting IL-11Rα expression levels.

In conclusion, this study demonstrated that the Cy7-CGRRAGGSC probe can enable target-specific imaging of tumors expressing IL-11Rα. Therefore, targeting IL-11Rα via optical imaging can be potentially used for the non-invasive and highly sensitive detection and characterization of IL-11Rα-expressing lesions, thus enhancing our ability to assess the effects of therapeutic interventions in the near future.
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