Preclinical Evaluation of [^{99m}Tc]Tc-Labeled Anti-EpCAM Nanobody for EpCAM Receptor Expression Imaging By Immuno-SPECT/CT

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Abstract

Purpose Overexpression of epithelial cell adhesion molecule (EpCAM) plays essential roles in tumorigenesis and tumor progression in almost all epithelium-derived cancer. Monitoring EpCAM expression in tumors can be used for the diagnosis, staging and prognosis of cancer patients, as well as guiding the individualized treatment of EpCAM-targeted drugs. In this study, we described the synthesis and evaluation of a site-specifically [^{99m}Tc]Tc-labeled EpCAM-targeted nanobody for the SPECT/CT imaging of EpCAM expression.

Methods We first prepared the [^{99m}Tc]Tc-HYNIC- G_4K , then it was site-specifically connected to EpCAM-targeted nanobody NB4. The *in vitro* characteristics of [^{99m}Tc]Tc-NB4 were investigated in HT-29 (EpCAM-positive) and HL-60 (EpCAM-negative) cells, while the *in vivo* studies were performed using small-animal SPECT/CT in the subcutaneous tumor models and the lymph node metastasis model to verify the specific targeting capacity as well as the potential applications of [^{99m}Tc]Tc-NB4.

Results [^{99m}Tc]Tc-NB4 displayed a high EpCAM specificity both *in vitro* and *in vivo*. SPECT/CT imaging revealed that [^{99m}Tc]Tc-NB4 was cleared rapidly from the blood and normal organs except for the kidneys, and HT-29 tumors were clearly visualized in contrast with HL-60 tumors. The uptake value of [^{99m}Tc]Tc-NB4 in HT-29 tumors was increased continuously from 3.77 ± 0.39 %ID/g at 0.5 h to 5.53 ± 0.82 %ID/g at 12 h after injection. Moreover, the [^{99m}Tc]Tc-NB4 SPECT/CT could clearly image tumor-infiltrating lymph nodes.

Conclusion [^{99m}Tc]Tc-NB4 is a broad-spectrum, specific and sensitive SPECT radiotracer for the noninvasive imaging of EpCAM expression in the epithelium-derived cancer, and revealed a great potential for the clinical translation.

Introduction

Epithelial cell adhesion molecule (EpCAM) is a type I transmembrane glycoprotein which mediates Ca^{2+} -independent homotypic cell adhesion, acting as an important cancer biomarker [1, 2]. The high expression of EpCAM has been found on a great variety of human adenocarcinomas and squamous cell carcinomas like breast, lung, colon, renal, gastric, prostate and ovarian cancer [3, 4]. Its expression level in cancer correlates well with tumor progression and invasiveness of almost all epithelium-derived tumors. Overexpression of EpCAM in some tumors such as breast cancer, hepatocellular cancer, pancreatic cancer and ovarian cancer indicates poor therapeutic outcomes and prognosis [1, 5, 6]. Consequently, EpCAM is an ideal therapeutic target for the treatment of the most frequent EpCAM-positive human cancers. Monoclonal antibodies against EpCAM have been developed, from the earliest therapeutic antibody edrecolomab for gastrointestinal adenocarcinoma to the trifunctional, bispecific antibody catumaxomab that has been approved for clinical use today [7–9]. However, not all patients have good responses to these drugs, which may be related to the EpCAM expression level or heterogeneity of the tumor itself. We are unable to perform biopsy on each tumor, so the noninvasive assessment methods of

EpCAM expression are essential, which is of great significance for the diagnosis and prognosis of tumors, as well as guiding the subsequent treatment.

Molecular imaging can noninvasively detect dynamic molecular processes in normal or pathological conditions *in vivo*, offering unlimited possibilities for accurate diagnosis and sensitive tumor detection [10]. Accordingly, molecular imaging agents that target EpCAM would be highly useful in the detection of epithelium-derived tumors. Recent studies have been successfully performed on the development of radiotracers for *in vivo* EpCAM imaging, such as antibodies [11–13]. Variable heavy chain domain of heavy-chain-only antibodies (VHH), also known as nanobody, was derived from heavy-chain-only antibodies which were found naturally in Camelidae [14]. Nanobodies have a small molecular weight of 15 kDa, which makes it a better penetration of tissue, a rapid diffusion and a faster biodistribution [15]. Compared to the current EpCAM-targeted antibody molecular probes, the nanobody-based molecular probes have better tissue permeability and faster body clearance. Meanwhile, owing to the moderate radioactivity half-life period of 6.02 h, ^{99m}Tc is well suited for capturing the *in vivo* pharmacokinetics of nanobodies. In order to ensure the high activity and high stability of the radiotracer, site-specific radiolabeling is a pretty good strategy for proteins. Sortases are bacterial transpeptidases that are used for the efficient protein engineering [16–18]. Herein, we chose a G_4K short peptide (sequence: GGGGK) for the sortase A-mediated site-specific modification of the EpCAM-targeted nanobody, then prepared a [^{99m}Tc]Tc-labeled nanobody to image tumors in the subcutaneous tumor models and the tumor lymph node metastasis model by immuno-SPECT/CT, and compared with [^{18}F]FDG PET/CT imaging to investigate its EpCAM targeting capability.

Materials And Methods

Preparation of [^{99m}Tc]Tc-NB4

The LPETG-His₆ modified EpCAM-targeted nanobody (NB4) was provided by Prof. Yakun Wan (Shanghai Institute of Materia Medica, Shanghai, China). Chemicals and solvents were purchased from *Sigma-Aldrich* (St. Louis, MO, USA). The synthesis process of GGGGK-HYNIC (shorted as HYNIC- G_4K) is shown in the supplementary Information. HYNIC- G_4K was labeled with ^{99m}Tc using N-tris-hydroxymethyl-methylglycine (Tricine) and trisodium triphenylphosphine-3,3',"-trisulfonate (TPPTS) as the co-ligands to obtain [^{99m}Tc]Tc-HYNIC- G_4K . HYNIC- G_4K (0.01 μmol) was mixed with combined solutions of 5 mg of tricine and 5 mg of TPPTS (in 150 μL of 50 mM succinate buffer, pH 4.9). [^{99m}Tc]NaTcO₄ (370 MBq) was then added to the solution and stirred at 100 °C for 20 min. The radiochemical purity of [^{99m}Tc]Tc-HYNIC- G_4K was determined by radio-HPLC. 500 μL reaction mixtures contained 50 mM Tris-HCl, 10 mM CaCl₂, 100 mM NaCl, 200 μM [^{99m}Tc]Tc-HYNIC- G_4K , 200 μM NB4, and 50 μM sortase A. The pH of the mixture was adjusted to 7.5 and the mixture was stirred for 20 min at 37°C. The radiochemical purity of [^{99m}Tc]Tc-NB4 was determined by Instant Thin Layer Chromatography Medium (iTLC, AR-2000 (Bioscan, USA)). Then the mixture was purified by size-exclusion chromatography HPLC using Superose 12 (GE Life

Science, Pittsburgh, USA). The radiochemical purity of [^{99m}Tc]Tc-NB4 was higher than 97% after purification.

Cell and Animal Models

The HT-29 human colorectal adenocarcinoma cell line was purchased from American Type Culture Collection (Manassas, VA, USA). The HL-60 human acute promyelocytic leukemia cell line was provided by Prof. Xiaoyan Qiu (NHC Key Laboratory of Medical Immunology, Peking University). Cells were maintained under standard conditions according to ATCC. HT-29 cells were transcribed for its luciferase gene. The HT-29 cells and HT-29-Luc cells grew in DMEM/F12 medium supplemented with 5% fetal bovine serum (FBS). The HL-60 cells grew in RPMI-1640 medium supplemented with 10% FBS. Both cancer cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO₂.

All animal experiments were performed in accordance with the guidelines of the Peking University Animal Care and Use Committee. BALB/c nude mice (4 weeks of age) were obtained from Department of Laboratory Animal Science of Peking University. To establish HT-29 subcutaneous tumor models, 5×10^6 HT-29 cells were inoculated subcutaneously into the right front flanks of female BALB/c nude mice. For the HT-29/HL-60 bilateral tumor model, 5×10^6 HL-60 cells (in 100 μL of PBS, which was mixed with 50% Matrigel (Corning, NY, USA)) were subcutaneously inoculated into the left front flanks of female BALB/c nude mice. After that, 5×10^6 HT-29 cells were subcutaneously inoculated into the right front flanks of the same female BALB/c nude mice 6 days later. The animals were used for *in vivo* studies when the tumor size reached 200-300 mm³ (2–3 week after inoculation).

For lymph node metastasis model, 2×10^5 HT-29-Luc cells were injected into the left front and hind footpads of female BALB/c mice. The growth of lymph node metastatic tumors was monitored by bioluminescence imaging (BLI) as previously described. Every 7 days, we used BLI to detect lymph node metastasis, and during each BLI we used black tape to completely cover the tumors in footpad to reduce in situ autofluorescence interference. Animal models were used for *in vivo* studies when tumor lymph node metastasis was detected.

Binding of NB4 to EpCAM

To verify the cross-species of EpCAM-targeted NB4, human, rhesus and mouse EpCAM proteins were used to bind with NB4. [^{125}I]I-NB4 was prepared by labeling NB4 with [^{125}I]NaI using the Iodogen method, as previously described. [^{125}I]I-NB4 (3.0 kBq) was added to 96-well Stripwell™ enzyme-linked immunosorbent assay (ELISA) plates (Costar, Cambridge, MA) coated with 0.2 μg human, rhesus or mouse EpCAM protein per well with or without an excess dose of cold NB4 (4 μg /well). After incubation for 1 h at 37°C, the plates were washed with ice-cold PBS containing 0.05% Tween-20 (PBST) to remove free radioactivity, and wells were collected and measured in a calibrated γ -counter (Wallac 1470-002, Perkin Elmer, Finland). All experiments were performed twice with four samples for each. Results were expressed as CPM per 1 pmol protein.

The identification method of EpCAM expression level on HT-29 and HL-60 cells is shown in the supporting information. For cell binding assays, [^{99m}Tc]Tc-NB4 was prepared as mentioned above. HT-29 and HL-60 tumor cells were digested to obtain single-cell suspensions and were transferred to Eppendorf tube (2×10^6 cells in 200 μL of PBS with 1% BSA per tube). [^{99m}Tc]Tc-NB4 (7.4 kBq) was added to each tube with or without an excess dose of cold NB4 (1000 folds excess) for 2 h at 4°C. The cells were washed with ice-cold PBST soon afterward to remove free radioactivity. Then the cells with bound radioactivity were collected, and the radioactivity was measured in a calibrated γ -counter. All experiments were performed twice with four samples for each. Results were expressed as CPM per 10^6 cells.

To evaluate the expression status of EpCAM in HT-29 and HL-60 cells, approximately 1×10^5 HT-29/HL-60 cells were seeded into confocal dishes. Cells were incubated with NB4 as primary antibodies for 2 h at room temperature after blocking with 5% FBS in PBS, followed by incubation with Anti-6X His tag antibody with DyLight 650 (Abcam, Cambridge, United Kingdom) as secondary antibody for 1 h at room temperature and visualized using a confocal microscope (Wetzler, Heidelberg, Germany).

Small-Animal PET/CT and SPECT/CT Imaging

The small-animal SPECT/CT imaging was performed on BALB/c nude mice bearing HT-29 cancer xenografts and HT-29/HL-60 bilateral cancer xenografts. Mice were allowed to wake up after probe injection. The pinhole SPECT images (peak: 140 keV, 20% width; frame time: 30s) were acquired for 27 minutes and subsequently CT images were acquired (50 kVp, 0.67 mA, rotation 210°, exposure time: 300 ms). All SPECT images were reconstructed and further analyzed with Fusion (Mediso, Budapest, Hungary).

For the HT-29 tumor model, each tumor-bearing nude mouse was injected via the tail vein with 18 MBq of [^{99m}Tc]Tc-NB4. At 30 min, 1 h, 2 h, 4 h, 8 h, and 12 h post-injection (p.i.), the mice were anesthetized by inhalation of 2% isoflurane and imaged using nanoScan following a standard protocol. For the HT-29/HL-60 bilateral tumor model, each tumor-bearing nude mouse was injected via the tail vein with 18 MBq of [^{99m}Tc]Tc-NB4. At 30 min, 1 h, 2 h p.i., the mice were imaged using the same method using nanoScan.

The small-animal PET/CT imaging was performed on BALB/c nude mice bearing HT-29/HL-60 bilateral cancer xenografts. Each tumor-bearing nude mouse was injected via the tail vein with 18 MBq of [^{18}F]FDG. Mice were kept unconscious under 2% isoflurane for 60 min p.i. to prevent tracer uptake in muscles. The body temperature of unconscious mice was maintained by a controllable electric blanket. At 1 h p.i., the mice were imaged using nanoScan (Mediso, Budapest, Hungary) following a standard protocol.

For the imaging of lymph node metastasis model, each HT-29-Luc tumor-bearing BALB/c nude mouse was injected via the tail vein with 18 MBq of [^{99m}Tc]Tc-NB4. SPECT images were acquired at 3 h p.i. using nanoScan. In order to determine the probe aggregation of the tumor-infiltrating lymph nodes, 10 μL 1% Evans Blue were injected into each paw pad, and the mice were sacrificed after 3 minutes p.i. The

bilateral axillary lymph nodes and popliteal lymph nodes of the mice that were stained blue by Evans Blue were removed and placed next to the mice to re-execute SPECT/CT imaging.

Biodistribution of [^{99m}Tc]Tc-NB4

For HT-29 tumor model's *ex vivo* biodistribution, female nude mice bearing HT-29 tumor xenografts were injected by tail vein with 0.37 MBq of [^{99m}Tc]Tc-NB4 to evaluate the distribution of [^{99m}Tc]Tc-NB4 in major organs and tumors (n = 4 per group). Meanwhile, cold NB4 was calculated and co-injected to ensure that the doses of SPECT/CT imaging and biodistribution were consistent. The mice were sacrificed and dissected at 30 min, 1 h, 2 h, 4 h, 8 h and 12 h p.i., and tumor, kidney, blood and other major organs were collected and weighted. Samples and prime standards were counted for radioactivity in a calibrated γ -counter. The *ex vivo* tissue activity is presented as the percentage of injection dose per gram of tissue (%ID/g). The blocking study was also performed in HT-29 mice by a co-injection of 0.37 MBq of [^{99m}Tc]Tc-NB4 with an excess dose of cold NB4 (1 mg). At 2 h p.i., the blocked mice were sacrificed and dissected. Then the organ biodistribution of [^{99m}Tc]Tc-NB4 was determined.

For HT-29/HL-60 bilateral tumor model's *ex vivo* biodistribution, mice bearing HT-29/HL-60 bilateral tumor xenografts were injected with 0.37 MBq of [^{99m}Tc]Tc-NB4 to evaluate the distribution of [^{99m}Tc]Tc-NB4 in major organs and tumors (n = 4 per group). Subsequently, organs and tissues were excised and weighed at 2 h p.i. Samples and prime standards were counted for radioactivity in a calibrated γ -counter. The *ex vivo* tissue activity is also presented as %ID/g.

Statistical Analysis

Quantitative data are expressed as the mean $\pm$ SD. Statistical analysis of image quantification and biodistribution were performed with one-way analysis of variance and Student's t-test with GraphPad Prism 6.0 (GraphPad Software, Inc.). $P < 0.05$ was considered statistically significant.

Results

Chemistry and Radiochemistry

HYNIC-G₄K (Fig. 1a) was obtained in 43.3% yield and was confirmed by mass spectroscopy analysis. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry detected 678.2(m/z) (C₂₇H₃₅N₉O₁₀S, Exact Mass: 677.22) (Fig. S1a). The [^{99m}Tc]Tc-labeling procedure of HYNIC-G₄K was done within 20 minutes, and the radiochemical purity of [^{99m}Tc]Tc-HYNIC-G₄K was greater than 98% as determined by radio-HPLC (Fig. S1b). The preparation of [^{99m}Tc]Tc-labeled EpCAM-targeted NB4 nanobody, [^{99m}Tc]Tc-HYNIC-G₄K-NB4(Tricine)(TPPTS) (shorted as [^{99m}Tc]Tc-NB4), was also done within 20 minutes. Using physiological saline as a developing agent, the radiolabeling yield determined by iTLC was about 60% (Fig. S2a). After the purification of Superose 12, the radiochemical purity of [^{99m}Tc]Tc-

NB4 was greater than 97% as determined by iTLC (Fig. S2b). The structure of [^{99m}Tc]Tc-NB4 is shown in Fig. 1b. The synthetic route for [^{99m}Tc]Tc-NB4 is shown in Fig. 1c.

***In vitro* verification of EpCAM-targeted specificity of NB4**

We first validated by flow cytometry that HT-29 cells highly expressed EpCAM, and HL-60 was EpCAM negative (Fig. S2c), consistent with previous experimental studies. Protein binding experiments showed that NB4 was highly selective for EpCAM proteins of different species (Fig. 2a). NB4 bound almost exclusively to the human EpCAM protein, while having little binding to murine and rhesus EpCAM proteins (1255.59 ± 25.11 vs. 14.89 ± 3.02 ; $P < 0.0001$, and 1255.59 ± 25.11 vs. 19.19 ± 7.25 ; $P < 0.0001$). The excessive cold NB4 could significantly inhibit the binding of ^{125}I -NB4 to human EpCAM (from 1255.59 ± 25.11 to 66.75 ± 7.97 ; $P < 0.0001$). In cell-binding assays, the binding value (CPM per 10^6 cells) of [^{99m}Tc]Tc-NB4 to HT-29 cells was much higher than that to HL-60 cells (73412.20 ± 5689.99 vs. 108.69 ± 30.65 ; $P < 0.01$) (Fig. 2b). Meanwhile, the binding of [^{99m}Tc]Tc-NB4 to HT-29 cells was significantly inhibited by the addition of an excess cold NB4 (from 73412.20 ± 5689.99 to 1337.61 ± 43.13 ; $P < 0.01$). Fluorescent staining revealed that NB4 accumulated on the cell membrane of HT-29 cells where EpCAM was expressed (Fig. 2c), whereas HL-60 cells could hardly be stained with NB4. These results suggest that NB4 has a great specificity for EpCAM *in vitro*.

SPECT/CT Imaging

The SPECT/CT imaging was performed in mice with HT-29 tumor xenografts at 30 min, 1 h, 2 h, 4 h, 8 h and 12 h after the injection of [^{99m}Tc]Tc-NB4, and the images showed a time-dependent tumor accumulation of radiotracer (Fig. 3a). At 2 h p.i., the uptake of [^{99m}Tc]Tc-NB4 in other organs was reduced to a lower level due to the rapid clearance of nanobody from the body, while the uptake of [^{99m}Tc]Tc-NB4 in kidneys and bladder was higher because nanobody is mainly metabolized by the urinary system.

***Ex Vivo* Biodistribution**

In order to evaluate the distribution of [^{99m}Tc]Tc-NB4 *in vivo*, animals were sacrificed at designated time points, and the results were summarized in Fig. 3b and Table S1. The increase of tumor uptake over time in imaging experiments was basically consistent with that in biodistribution. The HT-29 tumor uptake values of [^{99m}Tc]Tc-NB4 were 3.77 ± 0.39 , 4.02 ± 0.70 , 4.36 ± 0.46 , 4.33 ± 0.82 , 5.02 ± 0.67 and 5.53 ± 0.82 %ID/g at 30 min, 1 h, 2 h, 4 h, 8 h and 12 h p.i., showing an upward trend over time. Nevertheless, due to the rapid clearance of unbound probe *in vivo*, the uptake values of [^{99m}Tc]Tc-NB4 in blood and muscle tissue tended to decrease over time. Taking muscle tissue as an example, the uptake values were 0.47 ± 0.25 , 0.22 ± 0.08 , 0.13 ± 0.14 , 0.08 ± 0.06 , 0.10 ± 0.08 and 0.03 ± 0.01 %ID/g at 30 min, 1 h, 2 h, 4 h, 8 h and 12 h p.i. Accordingly, the values of Tumor/Muscle changed from 9.38 ± 3.38 at 30 min p.i. to 175.30 ± 79.66 at 12 h p.i. (Fig. 3c). In other words, the tumor uptake of [^{99m}Tc]Tc-NB4 was significantly higher than that in the blood and most other normal organs at almost all time points examined. The uptake value of kidneys was highest because [^{99m}Tc]Tc-NB4 is primarily excreted by the kidneys.

In the blocking study (Fig. 3d), mice were co-injected with [^{99m}Tc]Tc-NB4 and cold NB4 which was more than 100 times the dose of [^{99m}Tc]Tc-NB4. The tumor uptake (3.73 ± 0.37 %ID/g) in blocking group was not significantly different from the [^{99m}Tc]Tc-NB4 group. All organs showed similar uptake levels in two groups. Due to the large abundance of EpCAM expressed on the cell membrane, co-injection of 100-fold excess cold NB4 could not effectively block the uptake of [^{99m}Tc]Tc-NB4 in tumors.

[^{99m}Tc]Tc-NB4 SPECT/CT and [^{18}F]FDG PET/CT in the Bilateral Tumor Model

In order to validate the EpCAM-targeted specificity of [^{99m}Tc]Tc-NB4 imaging, we established the bilateral tumor-bearing mouse model according to the time line in Fig. 4a to keep the same size of tumors. We first performed the biodistribution experiment to compare tumor uptake on both sides, while the uptake in EpCAM-positive tumors was much higher than that in EpCAM-negative tumors (HT-29: 5.73 ± 1.45 %ID/g vs. HL-60: 0.18 ± 0.17 %ID/g, $P < 0.01$) (Fig. 4b). For the [^{99m}Tc]Tc-NB4 SPECT/CT imaging, the HT-29 tumors were clearly visible, whereas there was almost no radioactive signal in HL-60 tumors (Fig. 4c), which was consistent with the biodistribution result. For the [^{18}F]FDG PET/CT imaging, both HT-29 and HL-60 tumors could be clearly visualized (SUV 2.95 vs. 2.44) (Fig. 4c), which could not distinguish EpCAM-positive and EpCAM-negative tumors. As the immunofluorescence staining results (Fig. 4d), the HT-29 tumor tissues showed high Dylight-NB4 immunoreactivity. In contrast, HL-60 tumor tissues were hardly stained with Dylight-NB4.

[^{99m}Tc]Tc-NB4 SPECT/CT in lymphatic metastasis model

In order to verify the sensitivity of the probe, we established a lymphatic metastasis model by footpad inoculation of HT-29-Luc tumor cells into the left front and hind footpads of mice. When the *in vivo* BLI showed HT-29 tumor metastases in the left axillary lymph nodes and popliteal lymph nodes of mice on day 21, the [^{99m}Tc]Tc-NB4 SPECT/CT imaging was carried out in this model two days later (Fig. 5a). As showed in Fig. 5b, although the lymph nodes were very small, they were also clearly visualized, and the probe uptake in tumor-infiltrating lymph nodes was markedly higher compared to that of the control contralateral normal lymph nodes.

In order to further verify the probe aggregation in the lymph nodes, we used 1% Evans Blue by footpad injection to locate lymph nodes, then surgically removed axillary and popliteal lymph nodes and placed them next to the mouse to re-image. The radioactive signals of the original lymph nodes disappeared (Fig. 5c), which proved that the location of the probe aggregation was indeed the lymph node area.

Tumor-infiltrating lymph nodes and normal lymph nodes were collected after SPECT imaging and subjected to H&E staining and immunofluorescence staining. Compared with the normal lymph nodes, the tumor infiltrating lymph nodes showed obvious cancer nests, mitotic figures and atypia (Fig. 6a), as well as the higher Dylight-NB4 immunoreactivity (Fig. 6b).

Discussion

EpCAM plays a normal physiological function in normal tissues as a functional molecule, and its overexpression in tumor tissues makes it an excellent diagnostic and therapeutic target. Previous studies have shown that the expression level of EpCAM in breast cancer, liver cancer and pancreatic cancer is associated with poor prognosis, whereas the overexpression of EpCAM molecules in thyroid cancer and renal cancer has a good prognosis [23]. EpCAM can inhibit or promote tumor growth in different tumor types, thereby the detection of EpCAM can provide a guidance to make the treatment plan for cancer patients. In this study, we developed a new radiolabeled EpCAM-targeted nanobody [^{99m}Tc]Tc-NB4, which can be used as a broad-spectrum tumor imaging agent for the diagnosis, staging and prognosis. In the meanwhile, the development of EpCAM-specific antibodies in recent years offers the possibility of EpCAM-targeted immunotherapy. However, due to the tumor heterogeneity and different EpCAM expression levels of patients, not all patients can benefit from the immunotherapy [24]. By monitoring the expression level of EpCAM *in vivo* during immunotherapy, [^{99m}Tc]Tc-NB4 SPECT/CT can be used to guide the individualized treatment in clinic.

In this study, we used the site-specific radiolabeling strategy to prepare the nanobody probe [^{99m}Tc]Tc-NB4. The stable imaging quality up to 12 hours indirectly demonstrate the high stability of the [[^{99m}Tc]Tc-HYNIC(TPPTS)(Tricine)] site-specific radiolabeling system *in vivo* (Fig. 3a). This strategy is very important for reducing nonspecific radioactive signals in organs other than tumors. The rapid decrease of [^{99m}Tc]Tc-NB4 in blood and other organs ensures the low toxicity of [^{99m}Tc]Tc-NB4 as a imaging agent. We did not choose a radiolabeled monoclonal antibody for nuclear medicine imaging because its imaging time is 3-7 days p.i., which is not convenient for clinical application. [^{99m}Tc]Tc-NB4's rapid distribution and high signal-to-noise ratio *in vivo* (Fig. 3b-c) ensure the need for the fast noninvasive diagnostic imaging, which is a great advantage for clinical application. These make our radioactive probe can be used for the further confirmation when a clinically routine CT/MRI examination produces a suspected abnormality.

As the most widely used nuclear medicine imaging agent, [^{18}F]FDG is used to detect tumors in clinical, but it is not a specific imaging agent and cannot judge the expression of EpCAM in tumors (Fig. 4c). As an EpCAM-targeted tumor imaging agent, [^{99m}Tc]Tc-NB4 showed the high EpCAM specificity at protein and cellular levels. In animal models, [^{99m}Tc]Tc-NB4 SPECT/CT revealed the rapid accumulation and relatively high uptake in subcutaneous EpCAM-positive HT-29 tumors, while it was unable to image EpCAM-negative HL-60 tumors (Fig. 4c). This further showed the high specificity of the probe. The current study showed that the expression of EpCAM on the surface of each tumor cell exceeded 2×10^6 receptors/cell for HT-29 and some other tumors, which is a receptor with high abundance [13, 25], therefore the excessive unlabeled NB4 could not saturate binding sites in HT-29 tumors (Fig. 3d). The high specificity of NB4 was further proved by immunofluorescence staining of tumor tissues.

The HT-29 tumor uptake of [^{99m}Tc]Tc-NB4 increased with time, while the uptake in other organs except for the kidneys rapidly decreased (Fig. 3). We speculate that the increase of tumor uptake is related to cell internalization. Internalization experiments demonstrated that the internalization process of [^{99m}Tc]Tc-

NB4 was a concentration-dependent process rather than a receptor-mediated internalization process (Fig. S3). Meanwhile, the [^{99m}Tc]Tc-NB4 entered into the cells by internalization would not be released to the extracellular space by the exocytosis of the cells (Fig. S3D). The above results prove our guess to some extent.

Clinical staging and prognosis were evaluated by lymph node metastasis. SPECT/CT imaging with [^{99m}Tc]Tc-NB4 could accurately locate small lymph node metastasis tumors (< 5 mm in diameter) (Fig. 5b). The clear imaging of tumor-infiltrating lymph nodes showed a high sensitivity of [^{99m}Tc]Tc-NB4, which makes the early diagnosis of tumor and tumor metastasis possible. [^{99m}Tc]Tc-NB4 could accurately distinguish tumor lymph nodes from normal lymph nodes (Fig. 5c), which can help physicians to stage patients and further formulate surgery and treatment strategies.

The resolution and sensitivity of clinical SPECT/CT imaging were relatively lower than PET/CT imaging, and ^{99m}Tc drugs were relatively limited in technology development, so the overall development of ^{99m}Tc SPECT/CT for tumors was relatively slow [26]. However, in the past 20 years, on the one hand, the progress of technetium chemistry has promoted the development of new ^{99m}Tc radiopharmaceuticals; On the other hand, the development of detector technology and the progress of reconstruction algorithm in imaging equipment make the spatial resolution of SPECT closer to that of PET, and the sensitivity does not decrease [27]. These progresses bring new development opportunities for SPECT/CT technology. Furthermore, the optimal imaging time of nanobody probes is also perfectly consistent with the half-life of ^{99m}Tc , thus [^{99m}Tc]Tc-NB4 SPECT/CT has a great potential for the clinical translation.

Conclusions

The present study developed and evaluated a new nanobody-based molecular probe [^{99m}Tc]Tc-NB4 targeting EpCAM for SPECT/CT imaging. [^{99m}Tc]Tc-NB4 revealed the high specificity and sensitivity, and could clearly image EpCAM-positive tumors and lymph node metastasis *in vivo*. This probe can be used as a broad-spectrum probe for the diagnosis, staging and prognosis of cancer patients. Furthermore, it can be used to guide the individualized treatment of EpCAM-targeted drugs. [^{99m}Tc]Tc-NB4 SPECT/CT has a great potential for the clinical translation.

Declarations

Founding information

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Compliance with ethical standards

Conflict of interest

The authors declare no competing financial interest.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

Supplementary information

Results: internalization and externalization assays of [^{99m}Tc]Tc-labeled NB4; Experimental Section: Preparation of triglycine-containing peptide, Flow cytometry analysis, Internalization and externalization assays, Staining of tumor and lymph nodes tissues; Fig. S1-S3; Table S1.

All the information above was provided in supporting information.

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Figures

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(a-b) Structure of GGGGK-HYNIC and [^{99m}Tc]Tc-NB4. (c) Synthetic route of [^{99m}Tc]Tc-NB4.

Figure 2

(a) Protein binding of [125I]-NB4 to human, rhesus and mouse EpCAM. ****, $P < 0.0001$. (b) Cell binding of [99mTc]-NB4 to HT-29 (EpCAM-positive) and HL-60 (EpCAM-negative) tumor cells. **, $P < 0.01$. (c) EpCAM immunofluorescence staining in HT-29 and HL-60 tumor cells with NB4 as the primary antibody and anti-His tag antibody with DyLight as the secondary antibody.

Figure 3

(a) Representative static SPECT/CT images of the HT-29 tumor-bearing mice (EpCAM-positive, red dashed circle) administered with 18 MBq $[^{99m}\text{Tc}]\text{Tc-NB4}$. K, T and B indicate kidney, tumor and bladder, respectively. (b) Biodistribution of $[^{99m}\text{Tc}]\text{Tc-NB4}$ in HT-29 tumor-bearing mice. (c) Tumor/Muscle ratios of $[^{99m}\text{Tc}]\text{Tc-NB4}$ in HT-29 tumor-bearing nude mice. (d) Blocking study of $[^{99m}\text{Tc}]\text{Tc-NB4}$ biodistribution in HT-29 tumor-bearing nude mice at 2 h after injection. Blocking group was co-injected with an excess cold NB4 (1 mg) as a blocking agent. ns, $P > 0.05$.

Figure 4

(a) Schedule of $[^{18}\text{F}]\text{FDG}$ PET/CT and $[^{99\text{m}}\text{Tc}]\text{Tc-NB4}$ SPECT/CT imaging. (b) Biodistribution of $[^{99\text{m}}\text{Tc}]\text{Tc-NB4}$ in HT-29/HL-60 bilateral tumor-bearing mice at 2 h after injection. **, $P < 0.01$ (c) Representative images of $[^{99\text{m}}\text{Tc}]\text{Tc-NB4}$ SPECT/CT and $[^{18}\text{F}]\text{FDG}$ PET/CT in the HT-29/HL-60 bilateral tumor-bearing mice. (d) Immunofluorescence staining of EpCAM in HT-29 and HL-60 tumor tissues.

Figure 5

(a) Timeline illustration of tumor inoculation, BLI optical imaging and $[^{99m}\text{Tc}]\text{Tc-NB4}$ SPECT/CT imaging. (b) BLI of the HT-29-Luc lymph node (LN) metastasis model on day 21, and representative $[^{99m}\text{Tc}]\text{Tc-NB4}$ SPECT/CT images in this model at 3 h p.i. Coronal 2D images of axillary and popliteal LNs are shown. Yellow arrows refer to lymph node metastases. (c) Re-imaging of surgically removed LNs to verify the $[^{99m}\text{Tc}]\text{Tc-NB4}$ accumulation in tumor-infiltrating lymph nodes.

Figure 6

H&E staining (a) and Immunofluorescence staining (b) of tumor-infiltrating lymph nodes and normal lymph nodes.

Supplementary Files

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