Tc-99m and Fluorescence-Labeled Anti-Flt1 Peptide as a Multimodal Tumor Imaging Agent Targeting Vascular Endothelial Growth Factor-Receptor 1

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Abstract

Purpose We developed a Tc-99m and fluorescence-labeled peptide, Tc-99m TAMRA-GHEG-ECG-GNQWFI, to target tumor cells, and evaluated the diagnostic performance as a dual-modality imaging agent for tumor in a murine model.

Methods TAMRA-GHEG-ECG-GNQWFI was synthesized using Fmoc solid-phase peptide synthesis. Radiolabeling of TAMRA-GHEG-ECG-GNQWFI with Tc-99m was done using ligand exchange via tartrate. Binding affinity and in vitro cellular uptake studies were performed. Gamma camera imaging, biodistribution, and ex vivo imaging studies were performed in murine models with U87MG tumors. Tumor tissue slides were prepared and analyzed with immunohistochemistry using confocal microscopy.

Results After radiolabeling procedures with Tc-99m, Tc-99m TAMRA-GHEG-ECG-GNQWFI complexes were prepared in high yield (> 95%). The K_d of Tc-99m TAMRA-GHEG-ECG-GNQWFI determined by saturation binding was 29.5 ± 4.5 nM. Confocal microscopy images of U87MG cells incubated with TAMRA-GHEG-ECG-GNQWFI showed strong fluorescence in the cytoplasm. Gamma camera imaging revealed substantial uptake of Tc-99m TAMRA-GHEG-ECG-GNQWFI in tumors. Tumor uptake was effectively blocked by the co-injection of an excess concentration of GNQWFI. Specific uptake of Tc-99m TAMRA-GHEG-ECG-GNQWFI was assessed by biodistribution, ex vivo imaging, and immunohistochemistry stain studies.

Conclusions In vivo and in vitro studies revealed substantial and specific uptake of Tc-99m TAMRA-GHEG-ECG-GNQWFI in tumor cells. Tc-99m TAMRA-GHEG-ECG-GNQWFI could be a good candidate dual-modality imaging agent for tumors.

Keywords Vascular endothelial growth factor-receptor 1 · Anti-Flt1 peptide · Tc-99m · TAMRA · Multimodal imaging

Introduction

Vascular endothelial growth factor (VEGF) was identified as an endothelial cell-specific mitogen that has the capacity to induce physiological and pathological angiogenesis, and VEGF is one of the key angiogenic factors involved in the growth and malignant progression of tumors [1, 2]. This VEGF is now known as VEGF-A and is a member of a larger

family of growth factors, and VEGF-A induces various intracellular signaling and physiologic responses that are essential for angiogenesis [3]. VEGF-A acts mainly through two receptor tyrosine kinases: VEGF receptors 1 (VEGFR1, Flt1) and receptors 2 (VEGFR2, Flk-1/KDR), which are mainly expressed in endothelial cells [4]. Although the expression of these receptors was initially thought to be limited to endothelial cells, both receptors are overexpressed on the endothelium of tumor vasculature, yet they are almost undetectable in the vascular endothelium of adjacent normal tissues [5, 6]. These unique characteristics make the VEGFR1 and VEGFR2 as an attractive target for molecular imaging of tumor.

Bae et al. had identified a VEGFR1-selective hexapeptide, Gly-Asn-Gln-Trp-Phe-Ile (GNQWFI, named as anti-Flt1), through screening of synthetic peptide library, and a selected peptide, anti-Flt1, was investigated for binding specificity with various receptors and ligand peptides [7]. They had

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reported that the VEGFR1-specific anti-Flt1 peptide inhibits VEGF-induced endothelial cell migration and morphogenesis. Furthermore, this peptide inhibits growth and metastasis of tumor cells in mice. After then, several researchers had reported about specific binding affinity of the anti-Flt1 peptide to VEGFR1, and this peptide had been evaluated as a therapeutic agent for arthritis, retinal neovascularization, and diabetic nephropathy [8–11]. Considering the results of prior reports, the anti-Flt1 peptide is an excellent candidate as a target molecule for molecular imaging of tumor cells. Despite the desirable specific binding of the anti-Flt1 peptide to VEGFR1, there are no earlier efforts to develop the imaging agent targeting tumor cells using the anti-Flt1 peptide. Molecular imaging targeted to tumor cells has strong potential for both clinical use and as a research tool in the molecular biology of tumor cells and the development of therapeutic agents.

Multimodality imaging, a combination of two or more imaging modalities, may provide a better solution to overcome the limitations of the independent techniques [12]. The present study had developed a dual-modality imaging agent which is combining a radionuclide (Tc-99m) and a fluorescent agent (5-carboxytetramethylrhodamine, TAMRA). TAMRA allows optical imaging for high-resolution and real-time imaging [13]. Fluorescence agents like TAMRA, green fluorescent protein (GFP), and 7-diethylaminocoumarin-3-carboxylic acid (DEAC), which is having shorter emission wavelength than near-infrared (NIR) fluorescent dyes, are limited by poor tissue penetration. Still, the fluorescence can be visualized by fluorescent endoscopy [14, 15]. Nuclear imaging with Tc-99m offers excellent sensitivity and exhibits no limitation for tissue penetration [16, 17].

In the present study, we developed Tc-99m TAMRA-Gly-His-Glu-Gly-Glu-Cys-Gly-Gly-Asn-Gln-Trp-Phe-Ile (Tc-99m TAMRA-GHEG-ECG-GNQWFI) to target the tumor cells. Simultaneous labeling with Tc-99m and TAMRA could provide both nuclear and optical imaging, and the GHEG ligand was incorporated as a linker or spacer. The ECG ligand could provide strong and stable chelation with Tc-99m. The present study had evaluated the diagnostic performance of Tc-99m TAMRA-GHEG-ECG-GNQWFI as a dual-modality imaging agent for the tumors in a murine model.

Experimental Section

Materials

1N-HCl, acetone, SnCl₂, and sodium tartrate were purchased from Sigma Aldrich Korea (Seoul, Korea). Tc-99m pertechnetate was eluted from a commercial technetium generator (Tyco Healthcare, Mallinckrodt, Dublin, Ireland) at our institution. Instant thin-layer chromatography-silica gel (ITLC-SG) capillary column was purchased from

FutureChem (Seoul, Korea). U87MG human glioblastoma cells were obtained from the Korean Cell Line Bank (Seoul, Korea).

Synthesis and Characterization of Peptides

TAMRA-GHEG-ECG-GNQWFI, TAMRA-GHEG-ECG-QIFGWN (scrambled peptide), and GNQWFI (free target peptide) were synthesized commercially by Pepton, Inc. (Daejeon, Korea) at a purity grade exceeding 96%. Briefly, the peptides were synthesized using Fmoc solid-phase peptide synthesis (SPPS) with ASP48S (Pepton Inc.). The resulting peptide-bound resin was treated with TAMRA-succinimidyl ester and diisopropylethylamine in N-methyl-2-pyrrolidone. The synthesized compound was purified using reverse-phase high-performance liquid chromatography (RP-HPLC) with a Vydac Everest column (C18, 250 × 22 mm, 10 μm). Gradients used for elution were linear from 0 to 70% acetonitrile in water containing 0.1% trifluoroacetic acid (TFA). Liquid chromatography/mass spectrometry (LC/MS) was used to confirm the purified peptide molecular weight.

Radiolabeling with Tc-99m

Radiolabeling of TAMRA-GHEG-ECG-GNQWFI and TAMRA-GHEG-ECG-QIFGWN with Tc-99m was done as described previously [18–20]. Briefly, a mixed solution of peptide solution (0.005 mg/ml in 300 μl nitrogen-purged water) and sodium tartrate (100 mg/ml in 300 μl nitrogen-purged water) was prepared in a microcentrifuge tube. This solution was amended with fresh Tc-99m pertechnetate (1.0 ml, about 1110 MBq) and SnCl₂ (1 mg/ml in 30 μl nitrogen-purged 0.01 M HCl). The solution was heated at 95 °C for 15 min and cooled at room temperature. Characterization of radiolabeled peptides was done by radio-RP-HPLC (Alltech 305, Deerfield, IL) using a 4.6 × 250-mm C18 column with a particle size of 5 μm. A linear 30 to 70% gradient used for elution consisted of acetonitrile in water containing 0.1% TFA for 30 min at a flow rate of 2 ml/min. An ultraviolet detector (220 nm) and a gamma radiodetector were used for monitoring. Because only a single peak was detected, radio-RP-HPLC of the radiolabeled peptides was not performed for in vivo and in vitro studies.

The radiolabeled peptide was assessed for in vitro stability in saline and serum. The labeled complex (0.1 ml) was incubated with saline (1 ml) at room temperature (25 °C) and freshly collected human serum (1 ml) at 37 °C. The samples were withdrawn at 30 min and 1, 3, and 24 h and analyzed using ITLC-SG with saline and acetone as the mobile phase. All experiments were conducted in triplicate ($n = 3$).

Measurement of Tumor Cell Binding Affinity

U87MG cells were cultured in a humidified atmosphere with 5% CO₂. The medium was RPMI 1640 supplemented with

10% fetal bovine serum (FBS), L-glutamine (300 mg/l), 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 25 mM NaHCO₃. The binding affinities of Tc-99m TAMRA-GHEG-ECG-GNQWFI and Tc-99m TAMRA-GHEG-ECG-QIFGWN (scrambled peptide) for U87MG tumor cells were determined by saturation binding experiments as described previously [21]. Briefly, U87MG cells were seeded in a 24-well plate with 1×10^6 cells/well and cultivated overnight. The cells were washed twice for 5 min with ice-cold binding buffer (25 mM HEPES and 1% bovine serum albumin [BSA]). Then, a concentration gradient (0–500 nM) of Tc-99m TAMRA-GHEG-ECG-GNQWFI and Tc-99m TAMRA-GHEG-ECG-QIFGWN was added to the wells and incubated with cells at 37 °C for 1 h. The total volume of each well was 300 μ l. Nonspecific binding was performed by adding the same amount of radiolabeled peptides to each well without cells at different concentrations. Cells were rinsed twice with ice-cold binding buffer. Then, cells of each well were collected for gamma counting and radioactivity was measured using a 1480 Wizard 3 gamma counter (PerkinElmer Life and Analytical Sciences, Wallingford, CT, USA). The dissociation constant (K_d) and maximum number of binding sites (B_{max}) were determined using nonlinear regression models in GraphPad Prism software (version 5.03, GraphPad Software, La Jolla, CA, USA). Each data point is the mean of four determinations.

Cellular Uptake Using Confocal Microscopy

U87MG cells (1×10^5 cells/well) were cultured on the top of a coverslip slide at 37 °C for 24 h. The medium was removed and replaced with fresh serum-free medium (500 μ l), containing Tc-99m TAMRA-GHEG-ECG-GNQWFI and Tc-99m TAMRA-GHEG-ECG-QIFGWN (200 nM), and the cells were incubated for 1 h at 37 °C. Then, the cells were washed three times with phosphate-buffered saline (PBS). The coverslips were placed on slides containing one drop of fluorescent mounting medium (Dako, Glostrup, Denmark). Confocal laser scanning microscopy was performed with a FV1200 confocal microscope (Olympus, Pittsburgh, PA, USA) with a $\times 100$ oil immersion lens.

Murine Models with Tumor

Six-week-old female homozygous athymic BALB/c nu/nu mice (weighing 16–18 g) were purchased from Damul Science (Daejeon, Korea). The mice were subcutaneously inoculated with 1×10^7 U87MG cells (0.1 ml PBS) in the right side of the anterior chest region. The tumors were allowed to grow to about 400 to 550 mm³ in volume by caliper measurements of the perpendicular dimensions. Approximately 14 days after inoculation, the gamma camera imaging and biodistribution studies were performed.

Gamma Camera Imaging

The tumor-bearing mice were anesthetized by intraperitoneal injection of ketamine (60 mg/kg)/xylazine (5 mg/kg). Once the animal was in the surgical plane of anesthesia, 55.5 MBq (200 nM in 150 μ l) of Tc-99m TAMRA-GHEG-ECG-GNQWFI was intravenously administered via the tail vein. In vivo imaging was performed at 1, 2, and 3 h after injection using a gamma camera (Vertex; ADAC Laboratories, Milpitas, CA, USA) equipped with a pin-hole collimator. The images were digitally stored in a 512×512 matrix size. The regions of interest (ROIs, 15×15 pixels) were drawn at the sites of the tumors on the right chest walls, and additional ROIs were drawn at the left arm muscle for normal muscle uptake measurement ($n = 5$, respectively). The average counts per pixel within the ROIs were measured and target to nontarget ratios were calculated. Gamma camera imaging study with Tc-99m TAMRA-GHEG-ECG-QIFGWN (scrambled peptide) was performed as described above.

Competition Study with Free GNQWFI

Tc-99m TAMRA-GHEG-ECG-GNQWFI (200 nM) and free GNQWFI (5 mM) were co-injected intravenously via the tail vein ($n = 5$) to simulate the competitive effects of free GNQWFI during tumor uptake of Tc-99m TAMRA-GHEG-ECG-GNQWFI in U87MG tumors. Serial imaging was done as described above at 1, 2, and 3 h after injection.

Ex Vivo Optical Imaging and Postmortem Studies

Following the in vivo imaging study, mice were sacrificed ($n = 5$). Tumor tissue and several different organs were excised, and ex vivo optical imaging study was performed using fluorescence imaging system (FOBI-10BR, Neoscience, Suwon, Korea). Emission band for TAMRA (peak absorbance and peak emission wavelength = 565 and 580 nm, respectively) was 520 to 675 nm. Exposure time was 2.5 s per image and the image was analyzed using the dedicated software.

U87MG tumor was divided into two pieces after optical imaging, and each piece was assigned randomly to either the biodistribution study or confocal microscopy. The tumor tissue for confocal microscopy was immediately snap-frozen in OCT (optimal cutting temperature) solution, and another piece of tumor tissue was used for the biodistribution study.

Biodistribution Study

Tissues of tumor and selected organs were weighed and collected into preweighed gamma counter tubes. Radioactivities of tissues were counted in a gamma counter (1480 Wizard 3, PerkinElmer Life and Analytical Sciences). Counts per minute were decay-corrected, and results were expressed as a

Fig. 1 Chemical structure of the TAMRA-GHEG-ECG-GNQWFI and TAMRA-GHEG-ECG-QIFGWN (scrambled) peptides

percentage injected dose per gram of wet tissue (%ID/g). The total activities injected per animal were calculated by calculating the difference between the initial syringe counts and the remaining syringe counts after administration.

Immunohistochemistry Staining

The frozen tumor tissues were cut into slices (10 μ m). After thorough drying at room temperature, slices were fixed with ice-cold acetone for 10 min and dried in the air at room temperature for 20 min. The tumor sections were incubated with 5% goat serum for 30 min at room temperature to block nonspecific binding. Then, the tumor sections were incubated with the mouse anti-VEGFR1 antibodies (Flt1; sc-271789, 1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. After washing with PBS buffer, the tumor slides were incubated with Alexa Fluor® 488-conjugated goat anti-mouse secondary antibodies (cat. 115-545-062, 1:100 dilution; Jackson ImmunoResearch Inc., West Grove, PA, USA). After washing with PBS three times, the tumor slides were mounted with Prolong® Gold Antifade Reagent with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen, Carlsbad, CA, USA) and cover glass. Confocal laser scanning microscopy

was performed with a FV1200 confocal microscope (Olympus) with a $\times 60$ oil immersion lens. All photographs were taken with the same exposure time. Brightness and contrast adjustments were made equally to all images.

Statistical Analysis

Data are presented as means plus/minus standard deviation (SD). The target to nontarget ratios obtained from the gamma camera imaging and competition studies were compared using one-way analysis of variance and appropriate post hoc tests. A p value < 0.05 was considered statistically significant. The data were analyzed by SPSS version 18.0 (SPSS Inc., IBM Co., Armonk, NY, USA).

Results

Synthesis and Characterization of Peptides and Radiolabeling with Tc-99m

TAMRA-GHEG-ECG-GNQWFI ($C_{87}H_{104}N_{20}O_{24}S_1$, mol. wt. 1845.01) and TAMRA-GHEG-ECG-QIFGWN

Fig. 2 Cell binding affinity curves for Tc-99m TAMRA-GHEG-ECG-GNQWFI (a) and Tc-99m TAMRA-GHEG-ECG-QIFGWN (b) reveal saturation of Tc-99m TAMRA-GHEG-ECG-GNQWFI within the U87MG tumor cells, while Tc-99m TAMRA-GHEG-ECG-QIFGWN was not saturated

Fig. 3 Confocal microscopy images of U87MG cells incubating with TAMRA-GHEG-ECG-GNQWFI showed strong fluorescence activity in the cytoplasm (a). In contrast, U87MG cells incubating with TAMRA-GHEG-ECG-QIFGWN showed weak fluorescence activity (b)

($C_{87}H_{104}N_{20}O_{24}S_1$, mol. wt. 1845.01) were successfully synthesized using Fmoc SPSS (Fig. 1). A single radio-peptide species was obtained by radio-RP-HPLC (retention time = 6.6 and 6.4 min for Tc-99m TAMRA-GHEG-ECG-GNQWFI and Tc-99m TAMRA-GHEG-ECG-QIFGWN, respectively) after Tc-99m radiolabeling. The Tc-99m TAMRA-GHEG-ECG-

GNQWFI complex was prepared in high yield (>95%) and showed high stability in saline and serum. The intact percentages of Tc-99m TAMRA-GHEG-ECG-GNQWFI incubated in saline measured by ITLC-SG were 95.2 ± 0.6 , 94.1 ± 0.5 , 92.8 ± 1.2 , and $90.4 \pm 1.7\%$ at 30 min and 1, 3, and 24 h, respectively. The intact percentages of Tc-99m TAMRA-GHEG-ECG-GNQWFI

Fig. 4 (a) Serial gamma camera images of U87MG tumor-bearing (arrows) mice showed significant uptake of Tc-99m TAMRA-GHEG-ECG-GNQWFI. (b) The scrambled peptide, TAMRA-GHEG-ECG-QIFGWN, was not significantly accumulated into tumors. (c) Co-injection of excess concentration of GNQWFI reduced the tumor uptake of Tc-99m TAMRA-GHEG-ECG-GNQWFI

incubated in serum were 94.2 ± 1.0 , 93.4 ± 0.8 , 91.2 ± 1.3 , and $90.1 \pm 1.8\%$ at 30 min and 1, 3, and 24 h, respectively.

Measurement of Tumor Cell Binding Affinity

The K_d of Tc-99m TAMRA-GHEG-ECG-GNQWFI determined by saturation binding was 29.5 ± 4.5 nM ($B_{\max} = 69.1 \pm 2.8$, Fig. 2a). In contrast, the K_d of Tc-99m TAMRA-GHEG-ECG-QIFGWN was 2701 ± 1626 nM ($B_{\max} = 3091 \pm 1617$, Fig. 2b), which was 91 times higher than that of Tc-99m TAMRA-GHEG-ECG-GNQWFI ($p < 0.005$). These results demonstrate that the scrambled peptide without the GNQWFI motif may lose affinity for U87MG tumor cells.

Cellular Uptake Using Confocal Microscopy

Confocal microscopy images of U87MG cells incubated with TAMRA-GHEG-ECG-GNQWFI revealed strong fluorescence activity in the cytoplasm (Fig. 3(A)). In contrast, U87MG cells incubated with TAMRA-GHEG-ECG-QIFGWN showed weak fluorescence (Fig. 3(B)). The ratio of TAMRA to DAPI activity of TAMRA-GHEG-ECG-GNQWFI was significantly higher than that of TAMRA-GHEG-ECG-QIFGWN ($p < 0.005$, the ratio of TAMRA to DAPI activity of TAMRA-GHEG-ECG-GNQWFI and TAMRA-GHEG-ECG-QIFGWN = 0.51 ± 0.22 and 0.21 ± 0.09 , respectively).

Gamma Camera Imaging and Competition Study with Free GNQWFI

The most intense activity was observed in the kidneys, suggesting Tc-99m TAMRA-GHEG-ECG-GNQWFI was mainly excreted through the renal systems. In mice, Tc-99m TAMRA-GHEG-ECG-GNQWFI accumulated substantially in the U87MG tumors (Fig. 4(A)). The tumor to normal muscle uptake ratio of Tc-99m TAMRA-GHEG-ECG-GNQWFI increased with time (2.7 ± 0.4 , 3.3 ± 0.5 , and 4.3 ± 0.8 at 1, 2, and 3 h, respectively). The scrambled peptide Tc-99m TAMRA-GHEG-ECG-QIFGWN did not significantly accumulate in the tumors (Fig. 4(B)). The tumor to normal muscle uptake ratios of Tc-99m TAMRA-GHEG-ECG-QIFGWN (1.9 ± 0.2 , 2.1 ± 0.3 , and 2.0 ± 0.5 at 1, 2, and 3 h, respectively) were significantly lower than those of Tc-99m TAMRA-GHEG-ECG-GNQWFI ($p < 0.05$, Fig. 5).

In the competition study, when excess GNQWFI and Tc-99m TAMRA-GHEG-ECG-GNQWFI were co-injected, the tumor to normal muscle uptake ratios of Tc-99m TAMRA-GHEG-ECG-GNQWFI were decreased (1.8 ± 0.3 , 1.9 ± 0.3 , and 2.2 ± 0.6 at 1, 2, and 3 h, respectively, Fig. 4(C)). The observations indicated that the co-injection of excess concentration GNQWFI could block the uptake of Tc-99m TAMRA-GHEG-ECG-GNQWFI into U87MG tumors ($p < 0.05$, Fig. 5).

Ex Vivo Optical Imaging, Biodistribution, and Immunohistochemistry Staining

In ex vivo optical images, high fluorescent activity of TAMRA was detected in the liver and kidney among the healthy organs (Fig. 6). U87MG tumors showed significantly higher fluorescent activity of Tc-99m TAMRA-GHEG-ECG-GNQWFI than TAMRA-GHEG-ECG-QIFGWN. These observations were correlated strongly with the in vivo gamma camera images.

The %ID/g biodistribution values 3 h after injection of Tc-99m TAMRA-GHEG-ECG-GNQWFI and Tc-99m TAMRA-GHEG-ECG-QIFGWN are summarized in Table 1. The kidneys showed the highest activity in the murine model with injection of both peptides (8.87 ± 2.80 and 9.64 ± 2.73 for Tc-99m TAMRA-GHEG-ECG-GNQWFI and Tc-99m TAMRA-GHEG-ECG-QIFGWN, respectively). Most organs, except the kidneys, blood, and liver, showed minimal activity 3 h after injection. The tumor to normal muscle ratios of the %ID/g values were 6.39 ± 1.02 and 1.68 ± 0.70 for Tc-99m TAMRA-GHEG-ECG-GNQWFI and Tc-99m TAMRA-GHEG-ECG-QIFGWN, respectively.

Confocal microscopy with immunohistochemistry staining detected strong Tc-99m TAMRA-GHEG-ECG-GNQWFI fluorescence within the tumor cells, and the fluorescence was strongly correlated with the fluorescent activity of the

Fig. 5 At 1, 2, and 3 h after injection, the tumor to normal muscle uptake ratio of Tc-99m TAMRA-GHEG-ECG-GNQWFI was significantly higher than those of Tc-99m TAMRA-GHEG-ECG-QIFGWN and Tc-99m TAMRA-GHEG-ECG-GNQWFI with co-injection of excess GNQWFI ($p < 0.05$, asterisks)

Fig. 6 On ex vivo optical images of excised organs, U87MG tumor showed significantly higher fluorescent activity of Tc-99m TAMRA-GHEG-ECG-GNQWFI (arrows) than that of TAMRA-GHEG-ECG-

QIFGWN (arrowheads). (Lu, lung; Hr, heart; Lv, liver; St, stomach; Co, colon; Sp, spleen; Kd, kidney; Mu, muscle; Tu, U87MG tumor)

anti-VEGFR1 antibody (Fig. 7). Relatively weak fluorescence activity of Tc-99m TAMRA-GHEG-ECG-QIFGWN was detected on confocal microscopy.

Discussion

We developed Tc-99m TAMRA-GHEG-ECG-GNQWFI as a dual-modality imaging agent for targeting tumor. In vivo and

in vitro studies demonstrated substantial uptake of Tc-99m TAMRA-GHEG-ECG-GNQWFI into U87MG tumors. The scrambled peptide Tc-99m TAMRA-GHEG-ECG-QIFGWN did not accumulate in tumor cells and tissues. In a competition study, the tumor uptake of Tc-99m TAMRA-GHEG-ECG-GNQWFI was effectively blocked by the co-injection of excess concentration of GNQWFI. These results support the view that Tc-99m TAMRA-GHEG-ECG-GNQWFI and free GNQWFI bind to tumor cells using the same pathways. Also,

Table 1 Biodistribution data 3 h after injection of Tc-99m TAMRA-GHEG-ECG-GNQWFI and Tc-99m TAMRA-GHEG-ECG-QIFGWN in mice bearing U87MG tumor

Organs	Mean %ID/g (SD)	
	Tc-99m TAMRA-GHEG-ECG-GNQWFI (original peptide)	Tc-99m TAMRA-GHEG-ECG-QIFGWN (scrambled peptide)
Lungs	1.09 (0.34)	1.36 (0.67)
Heart	0.36 (0.07)	0.74 (0.37)
Blood	2.06 (0.31)	2.77 (1.24)
Liver	5.13 (0.35)	2.67 (0.38)
Stomach	0.80 (0.35)	0.93 (0.10)
Colon	0.61 (0.23)	0.73 (0.23)
Spleen	2.07 (0.67)	0.93 (0.24)
Kidneys	8.87 (2.80)	9.64 (2.73)
Muscles	0.21 (0.07)	0.27 (0.08)
Tumor	1.28 (0.28)	0.43 (0.14)

Fig. 7 On the confocal microscopy with immunohistochemistry stain, strong fluorescent TAMRA activities of Tc-99m TAMRA-GHEG-ECG-GNQWFI (a) were detected within the tumor tissue and it was well

correlated with anti-VEGFR1 antibody stain. Tc-99m TAMRA-GHEG-ECG-QIFGWN (b) showed relatively weak fluorescent activity of TAMRA compared to that of Tc-99m TAMRA-GHEG-ECG-GNQWFI

tumor uptake of Tc-99m TAMRA-GHEG-ECG-GNQWFI could be detected by ex vivo optical imaging. Taken together, the present study suggests that Tc-99m TAMRA-GHEG-ECG-GNQWFI is a good candidate as a dual-modality imaging agent for tumor.

After identification of GNQWFI as a specific binding ligand to VEGFR1 by Bae et al., GNQWFI had been evaluated as a therapeutic agent for arthritis, retinal neovascularization, and diabetic nephropathy [7–11]. The results had revealed that the GNQWFI peptide could be an effective strategy for the treatment of rheumatoid arthritis in mice and reduce the retinal vascular hyperpermeability in rat model with diabetic retinopathy. Despite the desirable specific binding of the GNQWFI peptide to VEGFR1, there are no earlier efforts to develop the imaging agent for tumor using the GNQWFI peptide as a target ligand. To our knowledge, this is the first study to develop a molecular imaging agent for tumor using GNQWFI peptide.

NIR fluorescent dyes such as Cy 5.5 and Cy 7.0, which have a longer emission wavelength, could provide better tissue penetration in vivo. However, these fluorescent dyes suffer from poor water solubility. Thus, the incorporation of these fluorescent dyes into the peptide sequence can severely compromise water solubility [22]. High water solubility is crucial for biocompatibility, high uptake efficiency, and low toxicity and is an important aspect of molecular imaging agent [23]. Thus, we decided to use TAMRA as the fluorescent agent to increase water solubility and TAMRA was adequate for ex vivo optical imaging and in vitro immunohistochemistry studies.

The GHEG sequence of TAMRA-GHEG-ECG-GNQWFI was incorporated as a linker or spacer. King et al. had reported that the insertion of histidine into the sequence of peptide-hydrazinonicotinamide (HYNIC) conjugates can improve tumor-targeting performance in vitro and in vivo [24]. They indicate that histidine would displace one ligand from another,

and the additional distance between two ligands could reduce the effect or disturbance of one ligand to another. Thus, we decided to insert a histidine-containing sequence, GHEG, into the TAMRA-ECG-GNQWFI sequence as a linker or spacer to reduce the disturbance of the fluorescence (TAMRA) on the chelating and targeting ligands (ECG-GNQWFI).

The ECG ligand of the TAMRA-GHEG-ECG-GNQWFI tripeptide including multiple nitrogen and single sulfur atoms showed strong and stable chelation with Tc-99m and is considered as a good candidate for the Tc-99m chelating ligand [18–20]. Tc-99m is the most widely used radioisotope for single-photon emission computed tomography (SPECT) imaging. The radionuclide has excellent physical characteristics for in vivo imaging, and Tc-99m-based imaging is very sensitive and highly quantitative [25].

This study has several limitations. Unfortunately, in vivo fluorescent imaging study was not performed due to relatively low fluorescent signal of the tumor lesion and was replaced by ex vivo optical imaging and immunohistochemical studies. Further study to improving the uptake of agent in the tumor lesion could actualize the simultaneous multimodal imaging. Secondly, the blood activity of Tc-99m TAMRA-GHEG-ECG-GNQWFI was relatively high, and this is responsible for the high uptake of the agent in the lungs and liver. These may cause increased radiation exposure of the subject and decreased target to nontarget uptake ratio. The modification of the linker ligand could decrease the uptake level of the blood in the further study.

Conclusions

In the present study, in vivo and in vitro studies revealed the substantial uptake of Tc-99m TAMRA-GHEG-ECG-

GNQWFI on the tumor. Fluorescence (TAMRA) and radioisotope (Tc-99m) molecules were successfully conjugated to the novel imaging agent to facilitate optical and nuclear imaging, respectively. Fluorescence could provide microscopic and ex vivo optical imaging, and nuclear imaging could allow quantitative assessment of organ biodistribution and tumor accumulation. Thus, Tc-99m TAMRA-GHEG-ECG-GNQWFI could be a good candidate dual-modality imaging agent for tumors.

Compliance with Ethical Standards

Conflict of Interest Dae-Weung Kim declares that this work was supported by the National Research Foundation of Korea (NRF) funded by the Korean government (MSIP: Ministry of Science, ICT and Future Planning) (2017R1C1B2001886). Myoung Hyoun Kim and Seul-Gi Kim declare that they have no conflict of 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.

Informed Consent The institutional review board of our institute approved this retrospective study, and the requirement to obtain informed consent was waived.

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