^{99m}Tc -3P-RGD₂ Micro-Single-Photon Emission Computed Tomography/Computed Tomography Provides a Rational Basis for Integrin $\alpha_v\beta_3$ -Targeted Therapy

Tong Fu,^{1,2} Wei Qu,¹ Fan Qiu,^{1,3} Yan Li,¹ Guoqiang Shao,¹ Wei Tian,¹
Zichun Hua,³ Yingjian Zhang,^{4,5} and Feng Wang¹

Abstract

Purpose: This study was to demonstrate the utility of ^{99m}Tc -3P-RGD₂ micro-single-photon emission computed tomography/computed tomography (SPECT/CT) for the integrin $\alpha_v\beta_3$ expression quantification in NCI-H446 and A549 lung cancer xenografts.

Materials and Methods: ^{99m}Tc -3P-RGD₂ was prepared with high radiochemical purity ($97\% \pm 2\%$) and showing high *in vitro* stability. The *in vitro* affinities of ^{99m}Tc -3P-RGD₂ to NCI-H446 and A549 tumor cells were analyzed with γ -counter, while the *in vivo* uptakes in NCI-H446 and A549 xenografts were evaluated with micro-SPECT/CT. The region of interest was drawn over the tumor site and contralateral muscle on the SPECT/CT image, and the tumor to nontumor (T/NT) ratio was calculated to estimate $\alpha_v\beta_3$ expression and tumor uptake. The expressions of integrin $\alpha_v\beta_3$ *in vitro* and *in vivo* were analyzed using a flow cytometer and immunofluorescence.

Results: Micro-SPECT/CT demonstrated focal uptake in the tumors. T/NT ratio in NCI-H446 xenografts was significantly higher compared with the A549 tumor model, as 5.92 ± 0.82 and 3.62 ± 0.91 , respectively, with $p < 0.05$. In addition, integrin $\alpha_v\beta_3$ expression in NCI-H446 cells was significantly higher compared with the A549 cells, which was consistent with the imaging data. A linear relationship was observed between ^{99m}Tc -3P-RGD₂ uptake and $\alpha_v\beta_3$ expression ($R^2 = 0.7667$, $p < 0.001$).

Conclusion: ^{99m}Tc -3P-RGD₂ SPECT/CT could be used to quantify integrin $\alpha_v\beta_3$ expression within tumors, providing a rational basis for integrin $\alpha_v\beta_3$ -targeted cancer therapy.

Key words: ^{99m}Tc -3P-RGD₂, anti-angiogenesis, integrin $\alpha_v\beta_3$, NSCLC, quantification

Introduction

Lung cancer is one of the leading causes of death worldwide,¹ and early diagnosis and appropriate treatments are critical in improving lung cancer patients' quality of life. Radiology greatly contributes to lung cancer diagnosis. Computed tomography (CT) and magnetic resonance imaging are generally used for lung cancer screening and cerebral

metastasis detection; however, there are some limitations in solitary pulmonary nodule characterization and distant metastasis detection.^{2,3} [^{18}F]-fluorodeoxyglucose (FDG) position emission tomography (PET)/CT has played an increasingly important role in lung cancer diagnosis and staging. However, there are limitations for [^{18}F] FDG with respect to detecting tumors with a low metabolic activity and evaluating efficiency of antivascular therapies.

¹Department of Nuclear Medicine, Nanjing First Hospital, Nanjing Medical University, Nanjing, Jiangsu Province, People's Republic of China.

²Department of Radiology, No. 2 Affiliated Hospital of Soochow University, Suzhou, Jiangsu Province, People's Republic of China.

³State Key Laboratory of Pharmaceutical Biotechnology, Department of Biochemistry, Nanjing University, Nanjing, Jiangsu Province, People's Republic of China.

⁴Department of Nuclear Medicine, Fudan University Shanghai Cancer Center, Shanghai, People's Republic of China.

⁵Department of Oncology, Shanghai Medwd College, Fudan University, Shanghai, People's Republic of China.

Address correspondence to: Feng Wang; Department of Nuclear Medicine, Nanjing First Hospital, Nanjing Medical University; Changle Road 68, Nanjing 210006, Jiangsu Province, People's Republic of China
E-mail: fengwangcn@hotmail.com

Integrin plays a crucial role in facilitating angiogenesis, which is a vital process for tumor growth and metastasis.^{4,5} The RGD peptide (Arg-Gly-Asp), which selectively targets integrin $\alpha_v\beta_3$, is a biomolecule that accelerates the binding of radionuclide particles, including ^{64}Cu , ^{111}In , ^{18}F , $^{99\text{m}}\text{Tc}$, and ^{68}Ga ,^{6–8} to integrin $\alpha_v\beta_3$, thereby enabling imaging of $\alpha_v\beta_3$ -positive tumors with PET and single-photon emission computed tomography (SPECT) with high accuracy.⁶ The $^{99\text{m}}\text{Tc}$ -3P-RGD₂ tracer, which is characterized by rapid blood clearance and urinary system excretion,⁹ has been demonstrated to be more effective than $^{99\text{m}}\text{Tc}$ -RGD₂, $^{99\text{m}}\text{Tc}$ -P-RGD₂, $^{99\text{m}}\text{Tc}$ -2P-RGD₂, or $^{99\text{m}}\text{Tc}$ -3G-RGD₂.^{10–12}

Besides its diagnostic role, RGD is an important agent in cancer treatment. In recent years, the use of anti-angiogenesis therapy, anti-integrin $\alpha_v\beta_3$ therapy, RGD-related chemotherapy, and gene therapy has rapidly developed.^{13–18} However, the expression level of integrin $\alpha_v\beta_3$ varies in different tumors, and the limited knowledge of $\alpha_v\beta_3$ expression in patient tumors has restricted the broad application of anti- $\alpha_v\beta_3$ therapy in clinical settings. Noninvasive imaging of $\alpha_v\beta_3$ -positive tumors and quantification of $\alpha_v\beta_3$ expression levels are important not only for diagnosis, but also for choosing appropriate treatment strategies and monitoring the response to therapy.

In this study, the efficacy of $^{99\text{m}}\text{Tc}$ -3P-RGD₂ for the detection of small-cell lung carcinoma NCI-H446 xenografts and nonsmall-cell lung carcinoma A549 xenografts was analyzed, and the correlation between $\alpha_v\beta_3$ expressions and tumor uptake rates was evaluated.

Materials and Methods

Chemicals and antibodies

The $^{99\text{m}}\text{Tc}$ -3P-RGD₂ compound represents [$^{99\text{m}}\text{Tc}$ (HYNIC-3P-RGD₂) (tricine) (TPPTS)], where HYNIC = 6:22 hydrazinonicotinyl, 3P-RGD₂ = PEG4-E[PEG4-c(RGDfK)]₂ (PEG4 = 15-amino-4,7,10,13-tetraoxapentadecanoic acid), and TPPTS = trisodiumtriphenylphosphine-3,3',3''-trisulfonate (Fig. S1).

Kit formation of the 3P-RGD₂ compound was kindly provided by Professor Shuang Liu of the School of Health Science, Purdue University (West Lafayette, IN). $\text{Na}^{99\text{m}}\text{TcO}_4$ was purchased from China Isotope Corporation (Nanjing, China). The anti- $\alpha_v\beta_3$ antibody was purchased from Abbiotec (San Diego, CA). Secondary antibodies AlexaFluor488-conjugated goat anti-rabbit antibody, Alexa594-conjugated goat anti-rabbit IgG, and nucleus-staining 4',6-diamidino-2-phenylindole (DAPI) were purchased from Life Technologies (Carlsbad, CA). The anti-CD31 antibody was acquired from BD (Heidelberg, Germany).

Cell culture

NCI-H446 and A549 cells were purchased from KeyGen Biology Company (Nanjing, China). They were cultured in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT), supplemented with 10% fetal bovine serum (Hyclone) and 100 IU/mL penicillin-streptomycin (Invitrogen, Burlington, ON, Canada), and grown at 37°C in 5% CO₂.

Animal model

The NCI-H446 and A549 lung carcinoma cell line-bearing athymic nude mice were purchased from KeyGen Biology

Company. Mice were housed under environmentally controlled conditions (22°C; 12-hour light/12-hour/dark cycle with the light cycle from 6:00 to 18:00 and the dark cycle from 18:00 to 6:00) and provided with pathogen-free food and water.

Animal care and use were performed strictly in accordance with the ethical guidelines of the Nanjing Medical University Animal Care and Use Committee, and the study protocol was approved by the local institutional review board.

$^{99\text{m}}\text{Tc}$ -3P-RGD₂ has been proven to be a safe and well-tolerated radiotracer in nonhuman primates. It shows high tumor uptake, rapid blood and renal clearance, and radiotracer accumulation in the bladder.⁹

Preparation of $^{99\text{m}}\text{Tc}$ -3P-RGD₂

$^{99\text{m}}\text{Tc}$ -3P-RGD₂ was prepared according to previously reported methods¹⁹ using a lyophilized kit formulation containing 20- μg HYNIC-3P-RGD₂, 5-mg TPPTS, 6.5-mg tricine, 40-mg mannitol, 38.5-mg disodium succinate hexahydrate, and 12.7-mg succinic acid. $^{99\text{m}}\text{Tc}$ -labeling was accomplished by adding 1–1.5 mL of $\text{Na}^{99\text{m}}\text{TcO}_4$ solution (1110–1850 MBq). The reconstituted mixture was incubated at 100°C for 20 minutes, and the radiochemical purity was determined using radio-high-performance liquid chromatography (HPLC).

Radio-HPLC analysis

Radio-HPLC was conducted on an LC-20AT system (Shimadzu, Japan). The flow rate was 0.5 mL/min. The gradient mobile phase was set up with solvent A (0.1% trifluoroacetic acid in Millipore water) and solvent B (0.1% trifluoroacetic acid in acetonitrile), 0–5 minutes 5% solvent B, 5–15 minutes from 5% to 70% solvent B, and 15–30 minutes from 70% to 5% solvent B. The ultraviolet/visible detector was set at $\lambda = 220$ nm, and a Zorbax-Rx C18 HPLC column (4.6 $\times$ 250 mm, 100 pore size) was used. The radiosensitivity was 200 K at room temperature.

Stability in vitro

The stability of $^{99\text{m}}\text{Tc}$ -3P-RGD₂ in newborn calf serum was determined after incubating the radiolabeled compound (37 MBq) in 2 mL newborn calf serum at 37°C. Every 20 μL mixture was injected directly into the radio-HPLC to analyze the radiochemistry purity, which was followed by radiolabeling efficiency analysis at 0, 3, 4, and 6 hours.

Immunofluorescence analysis for the expression of integrin $\alpha_v\beta_3$ in vitro

NCI-H446 and A549 cells (10⁶ cells/mL) were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) for 1 hour, and blocked for 1 hour with 3% bovine serum albumin. Then, the cells were incubated with the anti- $\alpha_v\beta_3$ antibody (Abbiotec) (diluted at 1:200) overnight at 4°C. After three washes with PBS, cells were incubated with the secondary antibody, Alexa Fluor488-conjugated goat anti-rabbit antibody (green) (Life Technologies), at 1:200 dilution for 1 hour at room temperature, followed by nuclei staining with DAPI (Life Technologies). The fluorescence was visualized

under a fluorescence microscope (Axioplan2; Zeiss, Jena, Germany) under 400 $\times$ magnification, with the same exposure time, equal brightness, and contrast adjustments used for all samples.

Flow cytometry assessment for integrin $\alpha_v\beta_3$ expression in vitro and in vivo

To analyze the expression of integrin $\alpha_v\beta_3$, cultured cells or cell suspensions from tumor tissues were incubated with the fluorescein isothiocyanate anti- $\alpha_v\beta_3$ antibody (Abbiotec) at the desired concentration for 30 minutes at room temperature. After three washes, the cells were analyzed using flow cytometry (FACScalibur; Becton Dickinson, Franklin Lanes, NJ).

In vitro binding of ^{99m}Tc -3P-RGD₂ to NCI-H446 and A549 cells

The binding affinities of ^{99m}Tc -3P-RGD₂ to H446 and A549 cells were determined using a γ -counter. Briefly, NCI-H446 and A549 cells were seeded in 24-well plates. After a 12-hour incubation, 3.7 kBq Na $^{99m}\text{TcO}_4$ or ^{99m}Tc -3P-RGD₂ was added to the control well or to RGD wells at intervals of 10, 60, 120, and 240 minutes. Then, the cells were washed twice with PBS and the bound radionuclide was removed from each well with 1 mol/L sodium hydroxide solution. Finally, all cells were transferred to tubes and analyzed using a γ -counter. The ^{99m}Tc -3P-RGD₂ uptake rate of cells was calculated using the following formula:

Binding rate = bound part / (bound part + unbound part) $\times$ 100%.

Whole-body micro-SPECT/CT imaging

SPECT/CT scans and images were obtained with a Micro-SPECT/CT PLUS system (Bioscan, Washington, DC) equipped with a 0.74-mm nine-pin-hole collimator: SPECT: 140 keV, 30 seconds/frame, 256 $\times$ 256 matrix, 20% window; CT scanner: 55 kVp, exposed time 1000 ms, 180 $^\circ$ plane. Static scans of 12 tumor-bearing mice (6 NCI-H446, 6 A549) were obtained 60 minutes after tail vein injection of $\sim$ 37–55.5 MBq of ^{99m}Tc -3P-RGD₂ in 0.1 mL saline. All 12 mice were anesthetized with 1.5% isoflurane for micro-SPECT/CT and throughout imaging. It took 30 minutes to complete the whole-body SPECT scan and 15 minutes to complete the whole-body CT scan. SPECT and CT data were reconstructed using InvivoScope software (Bioscan). The volumes of interest were drawn manually to cover the entire tumor. Based on the view in the CT image, the soft nontumor tissue reference (in the same transaxial plane, muscle) was also marked, and the tumor to nontumor (T/NT) ratios were calculated.

The correlation between the tumor uptake rates (measured by micro-SPECT/CT) and the integrin $\alpha_v\beta_3$ expressions (measured by flow cytometry) was analyzed.

Blocking experiments in vitro and in vivo

An *in vitro* blocking study was performed in NCI-H446 cells, ^{125}I -c(RGDyK) was prepared from Na ^{125}I (purchased from China Isotope Corporation) and c(RGDyK) (kindly provided by Dr. Shuang Liu of the School of Health Science, Purdue University, West Lafayette, IN). The radiochemical purity was 3.7×10^{13} Bq/mmol. Two microtiter

24-well vinyl assay plates were coated with 100 μL /well of a solution of NCI-H446 cell suspension (5×10^4 /well) in PBS in a coating buffer (500 mmol/L Tris-HCl, pH 7.4; 150 mmol/L NaCl; 2 mmol/L CaCl₂; 1 mmol/L MgCl₂; 1 mmol/L MnCl₂; and 0.1% bovine serum albumin 300 μL) for 24 hours at 4 $^\circ\text{C}$. The plates were washed twice with PBS. Then, 100 μL PBS containing 10 kBq of ^{125}I -c(RGDyK) and appropriate dilutions (0–1000 nmol/L) of 3P-RGD₂ in PBS were incubated in the wells at 4 $^\circ\text{C}$ for 2 hours. After incubation, the plates were washed thrice with PBS. The wells were cut out and counted in a gamma counter. The IC₅₀ value was calculated by nonlinear regression using a GraphPad Prism (Graph Pad Prism 4.0; GraphPad Software, San Diego, CA). Each data point is the average of three determinations. ^{99m}Tc -3P-RGD₂ was used as a radiotracer and 3P-RGD₂ as the blocking agent in the NCI-H446 lung cancer models; the same procedure was proceeded as in whole-body micro-SPECT/CT imaging, but 3P-RGD₂ (140 nmol, 100 μg /100 μL) was coinjected via tail vein as the blocking agent.

Immunostaining of tumor tissue sections

Tumor tissue sections (5- μm thick) were prepared according to standard protocols and then assessed for integrin $\alpha_v\beta_3$ expression. The sections were fixed in acetone and stained as described above. The primary antibodies used were rabbit anti-integrin $\alpha_v\beta_3$ and rat anti-CD31 (BD Biosciences, San Jose, CA). Secondary reagents used were Alexa488-conjugated goat anti-rabbit antibody (green) (Life Technologies) and Alexa594-conjugated goat anti-rabbit IgG (red) (Life Technologies). Sections were blocked for 1 hour using 4% goat serum and then labeled with primary antibodies followed by the secondary antibodies. Nuclei DNA was stained using DAPI (Life Technologies). Images were then visualized by Carl Zeiss Axioplan 2 microscopy (Zeiss, Welwyn Garden City, United Kingdom).

Statistical analyses

Data are expressed as the mean $\pm$ SD. Statistical analyses were carried out using the Student's *t*-test. All data were analyzed using SPSS software. For all analyses, $p < 0.05$ was considered statistically significant.

Results

The efficiency of HYNIC-3P-RGD₂ peptide labeling

The radiochemical purity of ^{99m}Tc -3P-RGD₂, determined by radio-HPLC, was $97\% \pm 2\%$ ($n = 10$), and the retention time was 14.15 minutes (Fig. 1; Fig. S2).

^{99m}Tc -3P-RGD₂ stability in vitro

Stability analysis of ^{99m}Tc -3P-RGD₂ in newborn calf serum revealed that the labeled HYNIC-3P-RGD₂ prepared from the kit was stable. The radiochemical purity of ^{99m}Tc -3P-RGD₂ in newborn calf serum was $>95\%$ at 0, 3, and 4 hours, and even after 6 hours of incubation (Fig. 2). The retention times were $\sim$ 14.5 minutes.

Binding studies

The binding abilities of Na $^{99m}\text{TcO}_4$ or ^{99m}Tc -3P-RGD₂ to NCI-H446 or A549 cells at different time points are shown

FIG. 1. ^{99m}Tc -3P-RGD₂ labeling efficiency was showing high radiochemical purity (over 95%), analyzed by radio-high-performance liquid chromatography (HPLC); the retention time was 14.15 minutes.

in Figure 3 and Table 1. There was no overall difference between the uptake of $\text{Na}^{99m}\text{TcO}_4$ in NCI-H446 and A549 cells; however, the overall uptake of ^{99m}Tc -3P-RGD₂ was significantly higher in NCI-H446 cells than in A549 cells. The difference between $\text{Na}^{99m}\text{TcO}_4$ and ^{99m}Tc -3P-RGD₂ uptake levels was significant at 4 hours in A549 cells

($t = -4.079$, $p = 0.015$) and NCI-H446 cells ($t = -11.818$, $p < 0.001$). Furthermore, the difference in ^{99m}Tc -3P-RGD₂ uptakes between A549 and NCI-H446 cells was also significant at 4 hours ($t = -4.005$, $p = 0.016$).

Expression levels of integrin $\alpha_v\beta_3$ in NCI-H446 and A549 cells

The expression levels of integrin $\alpha_v\beta_3$ in NCI-H446 and A549 cells are shown in Figure 3. Integrin $\alpha_v\beta_3$ was expressed at a higher level in NCI-H446 cells than in A549 cells, as analyzed by flow cytometry.

Uptakes of ^{99m}Tc -3P-RGD₂ in NCI-H446 and A549 xenografts

Representative whole-body scans of NCI-H446 and A549 tumor-bearing mice at 3 hours after intravenous administration of ^{99m}Tc -3P-RGD₂ are shown in Figure 4. In the NCI-H446 tumor models, the tumors appeared clear with high contrast to the contralateral background at 60 minutes postinjection, and the average N/NT ratio at 3 hours was 5.92 ± 0.82 . By contrast, in the A549 tumor models, the tumor could be visualized with moderate tumor-to-background contrast at 60 minutes postinjection with an average T/NT ratio at 3 hours of 3.62 ± 0.91 . The NCI-H446 tumor uptakes of ^{99m}Tc -3P-RGD₂ was significantly higher compared with A549 ($t = 4.557$, $p = 0.001$).

FIG. 2. ^{99m}Tc -3P-RGD₂ presented high radiochemical purity (over 95%) incubated after labeling in newborn calf serum at 0 (A), 3 (B), 4 (C), 6 (D) hours at 37°C analyzed by radio-HPLC; the retention times were similar, at around 14.5 minutes.

FIG. 3. (A) ^{99m}Tc -3P-RGD₂ uptake values in NCI-H446 and A549 cell assessment using a γ -counter. There is significant difference between NCI-H446 cells and A549 cells at the fourth hour, $**t = -4.005$, $p = 0.016$. (B) Representative images (original magnification $400\times$) of NCI-H446 and A549 cells with anti- $\alpha_v\beta_3$ antibody (AlexaFluo488, green) stained and nucleus stained (Hoechst, blue). (C) Representative images of flow cytometry analysis of $\alpha_v\beta_3$ expression on both cell lines.

Blocking experiments in vitro and in vivo

The authors determined the integrin $\alpha_v\beta_3$ binding affinity of 3P-RGD₂ by displacement of ^{125}I -c(RGDyK) bound to NCI-H446 cells. The IC₅₀ value was calculated to be 8.759 nmol/L. An *in vivo* blocking study was performed in NCI-H446 tumor models, unlabeled (cold) 3P-RGD₂ was injected (140 nmol, 100 $\mu\text{g}/100\ \mu\text{L}$) via the tail vein. The NCI-H446 tumor uptake in the control and blocking image was compared at 60 minutes postinjection. In blocking studies, the radioactivity uptake was significantly decreased ($T/NT = 1.93 \pm 0.36$), compared with controls (5.97 ± 0.42) (Fig. S3).

Integrin $\alpha_v\beta_3$ expression in NCI-H446 and A549 tumor tissues

Immunofluorescence staining was performed to examine the integrin $\alpha_v\beta_3$ and CD31 (blood vessel marker) expression levels in tumor tissues of different types of lung carcinoma. As shown in Figure 5, integrin $\alpha_v\beta_3$ expression was

higher in NCI-H446 tumor tissues than in A549 tumor tissues.

Correlation of ^{99m}Tc -3P-RGD₂ in vivo uptake and integrin $\alpha_v\beta_3$ expression

As shown in Figure 6, NCI-H446 and A549 tumor uptake was positively correlated with integrin $\alpha_v\beta_3$ expression ($R^2 = 0.7667$, $r = 0.876$, $y = 5.3146x + 2.9934$, $p < 0.001$).

Discussion

RGD (Arg-Gly-Asp) is a tripeptide sequence that has been used as a vehicle for various chemotherapeutic agents, nanoparticles, and gene therapy targets^{17,20–22} due to its high affinity with integrin $\alpha_v\beta_3$. Integrin $\alpha_v\beta_3$ plays a crucial role in angiogenesis and in facilitating tumor cell metastasis and invasion and has been studied intensively as a molecular target for both tumor image tracers and tumor therapy.^{23,24}

TABLE 1. THE UPTAKE OF $^{99m}\text{TcO}_4$ AND $^{99m}\text{Tc-3P-RGD}_2$ IN NCI-H446 AND A549 CELLS

Cells line	0.5 hour		1 hour		2 hours		4 hours	
	$\text{Na}^{99m}\text{TcO}_4$	$^{99m}\text{Tc-3P-RGD}_2$	$\text{Na}^{99m}\text{TcO}_4$	$^{99m}\text{Tc-3P-RGD}_2$	$\text{Na}^{99m}\text{TcO}_4$	$^{99m}\text{Tc-3P-RGD}_2$	$\text{Na}^{99m}\text{TcO}_4$	$^{99m}\text{Tc-3P-RGD}_2$
NCI-H446	$3.36\% \pm 0.32\%$	$5.81\% \pm 1.42\%$	$3.48\% \pm 0.14\%$	$7.84\% \pm 2.20\%$	$3.19\% \pm 0.05\%$	$9.88\% \pm 1.55\%$	$3.98\% \pm 0.37\%$	$15.72\% \pm 1.68\%$ ^a
A549	$3.79\% \pm 0.65\%$	$4.79\% \pm 0.29\%$	$4.05\% \pm 0.39\%$	$6.77\% \pm 0.99\%$	$3.97\% \pm 0.16\%$	$7.73\% \pm 1.30\%$	$4.15\% \pm 0.16\%$ ^b	$9.33\% \pm 2.19\%$ ^c

Mean $\pm$ standard deviation percent.
^a $t_f = 11.818$, $p < 0.001$.
^b $t_f = -4.079$, $p = 0.015$.
^c $t_f = -4.005$, $p = 0.016$.

FIG. 4. Left: The 3D and transverse views of representative micro-single-photon emission computed tomography/computed tomography images of nude mice bearing NCI-H446 tumors ($\sim 0.27\text{ cm}^3$; mean tumor to nontumor [T/NT] ratio = 5.92 ± 0.82) 3 hours after intravenous injection of $^{99m}\text{Tc-3P-RGD}_2$; Right: nude mice bearing A549 tumors ($\sim 0.15\text{ cm}^3$; mean T/NT ratio = 3.62 ± 0.91) 3 hours after intravenous injection of $^{99m}\text{Tc-3P-RGD}_2$. Significant difference exists, $t = 4.557$, $p = 0.001$.

$^{99m}\text{Tc-3P-RGD}_2$ is a promising radiotracer for $\alpha_v\beta_3$ -positive tumors. Besides $^{99m}\text{Tc-NC100692}$ and [^{18}F]Galacto-RGD, recent clinical studies showed $^{99m}\text{Tc-3P-RGD}_2$ and SPECT had high sensitivity and accuracy in the diagnosis of lung cancer and thyroid cancer. *In vitro* and *in vivo* studies also showed $^{99m}\text{Tc-3P-RGD}_2$ with high affinity to glioma and breast cancer.^{15,25–27} Dimeric cyclic RGD peptides and 3PEG4 increases the uptake of $^{99m}\text{Tc-3P-RGD}_2$ compared to $^{99m}\text{Tc-NC100692}$.¹¹ In addition, its relatively simple one-step preparation, high yields, and better biodistribution make $^{99m}\text{Tc-3P-RGD}_2$ a superior radiotracer to [^{18}F]Galacto-RGD. In this study, $^{99m}\text{Tc-3P-RGD}_2$ was labeled in kit formulation with high radiolabeling yield and confident stability (Figs. 1 and 2). It showed good sensitivity for detecting A549 and NCI-H446 xenograft foci. Nevertheless, the affinity to A549 and NCI-H446 differed both *in vitro* and *in vivo*, which correlated with $\alpha_v\beta_3$ expression level

FIG. 5. (A) Representative images of flow cytometry analyses of $\alpha_v\beta_3$ expression in NCI-H446 and A549 tumor tissues. (B) Representative images (original magnification $200\times$) of NCI-H446 and A549 tumor slice stained with anti- $\alpha_v\beta_3$ (AlexaFluo488, green) and anti-CD31 (AlexaFluo594, red) antibody. Yellow color (green $\alpha_v\beta_3$ staining merged with red CD31 staining) indicates tumor neovasculation.

variation (Figs. 3 and 4). Correlation was present between the uptake of ^{99m}Tc -3P-RGD₂ and integrin $\alpha_v\beta_3$ expression, represented by the following linear equation: $y = 5.3146x + 2.9934$, $R^2 = 0.7667$. This indicates that ^{99m}Tc -3P-RGD₂ could be used not only to distinguish but also to quantify the expression level of integrin $\alpha_v\beta_3$ in a noninvasive manner. In general, the expression levels of integrin $\alpha_v\beta_3$ differ

among different tumor cells or among different patients for the same tumor type, which presents a challenge for clinicians when faced with the decision of whether or not to adopt anti- $\alpha_v\beta_3$ therapy. The extra time required for testing different treatment options until finding the most efficient therapy means that patients are likely to miss the best window of treatment time for anti- $\alpha_v\beta_3$ therapy. ^{99m}Tc -3P-

FIG. 6. NCI-H446 and A549 tumor uptakes ($n = 12$) of ^{99m}Tc -3P-RGD₂ at 3 hours postinjection correlated well with $\alpha_v\beta_3$ expressions obtained from flow cytometry analysis in a linear manner. $y = 5.3146x + 2.9934$, $R^2 = 0.7667$, $p < 0.001$.

RGD₂ micro-SPECT/CT provided quantitative data of integrin $\alpha_v\beta_3$ in tumors, which forms a basis for its use in $\alpha_v\beta_3$ -targeted therapy.^{13–17} Further studies are warranted.

Conclusions

^{99m}Tc-3P-RGD₂ images showed focal uptake in tumor sites in both NCI-H446 and A549 xenografts. The linear relationship between the tumor radiouptake and integrin $\alpha_v\beta_3$ expression in NCI-H446 and A549 tumors provides a significant foundation to justify a clinician's choice in using anti-integrin $\alpha_v\beta_3$ or RGD-related therapies. Further studies are warranted, as this study is expected to pave the way for increased development of radioisotope-labeled RGD analogue therapies such as ¹⁷⁷Lu-labeled RGD and ⁹⁰Y-labeled RGD.

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Disclosure Statement

The authors declare no potential conflicts of interest.

References

1. The evolution of lung cancer treatment. *Lancet* 2013; 382:286.
2. Field JK, Oudkerk M, Pedersen JH, et al. Prospects for population screening and diagnosis of lung cancer. *Lancet* 2013;382:732.
3. Paul NS, Ley S, Metser U. Optimal imaging protocols for lung cancer staging: CT, PET, MR imaging, and the role of imaging. *Radiol Clin North Am* 2012;50:935.
4. Brooks PC, Clark RA, Cheresh DA. Requirement of vascular integrin alpha v beta 3 for angiogenesis. *Science* 1994;264:569.
5. Hwang R, Varner J. The role of integrins in tumor angiogenesis. *Hematol Oncol Clin North Am* 2004;18:991.
6. Liu S. Radiolabeled multimeric cyclic RGD peptides as integrin alphavbeta3 targeted radiotracers for tumor imaging. *Mol Pharm* 2006;3:472.
7. Liu Z, Yan Y, Liu S, et al. (18)F, (64)Cu, and (68)Ga labeled RGD-bombesin heterodimeric peptides for PET imaging of breast cancer. *Bioconj Chem* 2009;20:1016.
8. Liu Z, Niu G, Shi J, et al. (68)Ga-labeled cyclic RGD dimers with Gly3 and PEG4 linkers: Promising agents for tumor integrin alphavbeta3 PET imaging. *Eur J Nucl Med Mol Imaging* 2009;36:947.
9. Jia B, Liu Z, Zhu Z, et al. Blood clearance kinetics, biodistribution, and radiation dosimetry of a kit-formulated integrin $\alpha_v\beta_3$ -selective radiotracer ^{99m}Tc-3PRGD2 in non-human primates. *Mol Imaging Biol* 2011;13:730.
10. Wang L, Shi J, Kim YS, et al. Improving tumor-targeting capability and pharmacokinetics of (99m)Tc-labeled cyclic RGD dimers with PEG(4) linkers. *Mol Pharm* 2009;6:231.
11. Shi J, Wang L, Kim YS, et al. Improving tumor uptake and excretion kinetics of 99mTc-labeled cyclic arginine-glycine-aspartic (RGD) dimers with triglycine linkers. *J Med Chem* 2008;51:7980.
12. Zhou Y, Kim YS, Lu X, et al. Evaluation of 99mTc-labeled cyclic RGD dimers: Impact of cyclic RGD peptides and 99mTc chelates on biological properties. *Bioconj Chem* 2012;23:586.
13. Shojaei F. Anti-angiogenesis therapy in cancer: Current challenges and future perspectives. *Cancer Lett* 2012;320:130.
14. Goodman SL, Picard M. Integrins as therapeutic targets. *Trends Pharmacol Sci* 2012;33:405.
15. Desgrosellier JS, Cheresh DA. Integrins in cancer: Biological implications and therapeutic opportunities. *Nat Rev Cancer* 2010;10:9.
16. Ulahannan SV, Brahmer JR. Antiangiogenic agents in combination with chemotherapy in patients with advanced non-small cell lung cancer. *Cancer Invest* 2011;29:325.
17. Vachutinsky Y, Oba M, Miyata K, et al. Antiangiogenic gene therapy of experimental pancreatic tumor by sFlt-1 plasmid DNA carried by RGD-modified crosslinked polyplex micelles. *J Control Release* 2011;149:51.
18. Zhang F, Huang X, Zhu L, et al. Noninvasive monitoring of orthotopic glioblastoma therapy response using RGD-conjugated iron oxide nanoparticles. *Biomaterials* 2012;33: 5414.
19. Ji S, Zhou Y, Voorbach MJ, et al. Monitoring tumor response to linifanib therapy with SPECT/CT using the integrin $\alpha_v\beta_3$ -targeted radiotracer ^{99m}Tc-3P-RGD2. *J Pharmacol Exp Ther* 2013;346:251.
20. Santulli G, Basilicata MF, De Simone M, et al. Evaluation of the anti-angiogenic properties of the new selective $\alpha_v\beta_3$ integrin antagonist RGDechiHCit. *J Transl Med* 2011;9:7.
21. Yonenaga N, Kenjo E, Asai T, et al. RGD-based active targeting of novel polycation liposomes bearing siRNA for cancer treatment. *J Control Release* 2012;160:177.
22. Chen K, Chen X. Integrin targeted delivery of chemotherapeutics. *Theranostics* 2011;1:189.
23. Decristoforo C, Faintuch-Linkowski B, Rey A, et al. [99mTc]HYNIC-RGD for imaging integrin alphavbeta3 expression. *Nucl Med Biol* 2006;33:945.
24. Liu S. Radiolabeled cyclic RGD peptides as integrin alpha(v)beta(3)-targeted radiotracers: Maximizing binding affinity via bivalency. *Bioconj Chem* 2009;20:2199.
25. Haubner R, Wester HJ, Weber WA, et al. Noninvasive imaging of alpha(v)beta3 integrin expression using 18F-labeled RGD-containing glycopeptide and positron emission tomography. *Cancer Res* 2001;61:1781.
26. Axelsson R, Bach-Gansmo T, Castell-Conesa J, et al. An open-label, multicenter, phase 2a study to assess the feasibility of imaging metastases in late-stage cancer patients with the alpha v beta 3-selective angiogenesis imaging agent ^{99m}Tc-NC100692. *Acta Radiol* 2010;51:40.
27. Zhao D, Jin X, Li F, et al. Integrin $\alpha_v\beta_3$ imaging of radioactive iodine-refractory thyroid cancer using ^{99m}Tc-3PRGD2. *J Nucl Med* 2012;53:1872.