Immuno-PET imaging of VEGFR-2 expression in prostate cancer with $^{89}$Zr-labeled ramucirumab

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Abstract: The detection and monitoring of prostate cancer (PrCa) malignancies using most of the conventional strategies is challenging. As an over-expressed biomarker of PrCa, the vascular endothelial growth factor receptor 2 (VEGFR-2) can be delineated by non-invasive imaging to address such issue. Herein, we report the positron emission tomography (PET) of VEGFR-2 expression in a PrCa mice models by composing a novel tracer, $[^{89}$Zr]$^\text{Zirconium}$-labeled clinical VEGFR-2 antibody (Ramucirumab), i.e. $^{89}$Zr-Df-R. The VEGFR-2 expression levels among three different PrCa cell lines (PC-3, LNCAP and LAPC-4) were confirmed by flow cytometry. The immuno-PET imaging and bio-distribution (Bio-D) study were conducted in subcutaneous PrCa mice models via the $^{89}$Zr-Df-R. The regions of interest (ROI) data showed that the uptake of $^{89}$Zr-Df-R in the positive PC-3 (9.5±3 %ID/g) tumors are obviously higher than those ones in the negative LNCAP (6.0±1.7 %ID/g) or LAPC-4 (4.3±0.7 %ID/g) tumors at 120 hours post-injection, while the accumulation of $^{89}$Zr-Df-R in PC-3 tumors (4.3±1.2 %ID/g) could be significantly reduced by the blockade of unlabeled Ramucirumab. These quantitative data coincide with the Bio-D data and proves the specificity. Additionally, the immuno-fluorescent staining results confirmed the expression pattern of VEGFR-2 among various PrCa tumors. Finally, the flow cytometry of PC-3 tumor tissue further proved that the binding of $^{89}$Zr-Df-R to VEGFR-2 primarily occurs on the PC-3 tumor cells. In summary, the description of the VEGFR-2 expression in PrCa by in-vivo PET with $^{89}$Zr-Df-R is feasible and it may shed light on the early detection of foci and dynamic monitoring of anti-VEGFR-2 therapy in PrCa.

Keywords: Positron emission tomography (PET), vascular endothelial growth factor receptor 2 (VEGFR-2), prostate cancer, ramucirumab, zirconium-89

Introduction

Prostate cancer (PrCa) is estimated as the second-leading cause for all the death induced by cancer in the United States (US), and is also the major cancer type around the world in male [1, 2]. The conventional methods ranging from the determination of serum prostate-specific antigen (PSA), digital rectal examination, transrectal ultrasound (TRUS)-guided biopsy, magnetic resonance imaging (MRI) to the MRI-guided biopsy have been used for detecting and monitoring PrCa [3]. However, it is still challenging to achieve a good tissue-specificity with fine patient-compliance and acceptable representativeness of sampling simultaneously by these ways. Besides, it is also difficult to obtain a pathologic specimen for early diagnosis and intervention during the onset or asymptomatic stage of PrCa [4]. Therefore, a targeted, non-invasive, comprehensive and dynamic imaging modality is highly demanded in the clinical practice of PrCa theranostics.

The vascular endothelial growth factor receptor 2 (VEGFR-2/Flk-1/KDR) is an over-expressed biomarker in both tumor neo-vasculature and PrCa malignancies [5]. As a trans-membrane kinase receptor expressed on the vascular and lymphatic endothelium, the VEGFR-2 is critical for the proliferation/migration of vascular endothelial cell via the VEGF signaling. The high expression of VEGFR-2 was found in both of PrCa tumor cells and cultured cell lines [6-10]. It has been well confirmed that VEGFR-2 is associated with PrCa progression [11-13], and the differentiation stage of PrCa tumor is also related to the expression level of VEGFR-2 [6,
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14]. So, VEGFR-2 is becoming a promising target for the theranostics of PrCa [4, 10, 15, 16].

Ramucirumab (i.e. IMC-1121B) is a recombinant humanized IgG1 monoclonal antibody which can target VEGFR-2 and further inhibit its activation by VEGF. It exhibited convincing results in the phase II clinical trial for the therapy of PrCa [17]. [64Cu]copper-labeled Ramucirumab (64Cu-R) has been successfully employed as a positron emission tomography (PET) tracer that dynamically evaluated the tumor angiogenesis in a murine model of non-small cell lung cancer (NSCLC) [18, 19]. More importantly, the uptake of 64Cu-R within the tumor kept increasing even at the last time-point of imaging, i.e. 72 h post-injection (p.i.) [18]. It implied that the contrast between the tumor and background tissue could be improved by an extended scanning timeframe. Alternatively, nuclides with longer decay half-life (t1/2) may show a great potential in enhancing the quality of immuno-PET imaging mediat-ed by the Ramucirumab.

In this study, the feasibility of characterizing VEGFR-2 expression in PrCa by using [89Zr]zirconium-labeled Ramucirumab (89Zr-Df-R) was verified in the nude mouse models bearing subcutaneous xenograft of different PrCa cell lines (PC-3, LNCAP and LAPC-4) [6-10]. For this purpose, 1) the expression of VEGFR-2 in PrCa cell lines and tumor tissue cells was measured by flow cytometry; 2) Ramucirumab was radio-labeled with 89Zr via chelation chemistry and evaluated in-vivo; 3) the uptake of radio-activity quantified from the regions of interest (ROI) in PET images and the bio-distribution (Bio-D) of 89Zr-Df-R within major organs were investigated; 4) ex-vivo immuno-fluorescent staining of PrCa tumor sections was conducted to depict the expressing profile of VEGFR-2.

Materials and methods

Chemicals

Ramucirumab (Cyramza®) was obtained from the Eli Lilly, Inc. (Indianapolis, IN). The 55B11 rabbit anti-human VEGFR-2 antibody was purchased from the Cell Signaling Technology, Inc. (Danvers, MA). 1-(4-isothiocyanatophenyl)-3-[6, 17-dihydroxy-7,10,18,21-tetraaxo-27-(N-acetylhydroxylamino)-6,11,17,22-tetraazahexaeco-sine]thiourea (p-SCN-deferoxamine or Df) was bought from the Macrocyclics, Inc. (Dallas, TX). AlexaFluor488-labeled goat anti-rabbit antibody was purchased from the Molecular Probes, Inc. (Eugene, OR). AlexaFluor488-labeled goat anti-human antibody was obtained from the Life Technologies of Invitrogen, Inc. (Eugene, OR). AlexaFluor647 labeled rat anti-mouse CD31 antibody was bought from Bio-Legend, Inc. (San Diego, CA). Pharmingen rat anti-mouse CD31 antibody was purchased from BD Bioscience, Inc. (San Diego, CA). Cy3-labeled donkey anti-rat antibody was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). PD-10 desalting cartridge was bought from GE Healthcare (Piscataway, NJ). All other buffers and reagents were bought from Thermo Fisher Scientific (Fair Lawn, NJ).

Deferoxamine-modification and 89Zr-labeling of ramucirumab

The p-SCN-Df dissolved in 10 μL of dimethyl sulfoxide (DMSO) and the Ramucirumab dissolved in the mixture (300 μL) of phosphate buffered saline (PBS 1 x; 10 mM, pH 7.4)/NaHCO3-Na2CO3 buffer (0.1 M, pH 9.2) = 1:1 (v/v) were mixed in the ratio of 20:1 (mol/mol). The mixture was oscillated at room temperature (RT) for 2 h, then purified by PD-10 cartridge with PBS 1 x as the mobile phase. The fractions of resulting Df-R product were collected.

Nuclide 89Zr was produced by an onsite PETtrace cyclotron (GE Healthcare, Milwaukee, WI) using a reaction of 89Y(p,n)89Zr. The [89Y] yttrium foil (250 μm, 99.9% in purity) was bombarded by 16.4 MeV proton with the current of 5 mA for 2 h. Then the foil was dissolved in concentrated HCl (Ultrex grade; Mallinckrodt; Dublin, Ireland) and the solution was loaded onto a hydroxamate-functionalized resin column. After a washing by 6 N HCl, the product 89Zr was eluted by 1 M oxalic acid. The 89Zr oxalate product was collected.

Approximately 61 MBq (1.65 mCi) of 89Zr oxalate was added into 500 μL of HEPES buffer (0.5 M, pH 7.0). The pH value was adjusted to 7.0-7.5 with 1 M Na2CO3 solution. Then 200 μg of Df-R (200 μg/mCi) was added into the mixture. The reaction was carried out in a constant temperature shaker at 37°C with oscillation (700 rpm) for 1 h. The reaction mixture was purified via the PD-10 cartridge. Finally, the
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fractions of $^{89}$Zr-Df-R product were collected and passed through 0.22 μm filter for in-vivo injection [20-22].

Cell culture

Three Human PrCa cell lines (PC-3, LNCAP and LAPC-4) were from the American Type Culture Collection (ATCC; Manassas, VA). The PC-3 and LNCAP cells were grown in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco by Life Technologies, Inc.; Grand Island, NY) with high glucose and supplemented with 10% fetal bovine serum (FBS; Gemini Bio-products, Inc.; West Sacramento, CA). The LAPC-4 cells were grown in the same RPMI-1640 medium (high glucose), 20% FBS, 1% sodium pyruvate (NaPyr) and 0.25 mmol/L beta-mercaptoethanol (β-MeOH). All the cells were incubated at 37°C in a humidified constant temperature incubator with 5% CO$_2$ and were harvested for flow cytometry or tumor implantation at the confluence of ~80%.

Flow cytometry

The Expression of VEGFR-2 on tumor cell surface along with the binding affinity of Df-R was verified among three different PrCa cell lines (PC-3, LNCAP and LAPC-4) by flow cytometry. Cells were harvested at 80% confluence and suspended in the cold flow buffer (~1 × 10$^7$ cells/mL). The cell suspension split into ~1.5 × 10$^6$ cells/tube was blocked and incubated with cold PBS 1 × (as blank cell control), the goat anti-rabbit/human antibody (as the controls of secondary antibody only; 5 μg/mL), 55B11 [23] and Df-R (both as the primary antibodies; 10 μg/mL) for 1 h in ice bath, respectively. The cells engaged with 55B11 and Df-R were washed with cold PBS 1 × and then incubated with the Alexa488 anti-rabbit/human antibodies (5 μg/mL) for 1 h in ice bath and darkness, respectively. All the samples engaged with secondary antibodies were washed with cold PBS 1 ×. Finally, cells in each sample were re-suspended in 300 μL of cold PBS 1 ×. The 5-Laser LSR Fortessa cytometer (Becton-Dickinson, Inc.; San Jose, CA) was employed to test the cells. On FlowJo software (ver. X.0.7; Tree Star, Inc.; Ashland, OR), shift values of the top 1% of the positive counts in fluorescent intensity were gated on the histograms of LAPC-4 blank cells as the baseline control. This gate was applied to all the histograms of other samples and the resulting data were analyzed in the GraphPad Prism software (ver. 7.0; GraphPad, Inc.; San Diego, CA).

All the procedures of animal study were in compliance with the regulations by the Institutional Animal Care and Use Committee (IACUC), University of Wisconsin-Madison (UW-Madison). The xenograft tumor block was collected right after the mouse bearing PC-3 tumor was sacrificed. Then the tumor tissue was immersed in cold PBS 1 ×, cut into tiny pieces, homogenized and filtered with Falcon cell strainer (25 μm in pore size; Corning; Corning, NY) for collection. The cells are washed with cold 2% bovine serum albumin (in PBS 1 ×) and re-suspended in cold PBS 1 ×. The following steps are similar to the staining of cell lines. AlexaFluo480 anti-mouse CD31 antibody was used for vasculature staining. Ramucirumab and AlexaFluo488 anti-human antibody was used as the primary/secondary antibody for the staining of VEGFR-2, respectively.

PET imaging and bio-distribution

PET imaging was conducted on an Inveon Micro-PET/CT scanner (Siemens Medical Solutions USA, Inc.). Nude mice bearing PrCa tumors were injected with 5-10 MBq (0.14-0.27 mCi) of $^{89}$Zr-Df-R via lateral tail vein. For the blockade group of nude mice bearing PC-3 tumors, 1.5 mg of unlabeled (cold) Ramucirumab was injected to each mouse at 24 h before the injection of $^{89}$Zr-Df-R. The original imaging was conducted by 5-15 min of static acquisition at predefined time-points p.i. without attenuation or scatter correction, respectively. The PET images were reconstructed by three-dimensional ordered subset expectation maximization (OSEM3D) algorithm. The outlining of region of interest (ROI) and quantification of uptake in major organ was performed on Inveon Research Workshop (IRW) software (Siemens, Inc.). The percentage of injected dose per gram (%ID/g) was calculated by dividing the tissue activity in MBq/g, which was converted from the uptake in ROI volume, with the total radioactivity of injection.

Right after the PET scanning at the last time point of 120 h p.i., all the mice were euthanized and dissected immediately. The blood, major organs and tumors were collected and weighed. The radioactivity of all the blood and tissue samples were determined using a Wizard 2480
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Tumor tissue was frozen immediately after the dissection for Bio-D study and cut into slices of 5 μm in thickness by the Experimental Pathology Laboratory in the Carbone Cancer Center, UW-Madison. Tumor sections were fixed in cold acetone for 10 min and dried in air for 3 min at RT. Then the sections were blocked with 5% donkey serum for 30 min at RT. The blocked sections were incubated with 55B11 as the primary antibody (5 μg/mL) for overnight at 4°C and then incubated with AlexaFluor488 anti-rabbit antibody as the secondary antibody for 1 h at RT. The adjacent sections of the same tumors were also stained with rat anti-mouse CD31 (vascular endothelium biomarker) as the primary antibody (10 μg/mL) overnight at 4°C, followed by Cy3 anti-rat antibody as the sec-

Immuno-histology

Tumor tissue was frozen immediately after the dissection for Bio-D study and cut into slices of 5 μm in thickness by the Experimental Pathology Laboratory in the Carbone Cancer Center, UW-Madison. Tumor sections were fixed in cold acetone for 10 min and dried in air for 3 min at RT. Then the sections were blocked with 5% donkey serum for 30 min at RT. The blocked sections were incubated with 55B11 as the primary antibody (5 μg/mL) for overnight at 4°C and then incubated with AlexaFluor488 anti-rabbit antibody as the secondary antibody for 1 h at RT. The adjacent sections of the same tumors were also stained with rat anti-mouse CD31 (vascular endothelium biomarker) as the primary antibody (10 μg/mL) overnight at 4°C, followed by Cy3 anti-rat antibody as the secondary antibody (5 μg/mL) for 1 h at RT. Coverslips were mounted to the sections using Vectashield medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc.). All the fluorescent imaging was carried out on an A1R confocal microscope (Nikon, Inc.; Melville, NY).

Statistical analysis

Quantitative data were presented as mean ± standard deviation (SD). Means of uptake were compared using the Student’s t-test. Means of cell count percentage in flow cytometry were compared using the one-way ANOVA. For both, $P < 0.05$ were considered as statistically significant.

Results and discussion

VEGFR-2 expression among PrCa cell lines and tumor tissue

The VEGFR-2 expression, the in-vitro binding profile of deferoxamine-conjugated Ramucirumab (Df-R) in PrCa cell lines and the in-vivo VEGF-2 expression in PrCa tumor tissue were all delineated by flow cytometry (Figures 1 and S1). As shown in Figure 1, the PC-3 cells after immune-staining (with 55B11 and Df-R respectively) showed significant shifts compared to those of LAPC-4, LNCAP and control samples, demonstrating the highest expression of VEGF-2 in PC-3 cells. Notably, the binding affinity of the Ramucirumab was not affected by the Df-conjugation. As shown in the Figure S2, the signal of CD31 from the PC-3 tumor tissue cells is much weaker than that of VEGF-2 in PC-3 cells. Notably, the binding affinity of the Ramucirumab was not affected by the Df-conjugation. As shown in the Figure S2, the signal of CD31 from the PC-3 tumor tissue cells is much weaker than that of VEGF-2 in PC-3 cells.

The product of synthesis and radio-labeling

The p-SCN-Df was successfully conjugated to Ramucirumab at a ratio of 20:1 (mol/mol), sh-
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owing a high radio-chemical yield (> 80%) for Zr labeling. After the PD-10 purification, the specific activity of final $^{89}$Zr-Df-R tracer is 11.7±3.1 mCi/mg (432.9±114.7 MBq/mg).

In-vivo PET imaging and ex-vivo bio-distribution using the $^{89}$Zr-Df-R

Cells suspended in cold PBS 1 × were mixed with Matrigel (Corning by Discovery Labware, Inc.; Bedford, MA) in the ratio of 1:1 (v/v). The mixture was incubated with an insulin syringe pre-cooled on ice, −5 × 10^6 cells in 100 μL of mixture for each male athymic nude mouse (4-5 weeks in age; Envigo; Cambridge shire, UK). The inoculation site was right under skin layer and ~10 mm from the injection point on the right hind limb of mouse. The soft bubble under skin form by the inoculated mixture was adsorbed by mice slowly in 1-2 days. Tumors emerged at ~10 days post-inoculation. The growth of tumor was monitored by palpation from then on. At ~4 weeks post-inoculation, tumors reached 5-10 mm in diameter and were utilized for in-vivo studies. In order to monitor the potential growth of $^{89}$Zr-Df-R uptake at the later stage, the final imaging time-point was extended to 120 h p.i.. The quantitative uptake kinetics of tumors and major organs are coincident with the typical style of antibody tracers (Figure 2). The accumulation of $^{89}$Zr-Df-R within the PC-3 tumors kept increasing and reached the peak at 96 h p.i. (9.6±2.3 %ID/g), from 3.5±1.4 (at injection time-point) to 9.5±3.0 %ID/g (120 h p.i.) (n = 3). The LNCAp and LAPC-4 tumors appear much weaker uptakes, with signals ranging from 4.1±1.4 to 6.0±2.0 %ID/g (LNCAp) and 2.1±0.5 to 4.3±0.7 %ID/g (LAPC-4), respectively (n = 4; P < 0.05). Moreover, the radioactivity accumulated in the blood pool and liver have an analogous kinetic characteristic among three PrCa models, indicating the non-specific accumulation of $^{89}$Zr-Df-R were similar (Table S1). The whole-body maximum-intensity projections (MIP) of mice are compared in Figure 3. As it can be illustrated, the $^{89}$Zr-Df-R uptake was pronounced within PC-3 tumors, while the uptake in LNCAp and LAPC-4 tumors were modest.

In the blocking study, with the administration of 1.5 mg unlabeled (cold) Ramucirumab 24 h in advance, the uptake of $^{89}$Zr-Df-R in PC-3 tumors (2.8±0.9, 4.7±1.3, 4.8±1.2, 4.6±1.2, 4.2±1.2, 4.3±1.2 %ID/g at 4, 24, 48, 72, 96, 120 h p.i., respectively; n = 3) declined significantly (P < 0.05). However, the radioactivity in blood pool (5.0±1.5-11.4±1.2 %ID/g) and liver (5.5±1.1-6.7±1.7 %ID/g) were still comparable to those among the un-blocking PC-3 groups (blood pool: 4.1±1.0-13.0±1.0 ID%/g; liver: 5.2±1.2-7.6±0.6 ID%/g) (Figure 2; Table S1). The apparent variation of the $^{89}$Zr-Df-R uptake between the two PC-3 groups (without/with pre-blockade, Figure 3) and the significant difference of uptake kinetics in the major organs (Figure 2) further confirmed the specificity of $^{89}$Zr-Df-R towards VEGFR-2.

The radioactive Bio-D data acquired validates the quantitative uptake withdrawn from the PET ROI (Figure 4). The radio-activity within PC-3 tumors without blockade (10.0±3.3
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%ID/g, n = 3) is notably higher than that in LNCAP/LAPC-4 tumors (5.4±1.6, 5.2±0.6 %ID/g, respectively; n = 4), while the uptake was greatly prohibited by the Ramucirumab blockage in PC-3 tumors (4.3±1.0 %ID/g, n = 3). These Bio-D data well matches the uptake in ROI quantification, indicating the novel specificity towards VEGFR-2 for $^{89}$Zr-Df-R as well. These data well demonstrate that the PET uptake values can reliably represent the in-vivo distribution of the tracer.

The replacement of $^{64}$Cu with $^{89}$Zr

To date, the $^{64}$Cu-R is the only antibody-based PET tracer that has been utilized for imaging VEGFR-2 [18]. The authors claimed that the increasing trend of $^{64}$Cu-R uptake in tumor was still observed at the last time-point (72 h p.i.) of scanning. In consideration that the $^{64}$Cu ($t_{1/2}$ 12.7 h) would quickly decay and an enhanced tracer uptake might be achieved after that, the $^{89}$Zr ($t_{1/2}$ 78.4 h), a positron emitter with a longer decay $t_{1/2}$ was employed in current study. In this work, the absolute uptake value of $^{89}$Zr-Df-R in the PrCa PC-3 tumors is comparable to that of $^{64}$Cu-R in the NSCLC HCC4006 tumors at 48 h p.i.. However, the uptake plot of $^{89}$Zr-Df-R at the later time-points successfully provided a clearer PET imaging by an enhanced contrast of target/non-target tissues. Additionally, there is a baseline accretion in the VEGFR-2-negative LAPC-4 tumor and pre-blocked VEGFR-2-positive PC-3 tumors, which may be contributed by an enhanced permeability and retention (EPR) effect. This is a common phenomenon in the PET imaging with antibody tracers [24].

The expressing profile of VEGFR-2 in tumor tissue confirmed by immuno-histology

The immuno-fluorescent staining and confocal microscopy were conducted for further confirming the VEGFR-2 expressions among three tumors. As shown in Figure 5, the fluorescence from VEGFR-2 (in green) overlays with PC-3 and LNCAP tumor cells (nucleus, in blue) and the tumor vasculature (CD31, in red), but the VEGFR-2 signal in LNCAP tumor is much weaker than that in PC-3 tumor. In LAPC-4 tumor, the VEGFR-2 signal is minimal. No overlay between the VEGFR-2 and the nucleus of tumor cells (in blue) or CD31 (in red) was observed. These immuno-histological results confirmed the expressing profile of VEGFR-2 in the tumor tissues are consistent with the results of previous PET/Bio-D studies.

The signal of CD31 from mouse vasculature is much weaker than that of the VEGFR-2 on tumor cells. It coincides with the results from the flow cytometry of PC-3 tumor tissue. In con-
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Consideration that the Ramucirumab is human-specific, we believe the deposit of $^{89}$Zr-Df-R tracer within PC-3 tumor tissue is primarily contributed by the ones binding to the surface VEGFR-2 on the PC-3 tumor cells, which is consistent with our previous study [18]. This explanation corresponds to the reported studies in terms of the targeting potency and human-specific binding ability of Ramucirumab [25, 26].

Discussion

VEGFR-2 as an ideal target for PrCa

The VEGFR-2 pathway is widely involved in various biological processes of PrCa ranging from the angiogenesis, tumor cell proliferation to bone metastasis [10-13, 15, 16]. Thus, the monitoring of the VEGFR-2 expression level within the solid tumors is valuable for the personalized management of PrCa: 1) the early detection of primary lesions would greatly improve the outcome, with a nearly 100% 5-year survival rate after a successful early diagnosis; 2) the localization of bone metastasis, which is the primary cause of mortality in PrCa, may help the optimization of the clinical decision-making; 3) it facilitates the stratification of patients who can benefit from a particular anti-VEGFR-2 therapy; 4) it promotes the evaluation of VEGFR-2 profile during intervention; 5) it can serve as a screening tool of ideal medication in pre-clinical phase [4, 27]. Undeniably, VEGFR-2 is not only a rational therapeutic target (e.g. for Cabozantinib), but also a diagnostic biomarker for PrCa.

Advantages exhibited by immuno-PET in the imaging of VEGFR-2

Compared with classical pathological approaches such as the biopsy and histo-chemical staining, the non-invasive molecular imaging shows great advantages in mapping in-vivo biomarkers, including: 1) dynamic and real-timely surveillance; 2) direct and selective collection of information; 3) comprehensiveness throughout the whole-body; 4) favorable patient-compliance. Since now, several imaging modalities including the ultrasonic imaging (via the BR55 micro-bubble) and fluorescent imaging (quantum dot) have been applied to the characterization of VEGFR-2 expression in PrCa [3, 28, 29]. However, either of them can be parallel with the nuclear medicine imaging in terms of the sensitivity and depth of tissue penetration. In other words, the selectivity is not nice enough. Among all the existed modalities of molecular imaging, PET is an optimally quantitative and the most sensitive technique. Traditional PET tracers for PrCa are mostly based on small molecules or ligands, such as choline or VEGF. Those tracers usually suffer from the sub-optimal accuracy, low affinity or unsatisfying selectivity in the detection of primary tumor and metastasis. Previously, the $^{64}$Cu-labeled peptoid was reported for the imaging of VEGFR-2, but its relatively weak affinity and the fast excretion from blood circulation result in a low uptake within the tumor [30, 31]. In sharp comparison, antibody (or its fragment)-based tracers are able to offer better specificity and pharmaco-kinetic performance simultaneously during the immuno-PET imaging [27].

The merits of ramucirumab tracer

Notably, with the approval by the Food and Drug Administration of the US, the Ramucirumab employed here is fully safe and translatable [32]. Meanwhile, the $^{89}$Zr-labeled Ramucirumab
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Well conserves the affinity and selectivity of Ramucirumab towards the VEGFR subtypes (VEGFR-1/-2) [18]. Moreover, the biological $t_{1/2}$ of intact antibody aligns to the longer decay $t_{1/2}$ of $^{89}$Zr better in circulation, leading to the higher contrast at tumor site and alleviated interference from the background (e.g. in blood poor) in the extended period (Table S1) [33]. Finally, the $^{89}$Zr-Df-R facilitates the imaging of primary PrCa foci, since the molecular size of intact antibody (typically ~150 kDa) exceeds the threshold of renal clearance (typically 40~50 kDa). It provides a minimal background radio-activity in urinary system (Table S1) [27]. Consequently, hepatic clearance becomes the dominant route for the elimination of $^{89}$Zr-Df-R from circulation and accounts for the non-specific accumulation in liver (Table S1). All these results demonstrate that the $^{89}$Zr-labeled Ramucirumab shows great potential as a novel PET tracer for the further study and translation.

Figure 5. The confocal imaging of tumor tissues from the three prostate cancer (PrCa) models after immuno-fluorescent staining. Sample groups: DAPI, the nucleus stained by DAPI; VEGFR-2, the expression of VEGFR-2 stained by 55B11 rabbit anti-human antibody as the primary antibody; CD31, the expression of vascular endothelium biomarker CD31.

Conclusion

Herein, the $^{89}$Zr-Df-R has been well proved to be utilized as a novel tracer for the in-vivo monitoring of VEGFR-2 expression in PrCa via immuno-PET. Prominent binding and specific uptake in the VEGFR-2-positive malignancy can be achieved in the PC-3 cell line and its xenograft model, which are validated by the results of in-vitro and ex-vivo studies. The profile of VEGFR-2 expression in PrCa presented by the immuno-PET with $^{89}$Zr-Df-R is feasible and superior to the reported imaging modalities. The potential application of this tracer may shed light on the early focal detection, patient stratification, the monitoring of anti-VEGFR-2 therapy and the medication screening in PrCa.

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Disclosure of conflict of interest

None.

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**Figure S1.** The histograms of VEGFR-2 expression in prostate cancer (PrCa) cell lines measured by flow cytometry. The expression in the three PrCa cell lines. Sample groups: Anti-r 2nd Ab only, the controls engaging with fluorescent anti-rabbit secondary antibody only; Anti-h 2nd Ab only, the controls engaging with fluorescent anti-human secondary antibody only; 55B11 as 1st Ab, the samples engaging with 55B11 rabbit anti-human antibody as the primary antibody; Df-R as 1st Ab, the samples engaging with Df-Ramucirumab as the primary antibody.

**Figure S2.** The VEGFR-2 expression in PC-3 tumor tissue cells measured by flow cytometry. Panels: (A) blank cells; (B) the control engaging with Alexa488 anti-human secondary antibody only; (C) the sample engaging with Alexa647 anti-mouse CD31 antibody only; (D) the sample engaging with both Alexa647 anti-mouse CD31 antibody and Ramucirumab (as the primary antibody). Vertical axis, the emission signal from the Alexa488 fluorophore; Horizontal axis, the emission signal from the Alexa 647 fluorophore.
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Table S1. The quantitative uptake values of $^{89}$Zr-Df-Ramucirumab from the region of interest (ROI) in positron emission tomography (PET) images of the tumors and major organs in the three prostate cancer (PrCa) models. The Values are reported as mean± SD

<table>
<thead>
<tr>
<th>Group</th>
<th>Tissue</th>
<th>Mean value of uptake at the time-point post-injection (%ID/g)</th>
<th>4 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
<th>120 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC-3 (positive, n=3)</td>
<td>Tumor</td>
<td>3.5±1.4</td>
<td>6.6±1.7</td>
<td>8.4±2.1</td>
<td>8.9±1.9</td>
<td>9.6±2.3</td>
<td>9.5±3.0</td>
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<tr>
<td></td>
<td>Heart</td>
<td>13.0±1.0</td>
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<td>7.3±1.1</td>
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