Anti-GD2 antibody for radiopharmaceutical imaging of osteosarcoma

Yingli Fu · Jing Yu · Ioanna Liatsou · Yong Du · Anders Josefsson · Jessie R. Nedrow · Hans Rindt · Jeffrey N. Bryan · Dara L. Kraitchman · George Sgouros

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Abstract

Purpose Osteosarcoma (OS) is the most frequently diagnosed bone cancer in children with little improvement in overall survival in the past decades. The high surface expression of disialoganglioside GD2 on OS tumors and restricted expression in normal tissues makes it an ideal target for anti-OS radiopharmaceuticals. Since human and canine OS share many biological and molecular features, spontaneously occurring OS in canines has been an ideal model for testing new imaging and treatment modalities for human translation. In this study, we evaluated a humanized anti-GD2 antibody, hu3F8, as a potential delivery vector for targeted radiopharmaceutical imaging of human and canine OS.

Methods The cross-reactivity of hu3F8 with human and canine OS cells and tumors was examined by immunohistochemistry and flow cytometry. The hu3F8 was radiolabeled with indium-111, and the biodistribution of [111In]In-hu3F8 was assessed in tumor xenograft-bearing mice. The targeting ability of [111In]In-hu3F8 to metastatic OS was tested in spontaneous OS canines.

Results The hu3F8 cross-reacts with human and canine OS cells and canine OS tumors with high binding affinity. Biodistribution studies revealed selective uptake of [111In]In-hu3F8 in tumor tissue. SPECT/CT imaging of spontaneous OS canines demonstrated avid uptake of [111In]In-hu3F8 in all metastatic lesions. Immunohistochemistry confirmed the extensive binding of radiolabeled hu3F8 within both osseous and soft lesions.

Conclusion This study demonstrates the feasibility of targeting GD2 on OS cells and spontaneous OS canine tumors using hu3F8-based radiopharmaceutical imaging. Its ability to deliver an imaging payload in a targeted manner supports the utility of hu3F8 for precision imaging of OS and potential future use in radiopharmaceutical therapy.

Keywords hu3F8 · SPECT · Osteosarcoma · Radiopharmaceutical imaging · Anti-GD2

Introduction

Osteosarcoma (OS) is the most common primary bone malignancy diagnosed in children and young adults [1]. Despite aggressive surgery and combination chemotherapy, long-term disease-free survival of patients with metastatic or recurrent OS has remained poor with the median 5-year survival rate being <30% [2]. Progress in developing new effective treatments has stagnated over the past three decades [3, 4]. Therefore, new treatment modalities that can improve the outcomes are urgently needed.

The disialoganglioside GD2 is a tumor-associated antigen widely expressed by pediatric solid tumors, including OS [5, 6] and neuroblastomas [7, 8]. In metastatic OS, GD2 expression is maintained [6, 9] or upregulated [10]. The intimate involvement of GD2 in tumor progression and restricted expression in normal tissues makes GD2 an ideal target for immunotherapy and imaging [9, 11]. Murine 3F8, a monoclonal antibody that selectively binds to GD2 and activates human complement and antibody-dependent cell-mediated cytotoxicity [5], has been used for patients with high-risk neuroblastoma and achieved long-term remissions without notable toxicities [12]. Humanized 3F8 (hu3F8) was constructed to circumvent the immunogenicity of its murine counterpart [13]. The recent FDA approval of hu3F8 for treating high-risk neuroblastoma in the bone or bone marrow [14] and its demonstrated 10 times higher
binding affinity to GD2 [13] highlight the potential of hu3F8 as a promising targeting vector for treating metastatic OS.

OS is also the most common bone tumor found in dogs, with the majority of dogs succumbing to the disease with or without a treatment [15]. Both canine and human OS are likely to be high-grade histologically, with rapidly metastasizing tumors primarily to the lungs [16]. At the molecular level, canine and human OS present overlapping transcriptional profiles and shared DNA copy number aberrations [15]. The higher prevalence of OS in dogs and the more rapid disease progression render spontaneously occurring OS in dogs an ideal model for human OS safety and efficacy studies. In addition, canine OS occurs predominantly in large breed dogs; thus, the imaging and pharmacokinetic studies that are essential to the implementation of a new therapy in humans can be directly translated.

Radiopharmaceutical therapy (RPT) is emerging as a promising therapeutic modality for a variety of cancers, including metastatic OS, by targeted delivery of high dose of radiation in the form of alpha- or beta-particle emitting radionuclides to tumor-associated targets [17–21]. Prior to disease treatment, especially in the early stages, it is essential to develop targeted radiopharmaceutical imaging probes that could be used to visualize primary and metastatic lesions with high sensitivity.

The purpose of this study is to evaluate the potential of a humanized anti-GD2 antibody, hu3F8, that was developed for neuroblastoma therapy, as a potential delivery vector for targeted radiopharmaceutical imaging of human and canine metastatic OS. We developed the methodology for efficient radiolabeling of hu3F8 with $^{111}$In without compromising its immunoreactivity and investigated the biodistribution of $^{111}$In-labeled hu3F8 in canine OS and human neuroblastoma xenografts. Furthermore, the ability of radiolabeled hu3F8 to target metastatic OS lesions was examined in spontaneously occurring OS dogs to facilitate future translation to human OS patients.

Materials and methods

Reagents, cell lines, and canine osteosarcoma tissue samples

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. S-2-(4-Isothiocyanatobenzyl)-diethylenetriamine pentaacetic acid (p-SCN-Bn-DTPA) and $^{111}$In ($^{111}$In InCl$_3$) were purchased from Macrocyclics (Plano, TX, USA) and BWXT ITG Canada, respectively. The hu3F8 antibody was provided by Dr. Nai-Kong Cheung at Memorial Sloan-Kettering Cancer Center. Canine OS cells, OSCA78 (Kerafast, Boston, MA, USA), human OS cells (U2OS), and human neuroblastoma cells (IMR32 and SK-N-SH, ATCC, Manassas, VA, USA) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 100 µg/ml Primocin.

Canine OS tissue was acquired from the Center for Image-Guided Animal Therapy at the Johns Hopkins University. OS tissue was either freshly frozen and embedded in OCT compound or was fixed with 10% formalin followed by paraffin embedding.

Antibody radiolabeling

The $^{111}$In-labeled hu3F8 and rituximab were prepared as previously described [22, 23]. Briefly, the antibody was conjugated to p-SCN-Bn-DTPA (molar ratio, 1:7–9 (hu3F8), 1:1 (rituximab)) at 37 °C for 1 h, and the conjugate was purified by size-exclusion centrifugation. The resulting antibody conjugate was then added to 40.7–129.5 MBq of $^{111}$In-InCl$_3$, 0.5 mL of 0.2 M HCl, and 0.06 mL of 3 M NH$_4$OAc, pH 4. The resulting mixture was incubated at 37 °C for 30–60 min and then purified by a PD-10 column. The radiochemical purity was determined by radio-thin layer chromatography, and the protein concentration was determined by spectrophotometry (Nanodrop, Wilmington, DE, USA).

In vitro studies

Immunocytochemical and immunohistochemical staining

The cross-species reactivity of hu3F8 with human (U2OS) and canine (OSCA78) OS cells and spontaneously occurring canine OS tumor tissue was first evaluated by immunocytochemistry (ICC) and immunohistochemistry (IHC), respectively. Cells grown in 8-well chambers were fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature. After washing with PBS, cells were blocked for 30 min by serum-free antigen blocking solution (Dako Agilent, Carpinteria, CA, USA), followed by overnight incubation with hu3F8 (1:200) at 4 °C. Cells were then washed and incubated with FITC-labeled anti-human Fc secondary antibody (1:1000, Thermo Fisher Scientific, Waltham, MA, USA) for 1 h. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA). For IHC of canine OS tissue, 6-µm-thick sections were deparaffinized. Endogenous peroxidase was quenched by 0.3% H$_2$O$_2$. The samples were incubated in an antigen retrieval solution (Dako) and pressure cooked for 3 min. The samples were blocked and incubated with hu3F8 (1:200) overnight. After washing with PBS, OS samples were incubated with a biotinylated anti-human Fc secondary antibody (1:800 dilution, Thermo Fisher Scientific), followed by incubation with streptavidin–horseradish peroxidase complex and developed with 3,3′-diaminobenzidine peroxidase substrate kit (Vector Laboratories). The OS sections were then counterstained with hematoxylin. Human IMR32 cells known to express GD2 were used as a positive control, and SK-N-SH cells and dog spleen tissue were used as GD2 negative controls. Images were captured...
at 200× magnification using an upright microscope (Eclipse Ti, Nikon Instruments Inc., Melville, NY, USA).

**Flow cytometry** Human IMR32, U2OS, canine OSCA78 cells, and canine white blood cells (negative control) (1 × 10⁶) were collected and fixed with 4% PFA. The cells were then incubated with hu3F8 (5 µg/mL) for 30 min at 4 °C. After washing with PBS, cells were incubated with Alexa Fluor 647-labeled goat anti-human IgG (Southern Biotech, Birmingham, AL, USA) for 30 min, washed with PBS, and analyzed on a FACS flow cytometer (BD Biosciences).

**Receptor binding assay** The binding affinity of [¹¹¹In]In-DTPA-hu3F8 was determined in IMR32 and OSCA78 cells as described previously with modifications [23, 24]. In brief, cells were seeded in 24-well plates at 1.5 × 10⁵ cells/well 24 h prior to the experiment. After 24 h, the cells were washed twice with PBS and treated with a serial dilution of [¹¹¹In]In-DTPA-hu3F8 (0.05–100 nmoL/L) for 4 h at 4 °C. Non-specific binding was assessed by pretreating cells with 10 µg/well of unlabeled hu3F8 for 30 min at 4 °C followed by [¹¹¹In]In-DTPA-hu3F8 incubation. Each assay was conducted in triplicate. After 4 h of incubation, cells were washed twice with ice cold PBS, dissolved in 0.5% sodium dodecyl sulfate solution, and counted on a γ-well counter (PerkinElmer 2470 WIZARD®). The protein concentration of the cell lysates was determined with a BCA protein assay kit (Pierce). The measured activity was normalized to the number of cells (sites/cell). The Michaelis–Menten equation was fitted to the binding curve to determine the dissociation constant (K_d) and maximum number of binding sites (B_max) using Prism 9 (GraphPad, La Jolla, CA, USA).

**Immunoreactivity** The immunoreactivity of [¹¹¹In]In-labeled antibodies was determined as previously described [24] by antibody adherence to IMR32 and OSCA78 cells. Two sets of tubes (1 × 10⁷ cells/tube) were prepared for each cell line, and 0.1 µg of [¹¹¹In]In-DTPA-hu3F8 or [¹¹¹In]In-DTPA-rituximab was used for immunoreactivity assessment: immunoreactivity (%) = (bound activity in cells / total activity) × 100.

**In vivo mouse studies**

All animal studies were approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine. Six- to 8-week-old healthy female Nu/Nu mice were obtained from Charles River Laboratories (Wilmington, MA, USA). Mice received subcutaneous injections of either 1 × 10⁷ IMR32 or 3 × 10⁶ of OSCA78 in Matrigel in the right flank for biodistribution studies or both flanks for SPECT imaging.

**Biodistribution** Biodistribution studies were carried out as previously described [23]. Following a growth period of approximately 8 weeks, IMR32 and OSCA78 tumor-bearing mice (n = 3–4) were injected intravenously with 100 µL of [¹¹¹In]In-DTPA-hu3F8 (3.03 MBq/10 µg). Mice were euthanized with isoflurane at either 24 or 72 h after injection. The major organs, including the blood, heart, lungs, liver, kidneys, spleen, stomach (with content), intestine (with content), femur, muscle, and tumors, were harvested, weighed, and measured in an automatic γ-well counter. The percentage of injected activity per gram (%IA/g) was calculated by comparison with a weighed, diluted standard. In addition, to examine the specific accumulation of radiolabeled hu3F8 in OS tumors, additional biodistribution studies were performed in OSCA78 tumor-bearing mice using an [¹¹¹In]In-labeled irrelevant antibody, [¹¹¹In]In-DTPA-rituximab (1.74 MBq/10 µg) (n = 3). These mice were sacrificed at 72 h after injection, and their organs were harvested and processed, as described above. After counting, fresh OSCA78 tumors were embedded in OCT compound and sectioned for IHC staining to detect radiolabeled hu3F8 using an anti-human Fc antibody.

**SPECT imaging** Mice with established OSCA78 tumors in both flanks were injected intravenously with either [¹¹¹In]In-DTPA-hu3F8 (3.03 MBq, 10 µg) or [¹¹¹In]In-DTPA-rituximab (1.74 MBq, 10 µg). IMR32 xenografted mice received intravenous injection of [¹¹¹In]In-DTPA-hu3F8 (3.03 MBq, 10 µg). At 24-, 48-, and 96-h post-injection, SPECT/CT images were acquired with a NanoSPECT/CT system (Bioscan Inc., Washington, DC, USA) using multiplexed multipinhole gamma detectors and high-resolution collimators. Images were acquired for 60 min at each time point and were reconstructed at voxel size of 0.6 mm³ isotropic using the vendor-supplied iterative algorithm. SPECT images were co-registered with CT images for an anatomic reference using PMOD, version 3.7 (PMOD Technologies LLC, Zurich Switzerland), and the voxel intensity was calibrated using images of a standard with known activity and volume.

**In vivo dog studies**

**Tumor-free dogs** Three healthy research dogs (13.6–17.0 kg) received intravenous injection of either the unlabeled hu3F8 (100 µg; n = 2) or [¹¹¹In]In-DTPA-hu3F8 (17.0 MBq/100 µg; n = 1) to assess the tolerance of the antibody. Fifteen minutes prior to injection, the fasted dogs received intravenous analgesia and antihistamine prophylaxis with 0.05 mg/kg hydromorphone and 1 mg/kg of diphenhydramine, respectively. The dog that received [¹¹¹In]In-DTPA-hu3F8 was sedated with 0.005 mg/kg fentanyl and induced with 0.25 mg/kg medazolam and 4 mg/kg propofol, intubated, and placed under isoflurane anesthesia.
with mechanical ventilation. Whole-body SPECT/CT imaging with two bed positions (SPECT, voxel size = 2.4 mm³, final matrix = 256×256×337; CT, matrix size = 512×512, pixel size = 0.9766×0.9766 mm², slice thickness = 1.0 mm, tube voltage = 130 kVp, tube current = 26 mA) was performed at 4 and 24 h after injection on the Symbia T6 Series scanner (Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA) using a medium energy collimator. SPECT images were reconstructed using vendor’s Flash3D OSEM algorithm with compensation for attenuation and resolution. The attenuation map was generated from CT images. Volume of interests (VOIs) were drawn for the liver, spleen, kidneys, and heart on the first 4-h CT image and were then transposed to the 24-h time point after deformable registration of the scans had been performed using Velocity software (version 3.1, Varian Medical Systems, Palo Alto, CA, USA). One syringe containing 18.35 MBq [¹¹¹In]In was imaged for 5 min to determine the calibration factor (MBq/CPM) of the system. VOI for urinary bladder was draw on both 4- and 24-h CT images due to the change of the bladder size with time.

**Spontaneous OS dogs** Two companion dogs (A, 30.0 kg, and B, 32.1 kg) diagnosed with spontaneously occurring OS were studied. Metastatic recurrence of OS after amputation and chemotherapy was confirmed by fine-needle aspirate at 5 weeks in dog A and [¹⁸F]fluorodeoxyglucose-positron emission tomography ([¹⁸F]FDG-PET) at 12 months in dog B. Dog A received 100 MBq of [¹¹¹In]In-DTPA-hu3F8 (0.35 mg) intravenously, while dog B received 36 MBq of [¹¹¹In]In-DTPA-hu3F8. Whole-body SPECT/CT imaging was performed 48 h post-injection using similar parameters to those for the tumor-free dogs. Dog A was euthanized after pain palliation failed, 4 months after [¹¹¹In]In-DTPA-hu3F8 injection. Necropsy was performed by a board-certified pathologist. The shoulder and lung metastatic tissues were obtained and processed for histological analysis as described above.

**Statistical analysis**

Statistical analysis was performed using Prism 9. All data are presented as mean ± standard deviation (SD). Normality was tested using the Shapiro–Wilk test. Biodistribution groups were compared using one-way ANOVA. Values were considered significant at a P value of < 0.05.

**Results**

**Cross-reactivity**

We first validated by immunostaining (Fig. 1) and flow cytometry analysis (Fig. 2) that human and canine OS cells and canine OS tumor tissues highly reacted with hu3F8. GD2 expression was seen in a characteristic, uniform distribution on the surface of human U2OS and canine OSCA78 cells, similar to that on human IMR32, a neuroblastoma cell line that is known to express GD2. Canine OS tumor tissue showed positive reactivity to hu3F8 with heterogeneous, moderate staining. In contrast, SK-N-SH cells and tumor-free dog spleen tissue showed no evidence of GD2 expression (Fig. 1). Flow cytometry analysis revealed high GD2 expression in human IMR32 (81%), human U2OS (85%), and canine OSCA78 (93%) cells with mean fluorescence intensity of 2807, 2589, and 1386, respectively (Fig. 2).

**Radiolabeling and immunoreactivity**

When starting with a molar ratio of hu3F8 to DTPA at 1:7–9, the [¹¹¹In]In-DTPA-hu3F8 was radiolabeled at an average specific activity of 27.75 MBq/nmol and high yield (> 80%) and purity (> 97%). The immunoreactivity of [¹¹¹In]In-DTPA-hu3F8 was 35–40% for OSCA78 cells and 44–60% for IMR32 cells. The immunoreactivities toward OSCA78 and IMR32 cells were markedly reduced as the amount of chelator (DTPA) in the reaction increased. Similarly, the irrelevant antibody, rituximab, was radiolabeled at an average specific activity of 20.5 MBq/nmol and > 95% yield. As expected, the immunoreactivity of [¹¹¹In]In-DTPA-rituximab was low (< 3%) for both cell lines.

**Saturation binding assay**

The saturation binding assay demonstrated that [¹¹¹In]In-DTPA-hu3F8 binds to GD2 with high affinity, having a Kₐ of 7.4 ± 1.0 and 6.2 ± 1.9 nM and a Bₘₐₓ of 51,900 and 288,300 sites/cell for OSCA78 and IMR32, respectively (Fig. 3).

**Biodistribution and SPEC/CT imaging of mice**

The 24-h biodistribution of [¹¹¹In]In-DTPA-hu3F8 in mice bearing flank tumors of either OSCA78 or IMR32 demonstrated the highest uptake in tumors, followed by the blood, spleen, lung, and kidneys (Fig. 4a). The tumor uptakes were 12.0 ± 1.4 and 15.0 ± 7.6% IA/g, tumor-to-muscle ratios 10.6 and 21.1, and tumor-to-blood ratios 1.1 and 2.4 for OSCA78 and IMR32 tumors, respectively. Normal tissue uptakes were similar between tumor-bearing and tumor-free mice. The uptake in OSCA78 tumors was significantly different from all the other tissues (p < 0.0001) except for the blood. Likewise, the IMR32 tumor had significantly higher uptake than all other tissues except for the spleen (10.7 ± 4.6% IA/g) (Fig. 4a). It is worth noting that one IMR32 tumor was significantly bigger and had a relatively lower uptake (6.3% IA/g) as compared to other IMR32 tumors (Fig. 4b).
At 72 h the highest uptake of $^{111}$In-DTPA-hu3F8 was observed in both OSCA78 (28.0 ± 3.6% IA/g) and IMR32 (51.6% IA/g) tumors, with a tumor-to-muscle ratio of 93.3 and 206.6 and a tumor-to-blood ratio of 6.7 and 8.4, respectively.

Fig. 1 Hu3F8 cross reacts with human and canine osteosarcoma cells and canine osteosarcoma tumor tissue. Immunofluorescence staining showed surface GD2 expression for both human (U2OS) and canine (OSCA78) osteosarcoma cells, similar to the very high GD2-expressing human neuroblastoma line, IMR32. Canine osteosarcoma tissue showed heterogeneous GD2 expression. Canine spleen and human neuroblastoma cells, SK-N-SH, were negative to hu3F8 immunostaining. Yellow bars represent 25 µm, and black bars represent 100 µm.

Fig. 2 Flow cytometry analysis showed all human IMR32 cells (81%), human U2OS cells (85%), and canine OSCA78 cells (93%) express GD2, while canine white blood cells (WBC) were negative to hu3F8.
Fig. 3  Binding affinity of \( ^{[111}\text{In}]\text{In-DTPA-hu3F8} \) to GD2 determined by saturation binding assay in IMR32 and OSCA78 osteosarcoma cells. \( K_d \) is 7.4 ± 1.0 nM for OSCA78 and 6.2 ± 1.9 nM for IMR32. \( B_{\text{max}} \) is 51,900 sites/cell for OSCA78 and 288,300 sites/cell for IMR32. Specific binding = total bound, non-specific uptake

Fig. 4  Biodistribution of \( ^{[111}\text{In}]\text{In-DTPA-hu3F8} \). a) Biodistribution of \( ^{[111}\text{In}]\text{In-DTPA-hu3F8} \) in IMR32 (n = 3) and OSCA78 tumor-bearing mice (n = 4) or tumor-free (n = 3) mice 24 h after i.v. injection (3.03 MBq/10 μg). Each symbol on the plot represents a mouse. The mean and standard deviation (SD) correspond to the height of the bar and the indicated error bars. b) Digital photograph of retrieved OSCA78 and IMR32 tumors. c) Biodistribution of \( ^{[111}\text{In}]\text{In-DTPA-hu3F8} \) (3.03 MBq/10 μg) and \( ^{[111}\text{In}]\text{In-DTPA-rituximab} \) (1.74 MBq/10 μg) at 72 h in mice bearing OSCA78 or IMR32 tumors. d) Immunohistochemical staining of retrieved OSCA78 tumors 72 h after \( ^{[111}\text{In}]\text{In-DTPA-hu3F8} \) injection was positive for radiolabeled-hu3F8 (brown staining within the tumor tissue). Bars represent 1 cm in b and 25 μm in d.
Conversely, the radiolabeled irrelevant antibody showed minimal uptake across all the tissues except for the kidneys (15.9 ± 3.0% IA/g) (Fig. 4c). Immunohistochemical staining of retrieved OSCA78 tumors confirmed the specific accumulation of \([^{111}\text{In}]\text{In-DTPA-hu3F8}\) within the tumors (Fig. 4d).

The maximum intensity projection SPECT/CT images of the tumor-bearing mice with \([^{111}\text{In}]\text{In-DTPA-hu3F8}\) are shown in Fig. 5. For \([^{111}\text{In}]\text{In-DTPA-hu3F8}\), at 24 h post-injection, most of the activity had accumulated in the tumor with a slight uptake in the liver and heart. At 48 h, most of the delivered activity cleared from the circulation, while OSCA78 and IMR32 tumors retained a high uptake, which was clearly shown in both flanks of the animals. In contrast, \([^{111}\text{In}]\text{In-DTPA-rituximab}\) injected OSCA78 tumor-bearing mice only showed high contrast to the background in the kidneys, which was consistent with the biodistribution data. By 96 h, normal tissue uptakes of \([^{111}\text{In}]\text{In-DTPA-hu3F8}\) including the liver, spleen, and kidneys were not distinguishable from the background, and only OSCA78 and IMR32 tumors still showed high contrast to the background. The OSCA78 tumor-bearing mice treated with \([^{111}\text{In}]\text{In-DTPA-rituximab}\) demonstrated very low tumor and normal tissue accumulation, which was not discernable above background (Fig. 5).

**SPECT/CT imaging of dogs**

No toxicities were noted from intravenous injection of hu3F8 in any dogs. The measured activities for the major organs of the tumor-free dog, post-injection of 17.0 MBq of \([^{111}\text{In}]\text{In-DTPA-hu3F8}\), from the SPECT images are shown in Table 1. The primary uptake was seen in vascular organs, with the highest in the liver, followed by the heart and the spleen at both 4 h and 24 h after injection (Fig. 6).
Subsequently, the targeting ability of $^{[111}\text{In}]\text{In-DTPA-hu3F8}$ to OS lesions was investigated in client-owned dogs with metastatic OS. In dog B, $[^{18}\text{F}]\text{F-FDG-PET/CT}$ revealed three metastatic OS lesions, two in the ribs and one in the fourth lumbar vertebra (Fig. 7a and supplementary video S1). SPECT/CT imaging of this dog showed avid uptake of the radiolabeled hu3F8 in all three metastatic lesions 48 h after injection (Fig. 7b and supplementary video S2). In dog A, $[^{111}\text{In}]\text{In-DTPA-hu3F8}$ selectively bound to metastatic OS lesions in the right shoulder area 48 h post-injection (Fig. 7c). No appreciable uptake in the lungs was observed. Dog A was euthanized per owner’s request 4 months after $[^{111}\text{In}]\text{In-DTPA-hu3F8}$ injection. Necropsy evaluation revealed metastatic spread in both the right shoulder and the lungs. Immunohistochemical staining of these tissues demonstrated extensive distribution of the radiolabeled hu3F8 throughout the shoulder and lung lesions (Fig. 7d), indicating its ability to target both metastatic bone and soft lesions.

### Discussion

Primary and metastatic OS is known to stably express cell surface disialogangliosides, particularly GD2 [5–7, 10, 25]. With the clinical success of GD2-targeted immunotherapy in high-risk neuroblastoma [8, 12], there is strong interest in repurposing anti-GD2 antibodies for radiopharmaceutical imaging and therapy of OS [10], in which GD2 expression is nearly ubiquitous but may be lower and more heterogeneous than in neuroblastoma [26]. In this study, we present preclinical assessment of a humanized anti-GD2 antibody, hu3F8, as a targeted delivery vector for radiopharmaceutical imaging of OS in both a tumor xenograft murine model and a spontaneous OS canine model and demonstrate the selective binding of $[^{111}\text{In}]\text{In-DTPA-hu3F8}$ to GD2-positive tumors. The detection of radiolabeled hu3F8 in canine skeletal and pulmonary metastatic tissues suggests its ability to target both osseous and soft tissue OS lesions.

The majority of preclinical testing of cancer radiopharmaceutical imaging and therapy largely relies on genetically

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**Table 1** Uptake of $[^{111}\text{In}]\text{In-DTPA-hu3F8}$ (of 17.0 MBq) in a tumor-free dog’s organs at 4 and 24 h after administration from the SPECT/CT images

| Time after injection | Liver (%IA) | Heart (%IA) | Spleen (%IA) | Left kidney (%IA) | Right kidney (%IA) | Urinary bladder content (%IA) |
|----------------------|-------------|-------------|--------------|-------------------|-------------------|-----------------------------|
| 4 h                  | 19.3        | 11.3        | 4.0          | 1.4               | 1.9               | 1.2                         |
| 24 h                 | 23.2        | 8.1         | 3.0          | 1.3               | 1.8               | 2.1                         |

**Fig. 6** Fusion SPECT/CT images of a tumor-free dog at 4 and 24 h after administration of $[^{111}\text{In}]\text{In-DTPA-hu3F8}$ (17.0 MBq/100 µg)
identical, inbred, tumor-bearing mouse models [18, 24, 27–29]. While these models provide critical information on in vivo biodistribution of the tested agents, they often underrepresent the heterogeneity of naturally occurring tumors in humans and the complex interplay of the immune response to such therapy [30]. The canine OS model provides an important link between murine models and human studies to directly address the safety and therapeutic efficacy of RPT in the setting of an intact immune system. Recently, Magee et al. has demonstrated the safety and feasibility of the combination of external beam radiotherapy, intratumoral immunocytokine, and targeted radionuclide imaging/therapy in companion dogs with advanced stage cancer, including OS [31]. The use of the theranostic pair of 86Y-NM600 (for PET imaging) and 90Y-NM600 provides the ability to confirm tumor-selective uptake of NM600 and perform dosimetry for 90Y-NM600. Similar to NM600, an alkylphosphocholine analog that preferentially incorporates into cancer cell cytoplasmic membrane agnostic of species, GD2 is a disialoganglioside and is not species specific. The immunohistochemistry and flow cytometry studies demonstrated that anti-GD2 antibody, hu3F8, was strongly reactive to canine OS cells and tumors, paving the way to use hu3F8 as a delivery vector for targeted radiopharmaceutical imaging and therapy in spontaneous OS canines.

Preferential uptake of [111In]In-DTPA-hu3F8 in GD2-positive tumors was demonstrated in biodistribution studies and was further validated by SPECT imaging. This is similar to several other studies in which the targeting ability of radiolabeled antibodies against either insulin growth factor receptor type 2 (IGF2R) [17, 28, 32, 33] or CD146 [18] was demonstrated in mouse models of OS xenografts. IGF2R is also expressed in spleen, whereas GD2 expression in normal organs and tissues is limited such that only tumors showed the high uptake of [111In]In-DTPA-hu3F8 at 72 h after injection. Consistent with the slow clearance of the full-length anti-GD2 antibody from the blood ($t_{1/2} = 1.7$ days) [34], we observed similar uptake of [111In]In-DTPA-hu3F8 in OSCA78 tumors (12.0 ± 1.4% IA/g) to the blood (10.5 ± 0.8% IA/g) at 24 h after injection. However, the significantly higher uptake in OSCA78 tumors than other blood rich organs, such as the heart, liver, spleen, and
kidneys, suggested a blood-independent uptake of $^{[111}\text{In}]\text{In-DTPA-hu3F8}$ in OSCA78 tumors. The improved uptake of $^{[111}\text{In}]\text{In-DTPA-hu3F8}$ in tumors at 72 h as compared to 24 h was supportive of selective binding of $^{[111}\text{In}]\text{In-DTPA-hu3F8}$ to GD2-expressing tumors.

Both the saturation binding assay and flow cytometry revealed lower expression of GD2 on OSCA78 than on IMR32 cells. Nevertheless, the highly specific uptake of $^{[111}\text{In}]\text{In-DTPA-hu3F8}$ within both types of tumors as demonstrated earlier indicates that even tumors with low GD2 expression could be targeted with hu3F8-based radiopharmaceuticals.

Using spontaneous OS canine as a model, we demonstrated the specific targeting of $^{[111}\text{In}]\text{In-DTPA-hu3F8}$ in metastatic OS lesions that closely mirror human OS. In both dogs with naturally occurring OS, avid uptake of $^{[111}\text{In}]\text{In-DTPA-hu3F8}$ in OS lesions was observed, which was correlated well to the findings of the standard $^{[18}\text{F}]\text{F-FDG-PET}$ (Fig. 7). In dog B, SPECT imaging revealed an extra lesion in the chest that was absent from $^{[18}\text{F}]\text{F-FDG-PET}$, which could be attributed to either the presence of metabolically non-reactive lesions or extension of disease due to the 1.5-month time interval between the PET/CT and SPECT/CT acquisitions. In OS dog A, the pulmonary uptake of $^{[111}\text{In}]\text{In-DTPA-hu3F8}$ 48 h after injection was not readily distinguishable on SPECT/CT images, despite the fact that metastatic spread to the lungs was identified postmortem. This discrepancy could arise from the fact that the metastatic lesions in the lungs at the time of imaging were too small to be detected by the SPECT system, due to its relatively poor spatial resolution ($\sim 14 \text{ mm}^3$). This suggests that treatment decision based on imaging alone may lead to a missed opportunity for early interventions in some patients whose tumor lesions are not appreciable during screening. Nevertheless, immunohistochemistry of postmortem canine OS tissues showed the accumulation of radiolabeled hu3F8 within both osteoblastic and soft lesions, suggesting the potential of targeting high-risk OS with hu3F8-based RPT beyond just imaging.

The present study has the following specific limitations besides poor spatial resolution of clinical SPECT/CT when applied to canine model. Due to the different growth rates of human IMR32 and canine OSCA78 tumor xenografts, some of the animals have no/minimal tumors, while others have tumors that are too large and may lead to ulcerations. Thus, the sample size for biodistribution study and SPECT imaging is relatively small with a short-term follow-up period. The use of immunocompromised mice with tumor xenografts is another inherent limitation that does not allow us to determine the effects of the native immune response. Nevertheless, the subsequent SPECT/CT imaging of spontaneous OS in dogs attested to the targeting ability of hu3F8 in naturally occurring OS lesions, highlighting the potential of using hu3F8-based radiopharmaceutical probes for imaging OS. Future studies will be warranted on developing this system for alpha-emitter targeting of metastatic OS which will introduce a fundamentally new therapy for this otherwise incurable pediatric/adolescent cancer.

**Conclusion**

Our findings demonstrate the potential of anti-GD2 antibody as a targeted delivery vector for radiopharmaceutical imaging of human and canine OS. The cross-species reactivity and high binding affinity of anti-GD2 antibody to canine OS cells/tissue and its ability to selectively deliver an imaging payload (Indium-111) to OS tumors support the utility of hu3F8 for precise radiopharmaceutical imaging of human and canine OS.

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**Author contribution** Y. F., J. Y., and I. L. designed and performed all the murine experiments and collected and analyzed the data. Y. F. and J. Y. wrote the original manuscript, and all authors reviewed and edited the manuscript.

Y. D., A. J., and J. N. collected, analyzed, and interpreted data from SPECT/CT images.

H. R. and J. B. collected and analyzed flow cytometry data.

D. L. K. and G. S. acquired funding, directed the research, and supervised in the design and interpretation of the experiments and the writing of the manuscript.

D. L. K. performed all the canine experiments.

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**Data availability** All data associated with this study are present in the paper or the Supplementary Materials.

**Declarations**

**Ethics approval and consent to participate** Animal protocols were approved by the Animal Care and Use Committee of the Johns Hopkins University, School of Medicine. Client-owned dogs with confirmed metastatic OS were recruited from owners with written informed consent.

**Conflict of interest** The authors report no relevant conflicts of interest.

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