Targeted Imaging of Ewing Sarcoma in Preclinical Models Using a $^{64}$Cu-Labeled Anti-CD99 Antibody

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Abstract

**Purpose:** Ewing sarcoma is a tumor of the bone and soft tissue characterized by diffuse cell membrane expression of CD99 (MIC2). Single-site, surgically resectable disease is associated with an excellent 5-year event-free survival; conversely, patients with distant metastases have a poor prognosis. Noninvasive imaging is the standard approach to identifying sites of metastatic disease. We sought to develop a CD99-targeted imaging agent for staging Ewing sarcoma and other CD99-expressing tumors.

**Experimental Design:** We identified a CD99 antibody with highly specific binding in vitro and labeled this antibody with $^{64}$Cu. Mice with either subcutaneous Ewing sarcoma xenograft tumors or micrometastases were imaged with the $^{64}$Cu-labeled anti-CD99 antibody and these results were compared with conventional MRI and $^{[18F]}$fluorodeoxyglucose–positron emission tomography (FDG–PET) imaging.

**Results:** $^{64}$Cu-labeled anti-CD99 antibody demonstrated high avidity for the CD99-positive subcutaneous tumors, with a high tumor-to-background ratio, greater than that demonstrated with FDG–PET. Micrometastases, measuring 1 to 2 mm on MRI, were not detected with FDG–PET but were readily visualized with the $^{64}$Cu-labeled anti-CD99 antibody. Probe biodistribution studies demonstrated high specificity of the probe for CD99-positive tumors.

**Conclusions:** $^{64}$Cu-labeled anti-CD99 antibody can detect subcutaneous Ewing sarcoma tumors and metastatic sites with high sensitivity, outperforming FDG–PET in preclinical studies. This targeted radiotracer may have important implications for the diagnosis, surveillance, and treatment of Ewing sarcoma. Similarly, it may impact the management of other CD99 positive tumors. *Clin Cancer Res; 20(3); 678–87.*

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Introduction

Ewing sarcoma is a tumor of the bone or soft tissue affecting approximately 250 children, adolescents, and young adults each year (1, 2). Patients with localized disease are treated with chemotherapy, surgery for local control, and radiotherapy in patients for whom surgical margins remain positive (3). For patients with metastatic disease, chemotherapy and radiotherapy are the mainstays of treatment (4). Although patients with localized disease have a good prognosis with 70% 5-year event-free survival (EFS), those with metastatic or relapsed disease fare poorly with only 15% to 20% 5-year EFS (1, 2, 5). Accordingly, accurate assessment of the extent of disease at the time of diagnosis plays a critical role in directing appropriate therapy and assessing prognosis.

Noninvasive studies including MRI, computed tomography (CT), or positron emission tomography (PET) are used to determine disease stage. Although these techniques are optimally used in different clinical scenarios, their limits of detection, constrained by system resolution, vary by modality: approximately millimeter range for CT and MRI, and 1 cm for PET (6–10). Apart from resolution limitation, the detection of micrometastases for molecular imaging modalities, such as PET, is highly dependent upon the signal-to-background ratio provided by the imaging probe. Micrometastatic disease that cannot be detected with existing imaging modalities is a known risk for distant recurrence, hence the need exists for new imaging approaches that can more accurately identify remote sites of disease with high specificity and sensitivity (11).

Imaging also plays a primary role in assessing the response of patients to therapy. After completion of therapy, patients typically undergo surveillance with MRI of the primary disease site as well as chest CT, or serial chest
Translational Relevance
In this article, we describe the creation of a CD99-targeted imaging probe that is highly sensitive for the visualization of CD99-expressing tumors. The 64Cu-labeled anti-CD99 probe is able to visualize 1- to 2-mm lesions, in xenograft models of Ewing sarcoma micrometastases, below the threshold of detection using [2]18Ffluoro-2-deoxy-d-glucose–positron emission tomography (FDG–PET). The performance of the current probe establishes a rationale for the development of a CD99-targeted imaging probe for clinical translation. This radiotracer may significantly impact the determination of disease extent at diagnosis, interpretation of conventional imaging findings, and ultimate approach to therapy for patients with Ewing sarcoma. It may similarly impact the management of other CD99 positive tumors.

X-rays, for evaluation of pulmonary nodules, a common site of disease recurrence (12). Although CT imaging is excellent at detecting abnormal lung lesions of small size, it cannot accurately distinguish among scar tissue, vascular structures, inflammation, infection, and malignant disease. A targeted imaging approach for Ewing sarcoma would help alleviate the diagnostic quandary posed by the finding of small lung nodules detected using existing imaging approaches, both at the time of diagnosis and during post-treatment surveillance.

By routine histologic staining, Ewing sarcoma cells are similar to other so-called 'small round blue cell tumors.' The most specific diagnostic marker used for the histopathologic diagnosis of Ewing sarcoma is CD99 (MIC2), a 32-kDa integral membrane glycoprotein that is highly expressed in Ewing sarcoma (13, 14). CD99 plays a key role in cell adhesion, migration, and apoptosis in lymphocytes, and seems to affect differentiation and malignancy of Ewing cells (15, 16). In clinical Ewing sarcoma cancer specimens, immunohistochemical staining of CD99 demonstrates high expression in a diffuse membrane distribution with nearly 100% of specimens staining positive, speaking to its use as a diagnostic tool (17). CD99 is also expressed on circulating leukocytes; however, the degree of CD99 expression in white blood cells as compared with Ewing sarcoma cells is not well described (18, 19).

Given a known high level of expression of CD99 at the cell surface in Ewing sarcoma, we sought to create an imaging probe targeting CD99 to allow detection of Ewing sarcoma micrometastases in a more sensitive and specific manner than is possible with existing imaging modalities. Although therapeutic studies have targeted CD221 (insulin-like growth factor receptor), a well-researched Ewing sarcoma cell-surface protein, we opted to focus on CD99 given that the expression of CD221 has been shown to be variable (20). Given the routine use of PET imaging at diagnosis and during surveillance, we chose to develop a 64Cu-labeled anti-CD99 antibody, which may have utility not only in Ewing sarcoma but also in other cancers in which CD99 is overexpressed, including lymphomas, synovial cell sarcoma, rhabdomyosarcoma, spindle cell tumors, and other soft tissue sarcomas (21, 22).

Materials and Methods
Cell culture and flow cytometry
TC32 Ewing sarcoma cells (CD99+) were cultured in RPMI (Gibco); A673 Ewing sarcoma cells (CD99+) and Kelly neuroblastoma cells (CD99−) were cultured in Dulbecco's Modified Eagle Medium, all supplemented with 10% FBS and 1% penicillin–streptomycin (Gibco) in 5% CO2 at 37°C. A673 and TC32 cell lines were provided by Dr. Todd Golub (Broad Institute, Cambridge, MA). The Kelly cell line was provided by Dr. Rani George (Dana-Farber Cancer Institute, Boston, MA). All Ewing sarcoma cell lines were authenticated via confirmation of a translocation of EWS and FLI1 genes by FISH for disruption of the EWS locus, and reverse transcriptase PCR cloning of the fusion junction (data not shown). TC32 cells were transduced with a lentivirus containing the FUW-Luc-mCherry-puro construct to enable bioluminescence imaging (BLI; ref. 23). In preparation for flow cytometry, cells were washed with PBS (Gibco; pH 7.0–7.2), dissociated from flasks using Cellstripper (Cellgro), a nonenzymatic dissociation solution, centrifuged, and resuspended in PBS. Approximately 1 million cells were aliquoted, filtered, and incubated on ice with 2 μg/mL anti-CD99 antibody (Abcam #23617 and #48530) or anti-CD221 antibody (BD-556000, Abcam #16890) for 1 hour. At the end of incubation, samples were again washed with PBS and maintained at 4°C until analyzed on a Becton Dickinson FACScan. A nonspecific IgG isotype control was obtained from Southern Biotech (#0107-01).

Human blood leukocytes for flow cytometry studies were isolated using Ficoll-Paque density gradient separation after centrifugation for 30 minutes. Cells were stained with 1 μg/mL fluorescein isothiocyanate (FITC)-conjugated secondary anti-mouse immunoglobulin (IgG; BD-554001) for 1 hour. The end of incubation, samples were again washed with PBS and maintained at 4°C until analyzed on a Becton Dickinson FACScan. A nonspecific IgG isotype control was obtained from Southern Biotech (#0107-01).

Human blood leukocytes for flow cytometry studies were isolated using Ficoll-Paque density gradient separation after centrifugation for 30 minutes. Cells were stained with 1 μg/mL phycoerythrin (PE)-conjugated anti-CD3 (Biolegend #317308), as per the manufacturer’s recommendations, and subsequently labeled with anti-CD99 and an FITC-conjugated secondary anti-mouse IgG as described earlier.

Inoculation of subcutaneous and micrometastatic lesions
TC32, A673, or Kelly cells in mid-logarithmic growth phase were trypsinized, centrifuged, and resuspended in PBS. NCr nude mice were obtained from the Charles River Animal Facility (Wilmington, MA). NOD/SCID- IL2Rnull (NSG) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). For subcutaneous xenografts, 2 to 3 million TC32 or A673 cells and 4 to 6 million Kelly cells were suspended in 100 μL of PBS and injected subcutaneously in NCr-nude mice. For the experimental metastasis model, 5,000 to 15,000 TC32 cells were suspended in 200 μL of PBS and injected via tail vein into NSG mice. On the
basis of pilot studies, 1 to 2 mm isolated liver metastases appeared approximately 4 weeks after injection, whereas larger secondary lung lesions grew at approximately 6 weeks after injection.

**Antibody radiolabeling with ⁶⁴Cu**

Chemicals and reagents were obtained from Sigma-Aldrich unless otherwise specified. EDC (1-ethyl-3-(3-dimethylamino)propyl)carbodiimide hydrochloride) was purchased from Pierce. Metal-naïve pipette tips were purchased from Rainin Instrument and were used to prepare all samples. Glass and plasticware were acid washed with 10% HNO₃ and rinsed thoroughly with ultrapure water (>15 MΩ resistivity; US Filter/Siemens Water Technologies). Ultrapure water was also used in all buffer preparations. Metal contaminants in buffers were decreased by passing through a Chelex-100 resin column (Bio-Rad Laboratories). ⁶⁴Cu was purchased from Washington University (St. Louis, MO).

The anti-CD99 antibody (Abcam #23617, DN16) and its nonpecific IgG control were conjugated with the bifunctional chelator p-NH₂-Bn-NOTA and radiolabeled with ⁶⁴Cu according to previously reported methods (24). Briefly, the antibodies were purified by high-performance liquid chromatography (HPLC) using a BioSep SEC-S3000 column (Phenomenex) with an isotropic aqueous phase consisting of 0.1 mol/L sodium acetate, pH 5.0, at a flow rate of 1 mL/min. The antibodies were then concentrated to 8 mg/mL in sodium acetate buffer using centrifugal filter units (3,000 MWCO; Millipore). They were then mixed with p-NH₂-Bn-NOTA (Macrocyclics) and later with EDC at reagent/antibody molar ratios of 250:1 and 500:1, respectively. The reaction was diluted to a protein concentration of 5 mg/mL with sodium acetate buffer, mixed by gentle pipetting, centrifuged to remove air bubbles, and placed in a water bath at 37°C for 30 minutes. The antibody was separated from unreacted p-NH₂-Bn-NOTA using the same HPLC conditions as before, concentrated in sodium acetate buffer, aliquoted, and stored at −80°C. Flow cytometry was performed on TC32 cells to confirm antibody binding after conjugation.

For radiolabeling, conjugated antibody aliquots were thawed, mixed with ⁶⁴Cu at a ratio of 4:1 (µCi/µg), and incubated for 30 minutes at 25°C. TLC was used to assess radiolabeling. An aliquot (1 µL) of the radiolabeling reaction was added to 9 µL of phosphate buffer (0.1 mol/L, pH 8, 100 mmol/L EDTA). After 5 minutes, a 1 µL aliquot of the mixture was spotted on an instant TLC glass microfiber strip (Biodex Medical Systems), allowed to dry, and developed using phosphate buffer/EDTA as the mobile phase. Using these conditions, the radiolabeled antibody remains at the baseline and free ⁶⁴Cu moves with the solvent front. The TLC strip was cut in half and the radioactivity in each half was assayed. If the TLC assay showed that the radiochemical purity was less than 95%, the product was purified using centrifugal filter units (as described earlier). The radioimmunoconjugate was diluted with saline and sterile filtered (0.2 µm filter) before injection.

**Tumor volume measurements and BLI**

The volumes of subcutaneous tumors were determined by caliper measurements using the formula \( V = 0.5 \times L \times W^2 \). For PET studies, mice with 50 to 150 mm³ tumors were used to minimize necrosis. For the experimental metastasis model, the mice that underwent tail vein injection of tumor cells were serially imaged using BLI (IVIS Spectrum; Caliper Life Sciences) as previously described to identify mice with lung and liver lesions (25).

**MRI**

Mice were imaged with a Bruker 7T BioSpec system. Mice were anesthetized with 1% to 1.5% isoflurane, and rapid acquisition with relaxation enhancement methods were used to obtain the images with time to repetition in spin = 1200 ms, effective time to echo in spins = 20 ms; in plane geometry: field of view (FOV) = 25.6 × 19.2 mm, matrix size = 256 × 192, and slice thickness = 1 mm.

**PET imaging**

For ²⁵⁸F-fluoro-2-deoxy-o-glucose (FDG) imaging, mice were fasted overnight (~12–16 hours) with free access to water. Mice were warmed for at least an hour, anesthetized with inhaled sevoflurane, and intravenously injected with approximately 14 MBq (~400 µCi) of ¹⁸F-FDG in a volume of 250 µL through the lateral tail vein. Mice were maintained under anesthesia for a one-hour uptake period and then scanned (350–650 keV energy window, 10 minutes listmode acquisition, three-dimensional (3D) rebinning followed by ordered subsets expectation maximization-maximum a posteriori (OSEM-MAP) reconstruction) on a multimodality preclinical imaging system (Inveon). For selected mice, CT acquisitions (80 kVp, 0.5 mA, 220° rotation, 600 ms/deg exposure time, 80-µm reconstruction pixel size) were also performed immediately before the PET imaging. The reconstructed PET/CT images were analyzed using Inveon Research Workplace (Siemens Healthcare). Where applicable, tumor uptake was quantified by the software and reported as PET standardized uptake values (SUV).

For ⁶⁴Cu PET studies, similar procedures were followed except that no fasting was performed, and imaging occurred at multiple time points after tracer injection. Each mouse was intravenously injected with approximately 4 to 7 MBq (~100–200 µCi, 50 µg) of radiotracer (anti-CD99- or IgG conjugates) in a volume of 250 µL via the lateral tail vein. At later imaging time points (e.g., 48 hours), an acquisition time of 20 minutes was used to compensate for diminished counting statistics due to radioisotope decay.

**Biodistribution and washout**

One mouse with bilateral subcutaneous tumors was imaged at 4, 14, 24, and 44 hours postinjection and time–activity curves (TAC) were generated from regions-of-interest. Terminal tracer biodistribution studies were also carried out at 24 and 48 hours after tracer injection (n = 3 at each time point). Tumor, blood, and other selected tissues were dissected and weighed. Radioactivity in the tissue
Immunohistochemistry of paraffin-embedded tissue

Expression of CD99 in human Ewing sarcoma in murine tissues was evaluated by immunohistochemical staining (MIC2 Covance antibody, dilution: 1/150) using a manual method. Formalin-fixed paraffin-embedded tissue sections were mounted on Superfrost plus charged microscope slides. Tissues were incubated with primary antibody for 60 minutes followed by secondary anti-mouse antibody linked to horseradish peroxidase (HRP; Envision) for 30 minutes, both at room temperature. Expression of CD99 in Ewing sarcoma and human lymph node specimens was evaluated by immunohistochemical staining (DN16 Abcam antibody, dilution: 1/100) using an automated method on a Ventana Discovery XT platform according to the manufacturer’s instructions. Following the Closed Loop Assay Development protocol (Ventana Medical Systems), antibody staining was developed using the OmniMap DAB anti-Mouse (HRP) detection kit (Ventana Medical Systems). In both murine and human tissue staining studies, standard quality control procedures were undertaken to optimize antigen retrieval, primary antibody dilution, secondary antibody detection, and other factors for both "signal and noise." All human tumor specimens were studied in accordance with an Institutional Review Board–approved protocol from Boston Children’s Hospital for the study of discarded human sarcoma tissue (protocol #S10-12-0617).

Statistical analysis

Statistical analysis was conducted using the Student two-tailed t test. Data are expressed as means of two or more experiments ± SEM.

Results

Immunostaining of Ewing sarcoma cells

To develop a Ewing sarcoma-targeted imaging probe, we first sought to identify an antibody with high affinity for Ewing sarcoma cells. On the basis of prior studies, we assessed antibodies targeting CD221 (insulin-like growth factor receptor) and CD99 (MIC2) using flow cytometric analysis after in vitro immunofluorescent staining (26, 27). Although the Ewing sarcoma cell line TC32 showed CD221 positive staining (Fig. 1A), the degree of immunofluorescence was less than that for CD99, which consistently demonstrated a 2.5-log shift in fluorescence (Fig. 1B). CD99 staining was consistently positive in all Ewing sarcoma cell
lines tested (Supplementary Fig. 1), whereas no immuno-reactivity was found in cells without CD99 expression, such as the neuroblastoma cell line Kelly (Fig. 1C). The mouse anti-CD99 monoclonal antibody DN16 was derivitized for all subsequent studies.

Creation of a 64Cu anti-CD99 probe

With the goal of enhancing tumor contrast and, in turn, maximizing image resolution and sensitivity, we chose to develop a PET probe for in vivo imaging. On the basis of our prior studies, we first conjugated the bifunctional chelator p-NH2-Bn-NOTA to the DN16 antibody (24). After purification, retention of antibody affinity for CD99 was verified by flow cytometry (Fig. 1D). Aliquots of NOTA-DN16 were radiolabeled with 64Cu to form a 64Cu-DN16 probe, and the product with >95% radiochemical purity was used for in vivo studies.

PET imaging of Ewing sarcoma xenografts

The 64Cu-DN16 probe was first evaluated in mice bearing subcutaneous xenograft tumors. Mice with A673 (CD99+) xenograft tumors between 50 and 150 mm3 were injected with either 64Cu-DN16 or 64Cu-IgG, and PET imaged 24 hours postinjection. Quantitated data, mean ± SEM for n = 5 animals. B, mice with TC32 (CD99+) xenografts on the left and Kelly (CD99-) xenografts on the right were PET imaged one hour after injection of 18F-FDG PET. Data, as mean ± SEM for n = 3 animals. C, the same mice were injected with 64Cu-DN16 and PET imaged 24 hours after injection. Data, mean ± SEM for n = 3 animals. Significance determined by the Student t test.

PET imaging with a 64Cu-labeled anti-CD99 antibody compared with 18F-FDG. A, mice with A673 (CD99+) xenografts (white arrows) were injected with either 64Cu-DN16 or 64Cu-IgG, and PET imaged 24 hours postinjection. Quantitated data, mean ± SEM for n = 5 animals. B, mice with TC32 (CD99+) xenografts were established on the left flank, and Kelly (CD99- neuroblastoma) xenografts were established on the right flank. Mice with similarly sized tumors (50–150 mm3) were injected with 18F-FDG PET imaging at 1 hour postinjection, to demonstrate the location of tumors and show equivalency for 18F-FDG uptake in the two tumor types (Fig. 2B). The same mice were then injected with 64Cu-DN16 and PET imaged 24 hours later. In contrast with the 18F-FDG results (Fig. 2B), uptake of the 64Cu-DN16 radiotracer was restricted to the CD99+ TC32 tumors (Fig. 2C). Although tracer activity in the brain, kidneys, and heart was apparent in animals injected with 18F-FDG (Fig. 2B), in mice injected with the 64Cu-DN16 probe, tracer background was restricted to the blood pool (Fig. 2C). Together, these results demonstrate that the 64Cu-DN16 is highly specific for CD99-expressing tumors.

PET imaging of micrometastatic disease

Having established the specificity of 64Cu-DN16, we next assessed the sensitivity of the probe. To establish a model of micrometastatic disease, mice were injected intravenously with TC32 cells (transduced with Luc-mCherry-puro), and
the distribution and size of lesions in the liver and lungs was serially assessed using bioluminescence and MRI. Animals with disease in the liver were first identified using BLI (data not shown). MRI was then used to identify animals with 1 to 2 mm nodules in the liver (Fig. 3A, Supplementary Fig. 2).

To determine whether FDG–PET imaging can detect lesions in this size range, mice with 1 to 2 mm liver nodules were imaged 1 hour after injection of 18F-FDG. We were consistently unable to visualize any liver lesions using 18F-FDG–PET imaging (Fig. 3B). Following FDG imaging, mice were immediately injected with 64Cu-DN16, and repeat PET imaging was performed 24 hours later. In contrast to the 18F-FDG–PET results, 64Cu-DN16 uptake within the micrometastatic lesions was readily visualized (Fig. 3C and D).

We performed histopathologic validation in a mouse with 64Cu-DN16 PET-positive liver lesions, in which we also noted a focus of uptake in the femur (Fig. 4A and B). Routine hematoxylin and eosin (H&E) staining of the liver confirmed the presence of a deposit of small round blue cells consistent with Ewing sarcoma that stained positive for CD99 (Fig. 4C). Of interest, a similar focus of cells was found infiltrating the marrow space in the distal femur (Fig. 4D), corresponding to the location of the lesion identified by 64Cu-DN16–PET imaging (Fig. 4A and 4B). Together, these results demonstrate that preclinical PET imaging with the 64Cu-DN16 probe improves signal-to-background ratio and enables detection of 1 to 2 mm CD99⁺ micrometastatic lesions that are below the threshold of detection using 18F-FDG.
Probe performance

To more thoroughly define the biodistribution and pharmacokinetics of the $^{64}$Cu-DN16 probe, we performed longitudinal PET imaging and ex vivo validation in a subset of animals with subcutaneous and metastatic xenograft tumors. TAC for major organs and xenograft tumors in one mouse demonstrated a progressive decrease in activity in the liver, heart, and spleen over time (Fig. 5A). The TC32 tumor had a progressive increase in activity over the 40 hours of imaging (Fig. 5A). There was likewise a small increase in activity within the CD99$^+$/C0 Kelly tumor over time, perhaps due to enhanced permeability and retention effects commonly associated with tumors (28). However, the absolute activity within the Kelly tumor was substantially less than that previously shown in the TC32 tumor at all points later than 4 hours (Fig. 5A).

Terminal tracer biodistribution studies were performed at 24 and 48 hours after probe injection, with three mice at each time point (Fig. 5B). Each mouse had a unilateral TC32 tumor, whereas one mouse sacrificed at 48 hours had bilateral tumors with a TC32 tumor on one flank and a Kelly tumor on the contralateral flank. Figures 5B and C validate our imaging results (Figs. 2–4), demonstrating specificity of probe for CD99$^+$ tumors and accumulation of probe over time.

Comparative staining of lymphocytes and Ewing sarcoma

Within normal tissues, CD99 expression is highly restricted to lymphocytes and endothelial cells. To compare the expression of CD99 in Ewing sarcoma cells with that in lymphocytes, we isolated normal human mononuclear cells and stained a normalized number of mononuclear and TC32 Ewing sarcoma cells with the DN16 antibody. Cells were also stained with a CD3 antibody to identify T cells. CD99 expression was found on both CD3-positive and -negative lymphocytes. The mean fluorescence in Ewing sarcoma cells (Fig. 6A) was nearly 100-fold higher than the staining of normal human lymphocytes (Fig. 6B).

To further compare the expression of CD99 in clinical specimens, we performed immunohistochemistry on four independent human Ewing sarcoma and human lymph node specimens. Staining in lymph nodes was limited to T-cell zones and occasional cells morphologically consistent with dendritic cells (Fig. 6C, bottom), whereas staining in Ewing sarcoma cells was diffuse, membranous, and qualitatively more intense (Fig. 6C, top).

Discussion

In the clinic, imaging plays a key role in the management of patients with solid tumors, helping to determine the primary site of disease, sites of dissemination, and response to therapy. Although surgical sampling is used to determine the extent of local spread in some forms of cancer (e.g., regional lymph node sampling), in most cases imaging is the only modality used to determine whether distant metastases are present. Despite tremendous advances in the last few decades, the clinical limit of detection of current
imaging modalities is on the order of 5 to 10 mm (6–10). Moreover, lesions in this size range are often indistinguishable from nonmalignant processes, resulting in uncertainty in interpretation. There is thus an unmet need for more sensitive and specific cancer imaging approaches.

Molecular imaging sensitivity is directly dependent on system resolution as well as other characteristics of the scanner and, equally important, the tumor (signal)-to-background ratio provided by the imaging probe. In these experimental models, our data show that small lesions detectable with preclinical MRI cannot be detected using preclinical FDG–PET but can be detected using the same PET scanner with the 64Cu-labeled anti-CD99 antibody probe. Preclinical imaging devices offer superior resolution to clinical PET scanners, therefore it may be difficult to extrapolate these results to the clinical realm. However, we anticipate that this targeted imaging approach will translate to a similarly improved signal-to-background ratio when used with human scanners, leading to enhanced detection of metastatic lesions, and ultimately tailored therapy for patients with Ewing sarcoma.

There are multiple requirements for the development of an antibody-targeted imaging probe. First, the epitope of interest must be extracellular and must be expressed at high levels. Second, the radionuclide should have a sufficiently long half-life to allow delayed imaging to match the pharmacokinetic properties of intact antibodies. Finally, for clinical use, the antibody must be humanized to allow repeated use. The radionuclide dose in humans, extrapolated from standard 18F-FDG dosing, is approximately 10 to 15 times that dosed to mice. Assuming a similar antibody-to-radionuclide ratio, the antibody dose in humans would be less than 5 mg, which is clinically acceptable. For targeted imaging of Ewing sarcoma, we have demonstrated that a 64Cu-labeled anti-CD99 radioconjugate is both sensitive and specific for PET imaging of Ewing sarcoma in experimental models. These encouraging first results establish the rationale for proceeding with the development of a human probe for future clinical translation.

Assessment of specificity in xenograft models is confounded by lack of cross-species reactivity for most antibodies. Moreover, CD99 is normally expressed on leukocytes and is highly expressed on thymocytes, in which it is believed to augment T-cell adhesion, apoptosis of double positive T cells, and migration and activation (18). The immunodeficient animals used in this study lack lymphocytes, limiting the ability to assess whether the antibody is cross-reactive. To this end, we stained normal human lymphocytes and found that only a small fraction of total lymphocytes stain positive for CD99, and that this staining

Figure 6. Comparative CD99 staining of normal lymphocytes compared with Ewing sarcoma cells. A, TC32 Ewing sarcoma cells were stained with DN16-FITC. B, normal human lymphocytes were stained with DN16-FITC and anti-CD3-PE. C, formalin-fixed paraffin-embedded tissue sections of human Ewing sarcoma (top row) and reactive human lymph node (bottom row) specimens were stained with anti-CD99 antibody (DN16). Each panel is from an independent patient.
is quantitatively lower than that for Ewing sarcoma cells. We also performed immunohistochemistry on paraffin-embedded human Ewing sarcoma versus human lymph node specimens to determine the relative expression of CD99 in clinical specimens. We again found that a much higher proportion of Ewing sarcoma cells stain positive for CD99 and that the staining is far more intense than that for lymphoid tissues. These results suggest that the level of expression of CD99 in Ewing sarcoma cells significantly exceeds that in normal human lymphocytes, and predicts that a CD99-targeted imaging agent would have potential utility for imaging of CD99-overexpressing tumors.

The scope of a CD99-targeted imaging probe extends beyond Ewing sarcoma. CD99 is expressed in tumors arising from immature mesenchymal tissue, including rhabdomyosarcoma, synovial sarcoma, clear cell sarcoma, and other soft tissue sarcomas (21, 22). T-cell leukemia and lymphomas likewise express CD99 as a lineage-specific marker. The opportunity to expand use to these additional cancer types helps bolster the rationale for development of a CD99-targeted imaging probe.

Finally, the current results suggest that an anti-CD99 antibody may have utility not only for delivering imaging moieties to CD99+ tumors, but also for delivering therapeutic payloads. Several therapeutic antibodies have been engineered into imaging probes, as the characteristics desired for an imaging probe are generally congruent with those of a therapeutic antibody. Future studies will explore the use of a radionuclide with a longer half-life (e.g., 89Zr), allowing an extended period of tracer imaging and longer period of tracer washout to further reduce background. We will likewise explore DN16 as a therapeutic radioconjugate and as an antibody–drug conjugate. Ultimate antibody modification, including the creation of antibody fragments and/or antibody humanization may aid in translation to the clinic.

Disclosure of Potential Conflicts of Interest
J C. Aster is a consultant/advisory board member of CytomX Inc. and Cell Signaling Technology Inc. No potential conflicts of interest were disclosed by the other authors.

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