Annotating MYC status with $^{89}$Zr-transferrin imaging

Jason P Holland$^{1,5,6}$, Michael J Evans$^{2,6}$, Samuel L Rice$^1$, John Wongvipat$^2$, Charles L Sawyers$^{3,3}$ & Jason S Lewis$^{1,4}$

A noninvasive technology that quantitatively measures the activity of oncogenic signaling pathways could have a broad impact on cancer diagnosis and treatment with targeted therapies. Here we describe the development of $^{89}$Zr-desferrioxamine–labeled transferrin ($^{89}$Zr-transferrin), a new positron emission tomography (PET) radiotracer that binds the transferrin receptor 1 (TFRC, CD71) with high avidity. The use of $^{89}$Zr-transferrin produces high-contrast PET images that quantitatively reflect treatment-induced changes in MYC-regulated TFRC expression in a MYC-driven prostate cancer xenograft model. Moreover, $^{89}$Zr-transferrin imaging can detect the in situ development of prostate cancer in a transgenic MYC prostate cancer model, as well as in prostatic intraepithelial neoplasia (PIN) before histological or anatomic evidence of invasive cancer. These preclinical data establish $^{89}$Zr-transferrin as a sensitive tool for noninvasive measurement of oncogene-driven TFRC expression in prostate and potentially other cancers, with prospective near-term clinical application.

Cancer cells generally express higher amounts of TFRC than do normal cells, presumably to accommodate the increase in iron usage required for various biological processes associated with cell proliferation. On this basis, there has been extensive interest in designing strategies to target TFRC therapeutically and in developing tools for noninvasive TFRC imaging. Recently, it has become evident that TFRC expression can be coupled to specific oncogenic signaling pathways. One example is the transcription factor MYC, which is broadly implicated as a driver oncoprotein in many cancers and pathways. Indeed, prostate cancers that develop in mice with prostate-specific MYC expression have elevated levels of TFRC mRNA, as do human prostate cancers with MYC gene amplification or overexpression. TFRC is also a direct target gene of the HIF-1α transcription factor, which is upregulated in kidney cancer as a result of loss of the VHL tumor suppressor gene and more broadly in tumors with phosphoinositide 3-kinase pathway activation. These data suggest that the level of TFRC protein expression in tumors may reflect activation of specific oncogenic pathways and could serve as a biomarker of pathway modulation.

Previous strategies to image TFRC expression have been plagued by problems of specificity and image resolution. The most widely used transferrin-based radiopharmaceutical is $^{67}$Ga$^{3+}$-citrate, which rapidly metallates transferrin in vivo. However, $^{67}$Ga$^{3+}$-citrate imaging with single photon-emission computed tomography (SPECT) results in qualitative, low-resolution data with high radiotracer uptake in many normal tissues, and $^{67}$Ga$^{3+}$ has also been shown to bind other serum proteins. Applying the PET nuclide $^{68}$Ga is also problematic, as the short half-life ($t_{1/2} = 67.7$ min) is insufficient to allow optimal distribution of a large biomolecule like transferrin (molecular weight 76–81 kDa). Transferrin is an endogenous serum protein that regulates iron transportation and homeostasis by binding Fe$^{3+}$, and many have shown that it is a versatile scaffold for several other radionuclides, including transition metal salts ($^{111}$In, $^{99m}$Tc) and halogens ($^{18}$F and $^{131}$I). Generally, images from these studies have been suboptimal due to radionuclide metabolism and accumulation in normal tissues (for example, bladder). In designing a radiotracer better suited to our goals, we noted that the radionuclide $^{89}$Zr produces quantitative data with PET imaging and has highly desirable physical properties (half-life $t_{1/2} = 78.4$ h; positron-emission yield $\beta^+ = 22.3\%$). Moreover, in our recent studies on $^{89}$Zr-labeled monoclonal antibodies (functionalized using the chelate desferrioxamine B (DFO)), we reported exceptionally low radiotracer uptake in normal mouse tissues (particularly in the abdomen), owing to the thermodynamic and kinetic stability of $^{89}$Zr-DFO. On the basis of the advantage of reduced tissue background observed with $^{89}$Zr-DFO antibody conjugates, we hypothesized that coupling $^{89}$Zr to transferrin via DFO might yield high-contrast images more reflective of TFRC expression levels (Supplementary Fig. 1).

RESULTS

Radiotracer development and validation studies

After conjugation of DFO to transferrin, we radiolabeled mouse or human apotransferrin with $^{89}$Zr (see Supplementary Methods for synthetic details; see also Supplementary Figs. 2 and 3 and Supplementary Table 1). DFO conjugates were functionalized with an average of two chelates per molecule of transferrin, and in all radiolabeling experiments the radiochemical purity was >99%, with specific activities in the range of 160–330 MBq mg$^{-1}$. In vitro and in vivo stability and metabolism studies confirmed the suitability of $^{89}$Zr-DFO–labeled transferrin for use in vivo (Supplementary Table 1 and Supplementary Figs. 4 and 5). Notably, the $^{89}$Zr-DFO
interaction was considerably more stable than the nonspecific binding
of 89Zr to the endogenous ferric binding sites of transferrin, a radiolabeling strategy we and others12 have found to be less suitable for in vivo studies (J.S.L. and C.L.S., unpublished observations). Before conducting in vivo experiments, we conducted radiotracer uptake assays in vitro (Supplementary Fig. 6). The holo (Fe3+-bound) forms of mouse 89Zr-transferrin and human 89Zr-transferrin were internalized by cancer cell lines 4- and 12-fold more than their respective apo (no Fe3+) forms, consistent with a specific biological interaction between the radiotracers and TFRC23.

Transferrin-based radiopharmaceuticals are known to localize to regions of inflammation due to increased TFRC expression on activated peripheral blood mononuclear cells24,25. Therefore, we first assessed the in vivo behavior of 89Zr-transferrin using a chemically induced acute phase response model26. Immunocompetent mice were treated with a subcutaneous (s.c.) injection of turpentine oil into the right hind limb. At 24 h after injection, we administered 18F-FDG to confirm inflammation (Fig. 1, Supplementary Figs. 7 and 8) and then administered the mouse or human 89Zr-transferrin radiotracers 24 h after 18F-FDG. 89Zr-transferrin localized to the inflamed tissue microenvironment 1 h after intravenous (i.v.) administration and persisted for over 24 h. The signal intensity of 89Zr-transferrin in the inflamed limb was 6.5 ± 0.3% injected dose (ID) g−1 compared to 0.8 ± 0.1% ID g−1 (s.d.) in the contralateral control limb (inflamed-to-control contrast ratio >8.1 ± 0.4 (s.e.m.)), yielding a contrast ratio significantly higher than that observed with 18F-FDG (inflamed-to-control = 1.7 ± 0.3 (s.e.m.); inflamed tissue uptake = 3.7 ± 0.9% ID g−1 (s.d.)). Notably, we observed little difference in the signal intensity between the inflamed and control limb using a radiolabeled albumin construct.
(89Zr-mAlb), a control previously invoked to assess nonspecific radiotracer accumulation in this model, was 2.1 ± 0.3% ID g⁻¹ (s.e.m.); inflamed tissue uptake = 2.5 ± 0.6% ID g⁻¹ (s.d.). Biodistribution studies at 24 h after administration of mouse 89Zr-transferrin, human 89Zr-transferrin, and 89Zr-mAlb corroborated the PET imaging data, with inflamed-to-control muscle contrast ratios of 6.6 ± 1.9, 6.7 ± 2.9, and 0.9 ± 0.2, respectively (Fig. 1b, Supplementary Table 2 and Supplementary Fig. 8).

PET imaging of MYC-driven prostate cancer xenografts

We next explored the ability of 89Zr-transferrin to measure aberrant TFRC expression in cancer, focusing on a MYC-driven prostate cancer model since TFRC is a well-established MYC target gene. We chose to study 89Zr-transferrin using MycCaP, a mouse prostate cancer cell line derived from the HI-MYC transgenic prostate cancer model in which MYC transgene expression is driven by an androgen receptor–dependent promoter. Both MYC and TFRC mRNA levels were substantially reduced in MycCaP cells grown in culture following androgen receptor siRNA knockdown (Fig. 2a) and in MycCaP xenografts following castration (Supplementary Fig. 9). TFRC expression was restored by constitutive expression of MYC following AR knockdown, confirming that MYC regulates TFRC in this model (Supplementary Fig. 10). Having documented the androgen receptor– and MYC-dependent expression of TFRC in MycCaP xenografts, we asked whether these
changes could be measured in vivo by $^{89}$Zr-transferrin PET imaging. Mouse $^{89}$Zr-transferrin was administered to intact male mice bearing MycCaP tumors 48 h after no treatment or castration. Temporal PET imaging revealed that at 5 and 24 h after radiotracer administration, mouse $^{89}$Zr-transferrin uptake in MycCaP xenografts was significantly lower in the castrated versus intact hosts ($P < 0.001$; Fig. 2b,c). Biodistribution studies confirmed the PET data. At 5 and 24 h, xenograft activity in the castrated mice remained low (1.2 ± 0.4% ID g$^{-1}$ and 1.3 ± 0.4% ID g$^{-1}$ (s.d.), whereas in the intact mice, tumor-associated activity was significantly higher (3.4 ± 0.7% ID g$^{-1}$ (s.d.; $P < 0.01$) and 3.2 ± 0.3% ID g$^{-1}$ (s.d.; $P < 0.001$), respectively) (Fig. 2d, Supplementary Tables 3 and 4 and Supplementary Figs. 11 and 12). These data demonstrate that $^{89}$Zr-transferrin is capable of measuring acute modulations in TFRC levels in vivo.

PET of prostate cancer in MYC-driven transgenic mice

Subcutaneous xenografts are idealized model systems for in vivo imaging because tumor tissue is relatively isolated from the mouse host, thereby minimizing signal-to-noise issues that plague many radiotracer studies. To determine whether $^{89}$Zr-transferrin can detect spontaneous prostate cancer, we conducted PET and ex vivo studies in Hi-Myc transgenic mice. All Hi-Myc mice develop invasive prostatic adenocarcinoma by 1 year of age that can be readily detected by magnetic resonance imaging (MRI; for representative images, see Supplementary Fig. 13). Co-registered PET/computed tomography (CT) images of 12-month-old Hi-Myc mice ($n = 7$) showed high PET signal in regions that were spatially discrete from the bladder and aligned with enlarged prostatic masses seen on CT (Fig. 3a). Dorsal-to-ventral stack plots and temporal PET imaging further highlight the enhanced contrast associated with prostatic masses (Supplementary Figs. 14 and 15 and Supplementary Video 1). Quantitative analysis of the PET data (Supplementary Table 5 and Supplementary Fig. 16) revealed that mouse $^{89}$Zr-transferrin uptake in the prostate tissue reached a mean value of 4.8 ± 0.4% s.d. ID g$^{-1}$ at 24 h, with minimal bladder activity of 4.3 ± 0.15% ID g$^{-1}$ (s.d.), and an average prostate-to-muscle contrast ratio of 7.3 ± 1.7 (s.e.m.; $n = 7$). In comparison, PET/CT imaging with mouse $^{89}$Zr-transferrin in wild-type (WT) mice showed no contrast versus muscle uptake in the region assigned as normal prostate by CT (Fig. 3e).

Ex vivo PET imaging and biodistribution studies confirmed the specific uptake of mouse $^{89}$Zr-transferrin in the prostates of Hi-Myc mice (Fig. 3, Supplementary Tables 6–8 and Supplementary Figs. 17–22). Modest activity was observed in the bladder with low uptake in the seminal vesicles and variable uptake in the different lobes of the prostate (Fig. 3b–d and Supplementary Fig. 17). Radiotracer uptake in the seminal vesicles of Hi-Myc mice was slightly higher than in WT mice, most likely due to the presence of invasive cancer. Quantitative image analysis revealed the highest uptake of mouse $^{89}$Zr-transferrin in the dorsal and lateral prostate lobes in Hi-Myc but not WT mice (Fig. 3h and Supplementary Table 8), consistent with the fact that the most dramatic histopathologic changes are observed in these lobes. Blocking studies combined with biodistribution studies and PET (Fig. 3g, Supplementary Figs. 18 and 19 and Supplementary Table 6) confirmed the specific uptake of mouse $^{89}$Zr-transferrin in the Hi-Myc dorsal, ventral and lateral prostate lobes (3.1 ± 0.2% ID g$^{-1}$ versus 1.6 ± 0.2% ID g$^{-1}$ (s.d.) in blocked mice), and significantly lower uptake in the anterior prostate (0.5 ± 0.03% ID g$^{-1}$, (s.d.; $P < 0.001$)). Radiotracer uptake in WT prostate was low (0.5 ± 0.2%ID g$^{-1}$ and 0.5 ± 0.1% ID g$^{-1}$ (s.d.) and statistically lower than the dorsal, ventral and lateral prostate lobes from the Hi-Myc mice ($P < 0.001$; Supplementary Tables 6 and 7 and Supplementary Fig. 18). A truth plot of mean tissue uptake from PET versus accurate biodistribution data further validated the use of imaging for measuring quantitative changes in mouse $^{89}$Zr-transferrin uptake (Supplementary Fig. 21).

$^{89}$Zr-transferrin PET detects aberrant MYC signaling in PIN

TFRC expression is increased in the prostate cells of Hi-Myc mice in advance of the development of invasive prostate cancer, well before any abnormalities can be detected by MRI. The earliest pathologic change is PIN, a well-established precursor to prostate cancer in humans that can only be detected by histopathology. To determine whether $^{89}$Zr-transferrin can detect aberrant MYC signaling in the prostate at this early, precancer stage, we obtained PET/CT images from 4-month-old Hi-Myc mice after administration of mouse $^{89}$Zr-transferrin. The penetrance of high-grade PIN at 4 months is essentially 100%. PET images revealed a region of high contrast discrete from the bladder, corresponding to an area defined by CT as the dorsal region of the prostate (Fig. 3c). Ex vivo PET studies showed higher radiotracer uptake in the dorsal prostate lobes with comparatively lower levels in the lateral, anterior and ventral prostate, consistent with the higher prevalence of PIN in the dorsal prostate (Fig. 3d). Quantification of the PET and biodistribution data showed a statistically significant difference ($P < 0.001$) in mouse $^{89}$Zr-transferrin uptake between WT and Hi-Myc dorsal, lateral and ventral prostate (Fig. 3g,h; Supplementary Tables 6 and 7). Collectively, these data suggest that mouse $^{89}$Zr-transferrin can detect a clinical precursor of prostate cancer enriched in high TFRC expression, even before the onset of physical changes in organ size that might be visualized by anatomic imaging.

DISCUSSION

In summary, our data document quantitative and high-resolution PET imaging of TFRC expression in vivo with minimal background interference using the $^{89}$Zr-transferrin radiotracer. We demonstrate proof of concept of specific targeting of TFRC in vivo in well-established mouse models of inflammation and of MYC-driven prostate cancer and show that judicious choice of the radionuclide and labeling strategy can provide high-fidelity images of spontaneous prostate tumors despite elimination of tracer in the bladder. Finally, we show that MYC-driven TFRC expression can be detected by PET before any evidence of disease using anatomic imaging technologies.

A large body of data generated over the decades has pointed to a generally increased avidity of cancer for transferrin, which resulted in many efforts to image TFRC expression, primarily using $^{67}$Ga$^{3+}$-citrate but also with other transferrin radioconjugates. Despite the abundance of circulating transferrin in animal models and humans (a precondition that ostensibly would disadvantage specific uptake of radiolabeled transferrin constructs in tissues), transferrin-based radiotracers have been successful in demarcating disease in preclinical models. On the basis of the extensive mechanistic work demonstrating the specificity of $^{18}$F-labeled transferrin, it is plausible that tissue contrast is also achieved in vivo with $^{89}$Zr-transferrin owing to the rapid turnover kinetics of TFRC. Consequently, although endogenous transferrin vastly exceeds the concentration of $^{89}$Zr-transferrin in blood, the rapid recycling of the transferrin-TFRC complex seems to ensure that some pool of TFRC is always available to engage the radioligand under normal physiological conditions. Consistent with this observation, blocking was achieved in the Hi-MYC model only with a very large excess of cold holotransferrin (~100× exogenous transferrin added in addition to endogenous serum transferrin).
In spite of our proof of concept in preclinical models, the fact that no previously developed radiolabeled transferrin constructs have been widely adopted in clinical practice underscores their shortcomings, which are largely based on poor signal-to-noise ratios, inappropriate imaging time points or nonquantitative, low-resolution images. It has been previously hypothesized that some of these failures may be due to competing demand for transferrin binding among normal tissues, but our results with 89Zr-transferrin suggest that this is not the case. Our studies reveal generally low uptake of 89Zr-transferrin in normal tissues after 24 h, with persistently high uptake only in the liver (the site of transferrin production and metabolism), kidneys and bone. (The observed bone uptake of 89Zr-transferrin is most likely due to the known tropism of Zr4+ salts for bone, rather than to an inherent quality of 89Zr-transferrin itself.) On the basis of our data, we suggest that in many cases, the low-contrast images obtained with other radiolabeled transferrin conjugates may be explained by properties of the radioconjugate moiety rather than the biological properties of transferrin. This conclusion is consistent with growing evidence showing excellent performance of 89Zr-DFO-biomolecule conjugates in PET imaging and may stimulate further application of this versatile radiolabeling strategy. Of note, this technology can be readily translated into the clinic, where its ultimate utility can be assessed.

One potential drawback of imaging with 89Zr-transferrin is its demonstrated affinity for inflammatory abscesses. Many solid tumors, including prostate cancer, are perfused with inflammatory cells, potentially complicating the analysis of foci avid for 89Zr-transferrin. It should be noted, however, that the cross-reactivity of 18F-FDG with inflammation has not impeded its widespread use in the detection and management of solid tumors. Further, previous histologic characterization of the Hi-Myc transgenic model found that the prostatic tissue was not heavily infiltrated by macrophages.

The novelty and effectiveness of using TFRC expression as a downstream reporter of MYC oncprotein activity opens up the possibility that 89Zr-transferrin could become a powerful imaging biomarker for cancer detection and for assessing response to therapy. In light of recent reports demonstrating that JQ1—a selective and potent inhibitor of the epigenetic protein BRD4—exerts its antitumor effects by downregulating MYC activity, our radiotracer may be particularly suitable for monitoring response to this promising new class of therapy.

This work is of immediate relevance to the mouse modeling community where PET imaging of mouse prostate cancer with traditional radiotracers, such as 18F-FDG, is not useful because the signal is obscured by bladder accumulation. Supplementary Fig. 11. Although high TFRC expression has historically been considered a general characteristic of cancer cells, more recent data have defined links to specific signal transduction pathways that have a driving role in oncogenesis. MYC is perhaps the most compelling example because of its direct transcriptional upregulation of TFRC expression, but other key oncogenic pathways such as increased phosphoinoside 3-kinase signaling in the context of phosphorylation and tension homolog loss also lead to increased transferrin uptake as a result of post-translational effects on TFRC protein. One can envision using 89Zr-transferrin PET scans to track early response to appropriately molecularly targeted cancer therapies in patients (or mice), much in the same manner that 18F-FDG PET is now being used to assess early response to imatinib in patients with gastrointestinal stromal tumor. Efforts toward the clinical translation of 89Zr-transferrin are currently under way.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

J.P.H. conducted all chemistry and radiochemistry, M.J.E. conducted all cellular assays, J.P.H., M.J.E., S.L.R. and J.W. conducted in vivo and ex vivo experiments. J.P.H., M.J.E. C.L.S. and J.S.L. designed the experiments, analyzed data and wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Stability and metabolism studies. Radiotracer stability was assessed in vitro by incubation in solutions of saline and PBS for 5 d at 37 °C. RCP was determined by radio–ITLC and γ-counting, and the protein-labeled fraction was measured at various time points by size-exclusion chromatography (PD-10, Sephadex G-25M, GE Healthcare). We conducted in vitro and in vivo metabolism studies in mouse urine and mouse and human blood by using size-exclusion, radiometric HPLC (radio-HPLC). The radio-HPLC system was equipped with a Tosoh Science G3000SWXL column (300 mm × 7.8 mm; 5 μm; Fisher Scientific) and eluted with a 0.02 M sodium acetate, 0.15 M sodium chloride, pH 6.4, mobile phase at flow rate of 1 ml min⁻¹ at ambient temperature.

In vivo models. All mouse experiments were conducted in compliance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Memorial Sloan-Kettering Cancer Center. Genetically engineered mouse (GEM) models of human prostate cancer (Hi-Myc mice at 4 and 12 months old) were used in these experiments. Mouse models of inflammation were developed by s.c. injection of turpentine oil (50 μl, neat) on the right hind limb of intact, immunocompetent male FVB mice (Taconic Farms). Xenograft models were induced on the right flank by s.c. injection of 2.0 × 10⁵ MycCap cells in a basement membrane (BD Matrigel, Collaborative Biomedical Products). Palpable MycCap tumors (50–250 mm³) developed after a period of 14–21 d. Surgical castration was performed under anesthesia in accordance with our IACUC-approved protocol.

Biodistribution studies. Biodistribution studies were conducted in accordance with previously reported methods [19, 39].

Small-animal PET imaging. PET imaging experiments were conducted on a microPET Focus 120 scanner (Concorde Microsystems). In repeated studies (n = 4), mice were given formulations of mouse ⁸⁹Zr-transferrin (11.6–13.7 MBq, (313–370 μCi), 35–41 μg of protein, in 200 μl sterile saline for injection) via i.v. tail-vein injection. Approximately 5 min before recording PET images, mice were anesthetized by inhalation of 1–2% isoflurane (Baxter Healthcare) in an oxygen gas mixture and placed on the scanner bed. PET images were recorded at various time points between 1 and 120 h after injection. Image reconstruction and processing details have been reported elsewhere [19, 48]. Manually drawn two-dimensional regions of interest (ROIs) or three-dimensional volumes of interest (VOIs) were used to determined the maximum and mean radiotracer accumulation in units of % ID g⁻¹ (decay corrected to the time of injection) in various tissues. Images were analyzed using ASIPro VM software (Concorde Microsystems).

Co-registered PET/CT. Computed tomography (CT) images were acquired on a small-animal Siemens/CTI microCAT II (Siemens Medical Solutions) scanner with an 8.5 cm axial by 5.0 cm transaxial field of view. Co-registered PET/CT images were recorded at 16 h after radiotracer administration. Images from the two separate modalities were mapped to a matrix and co-registered in accordance with previously reported methods [49].

Magnetic resonance imaging. Mouse prostate MRIs were acquired on a Bruker 4.7T Biospec scanner operating at 200 MHz and equipped with a 400 mT m⁻¹ ID 12-cm gradient coil (Bruker Biospin MRI).

Statistical analyses. Data were analyzed by using the unpaired, two-tailed Student’s t-test. Differences at the 95% confidence level (P < 0.05) were considered to be statistically significant.

Additional methods. Detailed methodology is described in the Supplementary Methods.

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