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Recombinant Carcinoembryonic Antigen as a Reporter Gene for Molecular Imaging

Vania Kenanova¹, Bhaswati Barat¹, Tove Olafsen¹, Arion Chatziioannou¹, Harvey R. Herschman¹, Jonathan Braun², and Anna M. Wu^{1,‡}

¹ Crump Institute for Molecular Imaging, Department of Molecular and Medical Pharmacology, David Geffen School of Medicine at the University of California Los Angeles, California

² Department of Cellular and Molecular Pathology, David Geffen School of Medicine at the University of California Los Angeles, California

Abstract

Purpose—Reporter genes can provide a way of non-invasively assessing gene activity *in vivo*. However, current reporter gene strategies may be limited by the immunogenicity of foreign reporter proteins, endogenous expression or unwanted biological activity. We have developed a reporter gene based on carcinoembryonic antigen (CEA), a human protein with limited normal tissue expression.

Methods—To construct a CEA reporter gene for PET, a CEA minigene (N-A3) was fused to the extracellular and transmembrane domains of the human FcγRIIb receptor. The NA3-FcγRIIb recombinant gene, driven by a CMV promoter, was transfected in Jurkat (human T cell leukemia) cells. Expression was analyzed by flow cytometry, immunohistochemistry (IHC), and microPET imaging.

Results—Flow cytometry identified Jurkat clones stably expressing NA3-FcγRIIb at low, medium, and high levels. High and medium NA3-FcγRIIb expression could also be detected by Western blot. Reporter gene positive and negative Jurkat cells were used to establish xenografts in athymic mice. IHC showed staining of the tumor with high reporter gene expression; medium and low N-A3 expression was not detected. MicroPET imaging, using an anti-CEA ¹²⁴I-labeled scFv-Fc antibody fragment, demonstrated that only high N-A3 expression could be detected. Specific accumulation of activity was visualized at the N-A3 positive tumor as early as 4h. MicroPET image quantitation showed tumor activity of 1.8(±0.2), 15.2(±1.3) and 4.6(±1.2) %ID/g at 4h, 20h and 48h, respectively. Biodistribution at 48h, demonstrated tumor uptake of 4.8(±0.8) %ID/g.

Conclusion—The CEA N-A3 minigene has the potential to be used as a reporter gene for imaging cells *in vivo*.

Keywords

CEA reporter gene; Jurkat xenograft; ¹²⁴I-labeled scFv-Fc antibody fragment; microPET/microCT imaging; T cells

[‡]To whom correspondence should be addressed. Anna M. Wu, Crump Institute for Molecular Imaging, Department of Molecular and Medical Pharmacology, David Geffen School of Medicine at UCLA, 700 Westwood Plaza, Los Angeles, CA 90095, USA, awu@mednet.ucla.edu; tel. (310)794-5088; fax (310)206-8975.

Introduction

The term “reporter gene” defines a gene with a measurable phenotype that can be distinguished over a background of endogenous proteins [1]. Enzymatic assays have been employed in cell culture to detect expression of reporter genes such as chloramphenicol acetyltransferase, β -galactosidase, and alkaline phosphatase (AP). Optical detection of fluorescent proteins (such as Green Fluorescent Protein) and luciferase has enhanced the sensitivity and ease of use of reporter gene technology [2].

More recently, great interest in using reporter genes for non-invasive *in vivo* imaging in living individuals has emerged. Nuclear imaging, using radiolabeled tracers, holds the greatest potential for translational and clinical use of reporter gene technology. PET is especially promising, due to its inherently superior sensitivity, resolution and quantitative power. PET reporter systems require an appropriate combination of a reporter/marker gene and a reporter/marker probe that, together result in accumulation of a radioactive signal when the reporter gene is expressed. For example, Herpes Simplex Virus type 1 (HSV1) thymidine kinase (TK) can be employed to trap HSV1-TK specific reporter probes, such as 9-[4-fluoro-3-hydroxymethyl]butyl]guanine ($[^{18}\text{F}]$ FHBG) or 5-iodo-2'-fluoro-2'-deoxy-1- β -D-arabino-furanosyl-uracil $[^{124}\text{I}]$ FIAU [3]. Alternatively, a reporter gene can encode a receptor that binds a radiolabeled probe. The human dopamine type 2 receptor (D2R) and the somatostatin receptor (SSTR) are examples of such reporter genes [4,5]. Transporters can also be used as reporter genes; two examples are the human sodium iodide symporter (hNIS) and the norepinephrine transporter [6,7]. This versatile collection of reporter/probe systems provides a powerful platform to closely monitor various cellular targets in a repeated and quantitative manner. Despite the advances achieved with these technologies, there are potential disadvantages associated with their use as reporter genes in humans [8,9]. The HSV1 protein is immunogenic because of its viral origin. While D2R, SSTR2 and NIS are innate human proteins, they have specific biological functions and expression profiles. A mutant, defunctionalized version of the D2R reporter gene has already been generated to overcome potential biological activity of ectopically expressed D2R [10]. NIS is endogenously expressed in the thyroid, stomach, salivary gland and lactating breast, and in low levels in the prostate, ovaries, adrenal gland, lung and heart [11–13]. This broad endogenous expression could limit the specificity and sensitivity of reporter imaging in these or surrounding tissues. Furthermore, the rapid efflux of radioiodide or pertechnetate (NIS substrates) from cells or tissues expressing NIS could result in loss of signal. Nevertheless, the wide availability of NIS substrates and their fast clearance from the body (diminishing the likelihood of interaction with the underlying cellular biology) suggest potential use of NIS as a reporter gene in future human studies [14].

In the translational/clinical arena, reporter gene technology is particularly promising as a means to monitor immunotherapy. Real time monitoring of the survival, homing, expansion and activity of therapeutic immune cells would greatly benefit the therapy process. Marking antigen-specific T lymphocytes with reporter genes will provide clinicians with this opportunity. The feasibility of this approach was demonstrated by isolation and transduction with the HSV1-sr39TK reporter gene of splenic T cells from mice that had successfully rejected a virally induced sarcoma. After injection of the tagged T cells in mice bearing the same sarcoma tumor, localization at the tumor could be observed over time by microPET imaging, using the $[^{18}\text{F}]$ FHBG PET reporter probe [15]. In a different study, the migration, survival and selective accumulation of Epstein-Barr virus (EBV)-specific T cells, transduced with the HSV1-TK reporter gene, were visualized via $[^{124}\text{I}]$ FIAU and PET [16].

We suggest that a modified carcinoembryonic antigen (CEA) gene can be employed as a reporter gene for clinical application. CEA is a seven-domain GPI-linked cell surface glycoprotein, proposed to function as a Ca^{2+} -independent, homotypic, intercellular adhesion

molecule important in organizing the structure of the fetal colon [17]. In healthy adults, CEA is virtually non-existent, only found in low quantities in the lumen of the colon [18]. During the process of carcinogenesis, colorectal and several other adenocarcinomas (e.g. breast, lung) resume robust CEA expression, possibly related to their differentiation and biological behavior [17]. Due to its GPI-linkage with the cellular membrane, CEA is labile and upon cleavage by phospholipases is shed in the circulation and captured by hepatocytes [19]. The full length, native human CEA molecule has already been utilized as a reporter gene for the purpose of improving radiopharmaceutical tumor localization [20]. In these studies, CEA transduced tumor xenografts were targeted by a radioiodinated intact mouse antibody and mice were imaged using a gamma camera. Tumor localization was noted; however a significant amount of radioactivity remained in the circulation five days post injection, resulting in a low tumor-to-blood ratio (0.9 ± 0.6). In contrast to what has already been done, we utilized two domains (N and A3), rather than the complete CEA molecule. Additionally, the GPI linkage of native CEA was replaced by a non-cleavable transmembrane domain, to avoid shedding of the reporter protein. Instead of using a radiolabeled intact antibody with extended serum persistence (~ 20 days or longer), an anti-CEA antibody fragment with optimized pharmacokinetics for PET imaging [21], was employed as a PET reporter probe. In this study, we evaluated membrane anchored, recombinant CEA as a reporter gene and an anti-CEA antibody fragment as a PET reporter probe for the purpose of visualizing CEA-expressing T lymphocytes *in vivo*.

To generate the reporter gene, we used the truncated version of CEA, termed N-A3, which retains the antigenic target for the anti-CEA T84.66 antibody probe [22]. The N-A3 “bud” was placed on an inert “stem”; a truncated version of the non-internalizing human FcγRIIb cell surface receptor that lacks the intracellular signaling domain. After establishing that the NA3-FcγRIIb recombinant protein is expressed on the surface of T lymphocytes in cell culture, we demonstrated that expression is maintained *in vivo*. Finally, we tested whether T-cell masses can be visualized via microPET imaging, using a ^{124}I -labeled anti-CEA single chain Fv-Fc antibody fragment probe [21].

Materials and methods

NA3-FcγRIIb design, gene assembly, and creation of an expression plasmid

The generation and expression of N-A3 in *Pichia pastoris* has previously been described [22]. The yeast leader sequence of the N-A3 gene was reverted back to the human CEA leader sequence via overlap PCR [23]. The FcγRIIb receptor gene, lacking its intracellular signaling domain, was isolated from Daudi cells (human B-cell lymphoma, American Type Culture Collection ATCC# CCL-213, Manassas, VA) using the Oligotex Direct mRNA Micro and OneStep RT-PCR kits (Qiagen, Valencia, CA) according to manufacturer instructions. Gene specific primers were designed based on the FcγRIIb subtype 1 sequence [24]. The FcγRIIb gene portion used in this work included the complete extracellular domain (ECD) and transmembrane domain (TMD) of the protein, followed by a truncated intracellular tail of 20 amino acid residues.

The N-A3 gene was ligated to the FcγRIIb gene, yielding the NA3-FcγRIIb construct. A mutation in the N-A3 portion of the gene was incorporated to remove a targeting site recognized by hepatic Kupffer cells. The N-A3 mutagenesis primer: 5'-GGCCAGTTCCGGGTATACCCAGACCTGCCCAAGCCC-3' exchanged the glutamic acid (E) in the PELPK motif to aspartic acid (D), yielding PDLPK. A mutation was also incorporated in the FcγRIIb gene and was necessary to prevent possible binding of endogenous IgGs to the FcγRIIb ECD. The mutagenesis primer 5'-CCACTGCACAGGAAACATAGCCTACACGCTATTCTCATCCAAGCC-3' exchanged a glycine (G) with an alanine (A) residue (NIGYT to NIA YT) in the extracellular portion of the FcγRIIb receptor. A Quick-Change Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA)

was used to introduce both mutations, in the N-A3 and FcγRIIb genes. The complete NA3-FcγRIIb fusion gene was transferred into the pcDNA 3.1TM/Zeo(-) vector with a CMV promoter (Invitrogen Corp., Carlsbad, CA).

Cell culture, expression and selection

Human Jurkat cells (T cell leukemia, ATCC# TIB-152) were maintained as recommended. After linearizing the pcDNA 3.1/Zeo(-)-NA3-FcγRIIb gene with the *PvuI* restriction enzyme (cutting in the Amp^R), 1×10⁶ Jurkat cells were transfected with 10 μg DNA [25]. Cells were grown under Zeocin selection (200 μg/mL). Single clones were expanded and assayed for N-A3 gene expression by a FACScalibur Flow Cytometer (Beckman Dickinson, UK). One million transfected or non-transfected Jurkat cells were incubated with the anti-CEA chimeric T84.66 mAb [26], detected with the secondary R-Phycoerythrin (PE)-conjugated goat anti-human (Fcγ-specific) mAb (Jackson Immunoresearch Laboratories, West Grove, PA). Non-transfected Jurkat cells were used to define a gate for identifying clones expressing the reporter gene. The percentage of cells expressing NA3-FcγRIIb at levels above the non-transfected Jurkat (negative) gate was used for relative ranking of reporter gene expression. The data were analyzed with the Cell Quest software (Becton Dickinson, San Jose, CA).

Western blot of selected clones

NA3-FcγRIIb expressing Jurkat clones (1×10⁶ cells), as well as the same number of non-transfected Jurkat and LS174T colon cancer cells (ATCC# CL-188), were washed with 1X PBS and lysed by triturating in 20 μL lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.1% SDS and 0.02% NaN₃; pH 7.4). The lysate was centrifuged at 500X g for 10 min at 4° C. The supernatant (~ 30 μL) was combined with 10 μL of 4X SDS loading buffer and boiled at 100°C for 5 min, loaded on a polyacrylamide gradient Ready Gel (4–20% Tris-HCl, BioRad, Hercules, CA) and analyzed by Western blot. Two out of four identical nitrocellulose membranes were probed with the anti-CEA cT84.66 mAb, followed by either AP- or Horseradish Peroxidase (HRP)-conjugated goat anti-human (Fcγ-specific) Abs (Jackson Immunoresearch Laboratories). The remaining two membranes were probed with a mouse anti-human β actin mAb (Sigma-Aldrich, St. Louis, MO), followed by an AP- or HRP-conjugated goat anti-mouse (Fcγ-specific) Abs (Jackson Immunoresearch Laboratories) as loading controls. For AP detection, NBT and BCIP substrates (Promega, Madison, WI) were used to develop the signal from the NA3-FcγRIIb/CEA and β actin proteins. For HRP detection, an enhanced chemiluminescent substrate (Pierce Biotechnology, Inc., Rockford, IL) was utilized according to the manufacturer instructions. The film exposure varied from 30 seconds (β actin) to 2 minutes (N-A3/CEA), after which the film was developed and analyzed by an AlphaImager (Alpha Innotech Corp., San Leandro, CA). Using the AlphaImager HP V.5.0.1 software, the signal from the NA3-FcγRIIb transfected Jurkat lysates was quantified by drawing regions of interest (ROI), encompassing the bands on the blot and obtaining integrated data values (IDV) of the signal intensity. The reporter protein signals were then compared to the LS174T CEA signal to generate signal ratios.

Establishment of tumor xenografts

All animal studies were conducted under protocols approved by the Chancellor's Animal Research Committee at the University of California Los Angeles. The establishment of Jurkat xenografts was modified from the method described by Neville et al. [27]. Briefly, seven-to-eight week old athymic mice (Charles River Laboratories, Wilmington, MA) were sublethally irradiated with 450 Rads in a ¹³⁷Cs source irradiator (Mark-1, Model 68A, JL Shepherd and Associates, San Fernando, CA). Three days later, two microfuge tubes containing 1×10⁶ HT-1080 feeder cells (human fibrosarcoma, ATCC# CCL-121) were irradiated with 6,500 Rads. One tube of irradiated feeder cells was combined with 1×10⁶ transfected Jurkat cells,

while the second was added to the same number of non-transfected Jurkat cells. Mice were injected subcutaneously (s.c.) in the left shoulder region with NA3-Fc γ RIIb transfected Jurkat/feeder cell suspension. The right shoulder area was injected with the non-transfected Jurkat/feeder cells as a negative control. Tumor masses were allowed to develop for about 20 days and reached approximately 100 mg weight.

Immunohistochemistry (IHC)

IHC staining was performed on paraffin-embedded sections, derived from NA3-Fc γ RIIb transfected and non-transfected Jurkat cell tumors. LS174T colon carcinoma xenografts were used as a positive control. Sections from non-transfected Jurkat tumors served as a negative control. All tumors were removed, and 4 μ m paraffin sections were cut and mounted on positively charged, poly-lysine coated slides. The slides were deparaffinized and re-hydrated. Antigen retrieval was accomplished by steaming with 10 mM Citrate buffer (pH 6.0). Cells were stained with the cT84.66 antibody [26] and the staining was developed by an avidin-biotin complex method, using biotinylated goat anti-human (H+L) Abs and avidin-conjugated HRP (Vectastain ABC Elite Kits, Vector Laboratories, Inc., Burlingame, CA). Sections were examined at 40X magnification and photographed.

Radioiodination and imaging with ^{124}I -labeled scFv-Fc

Radioiodination of the anti-CEA scFv-Fc H310A protein was performed by the Iodogen method, as previously described [21]. Labeling reactions (0.1–0.2 mL) contained 0.15 to 0.3 mg purified protein and 1 to 1.5 mCi Na ^{124}I (Advanced Nuclide Technology, Indianapolis, IN). The labeling efficiency was measured by instant thin layer chromatography (ITLC), using the Tec-Control kit (Biodex Medical Systems, Shirley, NY). Immunoreactivity was determined by incubation of labeled protein with excess LS174T cells as described [25]. The measurements of cell-bound and free ^{124}I -immunoconjugates were performed using a gamma counter (Willac Wizard 3', Perkin Elmer Life and Analytical Sciences, Shelton, CT). The imaging experiments utilized the microPET Focus 220 (Siemens Preclinical Solutions, Knoxville, TN) and micro Computed Axial Tomography (microCAT II, Concorde Microsystems, Inc., Knoxville, TN) instruments. Four xenografted mice for each cell line, carrying N-A3 positive (left) and N-A3 negative (right) Jurkat tumors were established. Prior to ^{124}I imaging, thyroid and stomach uptake of radioiodine were blocked as described [21]. Mice were injected in the tail vein with 150 μ Ci ^{124}I -labeled anti-CEA scFv-Fc H310A in saline/1% HSA solution. At 4, 20 and 48 hours, mice were anesthetized using 2% isoflurane, placed on the tomography bed, and imaged. Acquisition time was 10 minutes. After the 48h microPET scan, one of the four animals was also imaged by microCT for 10 min. The microPET and microCT images were then co-registered to yield a single image. Following the final scan, mice were euthanized. Blood, excised tumors and organs were weighed and counted in the gamma counter. After decay correction, the percent injected dose per gram (%ID/g) was calculated.

Analysis of microPET images and statistics

All images were processed using the FBP algorithm [28] and displayed by the AMIDE software [29]. The same color threshold was applied to all sets of images. Ellipsoid ROIs with depth of 4 mm (n=4) were drawn in the tumor and %ID/g was obtained after entering the injected dose in mCi and ^{124}I cylinder factor of 2×10^{-8} mCi/cc/Image Units. The variance among mice carrying the same type of tumor and imaged with the same tracer was represented using standard error (SE).

Results

Expression and selection of NA3-FcγRIIb reporter protein

The N-A3 fusion gene is composed of 726 nucleotides. To avoid the labile GPI linkage and the possibility of reporter protein release from the cell membrane, we utilized the ECD and TMD of the non-internalizing human FcγRIIb subtype 1 receptor (Fig. 1a) [24,30]. The FcγRIIb TMD is followed by a short intracellular tail that acts as a membrane anchor. The rest of the intracellular portion of the receptor, including the signaling immunoreceptor tyrosine-based inhibitory motif (ITIM), was omitted. The combined NA3-FcγRIIb recombinant gene is approximately 1.4 kb. Jurkat cells were transfected with a mammalian expression vector encoding NA3-FcγRIIb. Figure 1b illustrates both the proposed orientation of the reporter protein in the cell and the envisioned reporter protein-reporter probe interaction. Screening by flow cytometry identified clones with various levels of reporter gene expression above the background defined by non-transfected Jurkat cells. Three clonal lines, exhibiting low (26.3%), medium (59.8%) and high (97.1%) percentages of cells expressing the reporter protein were NA3-FcγRIIb reporter expression, were selected for further analysis (Fig. 2).

Western blots and quantitation of NA3-FcγRIIb

Western blots, using cell lysates from the same number of high, medium and low NA3-FcγRIIb expressing Jurkat cells were performed. The NA3-FcγRIIb total protein from the high and medium expressors was visible (Fig. 3a; Lane 1 and 2), while CEA from the low expressor could not be detected (Fig. 3a; Lane 3) with AP-conjugated probe. Equal protein loading was ensured by probing for the house keeping β actin protein (Fig. 3b). The combined NA3-FcγRIIb recombinant gene should be translated into a protein of approximately 50 kDa. This calculated molecular weight was based on the amino acid sequence alone, without taking into consideration any posttranslational modifications. In reality, the NA3-FcγRIIb protein migrated as a molecule of approximately 75 kDa (Fig. 3a). This discrepancy is likely due to both FcγRIIb and N-A3 glycosylation. N-A3 alone migrates at 40 kDa [22], while the entire CEA glycoprotein (45–55% carbohydrate content) has a molecular weight ranging from 180 to 200 kDa [31]. This heterogeneity is reflected by the smear observed for native CEA expressed in LS174T cells (Fig. 3a; Lane 5).

An HRP-based Western blot of cell lysates from all three transfected clones, LS174T (+) and non-transduced Jurkat T cell (–) controls, was developed by chemiluminescence, to quantitate the relative amount of recombinant NA3-FcγRIIb protein expression (film not shown). The band profiles on the film were identical to those seen on the AP-blots (Fig. 3). ROIs encompassing the NA3-FcγRIIb and CEA signal were drawn and IDVs were derived (Table 1). The ratios, based on IDV were: 1.6 (high-to-medium), 2.3 (medium-to-low) and 3.6 (high-to-low) N-A3 expression. The IDV analysis also showed that LS174T expression of CEA is 19.9, 31.2 and 70.9 times greater than that of the high, medium and low N-A3 expressing Jurkat cell (Table 1). Calculations, based on the quantitative Western blot results and a report that there are approximately 5×10^5 CEA molecules on the surface of LS174T cells [32], suggest that there are roughly 2.5×10^4 , 1.6×10^4 and 7.0×10^3 N-A3 binding sites on the high, medium and low reporter gene expressing Jurkat T cells, respectively.

Immunohistochemistry of NA3-FcγRIIb tumors

IHC of tumors produced by s.c. injection of transfected Jurkat cells demonstrate that only high reporter gene expression can be detected by this method (Fig. 4). The brown staining, prominent on the LS174T tumor slides, is also visible on the high NA3-FcγRIIb expressing tumor slides, though not as intense. These data also suggest that N-A3 gene expression of the high expressing Jurkat cells grown as tumors, is lower than the LS174T CEA expression *in vivo*. No staining was observed in the medium and low NA3-FcγRIIb tumor tissue (Fig. 4).

MicroPET/microCT imaging of tumors bearing the NA3-FcγRIIb reporter gene

The reporter probe used for all microPET imaging studies was the ^{124}I -labeled anti-CEA scFv-Fc H310A antibody fragment [21,33]. The ^{124}I labeling efficiency ranged from 98.4 to 84.4% and the specific activity associated with the injected protein was between 2.6 and 2.3 $\mu\text{Ci}/\mu\text{g}$. The *in vivo* imaging of nude mice ($n=4$), carrying both transfected (left shoulder) and non-transfected (right shoulder) Jurkat tumors demonstrated that only high NA3-FcγRIIb expressing tumors can be visualized by microPET (Fig. 5a). Mice bearing medium or low N-A3 expressing Jurkat xenografts did not have detectable microPET signal (Fig. 5b and 5c). The high NA3-FcγRIIb reporter gene expressing tumors were visible in all four animals. The mean tumor activity, derived from the microPET images, measured 1.8, 15.2 and 4.6 %ID/g for the 4h, 20h and 48h scan time points, respectively (Table 2). The average tumor-to-background ratios, also based on ROI analyses, were 2.2, 6.1 and 3.8-to-1 for the early (4h), middle (20h) and late (48h) time points, respectively. At 48h, the tumor-to-background ratio for the mouse shown on Figure 5a, was 8.2-to-1.

One animal from each group (low, medium and high) was scanned by microCT, immediately after the last time point of microPET imaging. The high expressing N-A3 positive tumor PET signal coincided with the anatomical tumor location, whereas the N-A3 negative tumor showed no detectable signal. This observation confirmed that the microPET signal was specific to the N-A3 expressing tumor. There was no signal originating from the anatomical location of the medium (Fig. 5b) and low N-A3 (Fig. 5c) expressing xenografts. Some thyroid uptake, attributed to incomplete blocking, was seen in the mouse with medium N-A3 expressing tumor (Fig. 5b). A biodistribution after the last scan (48h) revealed a tumor-to-blood ratio of $1.4(\pm 0.2)$ and an average of $4.8(\pm 0.8)$ %ID/g ($n=4$) in the N-A3 high expressing tumor (Table 2). This value compares well with the microPET image-derived $4.6(\pm 1.2)$ %ID/g ($n=4$) at 48h, thus validating the early and middle time point microPET quantitation. The animal in Figure 5a demonstrated tumor uptake of 7.1 %ID/g and tumor-to-blood ratio of 2.1 at 48h. The %ID/g for the medium ($n=4$) and low N-A3 ($n=4$) expressing tumors were $0.6(\pm 0.1)$ and $0.5(\pm 0.1)$ (Table 3), with tumor-to-blood ratios of 0.8 and 0.7, respectively. The average tumor weights for the three groups (high, medium and low) were $0.063(\pm 0.023)\text{g}$, $0.166(\pm 0.028)\text{g}$, and $0.137(\pm 0.019)\text{g}$. The activity measured in the liver, spleen, kidneys and lung of the mice bearing high N-A3 expressing tumors, was below 2 %ID/g, while that for the medium and low N-A3 expressing tumor bearing mice was below 1 %ID/g (Table 3). *In vivo* dehalogenation of antibodies labeled by the Iodogen method is a well documented phenomenon [34,35]. As the radioiodinated probe (^{124}I -scFv-Fc H310A) is being metabolized, free radioiodide and radioiodinated peptide fragments are quickly washed out of the tissues and excreted in the urine.

Discussion

The need for a sensitive, convenient and quantitative technology to monitor dynamic processes in living subjects has grown tremendously over the past few years. Reporter gene technology has already established itself as a mainstay of functional analyses both *ex vivo* and *in vivo*. Furthermore, the use of reporter genes has reached as far as the patient bedside, demonstrating its clinical potential [36,37].

Significant concerns pertaining to the clinical application of currently available reporter genes exist, with respect to toxicity, immunogenicity and ectopic biological activity. To reduce these problems, we focused on CEA, a well characterized human protein with limited tissue expression. CEA, as a reporter gene, can be paired with any of a number of radiolabeled, engineered anti-CEA antibody fragments for non-invasive imaging. To adapt CEA for use as a cell-surface reporter, several modifications were made. (1) CEA is naturally a GPI-linked protein, readily cleaved by phospholipases and released into the surroundings and circulation.

To circumvent this potential problem, CEA reporter gene constructs were engineered to contain an anchoring transmembrane domain. (2) For simplicity of construction, a “mini” version of CEA (N-A3) containing the epitope for the anti-CEA cT84.66 antibody was used. (3) If released into the circulation, CEA is accumulated rapidly in the liver due to specific interactions between a PELPK motif in CEA and a receptor on Kupffer cells (hepatic macrophages). This sequence (also part of the N-A3 molecule) was mutated to PDLPK, ablating that interaction [38]. (4) A spacer domain was introduced between the CEA A3 domain and the transmembrane region to facilitate access of the membrane-bound protein to antibody imaging probes.

FcγRIIb type 1 is expressed on macrophages, neutrophils, mast cells and B cell lymphocytes [39]. To ensure that the FcγRIIb transmembrane portion of the reporter gene is inert, the intracellular domain containing the ITIM motif was omitted. To eliminate any possibility of interactions with endogenous IgG molecules, G¹⁵⁶ was substituted with alanine [40]. This substitution also prevents possible crosslinking of B cell receptor (BCR) with the FcγRIIb, as well as any IgG-mediated FcγRIIb homodimerization events.

Our study was designed to be a proof of principle. Instead of therapeutic anti-tumor T cells, human tumorigenic T lymphocytes were transduced with the NA3-FcγRIIb reporter gene. Western blots confirmed reporter protein expression by the “high” and “medium” expressors; the “low” expressor did not produce a visible band. The three transfected clones (low, medium and high NA3-FcγRIIb expressors) were used to establish xenografts in mice. IHC analysis revealed that reporter NA3-FcγRIIb expression could only be detected in the high N-A3 expressing tumor. MicroPET imaging of mice carrying xenografts of the reporter gene-transfected and non-transfected T cells were consistent with the IHC conclusions; only the high reporter gene expressing tumor could be visualized in the living animals.

The anti-CEA ¹²⁴I-scFv-Fc H310A reporter probe exhibits a terminal, β phase serum half-life of approximately 21 hours in mice [33]. Previous studies in mice bearing LS174T (CEA positive) xenografts showed that the radioiodinated scFv-Fc H310A antibody fragment localizes to CEA expressing tumors with maximum uptake of 18.0(±2.7) %ID/g at 24 hours post injection. Similarly, in this work the microPET imaging studies of the N-A3 high expressing xenografts showed high tumor uptake [15.2(±1.3) %ID/g] and tumor-to-background ratio (6.1-to-1) at 20 hours. Clearly, optimal timing for imaging will depend on factors such as probe metabolism and pharmacokinetics. In comparison to the previously developed CEA reporter gene/reporter probe system [20], the combination of anchored recombinant CEA reporter targeted by a faster clearing antibody fragment produced higher tumor-to-blood ratio (1.4±0.2) at 48h, rather than five days post tracer administration. When the positron emitter was switched from ¹²⁴I to ⁶⁴Cu, the microPET signal from the high N-A3 expressing tumor was virtually non-existent at 21h; with 2.3 %ID/g in the tumor and a tumor-to-blood ratio of 1.2-to-1 (data not shown). An earlier scan (16h), on the other hand, demonstrated higher tumor-to-background ratio. However, the liver signal was also elevated, creating increased background in the abdominal area. These observations suggest that ¹²⁴I-scFv-Fc H310A antibody fragment is the better of the two probes we tested for imaging the NA3-FcγRIIb reporter gene.

There are several possibilities which can explain the observation that only T cells expressing “high” levels of this reporter gene can be visualized by PET *in vivo*. Reporter gene expression in the “medium” and “low” cell lines may simply be below the threshold of microPET detectability, as a result of either microPET instrument sensitivity and/or specific activity of the probe. A second possibility is target accessibility – when T cells are clustered tightly together to form a solid mass, a portion of their surface area may not be available for reporter probe binding. Flow cytometry preparations mimic the suspended state of T cells in serum, while xenografts are more representative of T cells homing at the tumor site. Flow cytometry

can identify all three cell populations while xenograft IHC and imaging were positive only for cells with a high level of reporter gene expression.

MicroPET has a superior sensitivity when compared to most imaging modalities, and a resolution of approximately 1 mm³. One can begin to address the issue of defining a threshold of PET detectability and ask how many available cell surface reporters are necessary to visualize tagged T cells with a stoichiometric, non-catalytic probe. Estimations based on our quantitative Western blot data suggested that the high N-A3 reporter gene expressing T cells, visible by microPET, have approximately 25,000 N-A3 surface molecules per cell. The medium N-A3 expressing cells, not visible as tumors by microPET, have approximately 16,000 N-A3 binding sites per cell. These imaging results suggest that the threshold number of PET reporter binding sites is around 25,000. Considering the sensitivity of the microPET instrument, this means that a sufficient number of ¹²⁴I positrons needs to be associated with T cells expressing roughly 25,000 reporter molecules per cells for a signal to be detected. Furthermore, this positron activity has to be concentrated in a volume equal or larger than the resolution element of the system (~ 1mm³). Although these results give semi-quantitative information, more systematic studies of the relationship between number of reporter binding sites and positron activity necessary for microPET detection need to be conducted.

The application of the N-A3 reporter gene is not limited to immune cells. This reporter could be useful for tracking any cell type of interest (e.g. stem cells). N-A3 may also be used as a transgene in animal models. In the clinic it may find utility as a reporter for a variety of gene therapy protocols. The potential broad applicability of a CEA-based reporter gene supports further effort to advance this work.

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References

1. Alam J, Cook JL. Reporter genes: application to the study of mammalian gene transcription. *Anal Biochem* 1990;188(2):245–54. [PubMed: 2121064]
2. Serganova I, Blasberg R. Reporter gene imaging: potential impact on therapy. *Nucl Med Biol* 2005;32(7):763–80. [PubMed: 16243653]
3. Tjuvajev JG, Doubrovin M, Akhurst T, Cai S, Balatoni J, Alauddin MM, et al. Comparison of radiolabeled nucleoside probes (FIAU, FHBG, and FHPG) for PET imaging of HSV1-tk gene expression. *J Nucl Med* 2002;43(8):1072–83. [PubMed: 12163634]
4. MacLaren DC, Gambhir SS, Satyamurthy N, Barrio JR, Sharfstein S, Toyokuni T, et al. Repetitive, non-invasive imaging of the dopamine D2 receptor as a reporter gene in living animals. *Gene Ther* 1999;6(5):785–91. [PubMed: 10505102]
5. Ur E, Bomanji J, Mather SJ, Britton KE, Wass JA, Grossman AB, et al. Localization of neuroendocrine tumours and insulinomas using radiolabelled somatostatin analogues, 123I-Tyr3-octreotide and 111In-pentetreotide. *Clin Endocrinol (Oxf)* 1993;38(5):501–6. [PubMed: 8101146]
6. Haberkorn U, Henze M, Altmann A, Jiang S, Morri I, Mahmut M, et al. Transfer of the human NaI symporter gene enhances iodide uptake in hepatoma cells. *J Nucl Med* 2001;42(2):317–25. [PubMed: 11216532]

7. Altmann A, Kissel M, Zitzmann S, Kubler W, Mahmut M, Peschke P, et al. Increased MIBG uptake after transfer of the human norepinephrine transporter gene in rat hepatoma. *J Nucl Med* 2003;44(6): 973–80. [PubMed: 12791828]
8. Bonini C, Ferrari G, Verzeletti S, Servida P, Zappone E, Ruggieri L, et al. HSV-TK gene transfer into donor lymphocytes for control of allogeneic graft-versus-leukemia. *Science* 1997;276(5319):1719–24. [PubMed: 9180086]
9. Verzeletti S, Bonini C, Marktel S, Nobili N, Ciceri F, Traversari C, et al. Herpes simplex virus thymidine kinase gene transfer for controlled graft-versus-host disease and graft-versus-leukemia: clinical follow-up and improved new vectors. *Hum Gene Ther* 1998;9(15):2243–51. [PubMed: 9794208]
10. Liang Q, Satyamurthy N, Barrio JR, Toyokuni T, Phelps MP, Gambhir SS, et al. Noninvasive, quantitative imaging in living animals of a mutant dopamine D2 receptor reporter gene in which ligand binding is uncoupled from signal transduction. *Gene Ther* 2001;8(19):1490–8. [PubMed: 11593362]
11. Ajjan RA, Kamaruddin NA, Crisp M, Watson PF, Ludgate M, Weetman AP. Regulation and tissue distribution of the human sodium iodide symporter gene. *Clin Endocrinol (Oxf)* 1998;49(4):517–23. [PubMed: 9876351]
12. Smanik PA, Ryu KY, Theil KS, Mazzaferri EL, Jhiang SM. Expression, exon-intron organization, and chromosome mapping of the human sodium iodide symporter. *Endocrinology* 1997;138(8): 3555–8. [PubMed: 9231811]
13. Spitzweg C, Joba W, Eisenmenger W, Heufelder AE. Analysis of human sodium iodide symporter gene expression in extrathyroidal tissues and cloning of its complementary deoxyribonucleic acids from salivary gland, mammary gland, and gastric mucosa. *J Clin Endocrinol Metab* 1998;83(5):1746–51. [PubMed: 9589686]
14. Serganova I, Ponomarev V, Blasberg R. Human reporter genes: potential use in clinical studies. *Nucl Med Biol* 2007;34(7):791–807. [PubMed: 17921031]
15. Dubey P, Su H, Adonai N, Du S, Rosato A, Braun J, et al. Quantitative imaging of the T cell antitumor response by positron-emission tomography. *Proc Natl Acad Sci U S A* 2003;100(3):1232–7. [PubMed: 12547911]
16. Koehne G, Doubrovina M, Doubrovina E, Zanzonico P, Gallardo HF, Ivanova A, et al. Serial in vivo imaging of the targeted migration of human HSV-TK-transduced antigen-specific lymphocytes. *Nat Biotechnol* 2003;21(4):405–13. [PubMed: 12652311]
17. Benchimol S, Fuks A, Jothy S, Beauchemin N, Shiota K, Stanners CP. Carcinoembryonic antigen, a human tumor marker, functions as an intercellular adhesion molecule. *Cell* 1989;57(2):327–34. [PubMed: 2702691]
18. Shively JE, Beatty JD. CEA-related antigens: molecular biology and clinical significance. *Crit Rev Oncol Hematol* 1985;2(4):355–99. [PubMed: 3886178]
19. Toth CA, Thomas P, Broitman SA, Zamcheck N. Receptor-mediated endocytosis of carcinoembryonic antigen by rat liver Kupffer cells. *Cancer Res* 1985;45(1):392–7. [PubMed: 2856899]
20. Raben D, Buchsbaum DJ, Khazaeli MB, Rosenfeld ME, Gillespie GY, Grizzle WE, et al. Enhancement of radiolabeled antibody binding and tumor localization through adenoviral transduction of the human carcinoembryonic antigen gene. *Gene Ther* 1996;3(7):567–80. [PubMed: 8818643]
21. Kenanova V, Olafsen T, Crow DM, Sundaresan G, Subbarayan M, Carter NH, et al. Tailoring the pharmacokinetics and positron emission tomography imaging properties of anti-carcinoembryonic antigen single-chain Fv-Fc antibody fragments. *Cancer Res* 2005;65(2):622–31. [PubMed: 15695407]
22. You YH, Hefta LJ, Yazaki PJ, Wu AM, Shively JE. Expression, purification, and characterization of a two domain carcinoembryonic antigen minigene (N-A3) in pichia pastoris. The essential role of the N-domain. *Anticancer Res* 1998;18(5A):3193–201. [PubMed: 9858883]
23. Schrewe H, Thompson J, Bona M, Hefta LJ, Maruya A, Hassauer M, et al. Cloning of the complete gene for carcinoembryonic antigen: analysis of its promoter indicates a region conveying cell type-specific expression. *Mol Cell Biol* 1990;10(6):2738–48. [PubMed: 2342461]

24. Brooks DG, Qiu WQ, Luster AD, Ravetch JV. Structure and expression of human IgG FcRII(CD32). Functional heterogeneity is encoded by the alternatively spliced products of multiple genes. *J Exp Med* 1989;170(4):1369–85. [PubMed: 2529342]
25. Olafsen T, Kenanova VE, Wu AM. Tunable pharmacokinetics: modifying the in vivo half-life of antibodies by directed mutagenesis of the Fc fragment. *Nat Protoc* 2006;1(4):2048–60. [PubMed: 17487194]
26. Neumaier M, Shively L, Chen FS, Gaida FJ, Ilgen C, Paxton RJ, et al. Cloning of the genes for T84.66, an antibody that has a high specificity and affinity for carcinoembryonic antigen, and expression of chimeric human/mouse T84.66 genes in myeloma and Chinese hamster ovary cells. *Cancer Res* 1990;50(7):2128–34. [PubMed: 2107969]
27. Neville DM Jr, Scharff J, Srinivasachar K. In vivo T-cell ablation by a holo-immunotoxin directed at human CD3. *Proc Natl Acad Sci U S A* 1992;89(7):2585–9. [PubMed: 1372981]
28. Defrise M, Kinahan PE, Townsend DW, Michel C, Sibomana M, Newport DF. Exact and approximate rebinning algorithms for 3-D PET data. *IEEE Trans Med Imaging* 1997;16(2):145–58. [PubMed: 9101324]
29. Loening AM, Gambhir SS. AMIDE: a free software tool for multimodality medical image analysis. *Mol Imaging* 2003;2(3):131–7. [PubMed: 14649056]
30. Ravetch JV, Bolland S. IgG Fc receptors. *Annu Rev Immunol* 2001;19:275–90. [PubMed: 11244038]
31. Thompson JA, Grunert F, Zimmermann W. Carcinoembryonic antigen gene family: molecular biology and clinical perspectives. *J Clin Lab Anal* 1991;5(5):344–66. [PubMed: 1941355]
32. Berk DA, Yuan F, Leunig M, Jain RK. Direct in vivo measurement of targeted binding in a human tumor xenograft. *Proc Natl Acad Sci U S A* 1997;94(5):1785–90. [PubMed: 9050856]
33. Kenanova V, Olafsen T, Williams LE, Ruel NH, Longmate J, Yazaki PJ, et al. Radioiodinated versus radiometal-labeled anti-carcinoembryonic antigen single-chain Fv-Fc antibody fragments: optimal pharmacokinetics for therapy. *Cancer Res* 2007;67(2):718–26. [PubMed: 17234783]
34. Zalutsky MR, Noska MA, Colapinto EV, Garg PK, Bigner DD. Enhanced tumor localization and in vivo stability of a monoclonal antibody radioiodinated using N-succinimidyl 3-(tri-n-butylstannyl) benzoate. *Cancer Res* 1989;49(20):5543–9. [PubMed: 2477144]
35. Geissler F, Anderson SK, Venkatesan P, Press O. Intracellular catabolism of radiolabeled anti-mu antibodies by malignant B-cells. *Cancer Res* 1992;52(10):2907–15. [PubMed: 1581908]
36. Yaghoubi S, Barrio JR, Dahlbom M, Iyer M, Namavari M, Satyamurthy N, et al. Human pharmacokinetic and dosimetry studies of [(18F)F]FHBG: a reporter probe for imaging herpes simplex virus type-1 thymidine kinase reporter gene expression. *J Nucl Med* 2001;42(8):1225–34. [PubMed: 11483684]
37. Voges J, Reszka R, Gossmann A, Dittmar C, Richter R, Garlip G, et al. Imaging-guided convection-enhanced delivery and gene therapy of glioblastoma. *Ann Neurol* 2003;54(4):479–87. [PubMed: 14520660]
38. Gangopadhyay A, Thomas P. Processing of carcinoembryonic antigen by Kupffer cells: recognition of a penta-peptide sequence. *Arch Biochem Biophys* 1996;334(1):151–7. [PubMed: 8837750]
39. Ravetch JV, Kinet JP. Fc receptors. *Annu Rev Immunol* 1991;9:457–92. [PubMed: 1910686]
40. Hulett MD, Witort E, Brinkworth RI, McKenzie IF, Hogarth PM. Identification of the IgG binding site of the human low affinity receptor for IgG Fc gamma RII. Enhancement and ablation of binding by site-directed mutagenesis. *J Biol Chem* 1994;269(21):15287–93. [PubMed: 8195166]

Fig. 1. Schematic representation of **(a)** the CEA reporter gene construct, including the restriction enzyme cut sites. ECD = extracellular domain; TMD = transmembrane domain. **(b)** positioning of the reporter protein (NA3-FcγRIIb) in the cellular architecture and its interaction with the ¹²⁴I-labeled reporter probe (anti-CEA scFv-Fc antibody fragment).

Fig. 2.
NA3-Fc γ RIIb reporter gene expression in Jurkat T cells.

Fig. 3.

Western blot of N-A3 expression in NA3-FcγRIIb transfected cells **(a)** Blot probed with anti-CEA cT84.66 primary and AP-conjugated anti-human Fcγ-specific secondary antibodies; **(b)** Loading control blot, probed with anti-β actin mouse and AP-conjugated anti-mouse Fcγ-specific antibodies. Lane 1: High N-A3 expression; Lane 2: Medium N-A3 expression; Lane 3: Low N-A3 expression; Lane 4: Molecular weight standard; Lane 5: LS174T (CEA +) control; Lane 6: Non-transfected Jurkat (-) control.

Fig. 4. Immunohistochemistry of N-A3/CEA expression in tumors (40x magnification). Parental Jurkat and LS174T tumors are included as negative and positive controls.

Fig. 5. MicroPET/microCT imaging of (a) High; (b) Medium; and (c) Low N-A3 expressing Jurkat xenografts, using ^{124}I -labeled anti-CEA scFv-Fc H310A antibody fragment at 4, 20 and 48h post tracer injection. (+) and (-) indicates transfected and control Jurkat tumors, respectively.

Table 1

Flow cytometry and Western blot quantitation.

N-A3/CEA Expressor ^γ	Flow Cytometry	Western Blot		
	Expression (%) ^ξ	IDV [*]	Ratio	Value
Low	26.3	41158636	High-to-Medium	1.6
Medium	59.8	93718214	Medium-to-Low	2.3
High	97.1	147004411	High-to-Low	3.6
LS174T		2919821713	LS174T-to-High	19.9
			LS174T-to-Medium	31.2
			LS174T-to-Low	70.9

^γRelative ranking of NA3-FcγRIIb reporter positive Jurkat cells.^ξPercent N-A3 expressing cells when gated as shown in Fig. 2 (versus non-transfected Jurkat cell as a negative control).^{*}Integrated Data Value (IDV).

Table 2
MicroPET image analysis and biodistribution of N-A3 high expressing T cell xenografts.

Animal	microPET Image Quantitation Tumor (%ID/g)				Biodistribution (48h)		
	Number	Early (4h)	Middle (20h)	Late (48h)	Tumor (%ID/g)	Tumor weight (g)	Tumor-to-Blood Ratio
1 2 3 4		2.4	15.0	3.4	3.6	0.037	1.1
		1.7	14.2	3.7	4.8	0.048	1.4
		1.7	19.0	8.3	7.1	0.131	2.1
		1.4	12.5	2.9	3.7	0.037	1.1
Mean		1.8	15.2	4.6	4.8	0.063	1.4
Standard Error		0.2	1.3	1.2	0.8	0.023	0.2

Table 3

Biodistribution of ^{124}I -labeled scFv-Fc H310A in mice bearing NA3(+) and NA3(-) xenografts at 48h.

Tissue	NA3 expressor (%ID/g)		
	High	Medium	Low
NA3(+) tumor	4.8 (0.8)	0.6 (0.1)	0.5 (0.1)
NA3(-) tumor	2.9 (0.06)	0.3 (0.03)	0.4 (0.02)
Blood	3.4 (0.7)	0.8 (0.1)	0.7 (0.08)
Liver	1.1 (0.1)	0.6 (0.08)	0.6 (0.1)
Spleen	1.4 (0.6)	0.5 (0.06)	0.4 (0.02)
Kidney	0.9 (0.07)	0.6 (0.1)	0.3 (0.03)
Lung	1.7 (0.1)	0.7 (0.09)	0.4 (0.02)

Values are the means (n=4) with corresponding SE shown in parentheses.