SSTR2-Based Reporters for Assessing Gene Transfer into Non–Small Cell Lung Cancer: Evaluation Using an Intrathoracic Mouse Model

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Abstract

The most common cause of cancer-related deaths in North America is lung cancer, 85% of which is non–small cell lung cancer (NSCLC). Gene therapy is a promising approach, but has been hindered by lack of methods for localizing and quantifying gene expression *in vivo*. Human somatostatin receptor subtype-2 (SSTR2)-based reporters can be used to follow gene expression *in vivo* using ligands with greater affinity for this subtype. NSCLCs can express SSTR subtypes, which may interfere with SSTR2-based reporters. We assessed whether a SSTR2-based reporter can serve as a reporter of gene transfer into NSCLCs. SSTR subtype expression was assessed in NSCLC cell lines A549, H460, and H1299 using RT-PCR. After infection with an adenovirus containing hemagglutinin-A-tagged-SSTR2 (Ad-HA-SSTR2) or control insert, expression was assessed by immunologic techniques and binding to clinically-approved ¹¹¹In-octreotide. *In vivo*, after magnetic resonance (MR) imaging, intrathoracic H460 tumors were injected with Ad-HA-SSTR2 or control virus ($n = 6$ mice/group) under ultrasound guidance. Intravenous injection of ¹¹¹In-octreotide 2 days later was followed by planar and single-photon emission computed tomography (SPECT) imaging. Biodistribution into tumors was assessed *in vivo* using anatomic MR and functional gamma-camera images and *ex vivo* using excised organs/tumors. In human lung tumor samples ($n = 70$), SSTR2 expression was assessed using immunohistochemistry. All three NSCLC cell lines expressed different SSTR subtypes, but none expressed SSTR2. Upon Ad-HA-SSTR2 infection, HA-SSTR2 expression was seen in all three cell lines using antibodies targeting the HA domain or ¹¹¹In-octreotide targeting the receptor domain ($p < 0.05$). Intrathoracic tumors infected with Ad-HA-SSTR2 were clearly visible by gamma-camera imaging; expression was quantified by both *in vivo* and *ex vivo* biodistribution analysis and demonstrated greater uptake in tumors infected with Ad-HA-SSTR2 compared with control virus ($p < 0.05$). Immunohistochemistry found that 78% of NSCLCs are negative for and 13% have low levels of SSTR2 expression. It is concluded that SSTR2-based reporters can serve as reporters of gene transfer into NSCLCs.

Introduction

LUNG CANCER is the leading cause of cancer-related death in the United States for both men and women, with an estimated incidence and mortality of 219,440 cases and 159,390 deaths in 2009, respectively (American Cancer Society, 2009). Lung cancer patients suffer an overall 5-year survival of approximately 16% despite advances in chemotherapy, radiation therapy, and surgery. This low survival rate is commonly due to advanced stage upon diagnosis (Jett,

1993; Nesbitt *et al.*, 1995). Chemotherapy and radiotherapy have had limited effect on improving survival even in patients with early-stage NSCLC, where some trials demonstrate that adjuvant chemotherapy prolongs overall survival by only approximately 5% at 5 years (Scagliotti, 2007). In patients with locally advanced NSCLC, frequent cross-sectional imaging does not alter survival after combined modality therapy (Benamore *et al.*, 2007). New therapies, such as gene therapy (Vattemi and Claudio, 2004; Cross and Burmester, 2006), are promising for improving outcome, but they are hampered by

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an inability to noninvasively assess gene expression in patients. Reporter imaging systems that have the potential for clinical translation are needed for assessing expression at the target, for efficacy, and off target, which may cause toxicity.

For assessing expression, reporter systems may be used. These generally consist of a gene that encodes a protein that can be visualized directly or, more commonly, visualized after binding and/or entrapping a substrate. Poor tissue penetration of up to approximately 1 cm in light-based systems, such as green fluorescent protein (GFP) (Cote *et al.*, 1997; de Martin *et al.*, 1997; Meyer *et al.*, 1998) and luciferase (Stables *et al.*, 1999), limits their utility for percutaneous imaging of larger animals and humans. Nuclear medicine-based reporters do not suffer such a limitation. Human somatostatin receptor type 2 (hSSTR2)-based reporters (Rogers *et al.*, 2000; Zinn *et al.*, 2000; Kundra *et al.*, 2002) have been proposed as reporter systems and have several advantages, including being of human origin, thus, limiting an immune response; change in expression can be quantified *in vivo* over time (Singh *et al.*, 2009), and such reporters can be made signaling-deficient to inhibit untoward effects on transduced cells or on linked genes of interest (Han *et al.*, 2007). We have previously demonstrated that hSSTR2 expression can be quantified using small animal cognates of clinical machines for functional nuclear imaging and anatomic imaging, such as single-photon emission computed tomography (SPECT) and magnetic resonance (MR) (Yang *et al.*, 2005; Singh *et al.*, 2009). Anatomic imaging of intrathoracic lung tumors is difficult due to poor tissue contrast using traditional methods, *e.g.*, computed tomography (CT), as well as rapid breathing and cardiac motion, especially in mice, but can be approached using echo-planar imaging (EPI)-based MR (Bankson *et al.*, 2008).

A potential limitation of using SSTR2-based reporter imaging in lung cancer is that there have been reports that lung cancers can express SSTRs (Fujita *et al.*, 1994; Papotti *et al.*, 2001; Herlin *et al.*, 2009). This has the potential to confound hSSTR2-based imaging. Among the SSTR subtypes, clinically approved ¹¹¹In-octreotide has high binding to SSTR2, intermediate binding to SSTR3 and 5, and poor binding to SSTR1 and 4 (Kluxen *et al.*, 1992; O'Carroll *et al.*, 1992; Yamada *et al.*, 1992; Bell and Reisine, 1993; Panetta *et al.*, 1994). We hypothesized that hSSTR2-based imaging could be performed in lung cancer using such a subtype-selective imaging agent. We further assessed the prevalence of subtype 2 in clinical lung cancer tumor samples. Thus, we assessed whether a SSTR2-based reporter can serve as a reporter of gene transfer into NSCLCs.

Materials and Methods

Cell lines and adenovirus infection

A549, H460, and H1299 human NSCLC cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin-streptomycin mixture. Adherent monolayer cells were cultured at 37°C in a humidified atmosphere with 5% CO₂ and 95% air. A549, H460, and H1299 (1 × 10⁶ cells of each) were infected with or without 10¹⁰ pfu Ad-CMV-HA-hSSTR2 (Singh *et al.*, 2009) diluted in 1 ml of serum-free RPMI medium in a six-well plate. Two milliliters of medium was

added after 4 hrs and the cells incubated for 48 hrs. Ad-CMV-GFP served as a control. After 48 hrs, the cells were divided for immunofluorescence, ELISA, Western blotting, and receptor-binding studies.

Reverse transcriptase–polymerase chain reaction (RT-PCR)

Total RNA was isolated from A549, H460, and H1299 human NSCLC by extraction with TRIzol Reagent. One microgram of total RNA was reverse-transcribed, and PCR reactions were carried out using Superscript One-Step RT-PCR kit according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Five SSTR primer sets (SSTR1–5) were used:

SSTR1 sense: AAATGCGTCCCAGAACGGGACCT

SSTR1 antisense: CAGGTTCTCAGGTTGAAGTCTT

SSTR2 sense: GATGATCACCATGGCTGTG

SSTR2 antisense: CAGGCATGATCCCTCTTC

SSTR3 sense: TCATCTGCCTCTGCTACCTG

SSTR3 antisense: GAGCCCAAAGAAGGCAGGCT

SSTR4 sense: CATGGTCGCTATCCAGTGCA

SSTR4 antisense: GTGAGACAGAAGACGCTGGTGAA CAT

SSTR5 sense: GCTCTTGGTGTTTCGCGGACGT

SSTR5 antisense: CAGGTTGACGATGTTGACGGTGAAG.

Ten microliters of each PCR product was subjected to electrophoresis on a 1% agarose gel and visualized under UV light.

Immunofluorescence and ELISA

Immunofluorescence, ELISA, and Western blotting were performed as described (Kundra *et al.*, 2002; Yang *et al.*, 2005). A mouse anti-HA primary antibody (Covance, Princeton, NJ) and fluorescein isothiocyanate (FITC)-labeled goat anti-mouse secondary antibody (Southern Biotech, Birmingham, AL) were used for immunofluorescence. A horseradish peroxidase (HRP) anti-HA antibody (clone 3F10; Roche, Indianapolis, IN) was used for ELISA, and positive cells exhibited a green-colored product after exposure to an HRP ELISA substrate (BioRad, Hercules, CA). Western blotting was performed using HRP anti-HA antibody and developed using a chemiluminescent HRP substrate (Pierce Biotech, Rockland, IL).

Binding assay

Receptor binding assays were performed in triplicate as described (Han *et al.*, 2007). Assay reagents were added on 0.1% polyethylene amine (PEI)-treated and washed GF/B multi-screen plates (Millipore, Bedford, MA) in the following order: 160 μ l of cell membrane (5 μ g/well), 40 μ l of ¹¹¹In-octreotide (100 nM) (Mallinckrodt, Houston, TX) or 160 μ l of membrane of SSTR2, 20 μ l of cold ligand (1 μ M unlabeled somatostatin), and 20 μ l of ¹¹¹In-octreotide (100 nM). After incubation for 40 min at room temperature and washing with Tris-HCl (50 mM), the plate filters were punched out and the associated radioactivity was quantified using a Beckman γ -counter.

Animal and orthotopic implantation of tumor cells

Male athymic nude mice (8 weeks old) were purchased from Charles River Laboratories (Wilmington, MA). All

animals were cared for in accordance with guidelines set forth by the American Association for Accreditation of Laboratory Animal Care International and the U.S. Public Health Service Policy on Human Care and Use of Laboratory Animals. All experiments were approved and supervised by the MDACC Institutional Animal Care and Use Committee.

To produce tumors, H460 cells were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.02% EDTA. Trypsinization was stopped by adding RPMI medium containing 10% FBS, washed once with PBS, then resuspended and counted in a Coulter counter (Beckman). H460 cells at 5×10^6 in 100 μ l of medium were injected into the right side of the thoracic space.

Biodistribution and imaging

Twenty-four hours prior to injection, 12 male nude mice were irradiated with 3 Gy. Then mice were injected intrathoracically with 5×10^6 H460 cells in 100 μ l of PBS. MR imaging was done at day 5 after inoculation to confirm tumor and at day 9 for measuring tumor size. Three days before mCAM (Siemens Medical Solutions, Brookfield, CT) and SPECT imaging (day 6 after tumor inoculation), the mice were randomized into two groups with six mice in each group. Under ultrasound guidance (Vevo 770, linear transducer, 40 mHz), the tumors in the first group were injected with 10^{10} pfu of Ad-CMV-HA-hSSTR2, and tumors in the control group were injected with 10^{10} pfu of Ad-CMV-GFP.

For MR imaging, a respiratory- and cardiac-gated, fat-suppressed EPI-based sequence was used (Bankson *et al.*, 2008). Animals were anesthetized with 2% isoflurane. The animals were imaged supine with a 4.7 T small-animal MR scanner (Biospec USR47/40 NMR/MRI; Bruker Biospin Corp., Billerica, MA). A T_2 -weighted spin-echo EPI sequence (TE = 35 msec, TR >> T_1 ; several respiratory cycles, bandwidth = 100 kHz, matrix size = 128×128 ; acquired in eight shots with 1.56-msec echo spacing in 25.27-msec EPI readout, field of view = 3 cm $\times$ 3 cm, slice thickness = 1.25 mm, and skip = 0.25 mm) with a frequency-selective fat-suppression pulse was synchronized with cardiac and respiratory cycles to acquire one shot from one slice at each of multiple triggers during the plateau of the respiratory cycle (with the lungs at full expiration) and at either the Q wave or R wave of the ECG signal, depending on their prominence. The spin-echo EPI sequence was optimized to maximize contrast between solid tumors, chest wall, and lung tissue while minimizing respiratory and cardiac motion artifacts. Using Image J program (version: Java 1.3.1-03, National Institutes of Health, Bethesda, MD), tumor volumes were obtained by drawing regions of interest (ROI) on each image containing tumor (Yang *et al.*, 2005; Bankson *et al.*, 2008; Singh *et al.*, 2009) and converted to weight in grams, assuming a tissue density of 1 g/ml.

After MR imaging at day 9, anesthetized mice were injected intravenously with 300 μ Ci (13 MBq) of 111 In-octreotide (Mallinckrodt, St. Louis, MO). Twenty-four hours later, planar and SPECT imaging was acquired as described (Yang *et al.*, 2005). In brief, planar 10-min acquisitions were performed of anesthetized, prone mice using a gamma camera (mCAM) fitted with a medium-energy parallel-hole collimator. SPECT imaging (120 views, 7.5 sec per view, pixel size of 2.4 mm, 128×128 matrixes) of each animal was ac-

quired using a 360° rotation of a fixed 1/15 rpm rotational device attached to the front of the medium-energy collimator (15-min acquisition) and reconstructed. For both the planar and SPECT images, ROIs were drawn over the periphery of the tumor and uptake was subtracted from the left lung, which did not have a tumor. The counts per pixel per minute obtained from the ROI were converted to microcuries by using a calibration constant derived from imaging of standard activity phantoms by the gamma-camera scanner. Uptake by each tumor was divided by the weight of the tumor derived from MR imaging to obtain the percentage of injected dose per gram (%I.D./g) *in vivo* biodistribution.

The mice were euthanized after imaging, and organs and tumors were excised and weighed. Samples of these tissues were then weighed, and associated radioactivity was determined using a Cobra gamma counter to determine %I.D./g *ex vivo* biodistribution.

Immunohistochemistry of mouse samples

Sections of the excised mouse tumors were fixed in 10% formalin. Paraffin-embedded sections were processed for immunohistochemistry using the Vector MOM immunodetection peroxidase kit (catalog no. PK2200; Vector Laboratories, Burlingame, CA). The sections were probed with a primary mouse anti-HA antibody (1:250) and stained with the DAB Peroxidase Substrate kit (catalog no. SK-4100; Vector Labs). They were counterstained with Mayer's hematoxylin (catalog no. H-3404; Vector Labs).

Immunohistochemistry of human samples

To determine the expression of SSTR2 in primary lung cancer tumors, we selected and evaluated 70 NSCLCs (48 adenocarcinomas, 22 squamous cell carcinomas). These samples were archived, formalin-fixed, paraffin-embedded tumor tissue from surgically resected lung cancer specimens from the Lung Cancer Specialized Program of Research Excellence Tissue Bank at The University of Texas M.D. Anderson Cancer Center. This study was approved by the M.D. Anderson Cancer Center institutional review board. Tumor tissues were histologically analyzed and classified using the 2004 World Health Organization classification system. Samples were placed in a TMA, using three 1-mm-diameter cores that included tissue from the center, intermediate, and peripheral areas of the tumor.

Five micron-thick formalin-fixed, paraffin-embedded tissue histology sections from TMAs were deparaffinized, hydrated, heated in a steamer for antigen retrieval for 30 min with 10 mM sodium citrate (pH 6.0), and washed in Tris buffer. Peroxide blocking was performed with 3% H_2O_2 in methanol at room temperature for 15 min, followed by 10% bovine serum albumin in Tris-buffered saline with Tween 20 for 30 min at room temperature. Next, samples were incubated in anti-somatostatin receptor 2A rabbit polyclonal antibody (SSTR2A) (catalog no. PA3-109; Affinity BioReagents, Inc., Golden, CO), at 1:2,000 dilution, for 1 hr at room temperature. After washing, incubation with the secondary antibody (EnVision+ Dual Link labeled polymer; DAKO, Carpinteria, CA) was performed for 30 min, followed by application of diaminobenzidine chromogen for 5 min. The slides were then counterstained in hematoxylin and topped with a coverslip. As positive control, formalin-fixed

and paraffin-embedded gastric mucosa tissue was used. As negative control, paraffin-embedded positive gastric mucosa tissue was subjected to SSTR2A omitting the primary antibody, which was replaced with PBS buffer. Immunostaining evaluation was performed by two pathologists (I.W. and L.S.) using a white light microscope. Positive immunostaining in control cells and lung cancer tumor cells was mainly cytoplasmic and membranous. The percentage of positive tumor cells was evaluated.

Statistical method

Groups for ELISA, RT-PCR, and *in vivo* or *ex vivo* biodistribution were compared by Student's *t* test. Linear regression was used to correlate tumor weight derived by MR versus that of excised tumors. The analyses were performed using Excel 2003 software (Microsoft Inc., Seattle, WA). For all the tests, $p < 0.05$ was considered statistically significant.

Results

Expression of hSSTR subtypes in lung cancer cell lines

Expression of the SSTR subtypes (1–5) was tested in three human NSCLC cell lines (H460, H1299, and A549) using RT-PCR (Fig. 1). Both A549 and H1299 showed mRNA expression of SSTR1, 3, and 5, whereas H460 cells only expressed SSTR1 and 3. None expressed the subtype SSTR2. SSTR2 expression was seen in the positive control cell line 309 (Yang *et al.*, 2005). RT-PCR of the “housekeeping gene” β -actin demonstrated the quality of the mRNA. Primer quality was confirmed by PCR of SSTR subtypes from subtype-specific plasmids.

In vitro expression of HA-hSSTR2 after adenoviral infection

Expression of HA-hSSTR2 in H1299, H460, and A549 cells was assessed *in vitro* before and after Ad-CMV-HA-hSSTR2 infection using antibodies targeting the HA domain. Background is seen in uninfected cells, but expression on the cell membrane is clearly seen in all three cell types after Ad-CMV-HA-hSSTR2 infection (Fig. 2A). Quantitative ELISA confirmed expression in all three cell types after Ad-CMV-HA-hSSTR2 infection, but not in controls ($p < 0.05$, Fig. 2B). Western blot analysis confirmed that protein of the correct size was made in Ad-CMV-HA-hSSTR2 infected cells. As seen in Fig. 2C, a distinct band (60–72 kDa) is seen in cells

expressing the HA-hSSTR2 gene product in all three cell lines infected with Ad-CMV-HA-hSSTR2, but not in uninfected cells.

In vitro receptor binding of ^{111}In -octreotide

^{111}In -Octreotide was used to target the receptor portion of the HA-SSTR2 fusion protein. Figure 3 shows the binding of ^{111}In -octreotide at a saturating concentration of 100 nM to uninfected cells and cells infected with Ad-CMV-HA-hSSTR2. As seen, the radioligand binding increased significantly after Ad-CMV-HA-hSSTR2 infection.

In vivo imaging of ^{111}In -octreotide and ex vivo biodistribution

Representative EPI-based MR images (Fig. 4A and B) demonstrate tumor as increased signal in the superior aspect of the thorax. Tumor weights derived from volume assessment of the MR imaging correlated highly with that from excised tumors ($r^2 = 0.99$, $n = 12$, coefficient of the x variable = 1.03; Fig. 4C), as we have reported previously (Bankson *et al.*, 2008). We found that we could visualize the intrathoracic tumor by ultrasound; therefore, we used this modality to guide injection of adenovirus into the tumor (Fig. 4D). Both planar and SPECT imaging (Fig. 4E and F) demonstrated expression of HA-SSTR2 in tumors infected with Ad-CMV-HA-hSSTR2, but only background signal was seen in tumors infected with control virus. As mentioned above, the tumor volume assessment by MR enabled us to derive tumor weight. As with classic biodistribution experiments that excise and weigh tumors to control for underlying tumor size, *in vivo* biodistribution also was normalized to tumor weight. *Ex vivo* and *in vivo* biodistribution analysis normalized to tumor weight correlated (%I.D./g *ex vivo* vs. %I.D./g *in vivo* using planar and MR imaging, $r^2 = 0.93$, $p < 0.05$, $n = 12$; Fig. 5A); %I.D./g *ex vivo* vs. %I.D./g *in vivo* using SPECT and MR imaging, $r^2 = 0.90$, $p < 0.05$, $n = 12$; Fig. 5B). *Ex vivo* and *in vivo* biodistribution analysis demonstrated greater uptake in tumors injected with Ad-CMV-HA-hSSTR2 compared with tumors injected with control virus, Ad-CMV-GFP ($p < 0.05$; Fig. 5C). This was confirmed by immunohistochemistry targeting the HA-tag (Fig. 5D and E). Thus, by both *ex vivo* biodistribution analysis and *in vivo* biodistribution analysis using small animal cognates of clinically used imaging devices, expression of SSTR2-based reporters can be imaged and quantified after *in vivo* gene transfer into intrathoracic NSCLC tumors.

SSTR2 expression in human lung cancer samples

We evaluated whether SSTR2 is expressed in human lung cancer samples (Table 1, Fig. 6). As we noted in the cell lines, most NSCLCs do not express SSTR2. Only six (9%) NSCLCs expressed SSTR2A in more than 30% of the tumor cells, and all of them were adenocarcinomas (Fig. 6C). Among all NSCLC tumors, 79% lacked expression for this marker. Only one squamous cell carcinoma had focal and mild SSTR2A expression (Fig. 6D).

Discussion

Due to the poor prognosis of patients with lung cancer, novel therapies such as gene therapy are being explored. A

FIG. 1. H1299, H460, and A549 cells express combinations of SSTR subtypes other than subtype 2. RT-PCR of SSTR1–5 mRNA in NSCLC cell lines is presented. Plasmid cDNA and β -actin were used as positive controls and to demonstrate mRNA quality, respectively. As a second positive control for SSTR2, 309 cells were used.

FIG. 2. Immunologic methods targeting the HA domain demonstrate that H1299, H460, and A549 cells express HA-SSTR2 after *in vitro* infection with Ad-CMV-HA-hSSTR2. *In vitro* HA-hSSTR2 expression by H1299, H460, and A549 cells with and without *in vitro* infection with Ad-CMV-HA-hSSTR2 using antibodies targeting the HA-domain was determined. (A) Immunofluorescent images of H1299, H460, and A549 cells without infection (a–c) or infected with Ad-CMV-HA-hSSTR2 (d–f). (B) ELISA of H1299, H460, and A549 cells with or without Ad-CMV-HA-hSSTR2 infection. Error bars represent SD of triplicate samples (* $p < 0.05$, uninfected cells vs. Ad-CMV-HA-hSSTR2 infected cells). (C) Western blots of H1299, H460, and A549 cells with or without Ad-CMV-HA-hSSTR2 infection. Cell protein extracted from cell lines was loaded at 20 μ g per lane.

limitation of gene therapy has been a lack of noninvasive, clinically applicable systems for assessing gene expression. Such tools are needed for multiple applications from vector development to assessing whether there is sufficient expression of a therapeutic gene product for efficacy. Reporters can be used for these purposes either alone or by linking to a

FIG. 3. Radioligand targeting of the receptor domain demonstrates that H1299, H460, and A549 cells express HA-SSTR2 after *in vitro* infection with Ad-CMV-HA-hSSTR2. Specific binding of 100 nM 111 In-octreotide to cell membranes of NSCLC cell lines with or without infection with Ad-CMV-HA-hSSTR2 is shown. Error bars represent SD of triplicate samples (* $p < 0.05$, uninfected cells vs. Ad-CMV-HA-hSSTR2 infected cells).

therapeutic gene. SSTR2-based reporters have several advantages, including being of human origin, thus limiting an immune response; being amenable to longitudinal imaging (Singh *et al.*, 2009); having clinically approved and new radioligands; and having a variant that is signaling-deficient to inhibit untoward effects on transduced cells or on linked genes of interest (Han *et al.*, 2007). A potential disadvantage of using hSSTR2-based reporters in NSCLC is that there have been reports that NSCLC can express SSTRs (Fujita *et al.*, 1994; Papotti *et al.*, 2001; Herlin *et al.*, 2009). This has the potential to confound hSSTR2-based imaging. We found that SSTR2-based reporters can be used for noninvasive imaging of *in vivo* gene transfer into intrathoracic NSCLCs.

The current work has several novelties, including showing SSTR subtype expression in three different NSCLC lines, using ultrasound guidance for gene transfer into an intrathoracic tumor, and demonstrating imaging and both *in vivo* and *ex vivo* quantification of SSTR2-based reporter gene transfer in an intrathoracic model of NSCLC. Moreover, our IHC dataset is the largest that assesses SSTR2 expression in human NSCLC tumor samples.

Data on SSTR expression in NSCLC is limited and has generally involved small numbers of samples. Papotti *et al.* (2001) found that 67% of NSCLCs were negative (6/12) or focally positive by immunohistochemistry for SSTR2 (2/12), whereas 33% were positive. Another report noted low (9/19)

FIG. 4. HA-SSTR2 expression can be imaged after *in vivo* infection of intrathoracic H460 tumors with Ad-CMV-HA-hSSTR2. (A, B) EPI-based MRI imaging demonstrated tumor as increased signal in the lung apices. The tumor in (A) was injected with Ad-CMV-HA-hSSTR2, and the tumor in (B) was injected with Ad-CMV-GFP. (C) *In vivo* MR-derived lung tumor weight correlated with *ex vivo* tumor weight ($n = 12$). (D) Representative image of ultrasound-guided intratumoral injection of adenoviruses. (E, F) Representative coronal gamma-camera SPECT images of uptake of ^{111}In -octreotide by lung tumors injected with Ad-CMV-HA-hSSTR2 (E) or Ad-CMV-GFP (F). Normal clearance through the kidneys and bladder is seen. There is uptake by tumor injected with Ad-CMV-HA-hSSTR2 (E), but only background signal is seen in the tumor injected with Ad-CMV-GFP (F). The same mouse injected with Ad-CMV-HA-hSSTR2 is shown by MR (A) and SPECT (E), and the same mouse injected with Ad-CMV-GFP is shown by MR (B) and SPECT (F).

or moderate SSTR2 expression (7/19) in 84% of patients with NSCLC, and high levels in the remainder (Herlin *et al.*, 2009). Using radiolabeled somatostatin or analogues, Reubi *et al.* (1990) found no SSTR expression in 12 cases by autoradiography. In cell lines, Fujita *et al.* (1994) evaluated SSTR1 expression, and Papotti *et al.* (2001) SSTR2 expression, by RT-PCR and noted low levels of SSTR1 and 2 expression in most of the six NSCLC lines tested. In comparison, we did not find SSTR2 expression in the three cell lines that we tested, but SSTR1 was noted in all three. We found that there can be expression of combinations of other SSTR subtypes in the NSCLC cell lines, depending on the cell line. One possible explanation for differences in observed SSTR2 expression in cell lines and histologic samples may be the degree of differentiation, because it has been reported that SSTR2 expression is greatest in well differentiated NSCLC (Herlin *et al.*, 2009). Our IHC dataset including 70 patients is the largest evaluating SSTR2 expression in human NSCLC tumor samples of which we are aware. We found no SSTR2 expression in 78.5% of samples and low expression in 7.1%. Moderate to high expression was seen in 14.4%.

Clinically, the pattern of SSTR2 expression and ^{111}In -octreotide background in patients is well known, and is mimicked in mice (Kundra *et al.*, 2002). Tumors expressing SSTR2 can be seen even in areas of higher background, such as the liver (Lebtahi *et al.*, 2002). By imaging, low levels of ^{111}In -octreotide uptake have been reported in 10/10 patients with NSCLC by Lau *et al.* (2000); in 10/13 patients by Kirsch *et al.*

(1994); and in 40/40 primary tumors, 5/15 lymph node metastases, and 1/7 distant metastases by Kwekkeboom *et al.* (1994). Using a ^{68}Ga -labeled octreotide-based radiopharmaceutical, ^{68}Ga -DOTATOC, Dimitrakopoulou-Strauss *et al.* (2006) reported moderate uptake in 7/9 primary tumors and no uptake in 8 metastases using PET. Thus, although there may be uptake of radiolabeled octreotide in NSCLC, it is generally at a low level in primary tumors, and often completely lost in metastases.

Herlin *et al.* (2009) noted low (9/19) or moderate (7/19) SSTR2 expression in 84% of patients with NSCLC. Upon imaging with the somatostatin analogue $^{99\text{m}}\text{Tc}$ -depreotide, uptake did not correlate with SSTR2 expression, likely because this agent has relatively equal affinity for subtypes 2, 3, and 5 (Virgolini *et al.*, 1998). In this article, to partner with gene transfer of SSTR2-based reporters, we used ^{111}In -octreotide because it preferentially binds SSTR2. In addition to subtype selectivity, our choice was also driven by the fact that neither $^{99\text{m}}\text{Tc}$ -depreotide nor other radiolabeled somatostatin analogues (Kwekkeboom *et al.*, 1994) are commercially available currently in the United States. With ^{111}In -octreotide, expression by intrathoracic NSCLC cells could be clearly visualized after *in vivo* gene transfer of an SSTR2-based reporter. Low uptake was seen in cells transfected with control virus, suggesting that expression of other SSTR subtypes, such as subtypes 1 and 3 by H460 cells, does not interfere with detection of the SSTR2-based reporter expression after gene transfer.

FIG. 5. Quantitative *in vivo* and *ex vivo* biodistribution analysis demonstrating HA-SSTR2 expression by intrathoracic H460 tumors after *in vivo* infection with Ad-CMV-HA-hSSTR2. (A, B) *In vivo* and *ex vivo* biodistribution into tumors correlate. Correlation of *in vivo* %I.D./g of ^{111}In -octreotide uptake into tumors by planar and MR (A; $n=12$) or SPECT and MR (B; $n=12$) correlated with %I.D./g assessed from excised tumors. (C) *In vivo* and *ex vivo* biodistribution analysis demonstrates increased uptake in tumors infected with Ad-CMV-HA-hSSTR2 compared with tumors infected with Ad-CMV-GFP. *Tumor infected with Ad-CMV-HA-hSSTR2 vs. tumors infected with Ad-CMV-GFP: $p < 0.05$, $n=6$; error bars represent SD. (D, E) Immunohistochemistry directed at the HA-tag demonstrates expression of HA-SSTR2 in tumors infected with Ad-CMV-HA-hSSTR2 (arrow, E), but background activity in tumors infected with Ad-CMV-GFP (D).

In vitro methods demonstrated expression by targeting either the HA domain or the receptor portion of the fusion protein after adenoviral infection in three different NSCLC lines. The HA domain allowed evaluation of expression regardless of any endogenous SSTRs that may bind to the radiopharmaceutical, using antibodies that are much less expensive than the radioligand. Expression of protein of the appropriate size and at the appropriate subcellular location was demonstrated. Next, the receptor portion of the fusion protein was targeted by ^{111}In -octreotide, and again expression of the fusion protein after Ad-HA-SSTR2 infection was confirmed. Thus, SSTR2-based reporter expression could be clearly demonstrated in three different NSCLC cell lines despite expression of other SSTR subtypes. Supporting our findings, gene transfer of SSTR2 in an NSCLC cell line, A427, has been reported previously using a subcutaneous model (Zinn *et al.*, 2000; Rogers *et al.*, 2003, 2005; Parry *et al.*, 2007), but SSTR subtype expression, an intrathoracic NSCLC model, and *in vivo* quantification were not demonstrated.

For *in vivo* studies, we used MR to identify the presence of tumors after intrathoracic injection in mice. In mouse models, CT does not provide sufficient soft-tissue contrast for distinguishing tumor from the chest wall or mediastinal structures. Use of MR has traditionally been limited for the evaluation of the thorax, and even more so in small animals

due to rapid breathing and cardiac motion. An EPI-based, respiratory- and cardiac-gated sequence with fat saturation was used to generate T2-weighted images of the thorax that depicted the tumor (Bankson *et al.*, 2008). This then allowed us to screen mice for tumors and measure tumor volume.

In a novel application of small-animal imaging, we used ultrasound to guide needle placement into intrathoracic tumors. Normally, one would consider the lung not to be in the realm of ultrasound imaging due to air that can disrupt propagation of sound waves. With the intrathoracic tumors, a window could be found for visualizing the tumors. A limitation of this technique is that small, deep tumors surrounded by lung will likely not be accessible due to surrounding air. In the current application, use of ultrasound ensured injection into the tumor.

After adenovirus injection, MR and gamma-camera imaging were performed. As we have demonstrated previously, MR image analysis correlated highly with tumor weight (Bankson *et al.*, 2008), and we used these data to normalize *in vivo* %I.D. The *in vivo* %I.D./g correlated with *ex vivo* % I.D./g. Both methods demonstrated increased uptake after Ad-HA-SSTR2 infection compared with control virus. *In vivo* monitoring of gene transfer can be approached using reporter genes either alone or in conjunction with a gene of interest, *e.g.*, for developing vectors (Hemminki *et al.*, 2001), evaluating promoters, and inducing two separate

TABLE 1. SSTR-2 EXPRESSION IN NON-SMALL CELL LUNG CANCER

Diagnosis	Negative	5–10% cells	11–30% cells	31–60% Cells	Total
Adenocarcinoma	34 (70.8%)	4 (8.3%)	4 (8.3%)	6 (12.5%)	48 (100%)
Squamous cell carcinoma	21 (95.5%)	1 (4.5%)	0 (0%)	0 (0%)	22 (100%)
Total NSCLC	55 (78.5%)	5 (7.1%)	4 (5.7%)	6 (8.6%)	70 (100%)

FIG. 6. Immunohistochemical analysis of SSTR2 expression in human lung cancer. (A, B) Lung cancers that lack SSTR2 expression. (C, D) Lung cancers with SSTR2 expression (arrow): adenocarcinoma (AC) with high and diffuse SSTR2 expression (C), and squamous cell carcinoma (SQM), with focal and mild SSTR2 expression (D).

genes (Sun *et al.*, 2001), such as an imagable reporter and a therapeutic gene. The therapeutic gene of interest and reporter may be induced by separate promoters (Yaghoubi *et al.*, 2001; Zinn *et al.*, 2002) or may be linked, *e.g.*, via an internal ribosome entry site (Tjuvajev *et al.*, 1999; Liang *et al.*, 2002) or a bidirectional promoter (Sun *et al.*, 2001). Using such techniques, expression of the gene of interest and reporter correlate (Tjuvajev *et al.*, 1999; Sun *et al.*, 2001; Liang *et al.*, 2002; Zinn *et al.*, 2002; Yang *et al.*, 2005). Furthermore, the vector itself may serve as a therapeutic; for example, SSTR2 imaging with ^{111}In -octreotide has been used to demonstrate infection by a therapeutic vaccinia virus (McCart *et al.*, 2004).

Because most lung cancers have no or little SSTR2 expression, they are good candidates for SSTR2-based reporter imaging. In the minority with SSTR2 expression, SSTR2-based reporter imaging should still be applicable if a prescan is performed to obtain baseline levels of radiopharmaceutical uptake. Then increased expression after gene transfer may be assessed, because different amounts of SSTR2-based reporter expression can be discerned (Kundra *et al.*, 2002; Yang *et al.*, 2005; Singh *et al.*, 2009). Given that there is heterogeneity in radiopharmaceutical uptake among patients and among tumors/metastases within the same patient, when this technique is used in patients, it would be prudent to obtain a prescan with the radiopharmaceutical as a baseline before imaging gene transfer of SSTR2-based reporters.

To meet the promise of gene therapy, noninvasive imaging methods for assessing gene expression are needed that have the potential for clinical translation. In this work, we used clinically applicable tools such as adenovirus and ^{111}In -octreotide to demonstrate the utility of an hSSTR2-based reporter in an intrathoracic model of NSCLC. The findings suggest that it should be possible to visualize and non-invasively quantify SSTR2-based reporter gene transfer in NSCLCs, enabling a host of gene therapy applications, such as developing vectors, designing dosing schedules, and as-

sessing expression of a linked therapeutic gene. Importantly, because gene expression can vary among individuals and among tumors within an individual, the use of SSTR2-based reporters should enable the personalizing of therapy to individual patients.

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Author Disclosure Statement

There is a patent on the reporter imaging technology held by the institution.

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