High Correlation of Whole-Body Red Fluorescent Protein Imaging and Magnetic Resonance Imaging on an Orthotopic Model of Pancreatic Cancer

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Abstract
We have developed genetically fluorescent orthotopic models of human pancreatic cancer. In these models, noninvasive fluorescent protein imaging (FPI) of internal primary tumors and metastatic deposits has been carried out. Whole-body tumor images are easily and inexpensively obtained using FPI, permitting both detection and quantification of tumor load. In this study, we simultaneously compared single mice with a highly fluorescent, red fluorescent protein–expressing orthotopic pancreatic cancer xenografts with both FPI and high-resolution magnetic resonance imaging (MRI). Images were acquired at multiple time points after tumor implantation in the pancreas. Indwelling pancreatic primary tumors and metastatic foci were detected by both FPI and MRI. Moreover, a strong correlation existed between images taken with these two technologies. FPI permitted rapid, high-throughput imaging without the need for either anesthesia or contrast agents. Both FPI and MRI enabled accurate imaging of tumor growth and metastasis, although MRI enabled tissue structure to be visualized as well. FPI has high resolution and is exceedingly rapid with instant image capture. We suggest a complimentary role for these two imaging modalities. (Cancer Res 2005; 65(21): 9829-33)

Introduction
Small-animal imaging has been an area of intense worldwide development over the past 5 years. Because orthotopically implanted tumors grow deep within the animal, a noninvasive method must be employed to effectively and reliably image and monitor their growth and dissemination over time. A variety of such systems have been described, including traditional imaging modalities such as computed tomography, ultrasound, and magnetic resonance imaging (MRI), as well as newer modalities utilizing bioluminescence and fluorescence imaging.

The most information about the efficacy of a therapeutic intervention in animal models of cancer can be obtained when tumor growth and progression are sequentially monitored in a single animal over the entire course of treatment in real time. In this way, real-time qualitative and quantitative characterization of the pharmacologic effects of an agent on tumor growth can be determined. Both fluorescent protein imaging (FPI; refs. 1–3) and MRI (4) have shown their usefulness for such studies.

Our laboratories have been at the forefront of small-animal imaging of orthotopic models with FPI and MRI (1–4). Although both MRI and FPI have shown to be very useful for whole-body imaging of small animals, they have not been directly compared.

We have developed models of human tumors which express high levels of either green fluorescent protein (5) or red fluorescent protein (RFP; ref. 6). Using these models, it is possible to visualize internally growing tumors in the intact live animal by illumination with light of an appropriate excitation wavelength. We have shown the ability of MRI to provide three-dimensional visualization of tumor growth and metastasis in a nude-mouse liver-metastatic model enabling real-time analysis of tumor response to therapeutics (4).

Our experience with these two modalities has led us to believe that a combined imaging approach using both MRI and FPI can be highly effective in the study of the effects of novel therapeutics on small-animal models of human cancer. In this study, we used a fluorescent, highly metastatic, orthotopic model of human pancreatic cancer, MIA PaCa-2, which has been engineered to selectively express high levels of RFP cloned from the Discosoma sp. sea coral (6–8). Imaging of orthotopically implanted primary tumors and metastases in the same animals by both FPI and high-resolution MRI, at multiple time points after implantation, permitted a comparison of these two techniques. Our data suggest a complimentary role for these two imaging modalities in preclinical studies of cancer in orthotopic mouse models.

Materials and Methods

Cell line. The MIA-PaCa-2 human pancreatic cancer cell line was obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in DMEM media supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin and streptomycin (Life Technologies, Inc., Grand Island, NY). Cells were cultured at 37°C in a 5% CO2 incubator.

Red fluorescent protein retroviral transduction and selection of MIA-PaCa-2-RFP pancreatic cancer cells. The pDsRed-2 vector (Clontech Laboratories, Inc., Palo Alto, CA) was used to engineer MIA-PaCa-2 clones stably expressing RFP, as described previously (8). This vector expresses RFP and the neomycin resistance gene on the same bicistronic message. pDsRed-2 was produced in PT67 packaging cells. RFP transduction was initiated by incubating 20% confluent MIA-PaCa-2 cells with retroviral supernatants of the packaging cells and DMEM for 24 hours. Fresh medium was replenished at this time and cells were allowed to grow in the absence of retrovirus for 12 hours. This procedure was repeated until high levels of RFP expression, as determined using fluorescence microscopy, were achieved. Cells were then harvested by trypsin-EDTA and subcultured into selective medium that contained 200 μg/mL G418. The level of G418 was increased to 2,000 μg/mL stepwise. Clones expressing high levels of RFP were isolated with cloning cylinders as needed, and were amplified and
transferred using conventional culture methods. High RFP-expression clones were isolated in the absence of G418 for 10 passages to select for stable expression of RFP in vivo.

Animals and animal care. Male nude mice (NCR-nu/nu) between 4 and 6 weeks of age were maintained in a barrier facility on HEPA-filtered racks. The animals were fed with autoclaved laboratory rodent diet (Tekland LM-485, Western Research Products, Orange, CA) and were maintained on a daily 12-hour light 12-hour dark cycle. Animal experiments were done in accordance with the Guidelines for the Care and Use of Laboratory Animals (NIH Publication no. 85-23) under assurance no. A3873-01 and were approved by the Institutional Animal Care and Use Committee at AntiCancer, Inc. (San Diego, CA) and Roswell Park Cancer Institute (Buffalo, NY).

Surgical orthotopic implantation of MIA-PaCa-2-RFP tumors. Red-fluorescent human pancreatic cancer xenografts were established in nude mice by surgical orthotopic implantation (9). Briefly, MIA-PaCa-2-RFP tumors in the exponential growth phase, grown s.c. in nude mice, were resected aseptically. Necrotic tissues were cut away, and the remaining healthy tumor tissues were cut with scissors and minced into 1 mm³ pieces in RPMI 1640. Mice were then anesthetized and their abdomens were sterilized with alcohol. An incision was then created through the left upper abdomen and two tumor pieces were transplanted onto the middle of the abdominal pararectal line and peritoneum. The pancreas was carefully exposed and two tumor pieces were transplanted onto the middle of the gland using a single 8-0 surgical suture (Davis-Geck, Inc., Manati, Puerto Rico). The pancreas was then returned into the peritoneal cavity, and the abdominal wall and the skin were closed in two layers using 6-0 surgical sutures. All procedures were done with a 7× microscope (Olympus) or standard surgical loupes.

Fluorescence protein imaging. In vivo fluorescence imaging was done weekly. This was done in a fluorescent light box illuminated by fiberoptic lighting at 470 nm (Lightools Research, Encinitas, CA). Emitted fluorescence was collected through a long-pass filter GG475 (Chroma Technology, Battleboro, VT) on a Hamamatsu C5810 3-chip cooled color CCD camera (Hamamatsu Photonics Systems, Bridgewater, NJ). High-resolution images of 1,024 × 724 pixels were captured directly on an IBM PC or continuously through video output on a high-resolution Sony VCR model SLV-R1000 (Sony Corp., Tokyo, Japan). Images were processed for contrast and brightness and analyzed with the use of Image Pro Plus 3.1 software (Media Cybernetics, Silver Spring, MD). Real-time determination of tumor burden was done by quantifying fluorescent surface area, as described previously (5, 8).

Mice were also sacrificed and explored through a midline laparotomy and sternotomy. Excitation of RFP in the light box described above facilitated identification of primary and metastatic disease by fluorescence visualization. After acquiring full-body, open images, the solid organs were removed and their surfaces were thoroughly examined for any evidence of metastases through a Leica fluorescence stereo microscope model LZ12 (Leica Microsystems, Inc., Bannockburn, IL) equipped with a mercury 50 W lamp power supply. Selective excitation of RFP was produced through a D425/60 band-pass filter and 470 DCXR dichroic mirror. Emitted fluorescence was collected by the Hamamatsu camera system described above.

Magnetic resonance imaging. Following the acquisition of whole-body fluorescence images, full-body MRI acquisitions were done on a weekly basis. Before scanning, each animal was anesthetized with 4% isofluorane and then placed within a 3-mm-diameter butyrate plastic carrier tube. Body temperature was regulated by a small heating pad within the carrier containing circulating water at 37°C. Constant anesthesia was maintained with 2% isofluorane via an inlet tube. A small vacuum applied to a second tube served to remove carbon dioxide and excess anesthetic. In some animals, gadolinium-based contrast enhancing agent (Magnevist, Berlex, Inc., Montville, NJ) was injected into the dorsal tail vein at a concentration of 0.3 mmol/kg to improve contrast in T1-weighted images.

High-resolution MRI scans were done using a General Electric CSI 4.7 T/33 cm horizontal bore magnet (GE NMR Instruments, Fremont, CA) with radiofrequency and computer systems incorporating AVANCE digital electronics (Bruker BioSpec platform with ParaVision Version 2.1 Operating System, Bruker Medical, Billerica, MA).

MRI data were acquired using a G060 removable gradient coil insert generating a maximum field strength of 950 mT/m, and a custom-designed 35-mm radiofrequency transceiver coil. Standard spin-echo and rapid acquisition with relaxation enhancement MRI pulse sequences were used to acquire multislice images (4).

T1-weighted and T2-weighted coronal and transverse axial multislice, two-dimensional images were acquired for each mouse. T1-weighted spin-echo images were acquired with the following parameters: echo time/
repetition time = 8/400 ms, number of excitations (averages) = 8. T2-weighted rapid acquisition with relaxation enhancement spin-echo images were acquired with the following parameters: echo time/repetition time = 40/2,750 ms, echo train length = 4, number of excitations = 8. T1- and T2-weighted images offer different but complementary contrast dependent on the different T1 and T2 relaxation rates within normal and malignant tissues. Using the imaging parameters outlined above, tumor and metastases appear hypointense (dark) on T1-weighted images and hyperintense (bright) on T2-weighted images.

Coronal images were acquired using a 256/256 acquisition matrix with 25 1-mm slices over a field of view of 4.8 cm x 3.2 cm. Axial images were acquired using a 192 x 192 acquisition matrix with 25 1-mm slices over a field of view of 3.2 cm x 3.2 cm.

Three-dimensional acquisitions were also acquired for a subset of animals to obtain isotropic voxels for volume rendering. Three-dimensional acquisitions were acquired with rapid acquisition with relaxation enhancement spin-echo sequences with acquisition parameters of echo time/repetition time = 28/750 ms, echo train length = 8, and number of excitations = 2. Image data were acquired using a 256 x 128 x 128 matrix over a field of view of 6.4 x 3.2 x 3.2 cm for an isotropic resolution of 250 μm per voxel.

Image analysis and three-dimensional volume rendering of MRI data were done using AnalyzePC Version 5.0 (Biomedical Imaging Resource, Mayo Foundation, Rochester, MN). Specifically, raw data from the magnetic resonance scanner were reformatted and displayed using AnalyzePC. Regions of interest (normal tissue, tan; tumor, red) were defined using seed-growing algorithms with manual adjustment on visual inspection. Tumor volumes were calculated by multiplying the number of tumor voxels by the individual voxel volume. Three-dimensional magnetic resonance data were rendered with two reflective ray-casting algorithms, volume-compositing and gradient shading with selective transparency, in AnalyzePC volume rendering tool (10). These algorithms were chosen to present contrasting views of tumor size and location. The gradient shading with transparency rendering allows for internal structures to be visualized regardless of depth, whereas the volume-composite rendering was chosen to simulate the attenuation of red fluorescent light emitting from tumors within the body. For volume-composite rendering, the α map, which defines color and transparency of voxels rendered based on voxel intensity value, was adjusted such that all voxels were rendered with an α value (opacity) = 0.05, allowing for portions of tumor near the surface of the animal to be visualized. For the gradient shading/transparency rendering, tumor and normal tissue were rendered with 50% opacity to allow for complete visualization of the tumor regardless of location within the animal.

Correlation of fluorescent protein imaging and magnetic resonance imaging measurements of tumor burden. To confirm a correlation between tumor burdens, as determined by externally visualized RFP fluorescence and MRI measurements of tumor volume, the primary tumor of each mouse was used. The externally visualized RFP fluorescent area and the tumor volume based on MRI were determined as described above. The correlation coefficient r was calculated between tumor volume and RFP fluorescence. P < 0.05 was considered significant.

Results and Discussion

Comparison of whole-body fluorescent protein imaging and magnetic resonance imaging of primary pancreatic tumors.

Whole-body FPI and MRI allowed longitudinal visualization of pancreatic malignancy in an orthotopic nude mouse model. Figure 1 shows examples of such imaging by FPI and T1- and T2-weighted

Figure 2. FPI area correlated strongly with tumor volume measured by MRI for tumors <1,500 mm³ (R² = 0.7035, P = 0.0024). Measurement of externally visualized FPI and calculated measurements of the primary tumor by MRI of each mouse (n = 10) were obtained, as described in Materials and Methods.

Figure 3. Comparison of FPI and MRI of metastatic pancreatic cancer. Frontal (A) and transverse (B) views of a single nude mouse with orthotopically implanted Mia-PaCa-2-RFP human pancreatic cancer. Tumor is clearly visualized as red using both FPI and MRI. Left, transparency rendering provides qualitative analysis of total tumor burden. Right, volume rendering provides a depth of penetration perspective for comparison with FPI (center).
MRI. Contrast agent was administered i.v. before MRI on day 7 but not on day 10, accounting for tumor enhancement on T1-weighted images on day 7. The primary tumor was clearly visible using each imaging modality.

To determine whether the two imaging modalities correlated, 10 mice with tumors <1,500 mm³ were analyzed. Tumor volume as calculated by MRI and tumor area as calculated by FPI were compared (Fig. 2). The FPI image area correlated strongly with tumor volume measured by MRI for tumors <1,500 mm³ ($R^2 = 0.7035$, $P = 0.0024$). For tumors <2,000 mm³, there was still a significant correlation ($R^2 = 0.3363$, $P = 0.0481$; data not shown) although it was not as strong as for smaller tumors. For tumors >2,000 mm³, parts of the pancreatic tumor had grown close to the skin, which then overexposed the camera from picking up the portions of the tumor deeper inside the animal, thereby precluding correlation between the two modalities.

**Comparison of fluorescent protein imaging and magnetic resonance imaging of metastatic tumors.** Figure 3 shows whole-body images of a mouse after surgical orthotopic implantation of MIA-PaCa-2-RFP human pancreatic cancer. Front and side views using FPI correlated well with front and side MRI volume-rendered images. Front and side MRI transparency renderings identified all areas of the tumor.

Figure 4 shows "open" views of a mouse with tumor visualized using direct FPI after opening the skin. Both open FPI and high-resolution T1- and T2-weighted high-resolution MRI could discern anatomic relationships between tumor and adjacent normal organs and tissues.

**Comparison of fluorescent protein imaging and magnetic resonance imaging on pancreatic tumors implanted on the liver.** Because pancreatic cancer can often metastasize to the liver, we examined the role of MRI and FPI in mice with RFP-expressing MiaPaCa-2 implanted directly on the liver. Fragments of MiaPaCa-2 RFP pancreatic tumors were implanted into both lobes of the liver and then imaged 3 weeks later. Figure 5 shows that these liver tumors could be clearly visualized by both modalities.

**Comparison of magnetic resonance imaging and fluorescent protein imaging on ex vivo metastases.** Comparison of whole-body MRI and FPI of ex vivo metastases was also carried...
Small, suspicious lesion in the liver on T2-weighted image (C) of kidney Bdemonstrating left kidney metastasis, confirmed by FPI (www.aacrjournals.org 9833 Cancer Res 2005; 65: (21). November 1, 2005 Yang M, Baranov E, Jiang P, et al. Whole-body optical image demonstrating left kidney metastasis was confirmed by FPI out, as shown in Fig. 6. A T2-weighted, axial magnetic resonance T2-weighted image was confirmed to be a metastatic tumor using FPI (D) of the liver ex vivo.

out, as shown in Fig. 6. A T2-weighted, axial magnetic resonance image demonstrating left kidney metastasis was confirmed by FPI of a kidney metastasis ex vivo. A suspicious lesion in the liver on T2-weighted image was confirmed to be a metastatic tumor using FPI of the liver ex vivo.

General observations. Selective tumor fluorescence enabled acquisition of whole-body images in a noninvasive manner using simple, inexpensive fluorescence imaging equipment. Neither anesthesia nor contrast agents are necessary to provide highly specific, high-resolution images of indwelling abdominal tumors. Fluorescence imaging of sacrificed animals showed small, deep metastases that may be missed using whole-body FPI and MRI. MRI of the same mouse permitted more complete evaluation of tumor and host organ anatomy. Further visualization of the depth and size of the tumor was obtained via three-dimensional rendering of the magnetic resonance data. As with whole-body FPI, MRI lost sensitivity in the visualization of small metastases such as those to portal or mesenteric lymph nodes. In addition, use of MRI required anesthesia to immobilize the mouse during the long acquisition times.

Our study shows the combined utility of an imaging protocol that uses both FPI and MRI. Both modalities can be used to obtain serial, high-resolution images of tumor growth in the live animal over the course of a preclinical study. FPI may be done efficiently and inexpensively in the live animal without contrast enhancing agents or anesthesia. This modality has 100% specificity for tumor detection. MRI has the advantage that organ and tumor anatomy is well defined but its use requires significant cost and the use of anesthesia. Contrast enhancing agents may be used to increase tumor visibility and thereby increase specificity. FPI has the advantage of simplicity of equipment, lack of need of anesthesia or contrast agents, and high sensitivity. The high degree of correlation in whole-body imaging suggests that both modalities can be used in a complementary fashion for screening, quantifying tumor growth and assessing therapeutic efficacy in preclinical, small-animal research.

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