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Reverse-contrast imaging and targeted radiation therapy of advanced pancreatic cancer models

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Abstract

Purpose—To evaluate the feasibility of delivering experimental radiotherapy to tumors in the mouse pancreas. Imaging and treatment were performed using combined CT (computed tomography)/orthovoltage treatment with a rotating gantry.

Methods and Materials—After intraperitoneal administration of radiopaque iodinated contrast, abdominal organ delineation was performed by X-ray CT. With this technique we delineated the pancreas, and both orthotopic xenografts and genetically engineered disease. CT imaging was validated by comparison with magnetic resonance (MR) imaging. Therapeutic radiation was delivered via a 1 cm diameter field. Selective X-ray radiation therapy (XRT) of the non-invasively defined orthotopic mass was confirmed using γ H2AX staining. Mice could tolerate a dose of 15 Gy when the field was centered on the pancreas tail, and treatment was delivered as a continuous

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360-degree arc. This strategy was then used for radiation therapy planning for selective delivery of therapeutic XRT to orthotopic tumors.

Results—Tumor growth delay after 15 Gy was monitored, using CT and ultrasound to determine the tumor volume at various times post-treatment. Our strategy enables the use of clinical radiation oncology approaches to treat experimental tumors in the pancreas of small animals for the first time. We demonstrate that delivery of 15 Gy from a rotating gantry minimizes background healthy tissue damage and significantly retards tumor growth.

Conclusions—This advance permits evaluation of radiation planning and dosing parameters. Accurate non-invasive longitudinal imaging and monitoring of tumor progression and therapeutic response in pre-clinical models is now possible, and can be expected to more effectively evaluate pancreatic cancer disease and therapeutic response.

Classifications

pancreatic cancer; image guided radiotherapy; molecular imaging

Introduction

Research to better understand and combat cancer has benefitted from animal models that more closely resemble human cancer. The use of subcutaneous xenografts has largely been replaced by orthotopic tumors and genetically engineered immune competent mice, which spontaneously develop tumors in relevant organs. These models offer various advantages including: a tumor microenvironment that more closely resembles that found in human tumors; possession of gene expression profiles that may match patient samples; and a clinically relevant primary site from which the disease may metastasize (1–5). These models are particularly valued for testing treatment strategies, as cancer researchers may have been misled about the likely value of experimental therapies by relying on the unique biology of subcutaneous tumors (6).

This development poses a problem for experimental radiotherapy in that it is difficult to deliver meaningful doses to an orthotopic tumor without serious damage to adjacent normal structures. Recent technical achievements have led to the development of small animal combined radiotherapy/CT units (7, 8). This brings two novel advances to experimental radiotherapy: 1) They allow delivery of radiation dose to tissue volumes identified by CT and 2) By enabling delivery of the treatment dose from multiple angles, or in a continuous rotational arc, they spare normal tissue, and thus allow larger doses to be delivered to the tumor. These two characteristics more closely recapitulate the clinical paradigm of irradiation planning and delivery. However, dose delivery to pancreatic tumor models is further complicated because of the lack of CT contrast of the organ. Anatomical localization and delineation of the pancreas in mice is very challenging as the organ is not a defined solid secondary retroperitoneal organ (as it is in humans), but rather a thin membrane spread throughout the upper abdomen. To date, the only radiation therapy in experimental abdominal tumors has been attempted using the xenobiotic luciferase reporter, as performed by Tuli et al. (9, 10).

Here we explore an alternative approach that does not require a reporter. By injecting a large volume of dilute CT contrast agent intraperitoneally (IP), we achieve both physical separation of the abdominal structures and CT visualization, as tissue is outlined by X-ray opaque medium. The technique enables us to rapidly define the pancreas and its enclosed tumor. The use of small volumes of intraperitoneal contrast agent has previously been described (11), allowing the detection of liver metastases, ovarian, and Wilms cancer (12–14), and large-volume injection of saline has been used in the context of ultrasound imaging (15). To the best of our knowledge this is the first report to apply a contrast agent IP to precisely deliver experimental radiotherapy and monitor pancreatic disease. We have called this technique “Reverse Contrast CT”, and we have developed it in the context of pancreatic cancer models, though in principle it is applicable to any anatomical structure or tumor in the retro- or intraperitoneal space. This non-invasive technique is repeatable, and can be used not only to provide image guidance for tumor dose planning, but also to accurately monitor tumor burden and regrowth following intervention.

Materials and Methods

Mouse and Tumor Models

All animal studies were conducted under approved guidelines set forth by the Institutional Animal Care and Use Committee in a protocol approved by the XXX (XXX) Animal Research Center. For dose limiting toxicity and orthotopic xenograft studies, female Balb/c nu/nu mice of 6–8 weeks of age were purchased from Harlan Laboratories (Indianapolis, IN). The average weight of mice at the outset of experimentation was 21.9 ± 1.7 g (SD) in 49 mice. Tumor xenografts were implanted into animals using standard protocols (16). Briefly, animals were anesthetized (isoflurane) and placed on their right side under surgical drape, within a bioguard safety hood. Carprofen (5 mg/kg subcutaneously) was given for pre-emptive analgesia. Following a standard aseptic preparation, a left flank incision was made into the peritoneal cavity. The spleen was identified and gently retracted to expose the pancreas. The tumor graft was secured to the native pancreas with a single, simple interrupted ligature (4-0 Vicryl, polyglactin 910, Ethicon, Bridgewater, NJ). The pancreas and spleen were replaced in the abdomen. Routine two-layer closure was performed with the same absorbable suture as above in the muscle. Stainless steel wound clips were used to appose skin edges. Tumor grafts were obtained from AsPC1 tumors, grown subcutaneously in nude mice. Tumor pieces were stored in liquid nitrogen (with 10% DMSO and 10 % fetal bovine serum). AsPC1 cells were originally obtained from the American Type Culture Collection (Manassas, VA).

Conditional LSL-Kras^{G12D/+}, LSL-Trp53^{R172H/+} (17), and Pdx-1-Cre (18) mouse strains, bred to BRCA2^{exon3–4/exon3–4} homozygosity, were obtained from the Thomas Ludwig lab (Department of Molecular and Cellular Biochemistry, The Ohio State University, Columbus OH, USA) and interbred to obtain LSL-Kras^{G12D/+};LSL-Trp53^{R172H/+};Pdx-1-Cre; BRCA2^{exon3–4/exon3–4} triple mutant animals.

Contrast Injection and Computed Tomography

Mice were brought to the plane of anesthesia and maintained under isofluorane in air for agent administration and imaging. Mice were evaluated using an image guided small animal micro-irradiator (XRad225Cx, Precision X-Ray, Inc., North Brantford, CT). This device utilizes a dual focal spot X-ray tube at 45 kVp with a flat-panel amorphous silicon imager mounted on a C-arm gantry. All subjects were imaged with an isotropic resolution of 100 μ m, using a volume that encompassed the entire abdomen. Animals imaged with contrast were given approximately 3 mL of 75 mg Iodine/mL (iohexol; Omnipaque, GE Healthcare) between 5 and 15 minutes prior to scan. Data visualization was accomplished with a combination of the open source FIJI (National Institutes of Health) (19) and Amira 5.3.3 (FEI, Hillsboro, OR) as previously described (20). Semi-automated generation of organ volumes was accomplished with the Amira region-grow tool.

Magnetic Resonance Imaging

Correlative magnetic resonance (MR) imaging was performed on a Bruker Biospin 4.7T (Bruker, Billerica MA). Subjects were scanned using one of two protocols. The first was performed on mice that were scanned in the MR immediately after having received iodine contrast injection. Here, mice were scanned using a T₂-weighted fast spin-echo rapid acquisition with relaxation enhancement (RARE) whole-body coronal sequence (TR/TE, 2069.9/48 msec) with a total imaging time of 6.6 minutes. Approximately 22 slices (0.8 mm thickness) with an in-plane resolution of 117 μ m were analyzed in FIJI and Amira. Fused CT/MR images were generated using Amira. In the second protocol, used for comparative detection of tumor burden in transgenic mice, an axial RARE acquisition was performed (4885/47.2 msec) for 48 slices, with the same slice thickness and resolution as above.

Image Guided X-ray Radiotherapy

Therapeutic radiation was delivered via the small animal micro-irradiator with a tube voltage of 225 kVp and 13 mA; Radiation dosimetry was performed by the dosimetry staff at MSKCC. Details of the commissioning of the unit will be published separately. Irradiation was performed immediately following the detection of the tumor by X-ray CT, and the mice were not moved between the imaging and therapeutic irradiation. Therapeutic radiation was delivered only to animals with orthotopic xenografts. Treatments were delivered using the manufacturer's software; to determine the full dose distribution throughout the animal on an after-the-fact basis, we imported the plan into Metropolis, in-house treatment planning system (TPS). Metropolis is the most recently developed version for external beam radiation TPS at XXX. For this work, it was modified to adapt it to treatment planning with the small-animal irradiator (21). Arc therapy was approximated in Metropolis by generating 72 beams, separated by 5°. Image display and precision of delineated volumes and DVHs have been adjusted to be compatible with animal sizes, and the kilovoltage X-ray beam energies and associated collimators have been added and graphically overlaid in the beam's-eye-view, 2D and 3D image displays. To achieve appropriate spatial resolution for volume dose calculation and DVHs, the calculation grid spacing was reduced to 0.1 mm.

Animals were irradiated under isoflurane anesthesia at a dose rate of approximately 3 Gy/minute, delivered through a circular collimator of 1 cm diameter. Radiation was delivered either as a single beam anterior to posterior or in a continuous arc, utilizing the rotatable gantry of the micro-irradiator. To examine the effect of hypoxia in the tumor, some animals were irradiated while breathing carbogen, a 95% O₂, 5% CO₂ mixture through the isoflurane vaporizer; the total exposure to carbogen was approximately 20 minutes from anesthesia induction to completion of radiotherapy.

Ultrasound Imaging

For the verification of tumor motion and tumor growth, tumors were imaged using the VeVo 2100 (VisualSonics, Toronto ON). Animals were anesthetized with isoflurane, injected with 3 ml saline (15), and imaged with a 550s transducer using the manufacturer's general imaging and abdominal presets. 3D tumor images consisting of sequential frames separated by 0.14 mm were assembled and the tumor volumes obtained by manually outlining the tumor area in successive images; volume was calculated by VisualSonics software. Video of real-time ultrasound imaging of the tumor were compiled also using VisualSonics software.

Biodistribution

Female Balb/c nu/nu mice of 6–8 weeks of age were implanted orthotopically with ASPC-1 xenografts. After three weeks, the animals were randomly divided into two groups (n=5). All animals received 100 µCi of ¹⁸F-FDG intravenously by tail vein. Group 1 served as a control for Group 2, which were injected IP with 3 ml of contrast solution. After 20 minutes, the animals were dissected and resected tissues were weighed and gamma counted (Wizard², Perkin Elmer). The percent of injected activity per gram of tissue was computed in order to evaluate the distribution of the radiotracer.

Histopathological Analysis

The immunofluorescent staining was performed at Molecular Cytology Core Facility of XXX using Discovery XT processor (Ventana Medical Systems, Tuscon, AZ). Antigen retrieval was performed with CC1 buffer (Ventana Medical Systems). Sections were blocked for 30 minutes with Background Buster solution (Innovex, Richmond CA). Anti-phospho- H2A.X (Millipore, clone JBW301 0.03ug/mL) antibody was applied and sections were incubated for 5 hours, followed by 60 minutes incubation with biotinylated horse anti-mouse IgG (Vector Labs, Burlington CA) at 1:200 dilution. Detection was performed with Streptavidin-HRP D (Ventana Medical Systems), followed by incubation with Tyramide Alexa Fluor 568 (Invitrogen, Carlsbad CA) prepared according to manufacturer instruction with predetermined dilutions. Slides were counterstained with DAPI (5 ug/mL Sigma Aldrich, St Louis, MO) for 10 min and coverslipped with Mowiol.

Statistical Analysis

Data are presented as single replicates whenever possible. When mean values are provided, standard error of the mean for groups greater than 5 are given.

Results

Pancreas and Tumor Delineation

Conventional X-ray computed tomography (CT) is unable to visualize abdominal organs in the mouse, as shown in Fig. 1A. Since differentiation of structures by CT is dependent on differences in radiodensity, lungs (air), bone (calcified tissue) and soft-tissue (predominantly water) can be separated. However, the radiodensity between the other organs varies little making identification difficult, a problem exacerbated by the size of mice.

We developed a strategy for organ delineation using intraperitoneal (IP) administration of radiopaque (iodinated) contrast to outline individual organs (Fig. 1B). Contrast was injected as a 25% iohexol solution in a volume of 3 mL. Planar images in each orientation demonstrate that structures in the abdomen can be separately identified, using a rapid semi-automated region growing technique. The pancreas, a critical endocrine and exocrine gland, is particularly difficult to localize in small animal imaging, as it is small, diaphanous and obfuscated by other anatomical structures. Figure 1C shows a schematic of the mouse pancreas and surrounding organs. Using our method, the pancreas can be clearly delineated in a surface-rendered negative contrast-CT image of a representative animal (Fig. 1D). The pancreas is shown in blue in both figures.

We determined the minimum concentration of the contrast agent required for visualizing abdominal organs. Mice were injected with varying concentrations of iohexol over a range of 2.5 – 25% v/v; representative images are shown in Supplementary Figure 1. While the contrast between soft tissue and the surrounding media was reduced as the concentration of iohexol was lowered, good visualization could be obtained with 5% iohexol. Minimizing the iohexol concentration is valuable for radiation therapy experiments, as beam attenuation by the contrast agent will affect dosimetry. At 5% iohexol however, attenuation of a 225 kVp beam is slight – $1.38\% \text{ mm}^{-1}$, compared to $1.12\% \text{ mm}^{-1}$ for water. When combined with the fact that only approximately 35% of the beam-path passes through contrast-containing peritoneal fluid (estimated from CT images), this suggests a negligible loss of dose for therapy planning. Furthermore, it is worth noting that at the 40 kVp energy employed for CT image acquisition, there is significantly more attenuation by the contrast agent than at the therapy energy of 225 kVp. Just as in clinical radiotherapy procedures, the experimental therapy planning CT is acquired at much lower exposure settings (both mA and kVp), to minimize overall radiation dose.

Animal Tolerance for IP Contrast

3 mL is the generally accepted maximum volume that can be safely injected into the mouse peritoneal cavity (22). Morbidity, if it occurs, would be likely to take the form of either subcutaneous or pulmonary edema (23), leading to changes in skin tone or labored breathing. These were not observed in the course of any of our experiments. However, we have imaged animals with smaller injected volumes, and acceptable images can be obtained using a 2 mL injection (Supplementary Figure 2A). We also administered daily injections of 3 mL of 0.5% contrast agent to mice each day for 5 days, without toxicity or observations of morbidity. The excess weight due to injection was lost rapidly, with approximately 70% lost

after 5 h (n=4; Supplementary Figure 2B). There was no evidence of animal deterioration in the 2 weeks following IP injections. Thus, the treatment is well tolerated and could be used to direct both single dose and fractionated radiation regimes.

Verification by MR and Histology

Used alone, CT imaging lacks the soft-tissue contrast needed to differentiate between the organ and tumor, but tumors are clearly visible as an anomalous mass that is easy to recognize in comparison to images of non-tumor bearing animals (Supplementary Figure 3).

To further validate that we were correctly identifying tumors in the CT images, we used two approaches. First, CT guided XRT was delivered to the supposed tumor followed by sacrifice and assessment of DNA damage on tumor sections by staining for γ H2AX. The volumes were irradiated with 10 Gy, delivered by a stationary beam with a 1 cm diameter field. Animals were euthanized 30 minutes after treatment. Irradiated tumors show widespread DNA repair activity, covering the entire area of the tumor sections we examined (Fig. 2A). Control non-irradiated tumors also exhibited some background signal for DNA repair, in line with our expectations for these cystic, rapidly growing adenocarcinomas. In this way we were able to link the presumed tumor on the CT image, with the actual tumor, removed *ex vivo*.

Secondly, we employed magnetic resonance (MR) imaging to validate the CT images. MR is more sensitive to tissue-specific differences, as contrast in this modality is predicated on local proton density and relaxation properties, which vary from tissue to tissue. However, issues of logistics and registration limit the utility of MR to plan X-ray radiation therapy. Therefore, we compared our reverse contrast CT delineation method of the pancreas and orthotopic tumors with MR. Animals were IP-injected with radiopaque contrast and imaged first by MR, and then immediately thereafter by CT. At equivalent coronal perspectives, the organs of the abdomen can be detected in both modalities including the pancreas (Fig. 2B). Both modalities reveal concordance in the detection of an ASPC-1 orthotopic xenograft in the tail of the pancreas. The organs and tumor can be delineated in slices throughout the volumetric acquisitions of each imaging modality.

Physiological Effect

The administration of a volume into the intraperitoneal cavity may have physiological consequences stemming from an increase in abdominal pressure. We investigated whether there were any perfusion changes to the tumor and other organs which might influence biological responses in these animal models of disease. Tumor bearing animals with and without IP contrast were dosed with radioglucose (^{18}F -FDG). The distribution of tracer in the tumor and abdominal organs was computed at 20 minutes after injection. No difference in uptake of the radiotracer between the IP and non-injected groups was observed (Supplemental Figure 4). These results indicate that the intraperitoneal administration of contrast does not significantly effect perfusion in these small animal models at the tumor or organ-level.

Determination of a Tolerable Radiation Dose

Prior to assessing tumor radiation response, we established a radiation dose which could safely be given to mice. These experiments were performed on tumor-free mice. After pancreas delineation by reverse contrast CT, animals received focused radiation to the tail of the pancreas and surrounding tissue. We evaluated two therapy plans: 1) Using a posterior-to-anterior fixed angle (P-A), and 2) Doses delivered in an arc by rotating the gantry in a full 360° around the mouse (Arc). Radiation in both plans was delivered using a circular-field brass collimator, 1 cm in diameter.

Radiation dermatitis, a common side effect of clinical XRT (24) was observed in mice with the use of the fixed angle P-A dose plan at 12 Gy (Supplementary Figure 5). Arc therapy was employed for higher doses; skin reaction was not present in subjects treated with the 360° arc plans, as the dose was distributed across the axial circumference of the animal. The data suggested that 15 Gy could safely be delivered to the tail of the pancreas; out of 9 mice treated in this way, none showed significant short-term weight loss (i.e. 20% in 7 days; Fig. 3A–C). At 18 Gy, 20% weight loss was observed in 2 out of 18 animals, which were euthanized. Some long term toxicity was evident, as a further 2 animals in the 18 Gy group died by 40 days after treatment with severe weight loss; upon examination they had greatly reduced spleen and pancreas mass. For all long-term survivors, necropsies were conducted at 60 days post-treatment, when animals were sacrificed and the pancreas weighed. There was clearly a trend for pancreas weight to be reduced in irradiated mice, but there was no outward evidence of morbidity, as reflected in the body weights (Fig. 3D).

Experimental Radiotherapy

In order to assess the capacity of this technique to retard orthotopic tumor growth, we first verified that the entire tumor mass was indeed irradiated with a therapeutic absorbed dose. We utilized Metropolis, absorbed dose modeling software developed in-house at XXX. The results for an arc treatment delivering 15 Gy show that the whole tumor volume received the full dose (Figure 4). As the orthotopic tumor is approximately 7 mm diameter in a 10 mm field, there is an appreciable normal tissue volume that also receives 15 Gy. Increasing the beam accuracy would presumably reduce normal tissue damage and thus allow for an increase in the total dose that could be given. However, the surrounding space around the tumor also takes into account tumor motion during treatment.

In order to estimate the required boundary, we then quantified the extent of tumor motion by ultrasound imaging. Cine loops of five tumors in the sagittal and transverse planes, under normal respiratory and abdominal motion were acquired (Supplementary Video). Tumor motion was quantified in all three directions: in any one direction, the average displacement was 0.4 mm, and the maximum movement we observed was 1 mm. Thus, tumors should remain comfortably within the full dose volume, provided they are centered in the beam and not greater than 9 mm diameter. In the growth delay experiments described below tumor diameters ranged from 5 – 8 mm.

Based on our ability to define, monitor and treat orthotopic pancreatic tumors, we next sought to demonstrate that it was possible to carry out a standard tumor growth delay

experiment. Tumor growth was followed in two separate experiments by either reverse contrast CT or ultrasound (Figure 5A–D). Ultrasound is a more informative imaging methodology as there is inherent echo-contrast between tumor and normal tissues. However, it is clear that repeated imaging by reverse contrast CT successfully discriminates between treated and control tumors (Fig. 5C).

We noted in the cohort of irradiated mice followed by ultrasound that a minority (2/7) of the tumors displayed only modest growth delay (reflected in the large deviation in response). We considered that while this might represent a problem with our methodology, it was also reasonable to consider that microenvironmental features may mediate robustness of response. Specifically, hypoxia might be the source of intra-tumor variation in terms of radiation response. (AsPC1 tumors do display hypoxia, as determined by pimonidazole immunohistochemistry, Supplementary Figure 6). To test this hypothesis, we performed an additional XRT experiment of animals bearing orthotopic tumors. Here, we randomized animals into groups that 1) were imaged by CT but did not receive treatment; 2) received radiation breathing air (as above); or 3) received radiation while breathing carbogen (a 95% O₂, 5% CO₂ mix) used to mitigate tumor hypoxia (25). As shown in Figure 5D, the use of carbogen resulted in all of the tumors in the cohort being suppressed. The variability of air-breathing animals' response could be ascribed to unsuccessful targeting of the tumor mass, but the fact that carbogen treatment was uniformly effective supports our contention that it is possible to identify and treat orthotopic pancreatic tumors with our imaging protocol.

Delineating Genetically Engineered Pancreatic Ductal Adenocarcinoma (PDAC)

The reverse contrast enhancement technique was then used to detect and measure pancreatic ductal adenocarcinoma in an advanced genetically engineered model of the disease. In a representative animal, MR and CT images are compared (Figure 6). The MR identifies tumor structures that can be readily located in the CT images. Compared to orthotopic models, the GEMM mice are more challenging as the tumors may originate at any point in the pancreas. We did not extend these studies to test whether CT imaging alone could be used to direct treatment to GEMM tumors. At this point, we only wish to observe that the information on tumor location carried in MR images can be readily transferred to the corresponding CT and that it should in principle be possible to extend the CT-based treatment protocol to GEMM mice.

Discussion

We have shown that the abdominal organs of the mouse can be imaged using CT with IP-administered contrast agent. It is possible to use these images to direct therapeutic radiation doses to organs and organs bearing tumors in the abdomen. We also demonstrated using this technique the ability to monitor response to radiation longitudinally and non-invasively by volumetric measurement. We were able to repetitively administer IP contrast without detection of adverse effects in the mice in order to monitor tumor burden. Finally, we have applied this technique to define and treat orthotopic pancreatic tumors under various dose-planning strategies (fixed angle and arc) to study differences in mouse response.

Sparing the surrounding structures for orthotopic and GEMM models of the disease is critical as it enables us to look specifically at response in a clinically relevant fashion. We have shown that large doses can be given to tumor tissue with acceptable background dose to the animal. This enables clinically and biologically relevant questions to be tested using any longitudinal assay of response: tumor growth delay, as demonstrated here, but also tumor control or animal survival.

While the bulk of our study concerned orthotopic tumors, we considered it important to extend our approach to GEMM of pancreatic cancer. GEMM are an emerging resource in experimental therapeutics, particularly since they recapitulate the relevant tumor microenvironment for study, and have helped to identify stromatous components as key mediators of treatment resistance (26). However, targeted XRT of these tumors has, until now, not been feasible.

Comparison of MR and CT images of tumor-bearing animals shows that the same structures can be identified in both image sets. However, to use CT alone requires the investigator to reliably identify tumors, and so the problem is as much one of perception and training, as it is of physics and anatomy. The perceptual problems are greater with GEMM than with orthotopic tumors, as tumor location in the mouse is less predictable. We also note that the GEMM model employed in this study is particularly challenging, as it contains a BrCA2 mutation, and these animals are prone to developing multiple tumors throughout the organ. Nevertheless, as we have shown in orthotopic models, we are able to define the organ in its entirety, enabling targeted pancreatic irradiation in the context of multifocal organ-defined disease.

The main advantage of our reverse-contrast CT technique is its ease of implementation. Dual imaging approaches combine the advantages of each modality (for example the soft-tissue contrast of MR and guidance capabilities of CT), but require additional instrumentation and complex logistics. Our group has studied issues of animal image registration procedures in detail (27); such a technique requires the mouse to be immobilized from the start of the MR to the end of radiation treatment. In the context of more difficult to identify GEMM disease, our method still provides soft-tissue structure contrast using CT. This creates the opportunity to acquire multimodal imaging individually (without the use of immobilized-animal protocols) to aid in target-selection and treatment guidance. Mutual information by Reverse Contrast CT would not necessarily require that MR (or US) imaging were even performed on the same day.

Thus, we have demonstrated that it is possible to identify and irradiate orthotopic tumors growing in the tail of the pancreas without severe morbidity. GEMM tumors are also identifiable by CT contrast, though the greater variability in tumor location may require prior imaging with MR for reliable radiotherapy. Mice can be given doses of up to 15 Gy in a 1 cm diameter field; such doses are commonly delivered to subcutaneous tumors and are required for vascular effects (28, 29). Perhaps most importantly, doses of this magnitude are now being employed in clinical practice (30), and we believe our findings allow more relevant association between experimental and clinical practice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Camphausen K, et al. Influence of in vivo growth on human glioma cell line gene expression: convergent profiles under orthotopic conditions. *Proc Natl Acad Sci U S A*. 2005; 102(23):8287–8292. [PubMed: 15928080]
2. Shankavaram UT, et al. Molecular profiling indicates orthotopic xenograft of glioma cell lines simulate a subclass of human glioblastoma. *J Cell Mol Med*. 2012; 16(3):545–554. [PubMed: 21595825]
3. Olive KP, et al. Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. *Science*. 2009; 324(5933):1457–1461. [PubMed: 19460966]
4. Tan MH, Holyoke ED, Goldrosen MH. Murine colon adenocarcinoma: syngeneic orthotopic transplantation and subsequent hepatic metastases. *J Natl Cancer Inst*. 1977; 59(5):1537–1544. [PubMed: 909110]
5. Nakamura T, Fidler IJ, Coombes KR. Gene expression profile of metastatic human pancreatic cancer cells depends on the organ microenvironment. *Cancer Res*. 2007; 67(1):139–148. [PubMed: 17210693]
6. Singh M, Murriel CL, Johnson L. Genetically engineered mouse models: closing the gap between preclinical data and trial outcomes. *Cancer Res*. 2012; 72(11):2695–2700. [PubMed: 22593194]
7. Clarkson R, et al. Characterization of image quality and imageguidance performance of a preclinical microirradiator. *Med Phys*. 2011; 38(2):845–856. [PubMed: 21452722]
8. Wong J, et al. High-resolution, small animal radiation research platform with x-ray tomographic guidance capabilities. *Int J Radiat Oncol Biol Phys*. 2008; 71(5):1591–1599. [PubMed: 18640502]
9. Tuli R, et al. Development of a novel preclinical pancreatic cancer research model: bioluminescence image-guided focal irradiation and tumor monitoring of orthotopic xenografts. *Transl Oncol*. 2012; 5(2):77–84. [PubMed: 22496923]
10. Tuli R, et al. Radiosensitization of Pancreatic Cancer Cells In Vitro and In Vivo through Poly (ADP-ribose) Polymerase Inhibition with ABT-888. *Transl Oncol*. 2014; 7(3):439–445.
11. Johnson EM, Price RE, Rivera B, Cody DD. Intraperitoneal administration of an iodine-based contrast agent to improve abdominal micro-computed tomography imaging in mice. *Contemp Top Lab Anim Sci*. 2005; 44(6):20–27. [PubMed: 16370575]
12. Flores LG 2nd, et al. Monitoring therapy with MEK inhibitor U0126 in a novel Wilms tumor model in Wt1 knockout Igf2 transgenic mice using 18F-FDG PET with dual-contrast enhanced CT and MRI: early metabolic response without inhibition of tumor growth. *Mol Imaging Biol*. 2013; 15(2):175–185. [PubMed: 22875335]
13. Graham KC, et al. Contrast-enhanced microcomputed tomography using intraperitoneal contrast injection for the assessment of tumor-burden in liver metastasis models. *Invest Radiol*. 2008; 43(7):488–495. [PubMed: 18580331]
14. Rampurwala M, et al. Visualization and quantification of intraperitoneal tumors by in vivo computed tomography using negative contrast enhancement strategy in a mouse model of ovarian cancer. *Transl Oncol*. 2009; 2(2):96–106. [PubMed: 19412425]
15. Sastra SA, Olive KP. Quantification of murine pancreatic tumors by high-resolution ultrasound. *Methods Mol Biol*. 2013; 980:249–266. [PubMed: 23359158]

16. Kim MP, et al. Generation of orthotopic and heterotopic human pancreatic cancer xenografts in immunodeficient mice. *Nat Protoc.* 2009; 4(11):1670–1680. [PubMed: 19876027]
17. Olive KP, et al. Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome. *Cell.* 2004; 119(6):847–860. [PubMed: 15607980]
18. Hingorani SR, et al. Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. *Cancer Cell.* 2003; 4(6):437–450. [PubMed: 14706336]
19. Schindelin J, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods.* 2012; 9(7):676–682. [PubMed: 22743772]
20. Thorek DL, et al. Non-invasive mapping of deep-tissue lymph nodes in live animals using a multimodal PET/MRI nanoparticle. *Nat Commun.* 2014; 5:3097. [PubMed: 24445347]
21. Jeong J, et al. Implementation of a Clinical Treatment Planning System for Use with a Small Animal Irradiation System. *Medical Physics.* 2013; 40(6):115–116.
22. Hirota, J.; Shimizu, S. Routes of Administration. In: Heinrich, H., editor. *The Laboratory Mouse.* 2. Academic Press; London: 2012. p. 709-725.
23. Wellman, ML.; DiBartola, SP.; Kohn, CW. Applied Physiology of Body Fluids in Dogs and Cats. In: DiBartola, SP., editor. *Fluid, Electrolyte, and Acid-Base Disorders in Small Animal Practice.* 3. Saunders Elsevier; St Louis, MO: 2006. p. 3-26.
24. Fisher J, et al. Randomized phase III study comparing Best Supportive Care to Biafine as a prophylactic agent for radiation-induced skin toxicity for women undergoing breast irradiation: Radiation Therapy Oncology Group (RTOG) 97-13. *Int J Radiat Oncol Biol Phys.* 2000; 48(5): 1307–1310. [PubMed: 11121627]
25. Chaplin DJ, Horsman MR, Aoki DS. Nicotinamide, Fluosol DA and Carbogen: a strategy to reoxygenate acutely and chronically hypoxic cells in vivo. *Br J Cancer.* 1991; 63(1):109–113. [PubMed: 1846549]
26. Michl P, Gress TM. Improving drug delivery to pancreatic cancer: breaching the stromal fortress by targeting hyaluronic acid. *Gut.* 2012; 61(10):1377–1379. [PubMed: 22661496]
27. Zhang M, et al. Accuracy and reproducibility of tumor positioning during prolonged and multi-modality animal imaging studies. *Phys Med Biol.* 2008; 53(20):5867–5882. [PubMed: 18827321]
28. Griffin RJ, et al. Simultaneous inhibition of the receptor kinase activity of vascular endothelial, fibroblast, and platelet-derived growth factors suppresses tumor growth and enhances tumor radiation response. *Cancer Res.* 2002; 62(6):1702–1706. [PubMed: 11912143]
29. Garcia-Barros M, et al. Tumor response to radiotherapy regulated by endothelial cell apoptosis. *Science.* 2003; 300:1155–1159. [PubMed: 12750523]
30. Goodman KA, Hajj C. Role of radiation therapy in the management of pancreatic cancer. *J Surg Oncol.* 2013; 107(1):86–96. [PubMed: 22532174]

Summary

The recent development of genetically engineered mouse models of pancreatic cancer constitutes a significant advance for experimental therapy development against this deadly disease. However neither these models nor orthotopic tumors can be easily irradiated, due to limitations in X-ray detection of abdominal organs. This hinders development of more effective protocols to treat pancreatic cancer by irradiation, a major modality for clinical management, or to elucidate resistance mechanisms. We demonstrate a noninvasive imaging technique, which is then used to deliver large radiation doses to tumors in the mouse pancreas. This opens the way for studies of radiotherapy and chemoradiation in the most appropriate disease models available.

Fig. 1.

X-ray computed tomographic images (CT) in three orthogonal planes and a three-dimensional rendering of the mouse abdomen (A) without and (B) with the addition of intraperitoneal iodinated contrast solution. (C) A schematic of the mouse abdominal organs, to highlight difficulty of non-invasive imaging of the pancreas. (D) Three dimensional surface rendering of the organs of interest visualized by reverse contrast technique, and an oriented coronal CT slice. Organs of interest and the skeletal compartment are delineated. (L, liver; GB, gallbladder; K, kidney; Sp, spleen; St, stomach; P, pancreas; Int, intestines).

Fig. 2.

Treatment validation. (A) γ H2Ax staining (red) on a DAPI (blue) counterstain in a control and a 10 Gy irradiated tumor. Complete sections are shown in the left hand images; right hand images represent the detail in the white rectangle. (B) Comparison of reverse contrast CT and MR images in the same tumor-bearing animal. MR images were acquired immediately after the animal had been injected with iohexol/saline (4 mL) and imaged by CT. Coronal slices throughout the animal are shown. Post-acquisition, the CT and MR images restrained mouse were aligned by rigid body transform. The pancreas and orthotopic

tumor are highlighted by the arrow. (organs are L: liver; K: kidney; Sp: spleen; St: stomach; S M: skeletal muscle; C: cecum; S Int: small intestine and P+T: pancreas and tumor).

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Fig. 3. Maximum tolerated dose of CT-guided pancreatic irradiation. Animals were weighed daily for a minimum of 7 days after treatment to assess acute toxicity of high dose irradiation. (A) Individual whole-body weight measurements for control non-irradiated animals, (B) a single posterior to anterior beam and (C) radiation delivered in a continuous rotating 360° arc. Dose values inset in bottom right corner of each plot. Red points and lines are used to indicate that an animal lost greater than 20% bodyweight. (D) The effects of radiation on body and pancreas weight, 60 days after arc treatment.

Fig. 4. Absorbed Dose Modeling. Dose distribution profiles, generated by Metropolis for a 15 Gy arc therapy with crosshairs showing the treatment center in coronal (A); transverse (B); and sagittal planes (C). The tumor is outlined in purple. (D) Dose volume histogram for the tumor in the arc treatment plan.

Fig. 5.

The effect of radiation on tumor growth. (A) Coronal CT image of a non-irradiated mouse (tumor at arrow). In the second panel, the tumor volume is shown in three dimensions rendered as a red volume. The intersection of the coronal CT slice (same as at left) is shown intersecting the volume in the sagittal and coronal perspectives. (B) Corresponding images of a mouse treated with 15 Gy arc (tumor at arrow in CT slice), with the tumor in blue. Both image sets were acquired 7 days after the start of the experiment. (C) Tumor volumes, as calculated by CT imaging of control and irradiated tumors (n=5). (D) In a subsequent

experiment, also employing a 15 Gy arc treatment plan (either under air or carbogen), tumor volume was followed by ultrasound.

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Fig. 6.

Comparison of CT and MR images in a genetically engineered mouse model of pancreatic cancer. The CT and MR slices were selected manually for similar views of the tumor. (A) CT images. Crosshair (yellow) centered on the tumor. (B) MR images were acquired before CT contrast agent injection. The coronal MR image is generated from multiple transverse slices, and the distortion is a reconstruction artifact, linked to the number of slices. Crosshair (green) centered on the tumor.