Effective Rat Lung Tumor Model for Stereotactic Body Radiation Therapy

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Stereotactic body radiation therapy (SBRT) has found an important role in the treatment of patients with non-small cell lung cancer, demonstrating improvements in dose distribution and even tumor cure rates, particularly for early-stage disease. Despite its emerging clinical efficacy, SBRT has primarily evolved due to advances in medical imaging and more accurate dose delivery, leaving a void in knowledge of the fundamental biological mechanisms underlying its activity. Thus, there is a critical need for the development of orthotopic animal models to further probe the biology associated with high-dose-per-fraction treatment typical of SBRT. We report here on an improved surgically based methodology for generating solitary intrapulmonary nodule tumors, which can be treated with simulated SBRT using the X-RAD 225Cx small animal irradiator and Small Animal RadioTherapy (SmART) Plan treatment system. Over 90% of rats developed solitary tumors in the right lung. Furthermore, the tumor response to radiation was monitored noninvasively via bioluminescence imaging (BLI), and complete ablation of tumor growth was achieved with 36 Gy (3 fractions of 12 Gy each). We report a reproducible, orthotopic, clinically relevant lung tumor model, which better mimics patient treatment regimens. This system can be utilized to further explore the underlying biological mechanisms relevant to SBRT and high-dose-per-fraction radiation exposure and to provide a useful model to explore the efficacy of radiation modifiers in the treatment of non-small cell lung cancer. © 2016 by Radiation Research Society

INTRODUCTION

Stereotactic body radiation therapy (SBRT), also known as stereotactic ablative radiotherapy (SABR), utilizes high-

dose-per-fraction radiation and numerous technologies to isolate dose in and around a targeted tumor. SBRT has changed the standard treatment of early non-small cell lung cancer (NSCLC). Lung cancer is the leading cause of cancer-related mortality in the United States, and according to the American Cancer Society in 2016, an estimated 158,080 Americans are expected to die from lung cancer. For these reasons, there is an ongoing need to develop new treatment modalities and improve on those that currently exist to increase the overall survival of lung cancer patients (1). NSCLC includes squamous cell carcinoma, adenocarcinoma and tumors of large cell origin, which account for 80–90% of all lung cancers. The standard-of-care treatment for early-stage NSCLC is surgery, resulting in 5-year survival rates between 60 and 70% for early-stage patients (2). However, surgery is not a viable option for most NSCLC patients who either cannot tolerate surgical stress or postoperative recovery, or for whom the disease burden is too extensive. Conventionally fractionated radiation therapy is considered an alternative, noninvasive treatment approach for many patients, and has reported 2- and 5-year overall survival rates of 39% and 13%, respectively, for early-stage patients, with a significantly improved 5-year survival rate of 32% when tumors were locally controlled (3).

Numerous studies have demonstrated that SBRT can cure early-stage lung tumors in outpatient settings (4–11). In a national prospective clinical trial published in 2010, the use of SBRT [54 Gy total (18 Gy $\times$ 3 fractions, each separated by at least 40 h), with maximum treatment time of 14 days] was explored in the treatment of inoperable stage 1 NSCLC, and a 98%, three-year primary tumor control rate was achieved (10). Despite these promising clinical results and an increasing enthusiasm for expanded use of SBRT to other organ sites (12, 13), the modality has mostly advanced due to the exploitation of technological innovations in diagnostic imaging and more accurate radiation delivery. The biological mechanisms involved in SBRT are still poorly understood (14). Mounting evidence suggests that the underlying biology of high-dose-per-fraction radiation therapy may be different than that of

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conventionally fractionated radiation therapy. Nevertheless, more advanced preclinical and orthotopic animal models are needed to enhance our understanding about SBRT. Improvement of SBRT and the identification of effective radiation modifiers (sensitizers, protectors or mitigators) will emerge from formal biological studies exploring the fundamental mechanisms, pathways and treatment responses using *in vitro* and *in vivo* models in a high-dose-per-fraction setting.

Rodent models utilizing image-guided, high-dose-per-fraction treatment of tumors and normal tissues may prove attractive for the biological study of SBRT delivery. Unfortunately, most orthotopic lung cancer models employ the tail vein injection technique, which results in a multinodal and metastatic tumor burden. The treatment of multinodal tumors and systemic disease involves a different treatment circumstance than that commonly observed in the clinical SBRT setting where disease is more isolated. Furthermore, animals with miliary dissemination have very short lifespans making observation and measurement of biological outcomes difficult. Methods for generating solitary intrapulmonary nodules and techniques for delivering image-guided radiation treatment similar to human stereotactic radiation treatment in rodent models are still not generally available. Therefore, investigation of the tumor response to simulated SBRT requires a rat model carrying a solitary intrapulmonary nodule, which better simulates early stage human NSCLC. Here we describe the surgical implantation of orthotopic tumors in nude rats and demonstrate successful targeting of lung tumors using image-guided SBRT. These findings will better enable the design and testing of new strategies for treating NSCLC patients and further our understanding of the biological mechanisms underlying high-dose-per-fraction radiation therapy.

MATERIALS AND METHODS

Cell Culture

A549-Luc cells were graciously provided by Dr. John Minna (University of Texas Southwestern Medical Center, Dallas, TX) and cultured in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) under standard culture conditions. The A549-Luc lung cancer cell line was established using a lentivirus encoding the luciferase gene driven by a ubiquitin promoter. Stable clones were then isolated by G418 selection (2).

Tracheal Intubation and Ventilation

Female nude rats (NIH-RNU; National Cancer Institute, Frederick, MD) were anesthetized using 5% isoflurane and in oxygen (1 L/min) in an induction chamber and placed onto a Hallowell tilting workstation (Hallowell EMC, Pittsfield, MA). Rats were secured with an elastic band over the top incisor. The rat tongue was pulled out to the side of mouth using a Q-tip®. One to two drops of 2% lidocaine were applied to the back of the mouth to locally anesthetize the epiglottis. An 18-gauge catheter over an introducer wire was inserted through the vocal cords. The tip of the endotracheal tube was advanced no deeper than the thoracic inlet and the wire was removed.

The correct placement of tracheal intubation was confirmed by observing fogging on a dental mirror due to correct breathing. The rats were removed from the intubation board and connected to the Y port of the ventilator tubing. Anesthesia was maintained with 2% isoflurane in oxygen, and the ventilator was set to achieve a tidal volume of approximately 10 ml/kg and a respiratory rate of 79–90 breaths/min for a 150–200 g rat.

Surgical Orthotopic Model

A total of 1×10^6 cells were mixed with Matrigel® (1:1) in a final volume of 15 μ l. The surgical area was shaved and cleaned using povidine-iodine followed by 70% ethanol. Before surgery, an analgesic was administered subcutaneously (s.c.) to the rat (1 mg/kg; Buprenorphine SR™) followed by toe pinching to ensure an adequate level of anesthesia. A 1–2 cm incision was made on the skin over the 5th and 6th ribs on the right area of the chest and the muscle was bluntly dissected. The chest wall was opened with an incision through the 5th and 6th intercostal space. The bottom lobe of the right lung was carefully exteriorized using curved forceps, and a portion of the tissue was clamped with a microvascular clamp. A549-Luc cell suspension was injected into the isolated lung using a 28-gauge insulin syringe. Ten seconds later the microvascular clamp was removed. The surgical area was cleaned with saline and the lung was gently placed back into the chest. The incisions on the chest, muscle and skin were sutured closed in layers. The animals were carefully weaned off the ventilator to enable independent breathing by turning off the isoflurane and delivering 100% oxygen. Once the animal was responsive to reflexes, the endotracheal tube was removed. Animals were carefully observed for 24 h and an analgesic was administered as needed. Each implantation took about 15–20 min total starting from intubation.

All experiments were conducted and approved under the Institutional Animal Care and Use Committee animal welfare guidelines at UT Southwestern Medical Center. Animals were monitored daily for any visible signs and symptoms. Rats were euthanized if the animals showed signs of distress, including labored breathing, severe body weight loss, hunched posture and lack of movement due to tumor burden, in compliance with IACUC policy. We also euthanized animals when the bioluminescence imaging (BLI) intensity reached $\geq 10^9$ photons/s, which indicated a large tumor (~ 1.5 – 1.8 cm in diameter) in the lung, severely compromising animal activity.

Bioluminescence Imaging

Bioluminescence imaging was performed weekly using an IVIS Lumina or Spectrum Imaging Systems (Xenogen/Caliper Life Sciences, Alameda, CA). Rats were anesthetized by 2% isoflurane inhalation followed by s.c. injection of D-luciferin (150 mg/kg; Gold Biotechnology®, St. Louis, MO). Bioluminescent images were acquired 10 min after luciferin injection.

Cone Beam Computed Tomography Scans

Tumor growth in animals was monitored by weekly bioluminescence imaging. When sufficient bioluminescence was observed, cone beam computed tomography (CBCT) scans were obtained using an X-RAD 225Cx (Precision X-Ray Inc., North Branford, CT) to confirm tumor localization. Rats were anesthetized with 2% isoflurane inhalation and positioned on the CBCT bed. Images were obtained through the entire thorax at an isotropic voxel size of 45 μ m (40 kV, 500 μ A and 400 ms integration time).

Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) was performed using a horizontal bore 4.7-T magnet (Varian Medical Systems Inc., Palo Alto, CA) with a Doty rat body coil. Animals were anesthetized with isoflurane (1.5%) in air (1 L/min) and kept warm with a circulating

FIG. 1. CBCT images of lung tumor and treatment planning. Panel A: Lung tumor (blue line) and peripheral tissue (green line) contouring based on CBCT images. Panel B: Treatment planning of 12 Gy in 9 dynamic beams. Panel C: Dose map on contoured tissues.

warm water blanket. Body temperature and respiration were monitored with a small animal physiological monitoring system (Small Animal Instruments Inc., Stony Brook, NY) throughout the experiment. T₁-weighted images were acquired after intravenous (i.v.) injection of gadolinium contrast (Gadovist®) at a dose of 0.1 mmol/kg body weight using a fast spin-echo (FSE) sequence: TR = 500 ms; effective TE = 10 ms; echo train length = 2; slice thickness = 2 mm; field of view 60 × 60 mm; and matrix size 128 × 128.

Microirradiation

Rats were anesthetized with 2% isoflurane by inhalation. CBCT images were acquired using the X-RAD 225Cx small animal irradiator and processed using the Small Animal RadioTherapy (SmART) Plan treatment system (Precision X-ray Inc.) (15) to contour the lung tumor (Fig. 1A). A treatment of 12 Gy containing 9 beams (40° dynamic rotation per beam; see Fig. 1B) was planned and calculated to mimic arc treatment (Fig. 1C). The treatment plan was imported into the X-

FIG. 2. Panel A: Surgical implantation of lung tumor cells. Adult female athymic nude rats were anesthetized by 3% isoflurane. Rats were intubated through the trachea and supported by a small animal ventilator. Lungs were surgically exposed and injected with A549-Luc cells. Incisions were closed with suture and staples, and animals were monitored for recovery. Panel B: Tumor growth was observed three weeks after tumor implantation using multimodal imaging including bioluminescent imaging, CBCT and MRI. Tumor presence was confirmed after animals were euthanized. Panel C: Tumor development was monitored weekly with bioluminescent imaging after implantation. The pie chart shows percentages of lung tumor formation 3 weeks after surgical implantation.

RAD 225Cx system and delivered to the animal. The tumor was irradiated with either 12 Gy (1 fraction) or 36 Gy (3 fractions, 12 Gy for each fraction every 36 h) mimicking the SBRT regimen used for lung tumors in the clinic.

The irradiator was calibrated in accordance with AAPM TG-61 protocol (16) using the PTW N31010 ionization chamber (PTW, Freiburg, Germany). The ionization chamber calibration factor is directly traceable to the National Institute of Standards and Technology (NIST; Gaithersburg, MD) through calibration services provided by the University of Wisconsin Accredited Dosimetry Calibration Laboratory (ADCL; Madison, WI). Furthermore, the numerical computations of the irradiator's Monte Carlo-based SmART treatment planning system were verified through measurements in various phantom settings using radiochromic film. This included the standard acceptance testing and additional commissioning treatment test plans for various collimator sizes in homogeneous and heterogeneous phantom geometries.

RESULTS

Tumor Development: Surgical Lung Tumor Implantation

A549 cells expressing a luciferase reporter gene (A549-Luc, 1 million cells in 15 μ l of 1:1 mixture of saline and Matrigel) were surgically implanted into the right lung of ten female, athymic nude 8-week-old rats (Fig. 2A). BLI was conducted weekly (Fig. 2B) to track tumor development and growth. Once the tumor bioluminescence intensity was greater than 1×10^7 (photons/s), the animal was immediately scanned using multiple imaging modalities (CBCT and MRI) to confirm tumor formation and location. The circled area in Fig. 2B indicates the orthotopic tumor in coronal views of CBCT and MRI images. After image acquisition, animals were euthanized and examined to

confirm tumor development and localization. The presence of an orthotopic tumor in the lung (Fig. 2B) suggested that all the imaging methods used were sufficient to detect and validate tumor localization.

Single Nodule Tumor Development

We are currently generating these single nodule lung tumors in a rat model as a core service for a multi-investigator research project. To date, we have surgically implanted A549-Luc cells into the lung of 182 female nude rats. BLI was performed weekly to monitor tumor growth. A small cohort of rats ($n = 10$) were sacrificed after 3 weeks to confirm single nodule tumor formation in the right lung. Despite the small percentage of deaths ($\leq 2\%$, 4/182) due to either ventilation or surgical complications, the majority of animals ($\geq 90\%$, 164/182) developed a solitary tumor in the lung, and less than 8% (14/182) of animals developed multiple nodules (Fig. 2C). This indicated the feasibility of using the described surgical lung tumor implantation method to obtain 90% engraftment success rate of solitary nodule tumors inside the right lung.

Tumor Response to Radiation

To determine the response of single nodule tumors to radiation using this model, A549-Luc cells were surgically implanted into adult female, athymic rats and confirmed to have solitary nodules using BLI. Animals were separated into three groups: control ($n = 4$) and two treatment groups. Three weeks after implantation, one treatment group ($12 \text{ Gy} \times 1$, $n = 5$) received a single 12 Gy dose, while the other group ($12 \text{ Gy} \times 3$, $n = 3$) received 3 fractions of 12 Gy each over 72 h. CBCT images of the lung tumors were obtained before treatment. The CBCT images were used to contour the orthotopic tumors and individual treatment plans were generated using the SmART planning system (Fig. 1). After treatment, the tumor BLI signal was monitored weekly to evaluate radiation response. One representative animal from each group is shown in Fig. 3A. Control animals showed an increasing BLI signal weekly, which was terminated after 7 weeks with the BLI signal saturated across the chest. Animals irradiated with a single 12 Gy dose demonstrated a significantly reduced tumor growth rate and were monitored up to 18 weeks before euthanasia. This result indicates that a single dose of 12 Gy is appropriate for tumor growth delay experiments. In contrast, animals irradiated with $12 \text{ Gy} \times 3$ fractions each demonstrated a decreasing BLI signal, and no signal was detected by week 7. The bioluminescent signal-free rats were euthanized at 6 months after implantation, and the lungs were dissected to confirm tumor resolution by histologic analysis. The average tumor growth rates across the different treatment groups using BLI were compared (Fig. 3B). The control group demonstrated an increasing BLI signal after week 3, and animals were eventually euthanized between weeks 7–10. While both treatment regimens induced significant tumor growth delay, the 12 Gy

$\times 3$ treatment regimen ablated most tumors by week 13. These data demonstrate that A549 tumors are highly aggressive in the lungs of nude rats and that, with image guidance, high-dose irradiation can achieve complete ablation of early-stage tumors.

DISCUSSION

Over the years, orthotopic lung tumor models in rodents have been established through xenografts. These models play an essential role in evaluating lung cancer treatment due to the tumor anatomical location and interactions with the surrounding microenvironment. Nevertheless, additional animal models are needed to study the formation of solitary nodules and simulate clinically relevant treatment modalities in humans, such as SBRT.

Existing orthotopic lung models, developed primarily in mice, are unsuitable for the described purpose due to the widespread growth of multiple nodules in both lungs and metastatic disease (17–19). Mordant *et al.* compared and refined bioluminescent orthotopic mouse models of localized human NSCLC (17). Among these models, percutaneous orthotopic injection and transpleural orthotopic injection of A549 cells successfully induced localized tumors with multiple nodules (17). We previously reported moderate success (60%) in developing a single nodule tumor in the rat lung when NSCLC tumor cells were implanted with the help of image guidance (2). However, this model also frequently induced multiple tumors in the chest cavity. To overcome this problem, we introduced the surgical implantation of NSCLC cells directly into the right lung of rats. The formation of solitary tumors and the location in the lung were validated by multimodal imaging followed by direct visualization of the tumor of the same euthanized rats. This led to the development of an appropriate model to study the effects of high-dose-per-fraction SBRT. This model achieved nearly 100% survival after surgical orthotopic implantation. Serial BLI is an excellent tool for monitoring tumor growth over several weeks, allowing the tumor to develop characteristics suitable for treatment. In a small percentage of animals in this study multiple nodules were observed, which likely spread throughout the chest cavity due to tumor cells leaking out of the injection site. However, because these multiple-nodule tumors were detectable using BLI, those animals were removed from the treatment groups.

The development of a solitary lung tumor model in rodents and the use of the X-RAD 225Cx platform to deliver image-guided, high-dose-per-fraction radiation to the target comprise an improved simulation system of clinical SBRT in humans compared to s.c. or i.v. injection. In this study, BLI was used to determine the optimal time to initiate treatment. CBCT images were acquired to localize, contour and target the tumor for SBRT delivery. The rotating gantry in the X-RAD 225Cx system allowed for treatment planning and delivery using 360° rotating arc

FIG. 3. Dose response of A549-Luc orthotopic lung tumors to radiation. A549-Luc cells were implanted in female athymic 8-week-old nude rats. Tumor growth was monitored weekly by bioluminescence and the tumor was treated with 12 Gy $\times$ 1 and 12 Gy $\times$ 3 fractions at week 3. Panel A: Representative images from each group. Panel B: Data analysis among different groups. Radiation treatments were started at week 3 for both treatment groups (12 Gy $\times$ 1 fraction; 12 Gy $\times$ 3 fractions over 72 h).

beams to simulate clinical radiation treatment. This model allowed for accurate delivery of radiation to the tumor while simultaneously sparing normal tissues from the effects of radiation toxicity, distinguishing itself from typical large-field or hemi-body laboratory irradiations.

One limitation of our current model is the inability to mimic the tumor response in immune-competent circumstances. We used nude rats with altered immune function,

which plays a large role in cancer treatment. We also found that the tumors are not uniform in shape in our models. Nevertheless, validation of this model may further its use as an excellent tool for a deeper and broader understanding of the biological mechanisms underlying tumor response to SBRT. This model also offers a suitable platform to investigate the effects of novel compounds designed to increase tumor radiosensitivity.

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