Purpose: In the past 3 decades, synchrotron microbeam radiation therapy (S-MRT) has been shown to achieve both good tumor control and normal tissue sparing in a range of preclinical animal models. However, the use of S-MRT for the treatment of lung tumors has not yet been investigated. This study is the first to evaluate the therapeutic efficacy of S-MRT for the treatment of lung carcinoma, using a new syngeneic and orthotopic mouse model.

Methods and Materials: Lewis Lung carcinoma-bearing mice were irradiated with 2 cross-fired arrays of S-MRT or synchrotron broad-beam (S-BB) radiation therapy. S-MRT consisted of 17 microbeams with a width of 50 μm and center-to-center spacing of 400 μm. Each microbeam delivered a peak entrance dose of 400 Gy whereas S-BB delivered a homogeneous entrance dose of 5.16 Gy (corresponding to the S-MRT valley dose).

Results: Both treatments prolonged the survival of mice relative to the untreated controls. However, mice in the S-MRT group developed severe pulmonary edema around the irradiated carcinomas and did not have improved survival relative to the S-BB group. Subsequent postmortem examination of tumor size revealed that the mice in the S-MRT group had notably smaller tumor volume compared with the S-BB group, despite the presence of edema. Mice that were sham-implanted did not display any decline in health after S-MRT, experiencing only mild and transient edema between 4 days and 3 months.

Acknowledgments—The authors wish to acknowledge Daniel Hausermann, Matthew Cameron, and Clare Scott for experimental support on site at the Australian Synchrotron; Liam Day and Stefan Bartzsch for Monte Carlo modeling of the lung irradiation set-up; Helen Forrester for postirradiation animal care and monitoring; the National Imaging Facility and Monash Biomedical Imaging, Australia, for scientific and technical assistance; Tara Sephrizadeh for technical support during the in vivo computed tomography scans; Benoit Petit for assistance during the establishment of the tumor implantation method; Alexander Ernst for image analysis. Microscopy was performed on equipment supported by the Microscopy Imaging Center of the University of Bern, Switzerland.
Introduction

Lung cancer is the deadliest type of cancer, responsible for 1.8 million deaths worldwide in 2020.1 Radiation therapy is indicated for around three-quarters of all patients with lung cancer2; however, minimizing acute and late radiation-induced side effects while controlling tumor growth remains a major challenge. Radiation-induced pneumonitis (RIP) is the most relevant acute toxic effect after conventional radiation therapy to the thorax,3 and the most prominent long-term toxicity is radiation-induced pulmonary fibrosis, which has an incidence of up to 28%.4 Radiation-induced pulmonary fibrosis is not only detrimental to quality of life but can also lead to fatal respiratory insufficiency.5

Stereotactic body radiation therapy (SBRT) is a cutting-edge radiation therapy approach for the treatment of early-stage non-small cell lung cancer (NSCLC) and is associated with excellent rates of local disease control.6 However, SBRT can induce severe collateral effects including pneumonitis, rib fracture, and esophagitis, especially when treating centrally located lung tumors.7 For this reason, SBRT is predominantly applied to small peripheral lesions.

Synchrotron microbeam radiation therapy (S-MRT) is an innovative technique that uses the spatial fractionation of a synchrotron-generated x-ray beam into microbeams, typically between 20 and 100 μm wide. This geometry exploits dose-volume effect, where the tissue tolerance-dose increases as the proportion of irradiated tissue decreases.8 Additionally, S-MRT utilizes ultrahigh dose-rates, most often providing the capability of delivering treatments in less than 200 ms9 and thereby triggering a FLASH normal tissue sparing effect. This combination of the FLASH effect and spatial fractionation makes S-MRT a unique cancer treatment tool.

A vast body of preclinical research demonstrates that S-MRT effectively delays tumor growth or even ablates malignancies while being very well tolerated by normal tissue.10 So far, S-MRT has been studied using a limited number of cancer types, most commonly in vivo models of brain tumors,11,12 mammary tumors,13 melanoma,14 and squamous cell carcinoma15 have also been investigated. Mechanisms underlying the tumoricidal efficacy of S-MRT are (1) direct cellular radiation damage in the path of the microbeams,16 (2) selective disruption of immature vessels,17 and (3) enhancement of immune-cell recruitment.14 Together with excellent tumor control, S-MRT shows pronounced tissue-sparing effects in several organs such as the skin,18 central nervous system,19 and mature vasculature.20 Results from these studies are highly encouraging, but additional cancer types must be investigated to promote clinical translation.

The first S-MRT feasibility study for lung irradiation was recently published, demonstrating that the C57BL/6 mouse model is well suited to explore the potential of S-MRT for the treatment of lung cancer.21 The lungs of healthy mice were irradiated with a peak S-MRT dose of 400 Gy or 40 Gy using microbeams with a width of 50 μm and a center to center spacing of 400 μm.21 Moreover, preliminary (unpublished) data from our group indicates that S-MRT causes only low-grade lung fibrosis in rats up to 12 months after irradiation. Given its superior tumor control and observed sparing of the normal lung, S-MRT could be a novel alternative for treating lung tumors, including central and ultracentral malignancies and cases where complete tumor resection is not possible.22

Here we show, for the first time, the successful application of S-MRT in a new preclinical mouse model of lung carcinoma. Tumor-bearing lungs were irradiated with either 2 cross-fired arrays of S-MRT or synchrotron-broad beam (S-BB) radiation, with a control group receiving no treatment. In addition, S-MRT-induced changes in sham-injected lungs of healthy mice were observed with in vivo computed tomography (CT) scans up to 6 months after irradiation. In parallel, the presence of fibrosis was also examined in S-MRT-irradiated lungs of naïve mice at the same time point.

Methods and Materials

Animals

Animal experimental procedures were performed under the Swiss license BE69/19 and the permits AS2019/003 and AS2019/007 approved by the Australian Animal Ethics Committee. C57BL/6 female mice were purchased from the Animal Resources Centre in Canning Vale, Western Australia. Mice were 8 weeks old on arrival and were acclimatized for 2 weeks at the Medical beamline (IMBL) animal facility of the Australian synchrotron. Mice were housed at 23°C with a 12-hour light/12-hour dark cycle and had access to food and water ad libitum. For the survival study, animals were culled on reaching interruption criteria. These criteria were assessed for each mouse on a daily basis using a score sheet. A composite score was calculated based on the

Conclusions: S-MRT is a promising tool for the treatment of lung carcinoma, reducing tumor size compared with mice treated with S-BB and sparing healthy lungs from pulmonary fibrosis. Future experiments should focus on optimizing S-MRT parameters to minimize pulmonary edema and maximize the therapeutic ratio. © 2021 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/)
domains of weight loss, overall appearance, passive behavior and behavior after stimulation. Severe loss of weight (>15% from the day of irradiation), evident breathing problems, no reaction after stimulation, or a radiologic tumor volume greater than 100 mm³ based on in vivo CT, were criteria for immediate euthanasia.

**Cell line**

The murine Lewis Lung carcinoma (LLC1, also known as LL/2) cell line was purchased from the American Type Culture Collection (ATCC CRL-1642). Cells were cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 10% (v/v) Fetal Bovine Serum and kept at 37°C in a humidified incubator with 5% CO₂. Lewis Lung carcinoma is a type of NSCLC and KRAS-mutated tumor. This cell line is clinically relevant because NSCLC accounts for 85% of all lung cancers and KRAS mutations are the most common in patients with lung adenocarcinoma. It was chosen because it was originally derived from C57BL/6 mice lungs and therefore allowed the development of a syngeneic and orthotopic mouse model, immunologically compatible with the organism. Cells were used before the seventh passage. On the day of tumor implantation, cells were detached from the flask with a Trypsin solution (0.25%) and counted with a disposable Haemocytometer C-Chip (Model: DHC-N01, NanoEntek Inc, Seoul, South Korea). Cells were finally resuspended in Fetal Bovine Serum-free Dulbecco’s Modified Eagle’s Medium at a concentration of $1 \times 10^4$ LLC1 cells per 3 μL.

**LLC1 inoculations**

The protocol for tumor inoculation was adapted from Mordant and colleagues. Paracetamol in drinking water (2 mg/mL) was given 48 hours before implantation. Each mouse was weighed and anaesthetized with an intraperitoneal (IP) injection of anesthetic cocktail comprising of fentanyl (0.05 mg/kg of body weight [BW]), midazolam (5 mg/kg BW), and medetomidine (0.5 mg/kg BW) in sterile water. The body temperature of each mouse was maintained at 37°C during the whole procedure. Each mouse was laid on its left side exposing its right flank. The area of implantation (under the right-scapular region) was shaved with a trimmer and disinfected with a chlorhexidine solution (2%). To provide local anesthesia, a solution of lidocaine (0.5%) was injected subcutaneously (SC) at the site of inoculation. To identify the injection site, a superficial imaginary line (subsequently referred to as the midaxillary line), was drawn along the midsagittal plane. A skin incision of approximately 5 mm was made with surgical scissors along the midaxillary line and the cutaneous maximus and latissimus dorsi muscles were gently separated to visualize the rib cage. LLC1 cells were injected with a Hamilton syringe between the third and the fourth rib. Images describing the main steps of the procedure are shown in Fig. E1. A 3 μL volume of cell culture medium containing $1 \times 10^4$ cells was supplemented with 2 μL of Thrombin solution (18 U/mL) immediately before the injection. The total injection volume was 5 μL. After the inoculation, the incision was closed with 2 interrupted sutures using a 6/0 polyamide monofilament nonabsorbable surgical suture. A solution of Buprenorphine (0.1 mg/kg BW) was injected SC on the right flank to provide systemic analgesia. Anesthesia was reversed with an IP injection of flumazenil (0.5 mg/kg BW) and atipamezole (2.5 mg/kg BW) in sterile water. Two mice were sham-injected: the animals underwent the whole surgical procedure but received an injection with no tumor cells.

**Irradiation set-up**

Irradiations were performed 11 days after LLC1 inoculations in Hut 2B on the IMBL at the Australian synchrotron. After induction of anesthesia (same protocol as used for the LLC1 inoculations), mice were orally intubated with a modified catheter under fiber optic guidance (Kent Scientific, Torrington, CT). Mice were then fixed with surgical tape onto a Perspex positioning frame and transferred onto the DynamicsMRT small-animal irradiation stage. A Physiosuite (Kent Scientific) ventilator/monitor calculated the tidal volume and respiratory rate from the weight of the mouse. Mice were aligned to the synchrotron beam using an established image guidance system. Deep inspiration breath-hold was used for both imaging and irradiation to ensure correct alignment of the lung tumors to the beam. Mice were irradiated with either 2 cross-fired S-MRT arrays (13 mice) or 2 cross-fired S-BB fields (13 mice). Breath-hold, image guidance, and irradiation were controlled from outside the irradiation hutch. S-MRT and S-BB were delivered using 2 anterior-oblique fields, centered on the tumor and separated by an angle of 25 degrees. This angle was chosen to spare the spinal cord and the heart of the mouse. Mice were then disconnected from the ventilator, removed from the Perspex frame and given an IP injection of reversal cocktail (same as used for the tumor inoculations). Thirteen carcinoma-bearing mice were used as a nonirradiated control group (CTR). In addition, 14 healthy mice were irradiated with 2 cross-fired S-MRT arrays; 6 mice were culled 12 hours postirradiation to verify that the microbeams were delivered correctly to the tumor via H2AX staining and 8 mice were killed 6 months postirradiation to evaluate lung fibrosis. Another 2 sham-injected mice underwent periodic in vivo CT imaging to evaluate changes in the lungs up to 6 months postirradiation.

**Dosimetry**

S-MRT and S-BB were delivered during operation at 3 GeV and with a ring current of 200 mA. The doses delivered to the tumor for both modalities were determined from Monte Carlo simulations using a phantom representative of the mouse and the plastic holder used for the irradiations. The mouse was modelled as a rectangular prism (100 mm long × 30 mm wide × 15 mm deep) composed of water,
lying on a plastic plate (200 mm long, 150 mm wide, 5 mm deep) composed of poly methyl-methacrylate. Mice were scanned vertically through a 2 mm high beam-slice using the DynamicMRT stage to deliver the prescribed doses to a total field size of 7 mm × 7 mm. Reference dosimetry was performed with a PTW (Freiburg, Germany) Pinpoint 31014 ionisation chamber under broad-beam conditions at both the S-MRT and S-BB energy spectrums. For S-MRT (average energy 93 keV) each field delivered a peak entrance dose of 400 Gy (valley entrance dose = 2.86 Gy), equating to a peak and valley dose to the tumor (depth of 8.5 mm) of 353.6 Gy and 4.76 Gy, respectively. Each S-MRT field consisted of 17 microbeams of 50 μm width with 400 μm center-to-center spacing. The in-slice peak MRT dose-rate was 991.7 Gy/s at tumor depth (in-slice delivery time of 361 ms). The vertical scan speed of the mouse through the beam was 5.63 mm/s, leading to a total delivery time of 1.24 seconds for the entire field. For S-BB (average energy 124 keV), each field delivered an entrance dose of 5.16 Gy and a dose to the tumor of 4.76 Gy. The in-slice S-BB dose-rate was 37.0 Gy/s at 8.5 mm depth (in-slice delivery time of 129 ms) and the vertical scan speed was 15.63 mm/s. The total time to deliver the prescribed dose to the entire field was 447 ms. The uncertainties (k = 1) associated with the delivered doses to the tumor were 4.1%, 3.9%, and 8.5% for S-MRT (peak), S-MRT (valley) and S-BB, respectively. The S-MRT valley dose matched the S-BB dose delivered to the tumor to distinguish the advantage of high peak doses in conjunction with those of homogenous radiation.

**In vivo CT scans**

In vivo lung CT scans were performed using a Siemens Inveon multimodality scanner at the Monash Biomedical Imaging facility (Clayton, Australia). Scans were performed in step-and-shoot mode with 360 projections at 80 kV and 500 μA with a 180 ms exposure for each projection for a total scan time of 5 minutes. The image field-of-view was 38.63 × 33.64 mm with an effective pixel size of 40 μm. Animals were anaesthetised with isoflurane (2%-2.5%) in 100% oxygen throughout the scan.

**Tumor growth curve**

The tumor growth curve for the LLC1 carcinoma model was obtained by measuring the tumor size from the in vivo CT scan images. Measurements were taken at day 8 and day 12 postimplantation and every third day thereafter. The maximum dimensions of the tumor in the sagittal, coronal, and transverse planes were recorded. Tumor volume (in mm³) was calculated with the formula:

\[
V = \frac{4}{3} \pi \left( \frac{a}{2} \times \frac{b}{2} \times \frac{c}{2} \right)
\]

where \(a\), \(b\), and \(c\) are the maximum recorded lengths of the tumor in the sagittal, coronal, and transverse planes, respectively. Tumor doubling times were calculated between days 8 to 12, 12 to 15, 15 to 18, and day 18 to 21 postimplantation. Tumor doubling time was calculated with the formula:

\[
\text{Doubling time} = (t_2 - t_1) \cdot \frac{\ln(2)}{\ln\left(\frac{V_2}{V_1}\right)}
\]

where \(V_2\) and \(V_1\) are the mean tumor volumes at the later \((t_2)\) and earlier \((t_1)\) time points, respectively.

**Survival curves**

Kaplan-Meier survival curves were generated and analyzed using Prism version 9.0.1 (GraphPad Software, San Diego, CA).

**Postmortem micro-CT scans**

Mice were culled with an IP injection of sodium pentobarbital (200 mg/kg BW) and lungs were fixed with 4% PFA postmortem. Fixation was followed by either critical point drying in the case of the model characterization study or paraffin embedding for the lungs coming from the survival study. Samples were imaged on a Bruker SkyScan 1272 high-resolution microtomography machine (Bruker micro-CT, Kontich, Belgium). The x-ray source was set to a tube voltage of 50.0 kV and a tube current of 200.0 μA, the x-ray spectrum was not filtered before incidence onto the sample. A set of 962 projections of 2452 × 1640 pixels were recorded every 0.2 degrees over a 180 degree rotation for each sample. Every individual projection was exposed for 273 ms and 5 projections were averaged to reduce image noise. This resulted in scan times of approximately 55 minutes and an isometric voxel size of 8.0 μm in the final data sets. The projection images were subsequently reconstructed into a 3-dimensional (3D) stack of images with NRecon version 1.7.4.2 (Bruker microCT, Kontich, Belgium). Lung carcinomas were manually segmented from the 3D stack and their volumes were calculated with Imaris image version 9.3 (Bitplane, Zürich, Switzerland).

**Immunohistochemistry**

Mice lungs were instilled with a 2% paraformaldehyde solution through a postmortem tracheotomy.21 The harvested lungs were fixed in 4% paraformaldehyde for 24 hours, dehydrated in ethanol and finally embedded in paraffin. Immunohistochemistry was performed on 5 μm thick paraffin sections of the lungs using rabbit polyclonal antibodies against pan Cytokeratin (Bioss Antibodies, Woburn, MA), CD31 (Abcam, Cambridge, UK), and rabbit monoclonal antibody against Ki67 (Clone SP6; ThermoFisher Scientific, Fremont, CA).
Deparaffinized sections underwent antigen retrieval using Tris-EDTA buffer (pH 9) for CD31, EDTA buffer (pH 8) for pan Cytokeratin and citrate buffer (pH 6) for Ki67. After a blocking step (3% skimmed bovine milk with 2.5% goat serum), the sections were incubated with the primary antibody (1 hour at 37°C). Endogenous peroxidase activity was blocked with 0.6% hydrogen peroxide in methanol. ImmPRESS goat antirabbit IgG (Vector Laboratories, Burlingame, CA) was used to detect the primary antibodies binding to CD31, pan Cytokeratin, and Ki67. For CD31 and pan Cytokeratin, the Biotin-Free Tyramide Signal Amplification System (DAKO, Carpinteria, CA) was used. The sections were stained with NovaRed (Vector Laboratories) and cell nuclei were counterstained with hematoxylin. Stained sections were evaluated and photographed in an IMAGER.M2 light microscope (Carl Zeiss Microscopy GmbH, Jena, Germany).

Quantification of Ki67 nuclear immunoreactivity

To quantify Ki67 nuclear immunoreactivity of tumor cells, the sections stained with anti-Ki67 antibody were analyzed. Two to 4 different areas per section were chosen using a 20× objective. The images obtained were analyzed with ImageJ/Fiji software, version 2.0.0-rc-69/1.52p. One or 2 regions of interest (ROIs) per image were defined so that each ROI contained at least 200 to 400 tumor cells. ROIs were defined within 500 μm from the edge of the tumor. Ki67 positive and negative tumor cells in each ROI were manually counted using the ImageJ Plugin Cell counter. Ki67 immunoreactivity was expressed as a percentage of positive cells over the total number of tumor cells analyzed. Data were analyzed with Prism version 9.0.1 (GraphPad Software).

Immunofluorescence

Twelve hours after S-MRT, normal mouse lungs were harvested, fixed in PFA and embedded in paraffin as described above. After deparaffinisation (protocol described above), the tissue sections underwent antigen retrieval (citrate buffer, pH 6). Sections were then incubated with 2% BSA/PBS/0.3% Triton for 15 minutes and a second blocking solution (4.5 mL PBS-Triton 0.3%, 0.5 mL donkey serum, 0.15 g milk powder) for 1 hour at room temperature. Sections were incubated overnight at 4°C with the primary antibody, rabbit antigamma H2A.X-phospho S139 (Abcam) in blocking solution. On the morning of the following day, sections were incubated with a donkey antirabbit Alexa Fluor488 secondary antibody (Life Technologies, Carlsbad, CA), and 4’,6-diamidino-2-phenylindole in blocking solution for 6 hours at 4°C. Images were acquired with a Zeiss LSM 880 confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany).

Quantification of lung fibrosis

The lungs of irradiated normal mice were harvested 6 months after S-MRT irradiation, fixed in 4% PFA, and embedded in paraffin as described above. Five sets of paraffin-embedded lungs were randomly selected for processing and analysis. The lungs were cut coronally from caudal to cranial. Then, 5 μm thick sections were obtained from 5 different locations separated by 200 μm throughout the tissue. Only the most caudal portion of the remaining 3 lungs were examined. Sections were deparaffinized and stained with Masson-Goldner trichrome. Lung fibrosis was scored in all sections according to the method described by Hübner et al. Grade 0 corresponds to the absence of any fibrotic alteration in the lungs. Grade 1 to 8 describe progressive lung fibrosis where grade 1
Fig. 2. Histologic characterization of the LLC1 tumor model. Pan cytokeratin staining (NovaRed) showed clear localization of the carcinomas in the lung tissue at 11 (A), 14 (B), and 17 (C) days postimplantation. Higher magnification images of pan cytokeratin staining revealed the tumor borders (dotted line in A', B', and C'). Images of tumors stained for CD31 (NovaRed) showed well-vascularised tumors with regular blood vessel distribution at 11 (D, D'), 14 (E, E'), and 17 days postimplantation (F, F'). Images for Ki67 staining (NovaRed) demonstrated persistent proliferative activity at 11 (G, G'), 14 (H, H'), and 17 days postimplantation (I, I').
indicates minimal fibrosis and grade 8 total fibrosis with destruction of the lung structure.

**Results**

**LLC1 model**

The tumor growth curve for the LLC1 model is shown in Figure 1A. Tumor doubling times were 1.62 days, 1.89 days, 2.55 days, and 4.19 days for the intervals between day 8 to 12, 12 to 15, 15 to 18, and 18 to 21 postirradiation, respectively. Reconstructed 3D images from postmortem micro-CT scans of the whole lungs at 11 (Fig. 1B) and 14 (Fig. 1C) days postirradiation showed solid tumors localized in the caudal lobe of the right lung. The choice of controlled tumor placement by injection over a spontaneous tumor model allowed for tumor development at a sufficient distance from the central airways and the heart.

Pancytokeratin staining at all time points (11, 14, and 17 days postimplantation) revealed a well circumscribed...
tumor with well-defined pushing borders, but not surrounded by a fibrous capsule isolating it from the adjacent lung tissue (Fig. 2A-C). Staining for the CD31 protein (also known as PECAM-1), a marker for endothelial cells of blood vessels, showed the tumors were well vascularized with regularly distributed intratumoral microvessels (Fig. 2D-F). Tumors stained for the Ki67 nuclear protein showed a homogenous proliferative pattern at all the evaluated time points (Fig. 2G-I). The ratio of proliferating cells, calculated as the percentage of Ki67 positive cells over the total number of carcinoma cells within the tumor slice, was statistically significant between day 11 and day 17 postimplantation ($P = .0419$) and increased over time (Fig. E2).

Dosimetry and irradiation set-up

A schematic representation of the irradiation set-up is shown in Figure 3A. Gafchromic EBT-XD films (Ashland, Bridgewater, NJ) confirmed the microbeam configuration (50 $\mu$m beam width and 400 $\mu$m on-center spacing) as well as the angle of 25° between the 2 cross-fired S-MRT arrays (Fig. 3B) and S-BB fields (Fig. 3C). Immunofluorescent $\gamma$H2AX staining for DNA damage on lung slices corroborated the correct delivery of the 2 cross-fired S-MRT arrays through the whole right lung at 12 hours postirradiation (Fig. 3D). The same staining was performed in S-BB irradiated lung slices (Fig. 3E). A table summarizing dosimetry parameters is reported in Figure 3F.

Survival

Kaplan-Meier survival curves (Fig. 4A) demonstrated that both S-MRT and S-BB significantly increased the survival of the carcinoma-bearing mice relative to the CTR group. There were no statistically significant differences in survival between the S-BB and S-MRT groups ($P = .3173$). Median survival times were 7, 11, and 12 days for the control, S-BB, and S-MRT groups, respectively (Fig. 4B).

Radiologic evaluation of LLC1 carcinomas with in vivo CT imaging

In vivo CT imaging of mice from the CTR group confirmed the presence of a well-defined pulmonary mass, identified as the LLC1 carcinoma, in the right lung from 3 days before irradiation to day 10 postirradiation (Fig. 5A). The increased contrast and defined borders of the pulmonary mass allowed for precise measurements of the tumor volume. This was also the case for mice in the S-BB treated group (Fig. 5B). However, the CT images of mice belonging to the S-MRT group started to show fluid opacity surrounding the carcinomas from day 4 postirradiation, which became evident on days 7 and 10 (Fig. 5C). The radiologic finding of fluid opacity is attributable to pulmonary edema. The presence of edema hindered the identification of the tumor borders and the ability to measure tumor size for the S-MRT group. Finally, RIP cannot be excluded from the CT imaging of the S-MRT group.

Postmortem evaluation of tumor size with micro-CT imaging and histologic analysis

Lungs from the same mice shown in Figure 5A-C were harvested at 10 days postirradiation after reaching the experimental endpoints. Postmortem perfusion to remove blood and dehydration (postfixation) of the lungs allowed for visualization of the tumor mass only, using micro-CT imaging, without the edema (Fig. 5D-F). These images clearly demonstrated that the LLC1 carcinomas subjected to S-MRT were the smallest of the 3 groups. The calculated
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Discussion

Previous S-MRT research demonstrates amelioration of survival and exceptional tumor control in preclinical models of glioma, melanoma, and mammary carcinoma.\textsuperscript{10} The present study adds to this body of research by showing, for the first time, the efficacy of S-MRT for the treatment of lung carcinoma. Although we did not demonstrate a significant increase in survival time after S-MRT relative to S-BB (Fig. 4), postmortem analysis found that S-MRT was the most effective modality for reducing tumor size (Fig. 5D-F and Fig. E4). However, because this was designed as a survival study, there was an insufficient number of samples harvested at pre-established time points to confirm whether this observation was statistically significant. For this purpose, future mechanistic studies should be performed.

A further key finding of this study was the development of edema, and possible acute RIP around the tumors, after S-MRT (Fig. 5). The detrimental effects of pulmonary edema on respiratory function and overall well-being is likely to be the reason why there was no survival advantage for the S-MRT group compared with the S-BB group. In contrast, serial in vivo CT scans of the sham-implanted mice showed only a minor and transient form of liquid effusion after S-MRT. A similar observation of mild, transient edema was made previously in a study of normal rat brains irradiated with doses up to 1000 Gy by an S-MRT array of 25 \( \mu \)m wide microbeams with a center-to-center spacing of 211 \( \mu \)m.\textsuperscript{35} Taken together, these observations suggest that the presence of the malignancy itself was a critical contributor to the extent of the observed pulmonary edema in the S-MRT group. It has been demonstrated that the vascular toxicity and consequent vascular disruption caused by S-MRT depends on the stage of vessel maturation.\textsuperscript{17,20}

Tumors have immature vasculature, and therefore would be more susceptible to significant tumor capillary disruption and pulmonary edema after S-MRT.

The present study also demonstrated that S-MRT did not cause long-term lung fibrosis, even after delivering a high total peak dose (400 Gy \( \times \) 2 = 800 Gy). This suggests that there may be a potential benefit for patients who require aggressive radiation treatment that might otherwise significantly deteriorate their long-term quality of life. Future
Fig. 6. Long-term study of healthy lungs after irradiation. Sham-implanted mice which received S-MRT were evaluated over 6 months by in vivo computed tomography (CT) scans. Scans were performed 3 days before irradiation (A), then at day 1 (B), day 4 (C), day 7 (D), day 37 (E), 3 months (F), 4 months (G), and 6 months (H) postirradiation. Fluid opacity is indicated with yellow arrowheads in C, D, E, and F. (I to I’’’) Masson-Goldner trichrome stained lung sections at increasing magnification demonstrate no evidence of lung fibrosis at 6 months postirradiation. Panel I’’’ is part of I’’’. Panels I’’ and I’ are parts of I’, that is part of I.
experiments should aim to prevent the formation of edema while maintaining the demonstrated tumor control and long-term absence of fibrosis. The concomitant administration of S-MRT with other strategies known to reduce pulmonary edema should be considered, including supplemental oxygen or pharmacologic treatment with glyceryl trinitrate or furosemide. In addition, careful selection of S-MRT delivery parameters, such as peak dose, microbeam width and spacing, dose-rate and temporal fractionation, may also be crucial for the success of S-MRT for treating lung carcinoma.

A peak-dose of 400 Gy was chosen for this study based on the demonstrated therapeutic efficacy of this S-MRT dose for several other preclinical cancer models. However, for these previous studies, a smaller microbeam center-to-center spacing of 200 μm was used. To compensate for the use of a 400 μm center-to-center microbeam spacing, the only available configuration at the Australian Synchrotron, we chose to cross-fire 2 S-MRT arrays, each delivering 400 Gy, for a total peak dose of 800 Gy peak to the tumor and adjacent areas of surrounding normal lung tissue. In retrospect, this very high total dose may have exceeded the structural tolerance of the lung tissue, contributing to the observed pulmonary edema. For future experiments, the total peak dose could be reduced and the use of multiple cross-fired S-MRT arrays avoided, if a smaller center-to-center spacing between the microbeams is used.

Increasing the S-MRT dose-rate further may also contribute to diminishing the acute edema observed in the tumor-bearing mice. There is evidence that the FLASH normal tissue sparing effect is proportional to the applied dose-rate. Therefore, using a dose-rate even higher than 991.7 Gy/s (in-slice delivery time of 361 ms for S-MRT) could help to reduce, or even prevent, the basal fluid effusion from normal lung tissue which was observed in the sham-implanted mice and could have contributed to the pulmonary edema. Increasing the dose-rate, in combination with using a narrower collimator and a reduced total peak dose, could help to balance effective vascular disruption in the tumor with sparing of the surrounding normal lung tissue. Repeating these experiments at the European Synchrotron Radiation Facility (ESRF), where dose-rates of up to 16,000 Gy/s can be used, will allow for an evaluation of the contribution of dose-rate amplitude to the prevention of acute pulmonary edema after S-MRT.

Finally, an additional strategy that should be adopted in future experiments is temporal fractionation of the total S-MRT dose. A recent study showed that fractionating the dose with respect to time by administering S-MRT as 3 consecutive daily doses of 133 Gy resulted in a 50% rate of complete tumor remission in a mouse model of melanoma. This approach is likely to favor the therapeutic efficacy of S-MRT on lung cancer by also reducing the risk of pulmonary edema.

For future in vivo studies, S-MRT parameters should be carefully determined by considering the sensitivity of the treated organ bearing the malignancy. For the treatment of lung carcinoma, we propose the use of a microbeam center-to-center spacing of 200 μm, a total peak dose of 400 Gy administered daily over 2 to 3 consecutive days (eg, 2 × 200 Gy or 3 × 133 Gy), and an ultrahigh dose-rate in the order of 1 × 10^4 Gy/s. These considerations aim to reduce collateral effects – particularly pulmonary edema in lung cancer models, and may increase the therapeutic effect of S-MRT in the treatment of other unexplored tumors types.

Conclusions

In the present study, we have made a significant step toward using S-MRT for the treatment of lung cancer by successfully targeting and treating a localized lung carcinoma in a preclinical model. We have demonstrated that S-MRT increases survival compared with nonirradiated controls and reduces the size of tumors while not causing fibrotic changes up to 6 months postirradiation. Optimizing S-MRT parameters to reduce the risk of pulmonary edema will maximize its therapeutic potential and clinical translation for the treatment of lung malignancies.

References


