Tumor Control in RG2 Glioma-Bearing Rats: A Comparison Between Proton Minibeam Therapy and Standard Proton Therapy

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Purpose: Proton minibeam radiation therapy (pMBRT) is a novel radiotherapy approach exploiting the synergies of the inherent advantages of protons for radiotherapy with the gain in normal tissue preservation observed upon irradiation with narrow,spatially fractionated beams. The net gain in normal tissue sparing that has already showed by pMBRT may lead to the efficient treatment of very radioresistant tumours, which are currently mostly treated palliatively. The aim of this study was to perform the first evaluation of the tumor effectiveness of proton minibeam radiation therapy for the treatment of RG2 glioma bearing rats so to confirm the widening of the therapeutic window thanks to pMBRT.

Materials and methods: Two groups (n=9) of RG2 glioma-bearing rats were irradiated with either standard (seamless) proton therapy or with pMBRT, with a dose prescription of 25 Gy in one fraction at the tumour position (Bragg peak). Five animals per groups were followed up by means bioluminescence Imaging (BLI). The animals were followed up for a maximum of 6 months. At the end of the study histopathological studies were performed to assess both the tumor presence and the possible side effects.

Results: A significant tumor control was achieved in the two irradiated series, being more important in the pMBRT group. A percentage of 22 % and 67 % of long-term survivals (>170 days) were obtained in the standard PT and pMBRT groups. Tumor sterilization was confirmed by the histology evaluation. However, while the long-term survivals in the standard RT exhibit substantial brain damage, including radionecrosis, only very reduced toxicity was observed in the pMBRT group.

Conclusions: Our results show a significant increase in the therapeutic index of pMBRT for brain tumours: a remarkable enhancement of lifespan was observed in glioma-bearing rats, without any significant toxicity. Even more, an important percentage of rats (67%) was cured without substantial side effects.

1. Introduction

Proton minibeam radiation therapy (pMBRT) is a novel therapeutic strategy that partners the inherent advantages of protons for therapy and the benefits of the spatial fractionation of the dose [1]. One of the main assets of this innovative approach is that, thanks to the multiple Coulomb scattering of protons, a (quasi) homogeneous target coverage could be obtained even with only one array of proton minibeams [1]. Additionally, thanks to the use of protons, a negligible dose is deposited in normal tissues after the Bragg peak (tumour position), further reducing the secondary effects. We implemented this technique at the Orsay proton therapy center in 2014 [2]. In parallel, Dilmanian et al. [3] confirmed the physical feasibility of the technique. Reduced side effects in the mouse ear after pMBRT with 20 MeV protons were also reported [4]. Even though the beam energy is not clinically relevant (a 20 MeV proton beam only penetrates 1 mm in the body), this work provided another indication of the advantages of this approach.

We have recently demonstrated that pMBRT notably increases the tolerances of normal rat brain [4]. Rats irradiated with pMBRT (whole brain irradiations excepting the olfactory bulb), with peak doses of 58 Gy in one fraction (corresponding to an average dose of 25 Gy) exhibited no substantial brain lesions after a long-term follow up of 6 months. In contrast, a group of rats irradiated with the same average dose (25 Gy in one fraction) with a seamless (standard) proton irradiation exhibited extensive brain damage [5]. This important reduction of toxicity can be used to increase the therapeutic index in the treatment of clinical cases with good rates of tumor control but accompanied of substantial side effects, such as reduced speech, motor function or intelligence quotient in the treatment of paediatric astrocytoma and meningioma. It also offers the possibility of using more aggressive dose escalation schemes in the case of very radio-resistant tumors, such as high-grade gliomas (GBM), still one of the most challenging cases in clinical oncology. The goal of this work was to perform a first comparison of tumor control effectiveness of pMBRT versus conventional (seamless) PT irradiations for the treatment of gliomas. To the best of our knowledge this is the first in vivo evaluation of the effectiveness of pMBRT for glioma treatments.

2. Materials and methods

All animal experiments were conducted in accordance with the animal welfare and ethical guidelines of our institutions. They were approved by the Ethics Committee of the Institut Curie and French Ministry of Research (permit no. 6361-201608101234488). Rats were anaesthetised with isoflurane (2.5% in air) during irradiation and magnetic resonance imaging (MRI). At the end of the study, the rats were terminally anaesthetised for brain fixation by the intracardiac perfusion of formalin zinc.

2.1. Tumor cell line and tumor implantation

A rat glioma cell line, RG2-[D74] (ATCC® CRL-2433™), was used. RG2 tumour model is known to be very aggressive in vivo, with a highly invasive growth pattern, similar to human glioblastoma multiforme [6]. RG2 cells which have been transfected with the luciferase gene were used to be able to perform Bioluminescence Imaging (BLI) at a IVIS spectrum (PerkerElmer). BLI was employed verify the presence of the tumor before irradiation as well as to perform one part of the follow up. BLI has proven to be an effective technique for assessing intracranial glioblastoma growth noninvasively in rodent models [7,8].

Male Fischer 344 rats (Janvier Labs) were used. A number of 5000 RG2-Luc cells were suspended in 5 µl DMEM and then injected using a Hamilton syringe through a burr hole in the right caudate nucleus (5 mm anterior to the ear-bars, i.e. at the bregma site, 3.0 mm lateral to the midline, and 5.5 mm depth from the skull).
For the BLI the rats are injected intraperitoneally with a concentration of 150 mg/kgr (P/N 122799) of D-luceferin (Perkinelmer) in 500 µl. The peak of luminesce is reached 25 minutes after injection. The presence of tumor is confirmed when the bioluminescent signal overcomes the background level.

2.2. Irradiations and dosimetry

Three groups of animals (n=9/each) of 7 weeks-old male Fischer 344 rats at the time of irradiation were considered: i) a control group (tumor bearing rats, non-irradiated); ii) a group receiving conventional proton therapy (PT); *iii)* a group receiving pMBRT. The rats had very similar weights and head sizes. A prior test on tumor implantation allowed us to measure the average tumor size by using Hematoxylin & Eosin coloration. The average diameter of the tumor along the beam direction 4 days after implantation was 4 mm. Since the irradiations were going to be performed in pristine Bragg peak conditions (95 % coverage in 5mm), we chose to irradiate 4 days after implantation. The total area covered by the radiation was 1.6x1.6 cm^2. The dose prescription was 25 Gy at the tumor position (Bragg peak) in a unique fraction. One single fraction scheme was used to avoid any possible blurring inter-fraction of the minibeam pattern due to positioning errors

The irradiations were performed at one of the horizontal beamlines (passive scattering) at the ICPO-Orsay with a proton beam energy of 100 MeV and a dose rate of 2 Gy/min. In order to have the Bragg peak in the tumor position, a thickness of 48 mm of polymethyl methacrylate (PMMA) was used as a "buildup" material placed in front of the rat head. See figure 1. Gafchromic films placed laterally on each side of the rat's head (beam entry and exit) and attached to the skin allowed assessment of the quality of the irradiation. For minibeam generation a multislit collimator (400 µm x 2 cm; center to center distance: 3200 µm) was used, as in Peucelle et al [2]. The collimator's exit to the PMMA entrance was fixed to 7 cm. This configuration results in a homogeneous dose distribution in the Bragg peak in the case of water irradiations [2].

Monte Carlo simulations (Gate v7.1 [9]) were used to calculate the needed PMMA thickness and dose distributions in the rats' brains. The whole proton beamline and the actual experiments irradiation setting were modeled. Dose distributions were calculated in a rat's computer tomography images with a voxel scoring size of 1 mm × 100 µm × 0.2 cm.

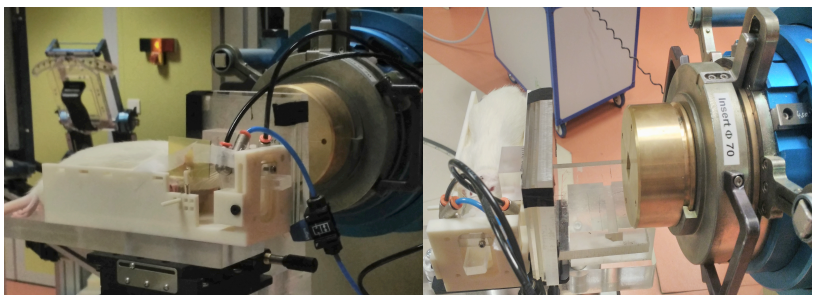

Figure 1. Photographs of the irradiation setup.

Figure 2 shows some 2D maps of the dose distributions inside a rat's head for a conventional (seamless) and a pMBRT irradiations. A quasi-homogeneous distribution was obtained at the target position in the case of the standard PT. In contrast some spatial fractionation is still maintained at the Bragg position in the pMBRT irradiations. The tissue heterogeneities present in the rat's head results in the loss of the dose homogenization that would be obtained in the case of water irradiations [2]. The peak-to-valley dose ratio (PVDR) values were 1.20 ± 0.05, 1.32 ± 0.05, 1.49 ± 0.06 and 2.22± 0.09 at the Bragg peak position, center of the brain, middle of the contralateral (normal) hemisphere and the skin, respectively. Thus the tumor received 25 ± 1 and 21 ± 1 Gy peak and valley doses.

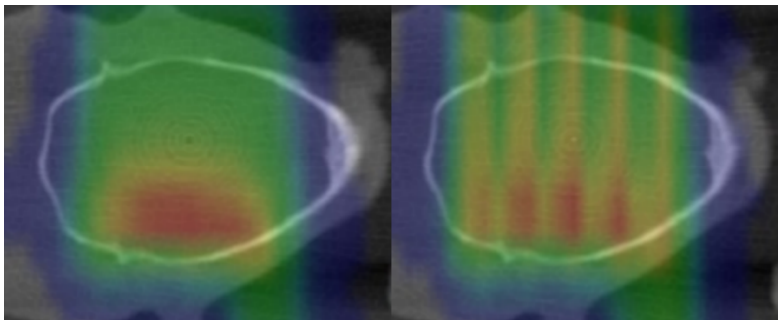

Figure 2. Examples of coronal 2D dose distributions in the computer tomography images of one rat's head corresponding to a conventional (seamless) irradiation (left) and a pMBRT one (right). Some spatial fractionation is still maintained at the target in the case of the pMRBT case, as reflected by the presence of areas of high dose (in red, peaks) and areas of lower dose (valleys).

2.3. Animals follow up

The animals were followed-up for almost 6 months to evaluate long-term effects. The clinical status of the animals was checked 5 times per week. Any rat showing the classical adverse neurological signs related to the tumour growth in the brain was humanely killed. These signs could be any of the following: loss of appetite and substantial weight loss (>10 % of the weight in 24h), periorbital haemorrhages, seizures or prostration.

BLI was used to monitor the evolution of 5 animals per group. The tumor is considered to be eliminated if there is a prolonged extinction of the IVIS signal (signal compatible with the background noise) for more than 90 days.

As explained before, any rats reaching the endpoints related to tumor growth was immediately sacrificed. The long-term survivals were follow up for 6 months (end of the study). For sacrifice,

the rats were terminally anaesthetised for brain fixation by the intracardiac perfusion of a fixative solution (formalin zinc). The brains were then removed, fixed in the fixative solution, and embedded in paraffin; 4-µm-thick sections were cut and stained in haematoxylin and eosin (HE) for the histopathological (double-blinded) analysis was carried out by ECVP (European College of Veterinary Pathologists) board certified pathologist. Immunohistochemistry analysis was performed to assess the networks and cell morphologies of microglia (anti-Iba-1 antibody, Wako Chemicals, dilution: 1:500) and astrocytes (anti-GFAP antibody, Sigma-Aldrich, dilution: 1:500). We focused on neuroinflammatory processes, characterized by a modification of microglial cell density and morphology: hyperplasia, thickening and shortening of cell processes.

3. Results

This section reports on the tumor control effectiveness as well as on the long-term side effects of irradiated rats.

3.1. Survival curves

Figure 3 shows the survival curves of tumor bearing rats. The median survival time post-implantation was calculated, and Kaplan Meier survival data were plotted versus time after tumor implantation. The survival curves were compared using the log-rank test between the irradiated group and the controls (Prism-GraphPad). The three curves showed to be statistically significantly different ($p < 0.0001$).

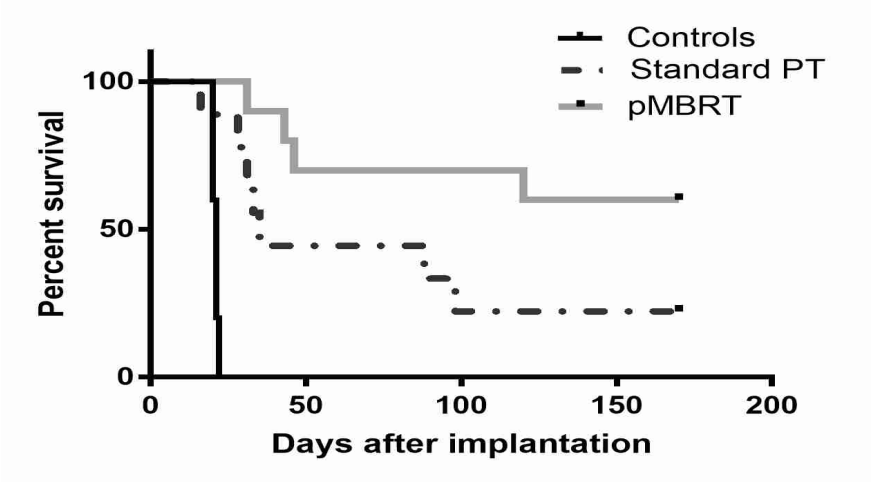

Figure 3. Survival curves for the controls, standard PT and pMBRT irradiated tumor-bearing rats. The irradiated rats presented a significant increase of mean survival time.

The mean survival time of the controls was 20 days as expected. A percentage of 22 % and 67 % of long-term survivals (>170 days) were obtained in the standard PT and pMBRT groups, respectively, which indicates tumor sterilisation.

Figure 4 shows the evolution of the average BLI signal for each of the three series as a function of the time after irradiation. The BLI signal of the untreated controls rapidly increases. In the irradiated groups there is a slower increase of signal after irradiation during the first 15 days, being smoother in the case of pMBRT. Then there is a rapid decrease of the BLI signal. This behaviour is present for every single irradiated rat. The average BLI signal is lower for the pMBRT than in the standard PT series, indicating a better tumor control. In contrast to the

standard PT series, the rats in the pMBRT series do not show any significant increase of BLI signal 30 days after irradiation.

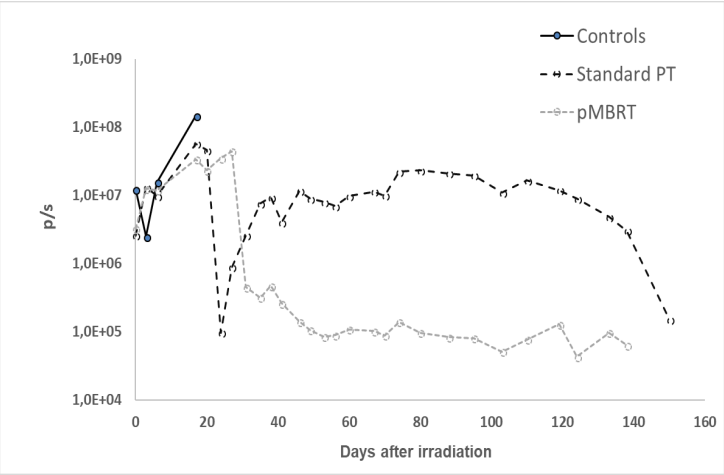

Figure 4. Average BLI for each of the three series.

Two long term survivals, one belonging to the standard PT series, the other one to the pMBRT irradiated group, still have some BLI signal 150 days after implantation, indicating the presence of some cellular senescence. Senescence is a cellular state characterized by several structural and functional features, including loss of mitotic activity resulting in cessation of cellular divisions. Therefore, to stop proliferation of cancer cells, it is not necessary to kill them all, instead induction of senescence pathway may be sufficient.

Histopathological analysis revealed clear differences between the two irradiation protocols at the long-term (6 months after irradiation). In both conditions, no glioma could be detected anymore but, after standard irradiation, few rats survived. Survivors (n=2/9) displayed different histological lesions, randomly distributed in the brain: necrotic foci, inflammation, oedema and calcifications. In contrast, after pMBRT, rats only displayed minimal inflammatory lesions, characterised by foci of microglial activation (Figure 4).

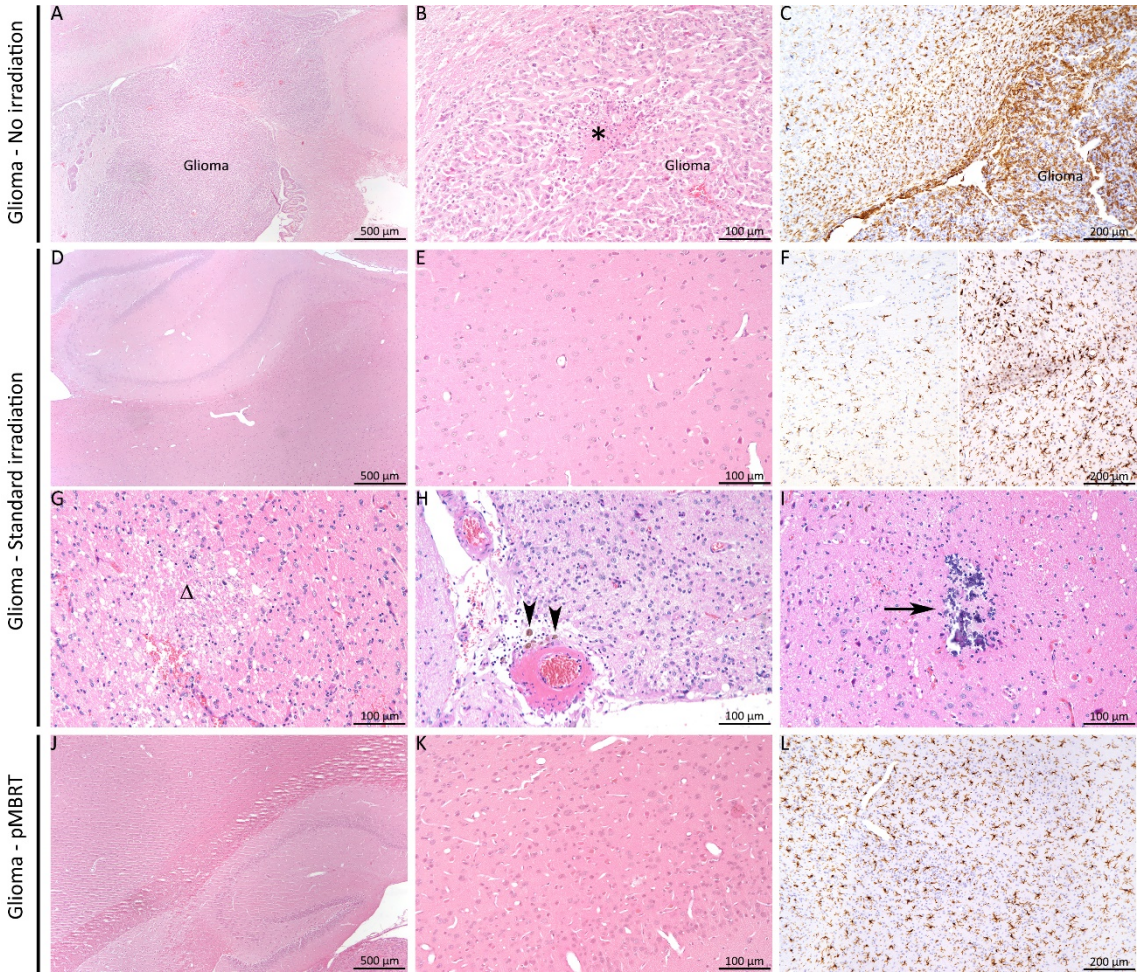

Figure 4: Long-term consequences of pMBRT clearly less severe than standard irradiation procedures.

A-C: Without irradiation, rats displayed large gliomas (**A**), sometimes with central necrosis/suppuration (**B**, star), and hyperplasia/activation of microglial cells in the tumor as well as at the periphery (**C**).

D-I: After standard irradiation procedures, two rats survived. For the first rat, we did not observe any histological lesion after HE analysis (**D-E**), but immunohistochemistry analysis revealed small foci of microglial cell activation (**F**: left panel normal but right panel with microglia activation). The second rat displayed different brain lesions: randomly distributed necrotic foci (**G**, Δ), perivascular oedema with inflammation and hemosiderin-filled macrophages (old haemorrhages; **H**, arrowheads), and foci of calcification (**I**, arrow).

J-L: In contrast, after pMBRT, almost no lesion was detected (**J,K**) except a mild multifocal increase in microglia density (**L**).

A-B, D-E, G-K: HE staining, C, F, L: Iba1 immunohistochemistry to assess microglial cell density and morphology.

4. Discussion

Despite the remarkable improvement in tumor dose conformation achieved in the last decades, the treatment of some radioresistant tumours, tumours close to a sensitive structure (e.g. central nervous system (CNS)) and paediatric cancers is still compromised due to the tolerances of normal tissues. This is especially critical in the case of radio-resistant brain tumors, such as high-grade gliomas. Proton minibeam radiation therapy is an innovative approach which has already proven its ability to drastically reduce neurotoxicity [5]. Average doses of 25 Gy (58 Gy peak dose) in one sole fraction were shown to be well tolerated by normal rat brain (whole brain irradiation, excepting olfactory bulb) when irradiated with pMBRT [5]. This is in contrast to conventional PT where severe damage was observed [5]. Radiation doses higher than 20-25 Gy are reported to be needed to obtain long-term survivals in glioma-bearing rats experiments [10,11] after conventional irradiations. The aim of this work was to assess whether this increase in normal brain tolerances could lead to a significant widening of the therapeutic window for high-grade gliomas. With that aim, the effectiveness of pMBRT for RG2 rat glioma tumor control with a dose prescription of 25 Gy was evaluated. The response of tumor bearing rats to standard and pMBRT irradiations was compared. To the best of our knowledge this is the first evaluation of the response of glioma-bearing rats to pMBRT. The dose was delivered in one single fraction to avoid any possible blurring inter-fraction of the minibeam pattern due to positioning errors. The same scheme is foreseen to be employed if clinical trials would be done.

A remarkable tumor control was achieved in the two irradiated series (conventional and pMBRT). The BLI reflected the tumor growth delay in the irradiated groups with respect to the controls, being this delay more important in the pMBRT series. The average BLI signal was lower in the animals in the pMBRT group with respect to the standard PT series, indicating a better tumor control. Furthermore, in contrast to the standard PT series, the rats in the pMBRT series do not show any significant increase of BLI signal 30 days after irradiation.

A percentage of 20 and 67 % of long-term survivals was obtained in the standard and pMBRT series, respectively. The histopathological analysis confirmed the tumor sterilization in those animals. However, while the long-term survivals in the standard RT exhibit substantial brain damage, including radionecrosis and important neuroinflammation, coherent with our prior work [5], the animals in the pMBRT showed a reduced neurotoxicity.

It should be highlighted that this reduced neurotoxicity is achieved even at very low PVDR values: the PVDR amounts less than 2 along the brain, with beam widths larger than 3 mm. These results have important implications for potential clinical trials, since it opens the door for future implementations with pencil beam scanning systems (millimetre-sized beams).

It also must be stressed that a remarkable tumor control and tumor sterilization (67%) was obtained in the pMBRT series despite the lack of homogenous coverage of the target. Indeed, the spatial fractionation is maintained at the target, with a PVDR of 1.2. This might indicate the participation of distinct biological mechanisms in pMBRT.

The remarkable enhancement of lifespan observed in glioma-bearing rats, with an important number of curations, without any significant toxicity confirms the widening of the therapeutic window thanks to pMBRT. The optimization of the irradiation parameters, such as the beam spacing the dose, the use of several entry ports, might further increase the number of curations

and further decrease toxicity. These studies were directly performed at a clinical centre, with clinically relevant energies, which makes the transfer to potential clinical trials a direct one.

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