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## **Proton minibeam radiation therapy leads to a superior tumor control than standard proton therapy in RG2 glioma-bearing rats**

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Y.P. conceived the project and supervised the study; Y. P and F. P. designed the experiments; C.N., A.P., D.L., F.P., R.D. and Y.P. participated in scientific discussions; C.N., A.P., D.L. and F.P. performed the irradiations; G.J. performed the histology analysis; W.G., M.J., D.L. and J.B. did the regular follow up of the animals; D. L. performed the BLI follow up; C. Guardiola performed the dose calculations; L. De Marzi modelled the proton beamline; Y.P. analysed the data and wrote the manuscript. All the authors have read and approved the manuscript.

### **Conflict of interest**

The authors have no conflict of interest to disclosure.

### **Running title**

pMBRT offers significant tumor control

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## **Proton minibeam radiation therapy leads to a superior tumor control than standard proton therapy in RG2 glioma-bearing rats**

### **Abstract**

**Purpose:** Proton minibeam radiation therapy (pMBRT) is a novel radiotherapy approach exploiting the synergies of proton therapy 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 been shown 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.

**Materials and methods:** Two groups (n=9) of RG2 glioma-bearing rats were irradiated with either standard proton therapy or with pMBRT, with a dose prescription of 25 Gy in one fraction. 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 tumour presence and the possible side effects.

**Results:** Tumor control was achieved in the two irradiated series, with superior survival in the pMBRT group compared to the standard PT group. Percentages of 22 % and 67 % of long-term survivals (>170 days) were obtained in the standard PT and pMBRT groups, respectively. No tumour was observed in the histopathological analysis. While the long-term survivals in the standard RT exhibit substantial brain damage, including marked radionecrosis, less severe toxicity was observed in the pMBRT group.

**Conclusions:** pMBRT offers a significant increase in the therapeutic index of brain tumours: the majority of the glioma-bearing rats (67%) survived 6-months with less severe side effects.

## **Keywords**

High-grade gliomas, radiotherapy, proton minibeam radiation therapy

### **1. Introduction**

Proton minibeam radiation therapy (pMBRT) is a novel therapeutic strategy [1] that notably increases the tolerances of normal rat brain compared to standard proton radiotherapy [2]. The important reduction in toxicity offers the possibility of using more aggressive dose escalation schemes in the case of very radio-resistant tumors, such as glioblastoma multiforme (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 (quasi-homogeneous dose distributions in the target) versus conventional (seamless) PT irradiations for the treatment of gliomas.

### **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 Ministry of Research (permit no. XXXX). Rats were anaesthetised with isoflurane (2.5 % in air) during irradiation.

#### **2.1. Tumor cell line and tumor implantation**

The RG2-[D74] (ATCC<sup>®</sup> CRL-2433<sup>™</sup>) rat glioma cell line was employed [3]. The cells were transfected with the luciferase gene to perform Bioluminescence Imaging (BLI) at a IVIS spectrum (PerkerElmer) in order to verify the presence of the tumor before irradiation as well as to perform one part of the follow up [4,5].

Male Fischer 344 rats (Janvier Labs) were used. A number of 5000 RG2-Luc cells were suspended in 5  $\mu$ l 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 were injected intraperitoneally with 150 mg/kg (P/N 122799) of D-luciferin (PerkinElmer) in 500  $\mu$ l.

## **2.2. Irradiations and dosimetry**

Three groups of animals (n=9/group) of 7 week-old male Fischer 344 rats at the time of irradiation were considered: *i*) a control group; *ii*) a group receiving conventional proton therapy (PT); *iii*) a group receiving pMBRT. A prior test on tumor implantation allowed us to measure the average tumor size (4 mm-mean diameter at 4 days after implantation) by using Hematoxylin & Eosin (HE) staining. The total irradiation area was 1.6x1.6 cm<sup>2</sup>. The dose prescription was 25 Gy at the tumor position (Bragg peak) in a unique fraction to avoid any possible blurring inter-fraction of the minibeam pattern due to positioning variations.

The irradiations were performed at one clinical beamline (passive scattering) 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 location, 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. For minibeam generation a multislit collimator (400  $\mu$ m x 2 cm; center to center distance: 3200  $\mu$ m [6]) was used.

Monte Carlo simulations (Gate v7.1 [7]) were used to calculate the dose distributions in the rat's computer tomography images. See figure 2. Some spatial fractionation is observed at the Bragg position in the pMBRT irradiations, with a peak-to-valley dose ratio of  $1.20 \pm 0.05$ .

## **2.2. Animals follow up**

The animals were followed-up for 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 (i.e. substantial weight loss (>10 % of the weight within 24h) was humanely killed (intracardiac perfusion of formalin zinc). Histopathological (double-blinded) analysis was carried out by a European College of Veterinary Pathologists board certified pathologist. During rat necropsy, the brains were removed and fixed in 10 % neural-buffered formalin; 4 (in the left part of the brain) to 5 (in the right part of the brain, where the tumors were implanted) longitudinal (from the olfactory lobes to beginning of the spinal chord) 4- $\mu$ m-thick sections were carried out at different levels, and stained in HE. Immunohistochemistry analysis was performed to assess the networks and cell morphologies of microglial cells (anti-Iba-1 antibody). BLI was used to monitor the evolution of 5 animals per group.

### **3. Results**

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

#### **3.1. Survival curves**

Figure 3 shows the survival curves. Kaplan Meier survival data were plotted versus time after tumor implantation. The survival curves were compared using the log-rank test (Prism-GraphPad) and are statistically significantly different ( $p < 0.0001$ ).

A percentage of 22 % and 67 % of long-term survivals (>180 days) were obtained in the standard PT and pMBRT groups, respectively, suggesting tumour eradication. The rest of the rats were sacrificed due to symptoms of tumour growth.

Figure 4 shows the evolution of the BLI signal for each of the three series as a function of the time after irradiation. In the case of standard PT, an increase in BLI signal occurs around 40



days after irradiation, suggesting a regrowth of the tumour. This contrasts with pMBRT treated rats, where the BLI signal is maintained at a background level indicating a better tumour control for pMBRT compared to standard PT. See figure 4.

Histopathological analysis revealed clear differences between the two treatments protocols in the long-term (6 months after irradiation). In both groups, no glioma was detected anymore in the surviving rats. In the left part of the brain, we observed similar minimal to mild lesions in both groups: multifocal meningeal calcifications, oedema, vacuolation of ependymal cells, rare activated microglial cells and microscopic foci of mineralisation, but numerous dark neurons. No significant differences were identified between the 2 groups.

Concerning the right hemisphere (where the tumor was implanted), survivors of standard PT (n=2/9) displayed severe histological lesions, characterised by: necrosis leading to cavitation (very large cavitation in one rat), microglia activation (neuroinflammation), and a probable dilatation of ventricles, oedema and calcifications. In contrast, after pMBRT, rats displayed clearly less severe lesions. For 3/5 rats, we indeed detected rare necrosis, probable ventriculomegaly and small foci of microglia activation. For 2/5 rats, we additionally detected one larger necrotic zone and one focus of mineralisation (below hippocampus, suggesting destruction of the tumor and then mineralisation). See figure 5.

#### **4. Conclusions**

pMBRT leads to a significant widening of the therapeutic window for high-grade gliomas in rats. A high survival rate (67 %) in the pMBRT group was obtained, being one of the highest achieved in glioma-bearing rats. In contrast to the standard PT series, the absence of any significant increase of BLI signal 30 days after pMBRT suggests the absence of tumour regrowth after the irradiation (further studies are needed to confirm it), which has rarely been observed in gliomas. Indeed, GBM recurrence occurs in nearly all tumour patients [8]. No

glioma was observed in the histopathological analysis in the long-term survivals. Rats receiving pMBRT showed reduced neurotoxicity compared to the substantial brain damage observed in the standard PT group. The optimization of the irradiation parameters, such as beam spacing or the dose, might further increase the therapeutic index by incrementing the number of long-term survivals while minimizing the side effects. Indeed, no significant lesions were observed in our previous work [9], in which both normal and tumoral tissues received highly heterogeneous dose distributions with minibeam patterns

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*Figure 1. Photographs of the irradiation setup.*

*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). The dashed line indicates the approximate central position of the tumor. Spatial fractionation is maintained at the target in the case of pMRBT, as reflected by the presence of areas of high dose (in red, peaks) and areas of lower dose (valleys).*

*Figure 3. Survival curves for the controls, standard PT and pMBRT irradiated tumour-bearing rats. Survival of pMBRT treated rats was significantly higher than that of PT treated rats ( $p < 0.0001$ ).*

*Figure 4. Left: Average BLI for each of the three series. Right: BLI signal for every single rat surviving more than 40 days after irradiation. The dashed black and gray lines are the BLI signal for each single rats of the standard PT and pMBRT, respectively. A significant difference between the two groups is observed.*

*Figure 5: Long-term side-effects of pMBRT are less severe than those of standard irradiation procedures. A-C: Without irradiation, rats displayed large gliomas (A), sometimes with central necrosis/suppurative (B, star), and hyperplasia/activation of microglial cells in the tumour as well as at the periphery (C). D-I: After standard irradiation, two rats survived long-term. They were sacrificed 6 months after irradiation (end of study). We detected marked necrosis, with cavitation and calcification, more severe for one rat (D-E, Δ) than for the other (G-H, black arrowheads). Marked activation of microglial cells was also observed (E,I). J-R: In contrast, after pMBRT, most rats (3/5) displayed only minimal to mild lesion (J-K), with foci of microglial cell activation (L). Only 2 rats displayed more severe lesions, characterised by foci of necrosis with destruction of the neuropil (M-O) or calcification (probably destruction and mineralisation of the tumor; P-Q, Δ and black arrows) with mild activation of microglial cells (R). Pictures from the right part of the brains: A-B, D-E, G-H, J-K, MN, P-Q: HE staining, C, F, I, L, O, R: Iba1 immunohistochemistry to assess microglial cell density and morphology.*







