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# First trial of spatial and temporal fractionations of the delivered dose using synchrotron microbeam radiation therapy

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The technical feasibility of temporal and spatial fractionations of the radiation dose has been evaluated using synchrotron microbeam radiation therapy for brain tumors in rats. A significant increase in lifespan (216%, $p < 0.0001$) resulted when three fractions of microbeam irradiation were applied to the tumor through three different ports, orthogonal to each other, at 24 h intervals. However, there were no long-term survivors, and immunohistological studies revealed that 9 L tumors were not entirely ablated.



**Keywords: synchrotron microbeam radiation therapy; brain tumors; temporal fractionation.**

## 1. Introduction

Microbeam radiation therapy (MRT) (Slatkin *et al.*, 1992) is a preclinical form of radiosurgery developed at Brookhaven National Laboratory (Upton, NY, USA) and the European Synchrotron Radiation Facility (ESRF, Grenoble, France) for brain tumor treatment. MRT uses high-flux synchrotron light fractionated into an array of parallel microbeams (25–75 µm wide, 100–200 µm spaced on centre) and delivers very high radiation doses (hundreds of Gy) to tumors within fractions of a second. Normal brain tissue exhibits particularly high radioresistance to microbeam irradiations (Laissue *et al.*, 1998, 2007; Slatkin *et al.*, 1995), mainly owing to vascular tissue sparing (Serduc, van de Looij *et al.*, 2008; Serduc *et al.*, 2006). Previous studies performed in rodents showed that microbeam irradiations significantly increased the lifespan of brain-tumor-bearing rats (Dilmanian *et al.*, 2002; Laissue *et al.*, 1998; Regnard *et al.*, 2008). Empirically, in preclinical animal experiments, MRT for brain tumors has been delivered in one session, by one or two arrays of microbeams. Although this technique resulted in improved therapeutic indices and efficient palliation, it often failed to eradicate those tumors completely. This led to the necessity of investigating different modes of delivering MRT alone, and/or using MRT together with radiation-enhancing substances or cytostatic drugs. Conventional clinical radiation therapy of solid cancer is based on temporal fractionation of the radiation dose (Castro *et al.*, 2003; Laperriere *et al.*, 2002) to allow normal tissue surrounding the lesion to recover between two fractions. In analogy, temporal fractionation of MRT might also enhance the therapeutic index of MRT.

In this study, we have investigated the technical feasibility of three-dimensional irradiation of rat brain tumors using MRT. We have analyzed the effect of three fractions of 400 Gy delivered to 9 L gliosarcomas from three different ports, at 24 h intervals, on the lifespan of the animals.

## 2. Methods

All operative procedures related to animal care strictly conformed to the Guidelines of the French Government with licenses 380324 and A3818510002.

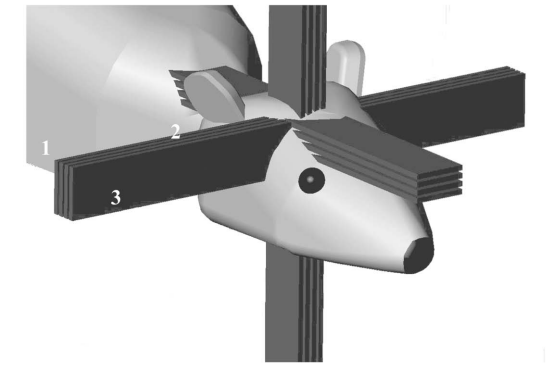
### 2.1. Brain tumor inoculation

The 9 L gliosarcoma cells were implanted in the brain of male Fisher 344 rats (Charles River, France, $n = 21$) as described previously (Regnard *et al.*, 2008). Anesthetized animals (xylazine/ketamine, 64.5/5.4 mg kg$^{-1}$) were placed on a stereotactic frame and $10^4$ 9 L cells suspended in 1 µl were injected through a burr hole in the right caudate nucleus. The skin incision was sewed and animals were placed in an incubator during wake-up before their return to the animal facility.

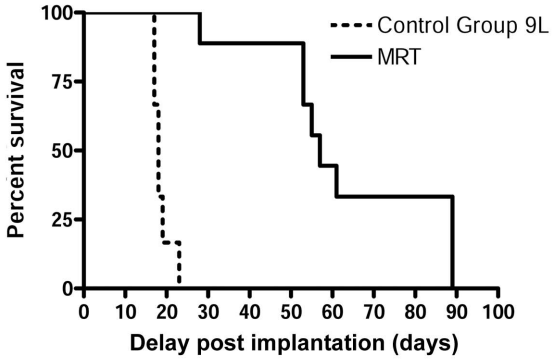
### 2.2. Brain tumor irradiation

MRT of rat brain tumors was performed at the ID17 biomedical beamline of the European Synchrotron Radiation Facility (Grenoble, France). The wiggler source produces a white spectrum of photons which extends after filtration from 50 to 350 keV with a maximum energy at 83 keV (Bräuer-Krisch *et al.*, 2003). The beam was spatially fractionated into an array of microbeams by using an adjustable multi-slit collimator (TECOMET; Bräuer-Krisch *et al.*, 2005). The dose rate was approximately 17000 Gy s$^{-1}$. At day 14 after tumor inoculation, the animals were positioned, fixed by the teeth, in front of the X-ray source on a $\kappa$-type goniometer (Huber, Germany).

The irradiation geometry is shown in Fig. 1(*a*). On the first day, rats ($n = 9$) were placed perpendicularly in front of the beam and received the first lateral irradiation, from the anatomically right to the left side




(*a*)




(*b*)

**Figure 1**
(*a*) Schematic representation of the irradiation geometry. The three arrays, administered at intervals of 24 h, produce a composite irradiation volume in the brain of 10.4 × 10.4 × 14.6 mm at the tumor site. (*b*) Survival curves of untreated (dashed line) and treated (solid line) Fisher rats bearing an intra-cerebral 9 L gliosarcoma.

of the head (port 1 on Fig. 1*a*, 10.4 mm-wide and 14.6 mm-high array of 50 microbeams, 50 µm-wide[1] and 211 µm on centre; entrance dose 400 Gy). Twenty-four hours later, animals were exposed again, but in the antero-posterior direction (port 2, 14.6 mm-wide and 10.4 mm-high array of 70 microbeams, identical to those described above; entrance dose 360 Gy). On the third day, the rats were placed vertically, perpendicularly to the beam and exposed (port 3) to a 10.4 mm-wide and 10.4 mm-high array of 50 microbeams identical to those mentioned before (entrance dose 400 Gy). The three irradiation ports produced a volume of 14.6 × 10.4 × 10.4 mm in which the three arrays overlapped (Fig. 1*a*). Each irradiation lasted less than 1 s. The three arrays produced a dose between two microbeams (valley dose) of 15 Gy in the centre of the tumor according to Monte Carlo calculations (Agostinelli & Cooperman, 2003). Animal immobility during exposure was checked on three control screens located in the control hutch.

### 2.3. Immunohistological study

After the irradiation, rats were followed up daily until their death. Body weight, behaviour and neurologic abnormalities were recorded. After the spontaneous death of the animals, the brain was excised and frozen in isopentane at 223 K. Horizontal frozen sections (20 µm thick) were obtained with a cryotome at 255 K and stained with haematoxylin and eosin (HE).

The labelling index of proliferating cells in the tumor was determined using immunochemistry for Ki67 and the brain vessels were stained for type IV collagen. Non-specific labelling was prevented by pre-incubating sections in normal donkey serum (NDS) for 1 h at room temperature (RT). Next, the sections were incubated overnight at RT with the primary antibody [rabbit monoclonal against Ki67 (1/200, Lab Vision Corporation, Fremont, CA, USA)/goat anti-collagen (1/1000, F-5202 VF83, UNLD)] with 0.5% NDS. Sections were then transferred to the secondary antibody [biotinylated donkey anti-rabbit IgG, 1/400/TRITC-conjugated donkey anti-goat F(ab′)2 1/100, Jackson Immunoresearch Laboratories] for 2 h at RT. Ki67 stained sections were incubated for 1 h at RT in streptavidin–peroxydase complex (streptABC, Dako cytomation, France) and Ki67 staining was obtained after an incubation in diaminobenzidine (SIGMA, France) procedure for revelation. The sections were examined using a NikonEclipse E600 microscope.

## 3. Results

Fig. 1(*b*) shows the survival curves of untreated ($n = 12$) and treated ($n = 9$) 9 L gliosarcoma-bearing rats. MRT significantly increased the median survival time (MST) and mean survival time of the treated group *versus* the untreated group (log rank test, $p < 0.0001$). The MST of untreated animals was 18 days after inoculation while that of the MRT-treated group was 57 days. Mean survival times were 18.6 days for the untreated group and 63.7 days for the MRT treated group. The longest survival time after tumor implantation was 23 days in the untreated group and 89 days in the MRT treated group. The increase in lifespan (ILS[2]) was 216%.

Histological observations revealed cellular damage and MRT effects on normal and tumoral cells and tissues. It appeared that tumor cell density was substantially reduced after radiation exposure (Figs. 2*c* and 2*d*) when compared with an untreated tumor (Figs. 2*a* and 2*b*). Tumors exhibited large necrotic regions, located at the tumor centre. Numerous mitotic figures were observed particularly at the periphery of the lesion. Ki67 immuno-staining showed a high labelling index at the tumor margin while the centre of the lesion was quasi-free of dividing cells (Figs. 2*c* and 2*d*). Type IV collagen immunochemistry revealed that the vessel density was markedly decreased at the centre of the lesion while the tumor margins presented an important number of large tumor vessels (Figs. 2*e* and 2*f*).

## 4. Discussion

This study reports the feasibility of a temporal and spatial fractionation of microbeam arrays applied for the treatment of rat brain tumors. This irradiation configuration yielded a significant increase in lifespan of 9 L gliosarcoma-bearing animals (+216%). However, despite the high dose deposition in the valley regions (∼45 Gy) where the beams of the three arrays intersected, the tumors were not ablated. Numerous mitotic figures were found at the tumor periphery and rats died from tumor recurrence.

The MST described in this study is the longest one obtained for the radiation treatment of this highly aggressive brain tumor at the ESRF. A similar MST has already been reported by Kim *et al.* (1999) using conventional radiotherapy ($^{60}$Co, three fractions of 18.6 Gy) but the

[1] The use of the 50 µm microbeam width was based on our results of previous studies which showed a significant increase in median survival time of 9 L gliosarcoma-bearing rats after irradiation with arrays of 50 µm-wide microbeams, compared with 25 or 75 µm-wide microbeams (Serduc *et al.*, 2009).

[2] $\mathrm{ILS} = (\mathrm{MST}_{\mathrm{treated\ animal}} - \mathrm{MST}_{\mathrm{control}})/\mathrm{MST}_{\mathrm{control}}$.

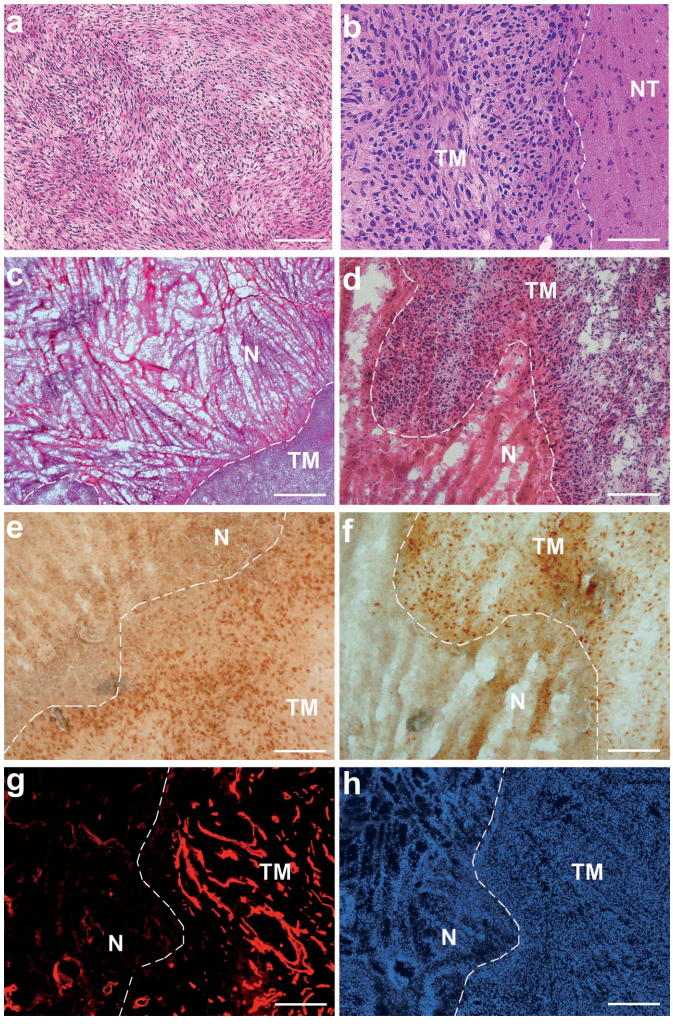



**Figure 2**
HE staining (*a*, *b*, *c* and *d*), Ki67 (*e*, *f*), type IV collagen immunochemistry (*g*) and DAPI stained nuclei (*h*) of sections of 9 L tumor implanted in rat brains. Images were taken from an unirradiated tumor (*a*, *b*) and from a rat which died 89 days after implantation (*c*–*h*). NT: normal tissue. N: necrotic area. TM: tumor margin. Ki67 positive cells (proliferating cells) appear in brown on images (*c*) and (*d*). Scale bars: 100 µm (*a*, *c*, *g* and *h*), 200 µm (*b*, *d*, *e* and *f*).

ILS was only 71% because of the lower aggressiveness of the tumor model (MST of the control group = 35 days). The 216% ILS described here demonstrates the efficiency of the microbeam radiation treatment. The valley doses (3 × 15 Gy) were close to the broad beam doses used by Kim *et al.* (1999) and we could expect that the main difference obtained in ILS would be due to high radiation doses deposited in the peak regions, *i.e.* the microbeam paths. The 360–400 Gy doses (1160 Gy at the microbeam intersections) provoke immediate cell death in the microbeam path and might have induced tumor vascular necrosis and tumor asphyxia. The three-dimensional irradiation geometry used here induced cubic segmentations of the tumor vessels and must have decreased nutrient supply to tumor cells. Indeed, MRT elicits differential effects on tumor vessels compared with normal vessels after exposure to high doses delivered in the microplanar tissue slices (Dilmanian *et al.*, 2002, 2003, 2007; Laissue *et al.*, 1998, 2007; Serduc, 2006). Several studies reported the weak and dose-dependent effect of MRT on normal brain vessel and nervous tissue (Dilmanian *et al.*, 2001, 2002; Laissue *et al.*, 1998, 2007; Serduc, van de Looij *et al.*, 2008; Serduc *et al.*, 2006) leading to the high cerebral radioresistance. Even if this has not been demonstrated in small cerebral tumors in mice (Serduc, Christen *et al.*, 2008), recent results obtained in our laboratory showed an important vascular necrosis at the centre of 9 L tumors implanted in rat brains after MRT (Serduc *et al.*, 2009). However, in the latter and in the present study, tumor cells were not totally sterilized and tumor recurrence took place at the periphery of the lesion. Ki67 positive (proliferating) cells were found at the tumor margin (Figs. 2*c* and 2*d*), where the tumor vessel density and most likely tumor angiogenesis activity are the highest (Figs. 2*e* and 2*f*).

The technical possibility of tumor reirradiations at the ESRF would allow new experimental designs for MRT brain tumor treatment. As has been suggested by Laissue *et al.* (2007), MRT might be used to inhibit the growth of brain tumor in infants, providing precious time to allow minimally toxic adjuvant therapies and to continue suppressing the tumor as the brain matures. One of the goals of MRT might be the safe control of tumor growth and three-dimensional reirradiation could become a powerful asset in brain tumor palliation. Preliminary work performed in our laboratory showed that a two-port irradiation (coplanar and crossfired) separated by one week significantly improved the MST and +50% increase in ILS of 9 L tumor-bearing rats compared with a crossfired microbeam radiosurgery (Regnard, 2008). According to these results, we expect that a third, non-coplanar, dose fraction using MRT would enhance again tumor treatment efficiency.

To conclude, we have demonstrated here for the first time the technical feasibility of 9 L brain tumor reirradiation using MRT. Although this irradiation configuration did not entirely sterilize the lesion, the treatment yielded the best ILS obtained for this highly aggressive brain tumor in our laboratory. The reirradiation schedule should now be optimized to find the appropriate delay between the irradiations in terms of normal tissue tolerance and tumor control.

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