



PET prediction of hypoxia

Predictive value of ^{18}F -FAZA PET imaging for guiding the association of radiotherapy with nimorazole: A preclinical study



Ly-Binh-An Tran^a, Anne Bol^b, Daniel Labar^b, Thanh-Trang Cao-Pham^a, Bénédicte Jordan^a, Vincent Grégoire^b, Bernard Gallez^{a,*}

^a Louvain Drug Research Institute, Biomedical Magnetic Resonance Research Group; and ^b Institut de Recherches Expérimentales et Cliniques, Center for Molecular Imaging, Radiotherapy and Oncology, Université catholique de Louvain, Brussels, Belgium

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ABSTRACT

Purpose: To assess the predictive value of hypoxia imaging by ^{18}F -FAZA PET in identifying tumors that may benefit from radiotherapy combined with nimorazole, a hypoxic radiosensitizer.

Material and methods: Rats of two tumor models (Rhabdomyosarcoma and 9L-glioma) were divided into two treated groups: radiotherapy (RT) alone or RT plus nimorazole. ^{18}F -FAZA PET images were obtained to evaluate tumor hypoxia before the treatment. Treatment outcome was assessed through the tumor growth time assay, defined as the time required for tumor to grow to 1.5 times its size before irradiation. **Results:** For rhabdomyosarcomas, the benefit of adding nimorazole to RT was not significant when considering all tumors. When stratifying into more and less hypoxic tumors according to the median ^{18}F -FAZA T/B ratio, we found that the combined treatment significantly improved the response of the “more hypoxic” subgroup, while there was no significant difference in the tumor growth time between the two treatment modalities for the “less hypoxic” subgroup. For 9L-gliomas, a clear benefit was demonstrated for the group receiving RT + nimorazole. However, the individual responses within the RT + nimorazole group were highly variable and independent of the ^{18}F -FAZA uptake.

Conclusions: ^{18}F -FAZA PET may be useful to guide hypoxia-directed RT using nimorazole as radiosensitizer. It identified a subgroup of more hypoxic tumors (displaying T/B ratio > 2.72) that would benefit from this combined treatment. Nevertheless, the predictive power was limited to rhabdomyosarcomas and ineffective for 9L-gliomas.

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Tumor hypoxia, the situation where tumor cells have been deprived of oxygen, is a common feature found in most solid tumors. Accumulated evidences demonstrated that this phenomenon is responsible for a poorer prognosis and has a negative impact on cancer treatment [1–3], especially radiotherapy (RT) [4–6]. Therefore, the clinical importance of hypoxia and the possibility to modulate it have been one of the most investigated issues in RT for many years. Many approaches have been tried to reduce hypoxia and preferentially enhance tumor radiosensitivity such as improving oxygen availability at the time of irradiation (by increasing the delivery and/or decreasing the consumption of oxygen) [7,8] or exploiting hypoxia by using cytotoxins killing selectively hypoxic cells or hypoxic radiosensitizers [9,10]. One of these promising methods is the combination of RT with nimorazole, a class of chemicals known as 5-nitroimidazoles. This

compound can mimic the effect of oxygen in promoting fixation of the free radicals produced by radiation damage and hence render cells more radiosensitive. Although nimorazole was shown to significantly improve the outcome of conventional RT in head and neck cancer, it is currently considered as a standard treatment only in Denmark [11]. That may be partly explained by the inability to identify the patients that could potentially benefit from hypoxia modification by nimorazole, and thus cannot reveal such a great effect to ensure a broader interest.

The importance of patient stratification is not only limited to the case of nimorazole but is a common issue for all hypoxia-driven interventions. In a systematic review, Overgaard identified 10,108 patients in 86 randomized trials designed to modify tumor hypoxia in patients treated with curative attempted primary RT alone [12]. From this meta-analysis, this author concluded that “Ample data exist to support a high level of evidence for the benefit of hypoxic modification. However, hypoxic modification still has no impact on general clinical practice”. And it appears that the variation in the results among the trials points toward a considerable

* Corresponding author at: Biomedical Magnetic Resonance Group (REMA), Louvain Drug Research Institute, Université catholique de Louvain, Avenue Mounier 73, B1.73.08, B-1200 Brussels, Belgium.

E-mail address: Bernard.Gallez@uclouvain.be (B. Gallez).

heterogeneity among tumors. In other words, not all tumors will benefit from such a hypoxia modification, and then it is mandatory to have reliable predictive biomarkers that will guide oncologists to select the optimal treatment intervention for each individual tumor.

So far, numerous methods have been developed to detect and measure hypoxia but only few clinical investigations concluded that hypoxia marker may be used as predictive test [13,14]. Recently, in a preclinical study [15], we provided compelling evidence that hypoxia imaging using ¹⁸F-FAZA PET may be used as a prognostic tool for the outcome following RT and could assess the response of tumor to carbogen breathing. Based on the tumor-to-background ratio of ¹⁸F-FAZA PET images, we were able to define the most appropriate hypoxia-driven intervention to potentiate the response to irradiation: carbogen breathing in responsive tumors or dose escalation in tumors non-responsive to carbogen.

Herein, we investigated further the predictive value of ¹⁸F-FAZA PET imaging for the outcome when using radiation in combination with the well-known hypoxia radiosensitizer nimorazole. For this purpose, ¹⁸F-FAZA PET scan was first carried out to provide the value of hypoxia before the treatment. Tumors from two tumor models were irradiated with or without nimorazole. The tumor growth time was monitored and used as the endpoint of treatment outcome. We finally tested the correspondence between the value derived from PET imaging and the tumor outcome to evaluate whether this technique could identify individual tumors having potential to benefit from RT + nimorazole.

Materials and methods

Animals and tumor models

Two rat tumor models were used for this study: rhabdomyosarcomas ($n = 28$) grown in male adult WAG/Rij rats (Charles River, Sulzfeld, Germany) and 9L-gliomas ($n = 22$) grown in male adult Fischer 344 rats (Charles River, Arbresle, France). The former (developed by Laboratory of Experimental Radiobiology of the Katholieke Universiteit Leuven, Belgium [16]) was induced through *in vivo* passage by grafting subcutaneously a syngeneic fragment of about 1 mm³ in the thigh of rats. For the latter inoculation, 5-10⁶ tumor cells (kindly provided by Dr. Olivier Bockstael, Université Libre de Bruxelles, Belgium) were diluted in 200 µl of culture medium and injected subcutaneously in the thigh of animals. All tumor implantations were performed under anesthesia with a mixed solution of ketamine and xylazine at a dose of 80 and 10 mg/kg, respectively. Rats were included in the study when tumor reached a diameter of 15–18 mm.

¹⁸F-FAZA PET imaging

Rats were injected in the tail veins with 18.5–25.9 MBq ¹⁸F-FAZA in a volume of 500 µL. This marker was produced as previously described [17]. PET acquisitions were performed 3 h after tracer injection on a dedicated small-animal PET scanner (MOSAIC, Philips) with a spatial resolution of 2.5 mm (FWHM). Rats anesthetized with 2 % isoflurane underwent first a 10 min emission scan followed by a 10 min transmission scan using a 370-MBq ¹³⁷Cs source. After the correction with attenuation factors obtained from the transmission scan, images were reconstructed using a fully 3D iterative algorithm (3D-RAMLA) in a 128 × 128 × 120 matrix, with a voxel of 1 mm³. The result of tracer distribution in tumor was expressed as tumor-to-background ratio (T/B ratio), calculated as the mean activity in the tumor region divided by the mean activity in the background region. Regions of interest (ROI) were delineated using the program PMOD (PMOD Technologies Ltd., Zurich,

Switzerland). The 2D ROIs were established on consecutive transversal slices using a 40% isocontour tool (ROI including the pixel values greater than 40% of the maximum pixel) that semiautomatically created a volume of interest (VOI) encircling the tumor. The background VOI of 1 cm³ (created from 2D ROIs of 5 mm in diameter on consecutive transversal slices) was chosen in the pelvic region to be representative of the tracer uptake within non-tumor tissue.

Treatment

Treatment was initiated immediately after PET imaging. The animals of both tumor models were randomly divided into two groups: RT alone or RT combined with nimorazole. For RT, single dose of irradiation was delivered by a ¹³⁷Cs irradiator IBL-637 (Oris, France), 20 Gy for rhabdomyosarcomas and 40 Gy for 9L-gliomas. Animals anesthetized with isoflurane (2% in air) were placed on a plexiglass and protected from the beam through a lead block of 3 cm thickness. Only the tumor was exposed to the beam via a hole of 25 mm in diameter. The animals were turned midway through the exposure time to ensure uniformity of dose distribution. Irradiation doses were selected on the basis of their respective radiation sensitivity to ensure a significant growth delay in the irradiation group compared to untreated rats. The hypoxic radiosensitizer nimorazole, kindly supplied by Prof. Jens Overgaard (Aarhus University Hospital, Denmark), was administered intraperitoneally at a dose of 100 mg/kg 30 min before irradiation in a subgroup of rats. The solution of nimorazole was freshly prepared on the day of treatment by dissolving the compound in NaCl 0.9% to a concentration of 20 mg/mL. Rats of the irradiated group without hypoxic sensitizer received IP injection of physiological solution. Dose of nimorazole and timing were chosen according to the result of previous studies to achieve the most effective radiosensitization [18,19].

Tumor growth assay

Tumor size was assessed by caliper measurement under isoflurane anesthesia and calculated as the mean of the longest and shortest perpendicular diameters. The tumors were first measured on the day of treatment to determine their diameter at the start of treatment (D0) and then at least twice a week during the follow-up (3 months post-irradiation or until tumor diameter reached 28 mm). Tumor growth time was defined as the time required for tumor to grow to 1.5 times D0. If a tumor failed to reach the endpoint by the time of sacrifice, the day of sacrifice was taken as the value of tumor growth time, though this may represent an underestimate of tumor response.

Statistical analysis

Results were expressed as mean value of parameter ± SEM (standard error of the mean). Group differences were assessed using *t*-test (including Levene's median test for homogeneity of group variances); Welch's *t* test replaced *t*-test for the situation of unequal variances. Pearson correlation coefficients were calculated to correlate the tumor growth time to tracer uptake. Statistical tests were performed using GraphPad Prism (version 5 for Windows) with $p < 0.05$ being considered as significant.

Results

The effect of radiation with and without using nimorazole on the tumor growth of rhabdomyosarcomas is presented in Fig. 1. When considering all tumors (Fig. 1, top panel), there was no benefit of adding nimorazole to RT: the tumor growth time of the

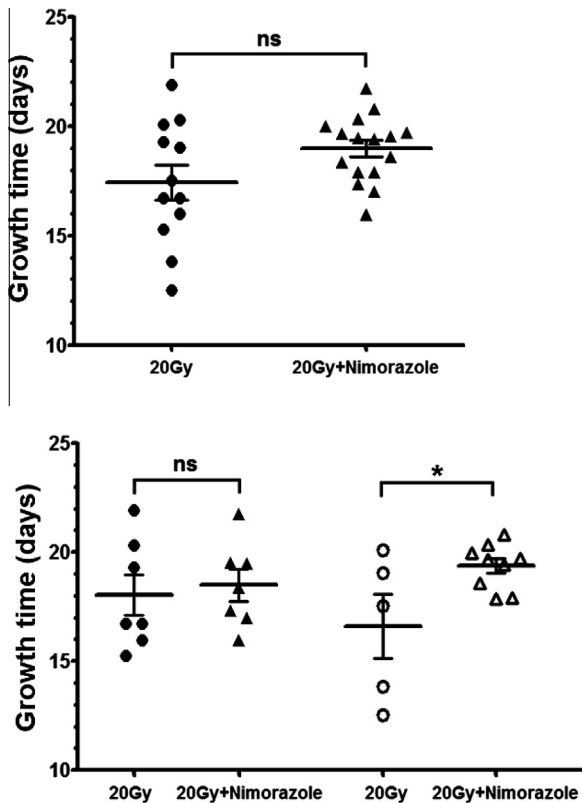


Fig. 1. Effect of RT with and without using nimorazole on the tumor growth time of rhabdomyosarcomas. Top panel: Analysis considering all tumors. There was no significant benefit from the combination of RT with nimorazole compared to RT alone. Bottom panel: Analysis according to the hypoxic status of the tumors using stratification into “less hypoxic” (filled symbol) and “more hypoxic” (open symbol) tumors based on the median of ^{18}F -FAZA T/B ratio. For the “less hypoxic” subgroup, there was no significant difference between the tumor growth time after RT alone and RT + nimorazole. For the “more hypoxic” subgroup, the combined treatment significantly improved the response to irradiation using a combination with nimorazole.

group irradiated with 20 Gy was 17.4 ± 0.8 days ($n = 12$), not significantly different from that of the group irradiated with the same dose plus nimorazole, 19.0 ± 0.4 days ($n = 16$), $p = 0.0709$. We analyzed the influence of tumor hypoxia on the tumor response by plotting the tumor growth time as a function of ^{18}F -FAZA uptake (Fig. 2). For the group receiving RT alone (Fig. 2, top panel), there was a trend (although not significant) showing that the more hypoxic the tumor is (high ^{18}F -FAZA uptake), the faster it is regrowing. However, this trend reversed in the treatment RT + nimorazole (Fig. 2, bottom panel), suggesting that the more hypoxic tumors were more responsive to the combined treatment. To further analyze this issue, we stratified the tumors into “more hypoxic” and “less hypoxic” subgroups based on the median value of ^{18}F -FAZA T/B ratio. The ^{18}F -FAZA T/B ratio of the 28 rhabdomyosarcomas included in this study varied from 1.69 to 3.49 with a median of 2.72. Hypoxia level in the two treated groups were quite equally distributed (RT alone: mean 2.62, range 1.93–3.33; RT + nimorazole: mean 2.69, range 1.69–3.49; $p = 0.70$; Sup. Fig. 1). Among the 14 tumors expressing T/B ratio below the global median (“less hypoxic”), 7 tumors received RT alone, 7 tumors received RT + nimorazole. In the group classified as “more hypoxic” (T/B ratio higher than the global median), there were 5 tumors subjected to RT alone and 9 tumors subjected to the combined therapy. No significant difference in tumor growth time was found between RT and RT + nimorazole in the “less hypoxic” group, 18.0 ± 0.9 days vs 18.5 ± 0.7 days ($p = 0.7094$) (Fig. 1, bottom panel,

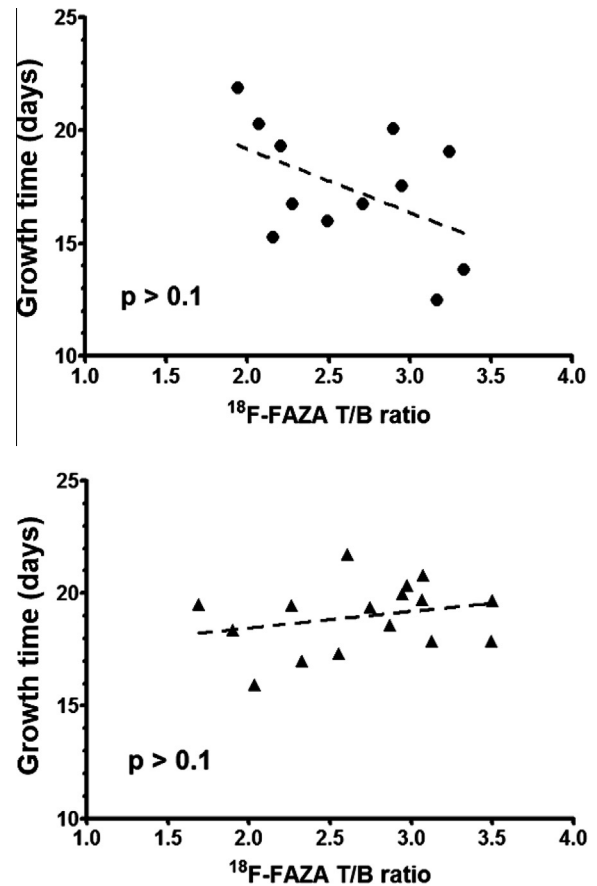


Fig. 2. Influence of tumor hypoxia on the tumor response in rhabdomyosarcomas: Individual growth times are plotted as a function of individual ^{18}F -FAZA T/B ratio. Top panel: tumors treated by RT alone. Bottom panel: tumors treated by RT + nimorazole. For the group receiving RT alone, there was a trend (although not significant) showing that the more hypoxic the tumor is (high ^{18}F -FAZA uptake), the faster it is regrowing. This trend was reversed in the treatment RT + nimorazole suggesting that the more hypoxic tumors were more responsive to the combined treatment.

filled symbols). In contrast, for the “more hypoxic” tumors, the combination of RT + nimorazole significantly enhanced the tumor response compared to RT alone, 19.4 ± 0.4 days vs 16.6 ± 1.5 days ($p = 0.0346$) (Fig. 1, bottom panel, open symbols).

In the case of 9L-gliomas, the combination of RT + nimorazole induced a tumor growth time (49.7 ± 10.9 days, $n = 10$) significantly longer ($p = 0.0437$) than for the group receiving RT alone (24.8 ± 4.5 days, $n = 12$) (Fig. 3). However, the variation between individual responses was quite pronounced within the group receiving RT + nimorazole. We also analyzed the influence of hypoxia on the tumor response in 9L-gliomas (Fig. 4). For the group treated with radiation alone, a significant correlation between the T/B ratio derived from ^{18}F -FAZA PET images and the response of 9L-gliomas was observed (Fig. 4, top panel, $r = 0.72$, $p = 0.0077$), an observation that is consistent with the result obtained in our previous study [15]. By contrast, the response of tumors receiving RT + nimorazole was independent of the FAZA retention (Fig. 4, bottom panel). Two different categories of response can be observed in this group. The mean T/B ratio on ^{18}F -FAZA PET images found in the four 9L-gliomas that responded well to RT + nimorazole was lower but not significantly different from that of the six tumors with a lower response (2.25 vs 2.50 , $p = 0.1624$). In terms of distribution of hypoxia level, there was a wider inter-individual variation in the value of ^{18}F -FAZA T/B ratio for the RT alone group compared to the group RT + nimorazole; still, the mean T/B ratio of

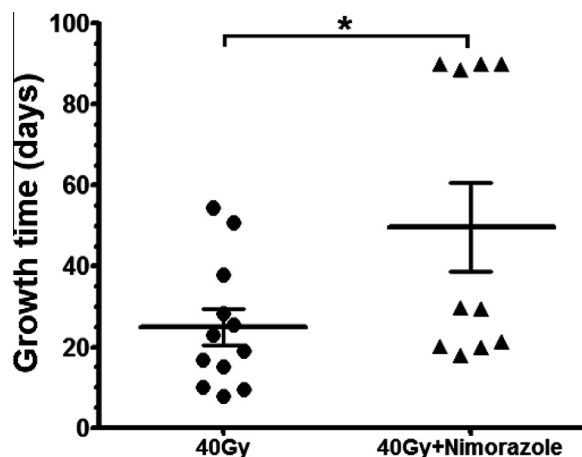


Fig. 3. Effect of RT with and without using nimorazole on the growth time of 9L-gliomas. RT + nimorazole significantly increased the tumor growth time compared to RT alone. It should be noted that the variation between individual responses within the group receiving the concomitant regime was highly variable and seems to be switched “on” or “off”.

two groups was quite comparable, 2.43 and 2.40, respectively ($p = 0.8744$) (Sup. Fig. 1).

Discussion

In an era in which individualized treatment is being proposed as the future for cancer therapy, tumor hypoxia should be considered as a highly relevant target. There is clear evidence showing that tumors sharing similar clinical characteristics can display different patterns in oxygenation status, resulting in variable response to therapies [20,21]. It is therefore important to consider the potential benefit of using tumor hypoxia as a tool for the identification of patients that would benefit of a specific treatment. Among the clinically applicable methods for detecting tumor hypoxia, ^{18}F -FAZA PET imaging is becoming more and more appealing. Being non-invasive and providing a three-dimensional image of hypoxia distribution within tumor, PET imaging is an attractive method to use for the delineation of hypoxic regions for dose painting strategy [22,23]. Furthermore, the prognostic value of ^{18}F -FAZA for outcome following RT was demonstrated in both preclinical and clinical studies [24,25]. Recently, we also showed that ^{18}F -FAZA imaging may serve as a predictive tool for guiding hypoxia-driven interventions (carbogen breathing or dose escalation) in RT [15]. The present study further investigated the predictive value of this biomarker for another hypoxia-targeted therapy, namely exploiting hypoxia by using the hypoxic radiosensitizer, nimorazole. High levels of plasma osteopontin and 15-genes hypoxia classifier were proved to be able to identify subpopulation of patients that benefit from radiation combined with nimorazole [26,27]. As far as we know, the value of ^{18}F -FAZA PET to predict the potential benefit of adding nimorazole to standard RT has not yet been investigated.

In the present study carried out on two hypoxic tumor models, we found to some extent a benefit of the addition of nimorazole to RT compared to RT alone. In the case of rhabdomyosarcomas, the growth delay was significantly extended for the “more hypoxic” group irradiated in the presence of nimorazole compared to the “more hypoxic” group receiving RT alone. By contrast, nimorazole was not effective in the tumors presenting a T/B ratio below the median value. This is likely due to the kinetic competition between oxygen and nitroimidazole for reaction with DNA base radicals [28]. Consequently, nimorazole would perform more effectively when oxygen is less present. According to our previous *in vivo* calibration [29], it is interesting to note that the T/B ratio

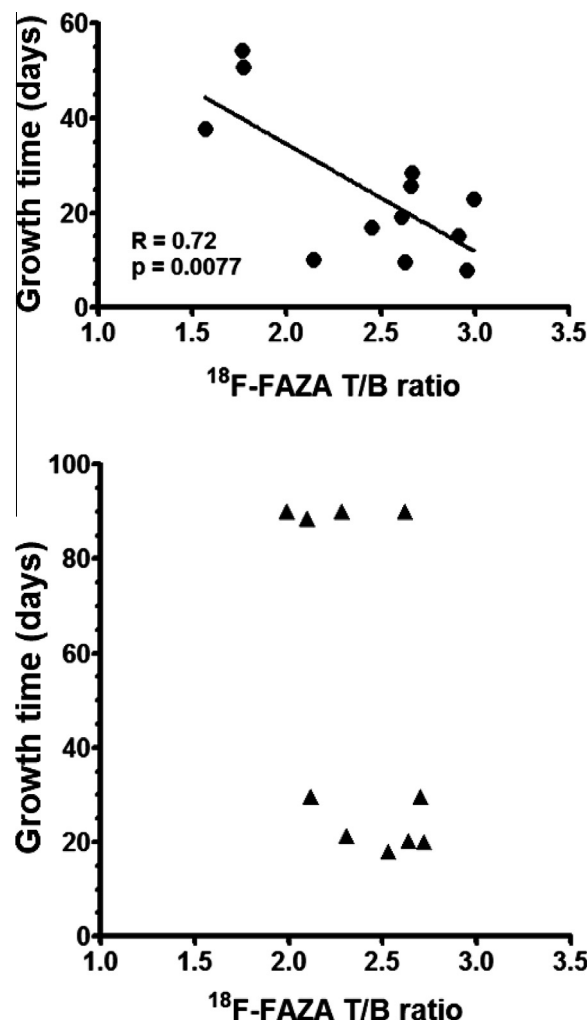


Fig. 4. Influence of tumor hypoxia on the tumor response in 9L-gliomas: individual growth times are plotted as a function of individual ^{18}F -FAZA T/B ratio. Top panel: tumors treated by RT alone. Bottom panel: tumors treated by RT + nimorazole. For the group receiving RT alone, there was a significant correlation between the T/B ratio derived from ^{18}F -FAZA PET and the tumor response. For the group receiving RT + nimorazole, the value of tumor growth time was categorized into two levels independently of the ^{18}F -FAZA uptake.

2.72 corresponds to 4 mmHg, a value comparable to the 5 mmHg level that was often used as a cut-off value for hypoxic fractions in many clinical studies conducted with the “gold standard” Eppendorf electrode [30,31]. However, given the high heterogeneity of tumor microenvironment, the classification into more and less hypoxic tumors based on the median pretreatment T/B ratio may be crude and oversimplified. Tumors displaying a T/B ratio below this median value may include small amounts of severely hypoxic cells that considerably impact the radioresistance. Still, this finding allowed us to show the proof of principle that the level of hypoxia varies between tumors and that this variation can be used to identify tumors that may benefit from a hypoxia-driven intervention. The result also indicated that only tumors with a “severe” hypoxic fraction seem to benefit from the combination of nimorazole together with radiation.

For 9L-gliomas, there were some tumors highly responsive to treatment in the group receiving RT alone; interestingly, these tumors displayed a T/B ratio below 1.7, the threshold of radiosensitivity suggested in our previous study [15]. In the group treated with RT + nimorazole, we did not record tumors expressing T/B ratio below 1.7 or above 2.7. As this group did not contain tumors that we can classify as “normoxic” (T/B below 1.7) nor “highly hypoxic”

(T/B above 2.7), we were unable to corroborate the predictive value of T/B ratio 2.72 found in the assay carried out with rhabdomyosarcomas. In addition, the tumor responses of this group were highly variable and seem to be switched “on” or “off” independently of the ^{18}F -FAZA uptake. This suggests that the improved outcome for 9L-gliomas after the combination therapy was not linked primarily to the hypoxic status but may be associated with other factors. Indeed, tumor hypoxia is not the only cause of radioresistance: intrinsic radiosensitivity and accelerated repopulation of tumor cells are also playing a major role in the response to radiation [32–34]. 9L-glioma is a highly radioresistant model: to induce a significant difference in growth delay between the irradiated tumors and those untreated, we had to deliver a single dose up to 40 Gy. Thus, this tumor model likely possesses the characteristics that might affect the efficacy of hypoxia-driven interventions. Another explanation may be due to the limitation of PET imaging that visualizes chronic hypoxia rather than acute hypoxia. The fluctuation in oxygenation could render some fraction of cells severely hypoxic at the time of treatment and become more radiosensitive in the presence of nimorazole. Consequently, it is difficult to observe such a relation between the hypoxia level measured by ^{18}F -FAZA PET and the tumor responses to RT plus radiosensitizer.

In our former study [15], we concluded that ^{18}F -FAZA PET imaging was able to identify the individual tumors that are responsive to hyperoxic challenge: 9L-gliomas benefit from carbogen breathing; whereas rhabdomyosarcomas did not respond to this modulation and needed a dose escalation to have a better outcome. In the present study, we found that highly hypoxic rhabdomyosarcomas benefit from the combination of RT + nimorazole. Taken together, these findings contributed to show that the tumor hypoxia that can be characterized by ^{18}F -FAZA PET imaging may serve as a guide to adapt the therapy by defining appropriate hypoxia-driven intervention: changing oxygen delivery or escalating the dose or adding hypoxic radiosensitizer at the time of irradiation. Put in a clinical context, our studies suggest that ^{18}F -FAZA PET imaging could help the oncologists to decide the best treatment option for each patient and therefore help to decrease the risk of treatment-related side effects as well as treatment costs.

Finally, it should be emphasized that our study suffers the weakness concerning the method to define PET volume where the tumors were drawn using the fixed threshold of 40% SUVmax (maximum standardized uptake value) (Fig. 5). This automatic process does not account for heterogeneity within tumor and may exclude peripheral thin area of tumor. However, there is no consensus regarding the optimal contouring method at the present [35]. Since the delineation of tumor based on the expert visual interpretation is still considered as the most practical approach in the absence of validated automatic tool, in the preliminary test, we examined the results analyzed by the manual delineation and those derived by the automatic ones using some arbitrary thresholds. We found that, for both tumor models, contouring tumor based on 40% SUVmax threshold could provide quite the similar value of ^{18}F -FAZA uptake compared to the manual method (data not shown). Moreover, the advantages of the automatic approach are providing the higher objectivity and reducing the time of image analysis. Besides the contouring method, the choice of PET parameter was another relevant strategy to effectively assess the results. The calculations using SUVmax and SUVmean showed to some extent to be related with the tumor growth time; however, they could not offer such a strong relationship as T/B ratio did (Sup. Fig. 2). T/B ratio was therefore the optimal parameter to quantify the tumor tracer uptake herein.

In conclusion, the present study has shown that ^{18}F -FAZA PET imaging may be useful to guide the use of hypoxic radiosensitizers as it identified a subgroup of more hypoxic rhabdomyosarcomas (T/B ratio > 2.72) where the combination RT + nimorazole was

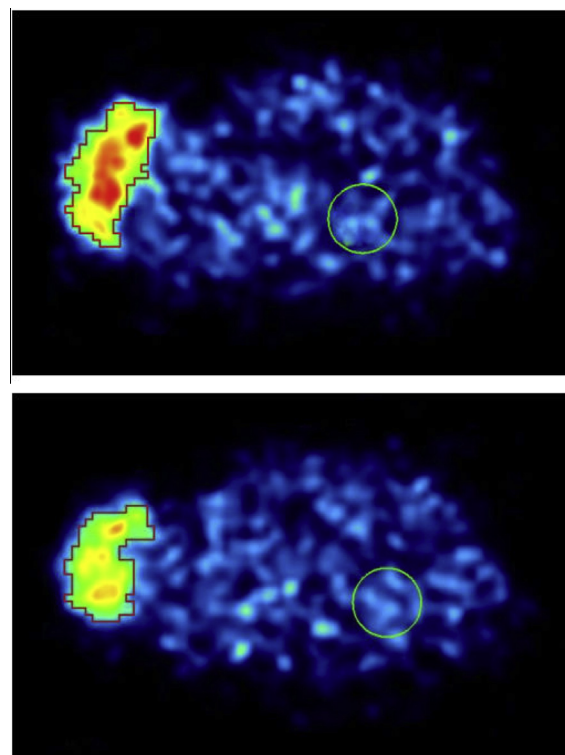


Fig. 5. Representative PET images illustrating ^{18}F -FAZA uptake in Rhabdomyosarcoma (top panel) and 9L-glioma (bottom panel). On the transversal slice, tumor delineated by 40% isocontour tool (including the pixel values greater than 40% of the maximum pixel) was marked by red ROI and reference tissue was marked by green ROI.

associated with a significant delay in tumor growth time. Nevertheless, the predictive power was limited to this tumor model and ineffective for the second model (9L-glioma). The conclusions should therefore be confirmed on other tumor models, using more clinical relevant endpoints (local tumor control, local tumor recurrence...) and RT protocols more close to the clinical practice (fractionated doses). Additionally, as ^{18}F -FAZA PET imaging is already clinically available, we believe it is timely to assess the predictive value of this imaging biomarker in clinical trials as well as its utility to select appropriate patients for hypoxia-modifying modalities and personalize treatment involving radiation.

Conflict of interest statement

The authors have no conflict of interest to disclose regarding the present study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.radonc.2014.12.015>.

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