FMISO-PET and immunohistochemistry verified tumor oxygenation, stemness, and immunosupportive microenvironment after preoperative neoadjuvant bevacizumab for newly diagnosed glioblastoma

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Research Article

Keywords: FMISO-PET, tumor oxygenation, tumor microenvironment, neoadjuvant bevacizumab, glioblastoma

Posted Date: February 8th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2545132/v1

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Abstract

Background

Gadolinium-enhanced magnetic resonance imaging and T2-weighted imaging/fluid-attenuated inversion recovery imaging are used to determine the efficacy of bevacizumab (Bev) against glioblastoma (GBM). Positron emission tomography (PET) using $^{18}$F-fluoromisonidazole (FMISO) reflects hypoxia in the tumor microenvironment (TME). This study compared FMISO-PET findings for alterations in tumor oxygenation in the TME of GBM during Bev treatment.

Materials and Methods

We retrospectively reviewed data from 7 patients with newly diagnosed IDH (isocitrate dehydrogenase)-wildtype GBM who underwent FMISO-PET during follow-up. Three patients received preoperative neoadjuvant Bev (neo-Bev) followed by radiation therapy and temozolomide, and subsequently underwent surgical resection. Re-operation was performed for recurrence. FMISO-PET was performed at each time point. Four patients who underwent tumor resection after FMISO-PET without any preoperative interventions were also included as a Control group. Surgically obtained tumor tissues were analyzed for expression of a marker of hypoxia (carbonic anhydrase; CA9), stem cell markers (nestin, FOXM1), and immunoregulatory molecules (CD163, FOXP3, PD-1, PD-L1) by immunohistochemistry (IHC).

Results

All 3 patients treated with preoperative chemoradiotherapy showed reduced FMISO accumulation (maximum tumor-to-blood ratio and hypoxic volume) and decreases in CA9 and FOXM1 compared with Controls. Two cases in the preoperative neo-Bev group showed recurrence with increasing FMISO accumulation. IHC showed increased CA9- and FOXM1-positive cells at recurrence in both cases. A trend toward fewer CA9-positive cells was seen in patients with low FMISO accumulation both with and without neo-Bev ($r = 0.90$, $p = 0.006$). Expressions of immunoregulatory molecules tended to be lower after neo-Bev compared with the Control group and increased at recurrence, but these differences were not significant.

Conclusion

FMISO-PET effectively visualized improvements in TME oxygenation after preoperative chemoradiotherapy including Bev. Increased FMISO accumulation at the time of recurrence, even under Bev treatment, suggests that FMISO-PET might be useful for monitoring the duration of Bev efficacy by reflecting tumor oxygenation.

Background

Hypoxic regions within the tumor microenvironment (TME) show reduced radiosensitivity and also increase the risk of refractory tumors via the activation of transcription factors such as hypoxia-inducible factor-1α (HIF1-α) and, further downstream, vascular endothelial growth factor (VEGF) [1, 2]. Bevacizumab (Bev), a monoclonal antibody against VEGF, is known to inhibit angiogenesis in tumor tissue and normalize vascular structures in tumor tissue to improve oxygenation and reduce brain edema around the lesion by decreasing vascular permeability. Bev is currently used in glioblastoma multiforme (GBM) treatment [3]. Recently, indirect effects have also been reported to reduce immunosuppressive immune cells such as regulatory T cells and tumor-associated macrophages within the TME of GBM by improving oxygenation [4, 5]. However, the efficacy of Bev therapy varies from person to person and the duration of efficacy is limited [3]. Furthermore, contrast enhancement on magnetic resonance imaging (MRI) disappears during the course of treatment with Bev, representing one of the issues to be addressed in the future for proper imaging evaluation of therapeutic efficacy [6].
Evaluating alterations in glioma metabolism has become important when anti-angiogenic therapies such as Bev have been included along with standard chemoradiotherapy. Hypoxia plays a pivotal role in tumor malignancy, aggressiveness, and refractoriness against chemoradiotherapy. Positron emission tomography (PET) using the tracer $^{18}$F-fluoromisonidazole (FMISO) detects hypoxic TMEs, which specifically accumulate the tracer. FMISO-PET has therefore been used to visualize hypoxic regions in GBM [7, 8]. Previous research has shown that alterations depicted on FMISO-PET have prognostic value due to their responsiveness to the induction of tumor oxygenation during Bev therapy for specific recurrent gliomas [9–11].

Although the optimal timing and duration of Bev administration remain controversial, we have proposed that preoperative neoadjuvant Bev (neo-Bev) for newly diagnosed GBM contributes to reducing intraoperative blood loss and improving preoperative performance status, and might increase sensitivity to postoperative radiation therapy (RT) [2, 12, 13]. To date, the only FMISO-PET studies that have evaluated the efficacy of Bev in the treatment of gliomas have been reports of recurrent cases. Furthermore, all observation periods to date have been short-term, and no reports have provided long-term observations of changes on FMISO-PET leading to the acquisition of resistance to Bev treatment in GBM over time.

The aim of the present study was to investigate whether FMISO-PET and immunohistochemistry (IHC) could verify our hypothesis that preoperative neo-Bev add-on neoadjuvant chemoradiotherapy might induce tumor oxygenation in patients with newly diagnosed GBM, while the TME in recurrent GBM would become hypoxic again when recurrent tumor becomes resistant to Bev therapy.

**Methods**

**Patients and Treatment**

This retrospective study included patients with primary IDH-wildtype GBM who underwent surgery and preoperative neoadjuvant and postoperative adjuvant therapies along with FMISO-PET at Kagawa University. Of these patients, those who underwent craniotomy with neo-Bev followed by RT and temozolomide (TMZ) as preoperative chemoradiotherapy were defined as the preoperative neo-Bev group, and those who underwent craniotomy without preoperative treatment were defined as the Control group.

In the preoperative neo-Bev group, all patients received two courses of Bev at 10 mg/kg, followed by RT (40 Gy in 16 fractions or 60 Gy in 30 fractions) and TMZ at 75 mg/m$^2$ for 42 days (Fig. 1). Craniotomy was performed at least 5 weeks after neo-Bev. After craniotomy, TMZ at 200 mg/m$^2$ and postoperative Bev at 10 mg/kg were continued as maintenance therapy (Table 1).
<table>
<thead>
<tr>
<th>Case/Control</th>
<th>Age/sex</th>
<th>Location</th>
<th>Preoperative treatment</th>
<th>Postoperative treatment</th>
<th>Recurrence</th>
<th>Treatment after recurrence</th>
<th>Additional therapy after reoperation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>65/F</td>
<td>left frontal</td>
<td>Bev × 2</td>
<td>Bev × 8</td>
<td>recurrence</td>
<td>reoperation</td>
<td>Bev × 6</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>TMZ; 42 days, RT; 60 Gy/30 fr</td>
<td>TMZ × 8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case 2</td>
<td>78/M</td>
<td>right temporal</td>
<td>Bev × 2</td>
<td>Bev × 5</td>
<td>recurrence</td>
<td>reoperation</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TMZ; 42 days, RT; 40 Gy/16 fr</td>
<td>TMZ × 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case 3</td>
<td>66/M</td>
<td>left occipitotemporal</td>
<td>Bev × 2</td>
<td>Bev × 26</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TMZ; 42 days, RT; 60 Gy/30 fr</td>
<td>TMZ × 25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>32/M</td>
<td>left cerebellum</td>
<td>–</td>
<td>TMZ; 42 days, RT; 60 Gy/30 fr</td>
<td></td>
<td></td>
<td>→ TMZ × 4</td>
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<tr>
<td>Control 2</td>
<td>76/M</td>
<td>left frontal</td>
<td>–</td>
<td>TMZ; 42 days, RT; 60 Gy/30 fr</td>
<td></td>
<td></td>
<td>(no maintenance treatment)</td>
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<tr>
<td>Control 3</td>
<td>55/M</td>
<td>right parietal</td>
<td>–</td>
<td>TMZ; 42 days, RT; 60 Gy/30 fr</td>
<td></td>
<td></td>
<td>(no maintenance treatment)</td>
</tr>
<tr>
<td>Control 4</td>
<td>76/F</td>
<td>left parietal</td>
<td>–</td>
<td>TMZ; 42 days, RT; 60 Gy/30 fr</td>
<td></td>
<td></td>
<td>(no maintenance treatment)</td>
</tr>
</tbody>
</table>

Bev, bevacizumab; F, female; fr, factions; M, male; RT, radiation therapy; TMZ, temozolomide.

**FMISO-PET**

FMISO-PET was performed: 1) before preoperative neo-Bev; 2) after preoperative neo-Bev (5 weeks after end of Bev, 1 week before surgery); 3) after surgery (only in some cases); and 4) at recurrence (only in cases that recurred) (Fig. 1). The examination protocols were performed using a previously described method [14, 15]. In the Control group, FMISO-PET was performed only preoperatively.
FMISO synthesis and PET acquisition

MRI was performed on a 3-T MAGNETOM Skyra scanner (Siemens Healthcare, Erlangen, Germany). Axial T2-weighted fluid-attenuated inversion recovery imaging (repetition time, 10,000 ms; echo time, 93 ms; slice thickness, 5 mm; matrix, 224 × 320), axial gadolinium-enhanced T1-weighted imaging (repetition time, 400 ms; echo time, 11 ms; slice, 5 mm; matrix, 230 × 384) and diffusion-weighted imaging (repetition time, 5300 ms; echo time, 69 ms; slice, 5 mm; matrix, 160 × 160; b-value 1000 s/mm$^2$) were performed.

PET studies were performed using a Biograph mCT PET/CT scanner (Siemens Medical Solutions, Knoxville, TN, USA). PET scans were acquired in the 3-dimensional model, and PET images were reconstructed as described in our previous study (simultaneous acquisition of 51 transverse images per field of view [FOV], with an intersection spacing of 3 mm, for a total axial FOV of 15 cm) [16]. PET radiotracers were produced using an HM-18 cyclotron (Sumitomo Heavy Industries, Tokyo, Japan). The radiochemical purity of FMISO was > 95% [17]. Transmission and regional emission images of the brain were obtained as described in our previous study [10]. Fasting was initiated 6 h before all PET studies, and the examination schedule was as follows: MRI, including contrast examination, on day 1; and FMISO on day 2.

PET data analysis

Uptake of FMISO in the brain tumor was semiquantitatively assessed by evaluating the maximum standardized uptake value (SUVmax). FMISO-PET images were converted into average venous blood concentrations of FMISO to obtain tumor-to-blood ratios (TBRs), allowing 3-dimensional pixel-by-pixel calculation of maximum TBR (TBRmax) for SUVmax. A cutoff value of 1.2 for FMISO TBR was used to determine the hypoxic volume (HV) [14, 18]. PET and MRI datasets were transferred to a Linux workstation and co-registration of FMISO/MRI was performed using Dr. View/Linux version R2.5 (AJS, Tokyo, Japan). Before reaching histopathological and molecular diagnoses, two radiologists analyzed the data to lower the risk of observer bias as much as possible.

Immunohistochemistry

Immunohistochemical analyses were performed on 4-µm sections of formalin-fixed, paraffin-embedded tissue from the tumors. Sections were stained using anti-CA9 antibody (1:1000, #ab15086; abcam, Cambridge, MA, USA), anti-CD34 antibody (1:100, #M7165; Dako, Glostrop, Denmark), anti-nestin antibody (1:1,000, MAB5326; Sigma-Aldrich, St. Louis, MO, USA), anti-FOXM1 antibody (1:250, #ab207298; abcam), anti-CD163 (1:500, #93498; CSTjapan, Tokyo, Japan), anti-PD-1 (1:200, #86163; CSTjapan), anti-PD-L1 (1:200, #13684; CSTjapan), and FOXP3 (1:100, #98377; CSTjapan). Antigen retrieval for FOXM1, HIF-1α, CD34, CD163, PD-1 and FOXP3 staining was performed in 10-mM citrate buffer (pH 6.0) using an autoclave. Antigen retrieval was performed in pH 9.0 retrieval buffer (Dako) using an autoclave for PD-L1 staining. Immunohistochemical staining was assessed by three authors (TS, NF, and TT) who were blinded to clinical information.

Immunohistochemical findings were assessed as previously described [12, 13, 19]. For CA9, the degree of expression was assessed as follows: 2, universal strong expression around necrotic regions; 1, occasional expression (typically around necrotic regions); and 0, negative staining. For quantitative evaluation of CD34+ vessels, stained sections were screened in a low-power field (×40), and the five middle-power fields (×200) showing the densest spots were assessed. For quantitative evaluation of nestin, both stain intensity and stain pattern were assigned numerical scores according to the Allred scoring system as Spence et al. reported [20]. Overall intensity is scored using a 4-point system: 0, no staining; 1, light staining; 2, medium staining; and 3, strong staining. Scoring for the extent of stain is given a numerical value and divided into six categories rather than three: 0, no stain; 1, ≤1/100 cells stained; 2, ≤1/10 cells stained; 3, ≤1/3 cells stained; 4, ≤2/3 cells stained; and 5, all cells stained. For quantitative evaluation of FOXM1, the percentage of tumor nuclei reactive to FOXM1 antibody was estimated following examination of a middle-power field (×200) using Gunma labeling index software [21]. For the assessment of PD-1, FOXP3, and CD163, stained sections were screened in a low-power field (×40) and five high-power fields (×400). Expression of PD-L1 was scored as described previously: 3+, ≥50%; 2+, ≥5% but < 50%; 1+, ≥1% but < 5%; and 0, ≤1%.
Statistical analyses

Paired-samples t-testing was used to compare FMISO TBRmax and HV before and after Bev treatment and before and after recurrence in recurrent cases. To compare FMISO TBRmax and HV between two groups, t-tests were used. For pathological markers, the Mann–Whitney U test was used for comparisons between two groups. Correlations between FMISO TBRmax and various markers were also analyzed using Spearman's rank correlation for the sample at the time of first surgery. All p-values were two-sided with the significance level set to p < 0.05.

Results

Patients

FMISO-PET was performed on 7 patients with newly diagnosed GBM during the observation period. Of these, 3 were included in the preoperative neo-Bev group and 4 in the Control group (Table 1).

The preoperative neo-Bev group included 2 men and 1 woman with a mean age of 69.7 years (range, 65–78 years). The Control group included 3 men and 1 woman with a mean age of 59.8 years (range, 32–76 years). In the preoperative neo-Bev group, two cases (Cases 1 and 2) showed recurrence during the observation period.

FMISO-PET findings

Comparing FMISO TBRmax and HV in PET scans taken before (pre-Bev) and after Bev (post-Bev) in the preoperative neo-Bev group, no significant difference was seen in TBRmax (p = 0.13), but HV was significantly lower after Bev (p = 0.03) (Table 1; Fig. 2a, b). No differences in FMISO TBRmax (p = 0.56) or HV (p = 0.09) were evident between the preoperative neo-Bev and Control groups on PET scans taken before craniotomy (Fig. 2c, d). The two patients in the preoperative neo-Bev group who developed recurrence showed higher values of FMISO TBRmax at the time of recurrence compared to before tumor removal (Table 2).
Table 2

<table>
<thead>
<tr>
<th>Case</th>
<th>Bev treatment</th>
<th>FMISO-PET</th>
<th>Immunohistochemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pre-op TBR</td>
<td>Pre-op HV</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case 1</td>
<td>Pre-neo-Bev</td>
<td>3.17</td>
<td>7.9</td>
</tr>
<tr>
<td></td>
<td>Post-neo-Bev</td>
<td>2.14</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td>Refractory Bev</td>
<td>2.56</td>
<td>2.5</td>
</tr>
<tr>
<td>Case 2</td>
<td>Pre-neo-Bev</td>
<td>2.62</td>
<td>6.41</td>
</tr>
<tr>
<td></td>
<td>Post-neo-Bev</td>
<td>1.96</td>
<td>5.42</td>
</tr>
<tr>
<td></td>
<td>Refractory Bev</td>
<td>2.77</td>
<td>1.41</td>
</tr>
<tr>
<td>Case 3</td>
<td>Pre-neo-Bev</td>
<td>4.02</td>
<td>6.26</td>
</tr>
<tr>
<td></td>
<td>Post-neo-Bev</td>
<td>3.86</td>
<td>4.59</td>
</tr>
<tr>
<td>Control 1</td>
<td>3.12</td>
<td>5.12</td>
<td>75</td>
</tr>
<tr>
<td>Control 2</td>
<td>1.55</td>
<td>10.8</td>
<td>45.2</td>
</tr>
<tr>
<td>Control 3</td>
<td>3.72</td>
<td>17.9</td>
<td>44</td>
</tr>
<tr>
<td>Control 4</td>
<td>4.36</td>
<td>13.8</td>
<td>29.8</td>
</tr>
</tbody>
</table>

Bev, bevacizumab; Control, without preoperative neoadjuvant therapy containing bevacizumab; HV, hypoxic volume; Post-neo-Bev, after neoadjuvant bevacizumab therapy and before surgery; Pre-op, preoperative; Pre-neo-Bev, before neoadjuvant bevacizumab therapy and surgery; Refractory Bev, refractory after bevacizumab therapy; TBR, tumor-blood ratio.

Immunohistochemistry analyses

To investigate alterations on FMISO-PET and in the TME of GBM during Bev therapy, expressions of a marker of hypoxia (CA9), markers of stemness (nestin, FOXM1), and immunoregulatory molecules (CD163, PD-1, and PD-L1) were evaluated (Table 2). Interestingly, CA9 and PD-L1 scores correlated with FMISO TBRmax (CA9: r = 0.90, p = 0.006; PD-L1: r = 0.88, p = 0.009) (Fig. 3a, b). Next, we analyzed differences between specimens from initial surgery in the preoperative neo-Bev and Control groups. All but one case in the Control group showed strong expression of CA9, corresponding with high FMISO TBRmax (Fig. 4a, b). A case showing negative CA9 expression in the Control group revealed lower FMISO TBRmax than cases appearing strongly positive for CA9. On the other hand, the neo-Bev group tended to show faint positive expression of CA9 with lower FMISO TBRmax compared with the Control group (Fig. 4d, e). Besides, after comparing other candidate markers, FOXM1 showed a trend toward higher expression in the Control group (p = 0.06; Fig. 4c, f). Next, we analyzed the difference between initial and reoperation specimens in the preoperative neo-Bev group. We found that CA9 was only faintly positive at initial surgery, although to the extent that expression of CA9 was not reflected in the score in Case 1, staining was more prominent at reoperation with increasing FMISO TBRmax (Fig. 4e, h). Moreover, FOXM1 in both cases increased at reoperation (Fig. 4f, i). However, alterations of other markers varied (Table 2).

Regardless of statistical significance, expressions of the endothelial marker (CD34) and the immunoregulatory molecules (CD163, FOXP3, PD-1, and PD-L1) tended to be lower in the preoperative neo-Bev group than in the Control group (Fig. 5a-f).
Discussion

We report here the first study using FMISO-PET in the follow-up of GBM patients treated with preoperative chemoradiotherapy including Bev before and after preoperative neo-Bev and for a long period of time up to the time of recurrence. In this study, FMISO-PET was used to confirm that even though initial treatment with Bev improved the oxygenation of hypoxic tumor tissues, hypoxic changes in TME occur at recurrence despite the administration of Bev. Similar changes were also confirmed pathologically.

As hypoxia in tumor tissues decreases radiosensitivity, FMISO-PET was initially used to identify hypoxic areas for systemic cancers before and after radiotherapy [1]. After Valk et al. first applied FMISO-PET to scanning brain tumors, this modality has often been used to delineate hypoxic regions within brain tumors, particularly gliomas [22]. FMISO-PET has been compared to and used in conjunction with other modalities such as other PET scans, dynamic susceptibility contrast MRI and contrast-enhanced MRI in relation to blood flow and metabolism. Previous studies have reported that the degree of FMISO accumulation is low in low-grade gliomas and high in high-grade gliomas, indicating consistency between grade and FMISO-PET findings [20, 23, 24]. Cher et al. suggested that FMISO-PET may be useful for detecting recurrent disease in clinical practice for patients with glioma [24]. Kawai et al. found that HV for FMISO correlated strongly with volume as assessed by contrast-enhanced MRI, and PET findings correlated with VEGF, reflecting a hypoxic environment [14, 15].

After Bev became available as an inhibitor of angiogenesis, vascular normalization by Bev was reported, then FMISO-PET was applied as a method to evaluate therapeutic efficacy [8]. Together with findings from contrast-enhanced MRI, Yamaguchi et al. reported that patients with improved oxygenation and loss of contrast enhancement effect after Bev administration show good prognosis [9]. According to Gerstner et al., the effects of Bev treatment varied among individuals, with no significant difference in FMISO accumulation before and after Bev treatment for recurrent GBM [10]. Similarly, in another study, we evaluated the relationship between prognosis of patients with recurrent GBM under Bev treatment and FMISO-PET findings. The results showed that median progression-free survival (PFS) and overall survival (OS) were longer in GBM patients with decreased TBR for FMISO after Bev therapy than in other GBM patients with increased TBR for FMISO. The patient group with reduced HV for FMISO after Bev therapy showed better results than the other group with increased HV in terms of both PFS and OS [in press]. In other words, decreased TBR and HV for FMISO may offer useful biomarkers and could better predict outcomes in GBM patients receiving Bev therapy. A recent study used FMISO-PET to evaluate the therapeutic effects of evofosfamide, a prodrug of nitromisonidazole that is used to improve hypoxic areas of recurrent GBM refractory to Bev treatment [11]. Thus, all reports related to the use of Bev for GBM have been for recurrent cases and have been limited to short-term observations of the course of treatment.

In our previous reports, preoperative neo-Bev was useful to control intraoperative hemorrhage due to reductions in tumor vascularity [12]. We have verified the safety and clinical benefits of preoperative neo-Bev in a multicenter exploratory prospective clinical phase I/II study [manuscript under preparation]. We have also compared the status of TME, including tumor oxygenation and stemness, by immunohistochemical analyses of paired samples obtained from the same patients who underwent repeated surgeries following preoperative neo-Bev administration as well as at the time of recurrence [4, 12, 13, 19]. In this context, we have described the effects of Bev therapy in improving hypoxic and immunosuppressive TMEs. By adding FMISO-PET to preoperative neo-Bev-based GBM therapeutics, we were able to obtain PET findings before and after Bev administration and clarify hypoxic changes during long-term follow-up, even in patients who initially responded well to treatment. In the current study, we identified a positive correlation between FMISO TBRmax and in situ CA9 expression. Furthermore, we were able to visualize alterations in tumor oxygenation throughout the clinical course. This finding may support therapeutic interventions.

Regardless of the statistical significance of immunohistochemical markers in the present study between the preoperative neo-Bev and Control groups, a hypoxic marker (CA9), stem cell markers (nestin and FOXM1), immunosuppressive cell markers (CD163 and Foxp3) and an immune checkpoint molecule (PD-L1) tended to decrease during effective Bev therapy. CA9 is known to correlate with FMISO accumulation, and the present results are also consistent with previous reports that PD-L1
correlates with hypoxia [25–27]. This study confirmed that CA9 and PD-L1 expressions correlated significantly with FMISO TBRmax. Thus, FMISO-PET could allow the identification of the hypoxic niche preoperatively and may effectively detect recurrent lesions during post-neo-Bev follow-up, which would presumably be useful for comprehensive evaluation in combination with contrast-enhanced MRI.

Some limitations to the current study need to be kept in mind. Since this was a retrospective study with a small number of cases, verification in a larger number of cases is essential to draw conclusions. Furthermore, the addition of RT and TMZ as preoperative therapeutic interventions makes the simple interpretation of the effects of preoperative neo-Bev more difficult.

Tumor oxygenation and associated alterations to the TME (including stemness, recruitment of immunosuppressive cells and induction of immune checkpoint molecules) should play a pivotal role in assessing the clinical benefits of Bev therapy. Given that RT and TMZ would have little effect on hypoxia and immunoregulatory molecules as determined by previously reported immunohistochemical findings [28], the current immunohistochemical and PET findings during Bev therapy might facilitate elucidation of the mechanisms underlying the effectiveness of and resistance to Bev therapy, and identification of suitable biomarkers for therapeutic response and clinical outcomes for the combination of immunotherapy with Bev.

**Conclusion**

This study revealed that preoperative neo-Bev induced tumor oxygenation that could be visualized on FMISO-PET. In addition, FMISO-PET was useful in monitoring the deterioration of oxygenation within the TME during the course of GBM treatment. This study strengthens the concept that preoperative neo-Bev may promote an immunosupportive TME with reductions in stemness markers and immunoregulatory molecules. Further research is required to confirm whether FMISO-PET is valid for assessing the duration and intensity of effects due to Bev treatment for GBM.

**Abbreviations**

Bev: bevacizumab  
FMISO-PET: $^{18}$F-fluoromisonidazole-positron emission tomography  
FOV: field of view  
GBM: glioblastoma  
HIF1-α: hypoxia-inducible factor-1α  
HV: hypoxic volume  
neo-Bev: neoadjuvant bevacizumab  
OS: overall survival  
PFS: progression-free survival  
RT: radiation therapy  
SUVmax: maximum standardized uptake value  
TBRmax: maximum tumor-to-blood ratio  
TBR: tumor-to-blood ratio  
TME: tumor microenvironment
Declarations

Ethics approval and consent to participate

This study was approved by the Ethical Committee of Jikei University and Kagawa University (The Jikei University; 27-239, Kagawa University; Heisei 28-163, Heisei 30-087, 2019-026, 2019-027). All procedures performed in the studies involving human participants were in accordance with the ethical standards of the institutional research committee of both institutes (The Jikei University; 27-239, Kagawa University; Heisei 28-163, Heisei 30-087, 2019-026, 2019-027) and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Written informed consent was obtained from the patients.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Consent for publication

Written informed consent was obtained from all seven patients for publication of this study and accompanying images.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by the Ministry of Education, Culture, Sports, Science, and Technology and the Japan Society for Promotion of Science (KAKENHI) (grant no. 21K09161), and a Grant for Multi-Institutional Collaborative Project by Jikei University School of Medicine (grant no. 2022-602DC).

Authors' contributions

TS analyzed and interpreted the patient data regarding FMISO-PET and IHC findings and was a major contributor in writing the manuscript. JT and YY performed the histological examination and contributed to editing the manuscript. NF and TT assessed the histological findings. KS, DO and KM performed the FMISO-PET examination. YA, YM, MS, KM and TT contributed to editing the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

References


**Figures**

**Figure 1**

![Figure 1](image-url)
Schema representing the schedule of treatment and FMISO-PET in the current study. In the preoperative neo-Bev group (neo-Bev group), all patients received two courses of Bev at 10 mg/kg, followed by radiation therapy (40 Gy in 16 fractions or 60 Gy in 30 fractions) and temozolomide (TMZ) at 75 mg/m² for 42 days. FMISO-PET was performed: 1) before preoperative neo-Bev; 2) after neo-Bev (5 weeks after end of Bev); 3) after surgery (only in some cases); and 4) at recurrence (only in cases that recurred). In the Control group, FMISO-PET was performed before surgery alone.

**Figure 2**

a, b) FMISO accumulation as represented by TBRmax (a) and HV (b) before and after neo-Bev. c, d) FMISO accumulation as represented by TBRmax (c) and HV (d) in the neo-Bev group compared with the Control group. *p ≤ 0.05
Correlation between preoperative TBRmax and a marker of hypoxia and an immune checkpoint molecule. Expressions of CA9 (a) and PD-L1 (b) correlate strongly with TBRmax (CA9: $r=0.90$, $p=0.006$; PD-L1: $r=0.88$, $p=0.009$).
Figure 4

FMISO-PET (a, d, g) and findings from immunohistochemical analysis (b, e, h: CA9; c, f, i: FOXM1) for representative cases from the Control group (a–c: Control 1) and preoperative neo-Bev group (d–f: Case 2 at initial surgery; g–i: Case 2 at second surgery). Comparing specimens from the preoperative neo-Bev group at initial surgery and reoperation, CA9, as a marker reflecting a hypoxic environment, was only faintly positive at initial surgery and was more prominent at reoperation. e, h) For FOXM1, a trend toward higher FOXM1 was seen in the control group, but the difference was not significant (c, f, i: p=0.06). Photomicrograph of immunohistochemistry (×200; bar = 200 μm). CA9, carbonic anhydrase 9; FOXM1, forkhead box M1.

Figure 5

Findings from immunohistochemical analysis (a, d: CD34; b, e: CD163; c, f: PD-L1) for representative cases from the Control group (a–c: Control 1) and Pretreatment group (d–f: Case 2 at initial surgery). Regardless of statistical significance, expressions of the endothelial marker (CD34) and immunoregulatory molecules (CD163, PD-L1) tended to be lower after neo-Bev compared to the Control group. Photomicrograph of immunohistochemistry (×200; bar = 200 μm)