Assessment of radiographic and prognostic characteristics of programmed death-ligand 1 expression in high-grade gliomas

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Abstract

Purpose Gliomas are characterized by immunosuppressive features. Programmed death-ligand 1 (PD-L1) is overexpressed and plays an important role in the immunosuppressive tumor microenvironments of gliomas. However, the radiographical and prognostic significance of PD-L1 expression remains unclear.

Methods Using tissue microarrays, we evaluated PD-L1 expression and the presence of tumor-infiltrating CD4+ and CD8+ T cells and CD204+ macrophages using immunohistochemical analysis. Contrast enhancement area and fluid-attenuated inversion recovery (FLAIR) hyperintensity area were evaluated by two-dimensional analysis. Kaplan–Meier analysis was performed to evaluate the overall survival time in 44 patients with isocitrate dehydrogenase (IDH)-wildtype glioblastoma.

Results We evaluated 71 patients with newly diagnosed high-grade gliomas who were treated between October 1998 and April 2012. PD-L1 expression was observed in 15 patients (21.1%). A significant association of PD-L1 expression with the CD4+ and CD8+ T cell densities, but not with CD204+ macrophage densities, was observed (p = 0.025, p = 0.0098, and p = 0.19, respectively). The FLAIR-to-enhancement ratio was significantly higher in PD-L1+ tumors than in PD-L1− tumors (p = 0.0037). PD-L1 expression did not show a significant association with the median survival time (PD-L1+ vs. PD-L1−: 19.2 vs 14.9 months; p = 0.39).

Conclusion PD-L1 expression was associated with CD4+ and CD8+ T cell infiltration, indicating a significant interplay between PD-L1 and immune cells. The positive correlation of PD-L1 expression with an increased FLAIR-to-enhancement ratio suggested that radiographical characteristics could reflect the immunological status. Our results did not support the prognostic impact of PD-L1 in patients with IDH-wildtype glioblastomas.

Keywords Programmed death-ligand 1 · High-grade glioma · Tumor-infiltrating lymphocyte · FLAIR-to-enhancement ratio · IDH-wildtype glioblastoma

Introduction

Glioma is the most common type of primary brain tumor, and glioblastoma is the most aggressive type of glioma. The standard management of glioblastomas include maximal safe resection, radiotherapy, temozolomide (TMZ) therapy, and tumor-treating fields. Patients with glioblastomas have a poor prognosis, with a 5 year survival rate of 15% in Japan [1]. Patient age, performance status, extent of resection, neurological function, isocitrate dehydrogenase (IDH) 1/2 mutations, and O-6-methylguanine deoxyribonucleic acid methyltransferase (MGMT) promoter methylation are the most consistently reported prognostic factors for such patients [2–4].
Glioma is characterized as having an immunosuppressive tumor microenvironment [5]. This microenvironment is mediated by a complex immune network of tumor cells and tumor-infiltrating immune cells (TIICs), such as tumor-infiltrating lymphocytes (TILs), tumor-associated macrophages, and myeloid-derived suppressor cells (MDSCs). Effector cells, including CD8+ T cells, attack the tumor cells and exert an anti-tumor activity. Conversely, suppressor cells, including regulatory T cells (Treg), M2 macrophages, and MDSCs, inhibit the anti-tumor activity, thereby allowing tumor progression and attenuating drug efficacy. The balance between the effector and suppressor cells could be a determinant of clinical outcomes [6, 7].

Programmed death-ligand 1 (PD-L1) is an immune checkpoint molecule and a co-inhibitory ligand expressed in many types of tumor cells. Its incidence in glioblastomas is 19–61% [2, 8–12]. Binding of PD-L1 to its receptor, programmed cell death 1 (PD-1), induces T cell dysfunction and apoptosis by increasing cytokine production; this results in immune escape of diffuse gliomas [13]. Furthermore, PD-L1 is associated with immunosuppressive cells, including Treg and M2 macrophages, and causes strong immune suppression [14–16]. Thus, the PD-1/PD-L1 axis plays an important role in facilitating immune escape of gliomas [17]. Although the prognostic impact of PD-L1 expression has been explored in patients with gliomas [2, 8–11, 18], the results have been heterogeneous and the prognostic value of PD-L1 remains controversial [17].

Immune checkpoint inhibitors elicit durable responses in a variety of solid malignancies; however, they have not shown significant efficacy against gliomas [19, 20]. One study in patients with recurrent glioblastomas showed that nivolumab did not show survival benefit over bevacizumab with an objective response rate of 7.8% [20]. In addition, neoadjuvant strategies of anti-PD-1 blockade showed heterogeneous survival benefits [21, 22]. These studies indicate the need to identify patient population who could have therapeutic benefit from immune therapy.

Radiographic modalities, such as magnetic resonance imaging (MRI), are widely used in the noninvasive diagnosis of gliomas. MRI is very useful for visualizing anatomical abnormalities and evaluating the pathological characteristics of tumors; however, whether radiographical assessment can determine the immune status remains unclear [23–25].

In this study, we aimed to examine the relationship of PD-L1 expression with TIICs and genetic alterations in 71 patients with high-grade gliomas and investigate imaging features reflective of PD-L1 expression and TIIC infiltration in 62 patients who showed a single enhancing lesion. We also determined the prognostic significance of PD-L1 expression in 44 patients with IDH-wildtype glioblastomas.

Materials and methods

Patients and clinical data collection

We used a tissue microarray (TMA) constructed from 100 high-grade gliomas in patients who were treated between October 1998 and April 2012 at our institute. Among these, 24 and 5 patients with recurrent tumors and tumors with poor immunohistochemical staining results were excluded, respectively. The remaining 71 patients with newly diagnosed tumors were included in this retrospective observational study. All tumors were diagnosed by neuropathologists at our hospital and were reclassified according to the World Health Organization (WHO) 2021 classification [26]. The patients’ charts were reviewed for clinical history, Karnofsky performance status (KPS), date of initial surgery, date of tumor recurrence, date of death, and last hospital visit.

Tissue microarray

Formalin-fixed, paraffin-embedded tissue blocks and the corresponding hematoxylin and eosin (H&E)-stained slides were overlaid for TMA sampling. A 2.0 mm cylindrical core was obtained for each case. Microarray blocks were sectioned to obtain 4 μm thick sections.

Immunohistochemistry

Immunohistochemical staining was performed using TMA with autostainers [Ventana BenchMark ULTRA platform (Ventana) and Autostainer Link 48 (Dako)] [27]. We assessed cell surface markers for TIICs (including CD4, CD8, and CD204) and PD-L1 expression in the tumor cells. The primary antibodies used were as follows: 4B12 (1:50; for CD4), 4B11 (1:50, for CD8; Leica Microsystems, Newcastle, UK), SRA-C6 (1:200, for CD204; Trans Genic Inc., Kumamoto, Japan), and E1L3N (1:800, for PD-L1; Cell Signaling Technology, Danvers, MA) [27, 28].

Immunohistochemical evaluation

The microscopic images were scanned and digitized using a NanoZoomer Digital Pathology system (Hamamatsu Photonics, Hamamatsu, Japan). The H&E-stained slides were viewed at × 40 magnification, and tumor areas were selected as the area of interest; non-neoplastic brain tissue, necrotic areas, and empty spaces were manually excluded from the analyses.

The numbers of CD4+ T cells, CD8+ T cells, and CD204+ macrophages were counted within the area of
interest using a Patholoscope (Mitani Corp). The cellular density was calculated as the number of stained cells divided by the area of the area of interest (1.512 mm²).

PD-L1 staining was evaluated by two neuropathologists (AY. and KS.) and classified as diffuse, focal, or absent; PD-L1+ tumors were defined as either focally or diffusely stained, while PD-L1− tumors were defined as unstained. For CD4+ and CD8+ T cell densities and CD204+ macrophage densities, cut-off values were arbitrarily defined as >75% percentile positive cells/mm²; for the CD8/CD204 ratio, the cut-off value was defined as >75% percentile of the ratio. These cut-off values for CD4+ T cell densities, CD8+ T cell densities, and CD204+ macrophage densities corresponded to 20, 20, and 600 cells/mm², respectively; for the CD8/CD204 ratio, the cut-off value corresponded to 0.02.

Tumors were classified into four groups based on PD-L1 positivity/negativity and CD8+ T-cell densities above/below the cut-off value (i.e., a combined PD-L1/CD8 classification).

### IDH1/2 mutation and MGMT promoter methylation analysis

**IDH1/2 mutation status** and **MGMT promoter methylation status** were evaluated in 50 and 55 tumors, respectively. Briefly, tumor DNA was extracted from frozen tumor tissues using a DNeasy Blood & Tissue Kit (Qiagen, Tokyo, Japan). The presence of hotspot and other known mutations in **IDH1** (R132) and **IDH2** (R172) was assessed by pyrosequencing, as described previously [29]. The **MGMT** promoter methylation status was analyzed using bisulfite modification of the tumor genomic DNA, followed by pyrosequencing, as described previously [30]; the status was defined as methylated and unmethylated when its mean levels at 16 CpG sites were ≥ 16% and < 16%, respectively [30, 31].

### Imaging assessment

Contrast enhancement area was calculated as the product of perpendicular diameters of contrast-enhancing lesions on T1-weighted images [32]. Fluid-attenuated inversion recovery (FLAIR) hyperintensity area was calculated as the product of the perpendicular diameters of hyperintensity areas on FLAIR or T2-weighted images [33]. The ratio of the FLAIR hyperintensity area to the contrast enhancement area (i.e., the FLAIR-to-enhancement ratio) was obtained by dividing the FLAIR hyperintensity area by the contrast enhancement area.

### Statistical analysis

The differences between PD-L+ and PD-L1-tumors with respect to CD4+ T-cell, CD8+ T-cell, and CD204+ macrophage densities were evaluated using the Mann–Whitney U test. The differences between PD-L+ and PD-L1− tumors with respect to IDH1/2 mutation status, and MGMT promoter methylation status were evaluated using the χ² test. The differences in imaging parameters among tumors categorized by their PD-L1 expression, CD4+ T-cell, CD8+ T-cell, and CD204+ macrophage densities were evaluated using the Mann–Whitney U test. A receiver operating characteristic (ROC) analysis was performed to determine an optimal cutoff value of the FLAIR-to-enhancement ratio to provide the highest combination of sensitivity and specificity for differentiating PD-L1 expression. Overall survival (OS) was defined as the interval between the date of the initial surgery and the date of death or the last follow up. Progression-free survival (PFS) was defined as the interval between the date of initial surgery and the date of progression, death, or last follow up. OS and PFS were calculated using the Kaplan–Meier method and compared using the log-rank test. Statistical significance was set at p < 0.05. Statistical tests were performed using JMP® ver. 16.2.0 for Mac (SAS Institute Japan, Tokyo, Japan), and GraphPad Prism® ver. 9.4.1 for Mac (GraphPad Software, La Jolla, CA, USA).

### Results

#### Patient characteristics

The patients’ characteristics are summarized in Table 1. The population comprised 31 men (43.7%) and 40 women (56.3%) with median age of 64 years. The tumors comprised 44 glioblastomas, IDH-wildtype, WHO grade 4 (62.0%), 4 astrocytomas, IDH-mutant, WHO grade 4 (5.6%), 20 glioblastomas, not otherwise specified (NOS; 28.2%), 2 oligodendrogliomas, IDH-mutant and 1p19q co-deleted, WHO grade 3 (2.8%); and 1 high-grade astrocytoma, NOS (1.4%). In 20 (28.2%) and 35 (49.3%) tumors, the MGMT promoter status was hypermethylated and hypomethylated, respectively.

Among the 44 patients with glioblastomas, IDH-wildtype, WHO grade 4, 34 patients (77.3%) received combined radiotherapy and temozolomide (TMZ)-based chemotherapy, 7 (15.9%) received combined radiotherapy and nimustine hydrochloride (ACNU)-based chemotherapy, and 3 (6.8%) received radiotherapy alone. Furthermore, 29 patients (65.9%) underwent total or subtotal resection, while 15 patients (34.1%) underwent biopsy or partial resection (Supplementary Table 1).
Correlation of PD-L1 expression with TIICs densities, IDH1/2 mutation, and MGMT promoter methylation status

PD-L1 staining was diffusely and focally positive in eight (Fig. 1A) and seven (Fig. 1B) tumors, respectively. Therefore, these 15 tumors (21.1%) were defined as PD-L1+ tumors; the remaining 56 tumors (78.9%) that showed no PD-L1 staining (Fig. 1C) were defined as PD-L1- tumors.

Immunohistochemical findings for CD4, CD8, and CD204 are shown in Fig. 2A–D, respectively. The median number of CD4+ and CD8+ cell densities was significantly higher in PD-L1+ tumors than in PD-L1− tumors [PD-L1+ vs PD-L1−: 24.5 vs 5.3 cells/mm² for CD4+ cells (p = 0.025, Fig. 2A); 19.8 vs 6.3 cells/mm² for CD8+ cell densities (p = 0.0098, Fig. 2B)]. There was no significant difference in the median number of CD204+ cell densities between PD-L1+ and PD-L1− tumors (332.7 vs 512.6 cells/mm²; p = 0.19; Fig. 2C). The frequencies of PD-L1+ tumors were 16.7% and 27.3% in IDH-mutant tumors (n = 6) and IDH-wildtype tumors (n = 44), respectively; however, this difference was not statistically significant (p = 0.58; Fig. 2D). Furthermore, 30.0% and 20.0% of the PD-L1+ tumors were MGMT hypermethylated (n = 20) and hypomethylated (n = 35), respectively; this difference was not statistically significant (p = 0.40; Fig. 2E).

Imaging characteristics

We evaluated the imaging characteristics of 62 patients who showed a single contrast-enhancing lesion. The contrast enhancement and FLAIR hyperintensity areas did not differ between the PD-L1− and PD-L1+ tumors (p = 0.18 and p = 0.84, respectively) and among tumors with low and high of CD4+ T cell densities (p = 1.00 and p = 0.86, respectively), CD8+ T cell densities (p = 0.33, and p = 0.17, respectively), and CD204+ macrophage densities (p = 0.23, and p = 0.39, respectively). The FLAIR-to-enhancement ratio was significantly higher in PD-L1+ tumors than in PD-L1− tumors (p = 0.0037), but not in tumors with low and high tumors of CD4+ T cell densities, CD8+ T cell densities, and CD204+ macrophage densities (p = 0.14, 0.44, and 0.85, respectively; Table 2). The optimal cutoff value of the FLAIR-to-enhancement ratio for differentiating between tumors with and without PD-L1 expression was 1.83, as calculated by an ROC curve with sensitivity of 78.6%, specificity of 72.9%, and area under the curve of 0.75. The PD-L1− tumors were observed in 92.1% (35/38) among the tumors with FLAIR to enhancement ratio below the cutoff value, whereas the PD-L1+ tumors were in 45.8% (11/24) of the tumors with FLAIR to enhancement ratio above the cutoff value (p = 0.0011) (Fig. 3B).

Prognostic impact in patients with IDH-wildtype glioblastomas

Next, we focused on the 44 patients with IDH-wildtype glioblastomas to evaluate the prognostic impact of PD-L1 and TIICs. Kaplan–Meier analysis did not show a significant association of PD-L1 expression with the OS or PFS [median survival time (MST): PD-L1+ vs PD-L1−, 19.2 vs 14.9 months (p = 0.39); median PFS (mPFS): PD-L1+ vs PD-L1−, 9.6 vs 8.1 months (p = 0.46)]. Tumor-infiltrating CD4, CD8, and CD204 cells densities and the ratio of CD8/CD204 cells densities were not significantly associated with patient outcomes (Supplementary Table 2). Moreover, 44 patients with IDH-wildtype glioblastomas were categorized into the PD-L1+/CD8 high (five patients, 11.4%), PD-L1+/CD8 low (five patients, 11.4%), PD-L1−/CD8 high (two patients, 4.5%), and PD-L1−/CD8 low (27 patients, 61.4%) groups. The MST and mPFS did not differ significantly among these groups (Supplementary Figure 1, Supplementary Table 2).

Discussion

In this study, we showed a significant association of PD-L1 expression with CD4+ and CD8+ T cell densities, but not with CD204+ macrophage densities. IDH1/2 mutation
status, and MGMT promoter methylation status. Our radiographical assessment revealed that PD-L1 expression was significantly associated with the FLAIR-to-enhancement ratio. Furthermore, PD-L1 expression, TIICs densities, and four groups categorized according to the combined PD-L1/CD8 classification were not associated with the PFS or OS.

We found a positive correlation between PD-L1 expression and CD4+ and CD8+ T cell densities, indicating a significant interplay between PD-L1 and the CD4+ and CD8+ T cells. Lee et al. also showed that PD-L1 expression was associated with CD3+ T cell infiltration [2]. PD-L1 is usually undetectable in most normal tissues, including the normal cortex and white matter [34, 35]. However, inflammatory cytokines, particularly interferons released by TIL, drive tumor PD-L1 expression; this in turn triggers TIL inhibition [35]. Therefore, the positive correlation between PD-L1 expression and CD4+ and CD8+ T cell densities could indicate an adaptive immune resistance mechanism in high-grade gliomas for suppressing the functions of the local effector T-cells and escaping immuno-surveillance. We did not observe any association between CD204+ macrophage densities and PD-L1 expression, but found an abundant CD204+ macrophage densities; this abundant CD204+ macrophage infiltration in gliomas might contribute to immunosuppressive activities independent of PD-L1 [7].

Previous studies have reported that PD-L1 expression was significantly reduced in IDH1/2 mutant tumors than in IDH1/2 wildtype tumors [2, 34, 36]. We also found a lower PD-L1 expression in IDH1/2 mutant tumors than in IDH1/2 wildtype tumors; however, our small sample size did not have enough statistical power to meet the significance. Few studies have investigated the relationship between PD-L1 expression and MGMT promoter methylation status [2, 34]; Holzl et al. showed no correlation between the two [34], which was similar to our results.

Few studies have shown that radiographical assessment can determine the immune status, and their findings are inconsistent [23–25]. Narang et al. demonstrated the relationship between MRI-derived textural features and intratumoral CD3+ T-cell infiltration, indicating the potential value of radiological assessment of the immunological

Fig. 1 Immunohistochemical staining of programmed cell death-ligand 1 (PD-L1) and CD4, CD8, and CD204 (A–C) Representative figures show immunohistochemical staining of PD-L1 as diffusely positive (A), focally positive (B), and absent (C). (D–F) Representative figures show immunohistochemical staining of CD4 (D), CD8 (E), and CD204 (F)
status [24]. Tamura et al. reported the positive correlation between FLAIR hyperintensity area and the number of CD163+ cells in both peritumoral brain zone and tumor core, suggesting that infiltration of immunosuppressive cells could be related to the radiographical features [25]. Conversely, Dubinski et al. did not show that the edema-to-tumor ratio had no significant association with lymphocytic and/or myelocytic cells infiltration and suggested that the imaging analysis on extent of edema could not predict immune cell infiltration [23]. In our study, we showed the positive correlation of PD-L1 expression with an increased FLAIR-to-enhancement ratio, with a sensitivity of 78.6% and specificity of 72.9%. This is a key finding of this study, because it indicates that the radiographic finding can reflect the immunological status (such as the PD-L1 expression). In addition, the high negative predictive value for detecting the PD-L1− tumors was 92.1%, which might be clinically useful in daily practice.

The FLAIR hyperintensity surrounding the contrast-enhancing tumor component of a glioma represents vasogenic edema and non-contrast-enhancing tumor infiltration [37]. Glioma-associated vasogenic edema is caused by

Fig. 2 Correlation of programmed cell death-ligand 1 (PD-L1) expression with densities of tumor-infiltrating immune cells and genetic alterations (A) The median number of CD4+ cell densities is 24.5 cells/mm² in PD-L1+ tumors and 5.3 cells/mm² in PD-L1− tumors (p=0.025) (B) The median number of CD8+ cell densities is 19.8 cells/mm² in PD-L1+ tumors and 6.3 cells/mm² in PD-L1− tumors (p=0.0098) (C) The median number of CD204+ cell densities is 332.7 cells/mm² in PD-L1+ tumors and 512.6 cells/mm² in PD-L1− tumors (p=0.58) (D) The frequencies of PD-L1+ tumors are 16.7% and 27.3% in IDH-mutant tumors (n = 6) and IDH-wildtype tumors (n = 44), respectively (p=0.58) (E) The frequencies of PD-L1+ tumors are 30.0% and 20.0% in MGMT hypomethylated tumors (n = 20) and MGMT hypomethylated tumors (n = 35), respectively (p=0.40)
increased vascular permeability [38], which is due to immature tumor vessels that are induced by an excessive secretion of angiogenetic factors, such as the vascular endothelial growth factor (VEGF) [39]. VEGF expression is shown to be positively associated with PD-L1 expression in several cancers, including gliomas [40–42]. Thus, one possible mechanism underlying the positive correlation between the FLAIR-to-enhancement ratio and PD-L1 expression is that upregulated VEGF induces both vasogenic edema and PD-L1 expression.

Previous studies have shown heterogeneous findings on the prognostic value of PD-L1 expression in patients with glioma [2, 8–11, 18]; some have revealed a negative correlation between PD-L1 expression and prognosis [9–11], while others have revealed that PD-L1 is not correlated with prognosis [2, 8, 18]. Our results did not support the prognostic impact of PD-L1 expression on the OS and PFS of patients with IDH-wildtype glioblastomas. We also did not find prognostic differences among groups created using the combined PD-L1/CD8 classification. These observations indicate that the PD-1/PD-L1 pathway might play a limited role in the prognosis of IDH-wildtype glioblastomas through a complex interplay of immunosuppressive mechanisms, including other checkpoint molecules, Treg and M2 macrophages. Furthermore, tumors in the PD-L1+/CD8 high subgroup are thought to be the most likely to benefit from single-agent anti–PD-1/L1 blockade [43]; however, we found that only 11.4% of the IDH-wildtype glioblastoma patients were categorized into this subtype. Collectively, the limited role of the PD-1/PD-L1 pathway and the small subset of tumors in the PD-L1+/CD8 high subgroup might explain the previously reported low response rates to nivolumab treatment [20].

This study has some limitations. First, it was performed retrospectively and the sample size was small. Therefore, our results need to be confirmed in larger studies. Second, due to lack of standardization, the immunohistochemical evaluation method and parameters in our study differed from those in other studies, including the antibodies and criteria of positivity used. Due to these differences, our results need to be interpreted with caution. Third, the FLAIR-to-enhancement ratio was not evaluated by volumetric analysis. Although we acknowledged that compared to two-dimensional analysis, volumetric analysis could provide more detailed data and might detect smaller differences, we prioritize the easier application of the two-dimensional analysis in daily clinical practice.

In summary, we found a positive correlation of PD-L1 with CD4+ and CD8+ T-cell infiltration, indicating a significant interplay between PD-L1 and TILs. The positive
correlation of PD-L1 expression with an increased FLAIR-to-enhancement ratio suggests that radiographical assessment could reflect the immunological status. Our results did not support the prognostic impact of PD-L1 in patients with IDH-wildtype glioblastomas.

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Author contributions MO, SK, and YN designed the study. MO and SK conducted the statistical analysis. AY and KS contributed to tissue microarray preparation, diagnosis, and data acquisition. MO, YM, MT, SY, YT, KI, and YN contributed to the patients’ management. MO, YM, MT, SY, YT, KI, and YN contributed to sample collection, molecular analysis, data acquisition, and interpretation. MO, KS and YN wrote the first draft of the manuscript, and all authors edited it. All authors read and approved the final manuscript.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations竞

Competing interests The authors have no relevant financial or non-financial interests to disclose.

Ethical approval All procedures performed in this study were in accordance with the ethical standards of the institutional review board and the 1964 Helsinki Declaration and its later amendments. This study was approved by the Institutional Review Board of the National Cancer Center (2004–066 or 2007–086).

Consent to publish No individual personal data is contained in this manuscript.

Consent to participate Written informed consent was obtained from all individual participants included in the study.

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