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ORIGINAL ARTICLE

Maintenance of WT1 expression in tumor cells is associated with a good prognosis in malignant glioma patients treated with WT1 peptide vaccine immunotherapy

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Abstract

We have previously revealed the overexpression of Wilms' tumor gene 1 (*WT1*) in malignant glioma and developed WT1 peptide vaccine cancer immunotherapy. A phase II clinical trial indicated the clinical efficacy of the WT1 peptide vaccine for recurrent malignant glioma. Here, we aimed to investigate the immunological microenvironment in glioma tissues before and after WT1 peptide vaccine treatment. Paired tissue samples were obtained from 20 malignant glioma patients who had received the WT1 peptide vaccine for > 3 months and experienced tumor progression, confirmed radiographically and/or clinically, during vaccination. We discovered that the expression of WT1 and HLA class I antigens in the tumor cells significantly decreased after vaccination. Maintenance of WT1 expression, which is the target molecule of immunotherapy, in tumor cells during the vaccination period was significantly associated with a longer progression-free and overall survival. A high expression of HLA class I antigens and low $CD4^+/CD8^+$ tumor-infiltrating lymphocytes (TIL) ratio in pre-vaccination specimens, were also associated with a good prognosis. No statistically significant difference existed in the number of infiltrating $CD3^+$ or $CD8^+$ T cells between the pre- and post-vaccination specimens, whereas the number of infiltrating $CD4^+$ T cells significantly decreased in the post-vaccination specimens. This study provides insight into the mechanisms of intra-tumoral immune reaction/escape during WT1 peptide vaccine treatment and suggests potential clinical strategies for cancer immunotherapy.

Keywords Glioma · Wilms tumor gene 1 · Immunotherapy · Intra-tumor immune response · Cancer vaccine

Abbreviations

AA	Anaplastic astrocytoma
AE	Anaplastic ependymoma
AOA	Anaplastic oligoastrocytoma
CI	Confidence intervals
CR	Complete response
CTL	Cytotoxic T lymphocyte
GBM	Glioblastoma
HLA	Human leukocyte antigen
HR	Hazard ratio
IL	Interleukin
OS-WT1	Overall survival from the start of the WT1 vaccination
PD	Progressive disease
PD-1	Programmed cell death-1
PD-L1	Programmed cell death ligand-1
PFS-WT1	Progression-free survival from the start of the WT1 vaccination
PR	Partial response
SD	Stable disease
TAA	Tumor-associated antigen
TGF	Transforming Growth Factor
TIL	Tumor-infiltrating lymphocytes
Treg	Regulatory T cell
TRM	Tissue-resident memory T cell
TSA	Tumor-specific antigen
WHO	World Health Organization
WT1	Wilms' tumor gene 1

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Introduction

Malignant gliomas are the most common primary adult brain tumors, accounting for 70% of the primary malignant central nervous system tumors diagnosed in adults [1]. Following the older scheme of tumor classification by the World Health Organization (WHO) [2], gliomas are graded on a scale of I–IV, depending on the clinical prognosis. Grade III [anaplastic astrocytoma (AA), anaplastic oligoastrocytoma (AOA), and anaplastic ependymoma (AE)] and grade IV [glioblastoma multiforme (GBM)] gliomas are considered high-grade gliomas that are associated with a worse prognosis than grades I and II gliomas. Despite the search for effective treatments, patients with GBM, one of the most malignant gliomas, have a poor median survival of 14.6 months when treated with the current standard therapeutic combination of temozolomide and radiotherapy [3].

Since Burnet proposed the cancer immuno-surveillance hypothesis in the 1950s [4], there has been a surge of studies on antitumor immune responses against solid tumors. It is known that the induction of immune response against tumor-associated antigens (TAAs) or tumor-specific antigens (TSAs) is one of the antitumor mechanisms, and several TAAs have been identified in gliomas. These TAAs get exposed to the host immune system at the time of tumor cell death due to the inflammatory response during tumor development, thus inducing TAA-specific cytotoxic T lymphocytes (CTLs) that suppress the tumor [5]. It is essential to induce abundant TAAs-specific tumor-infiltrating lymphocytes (TILs) for successful immunotherapy, and some studies have reported that the presence of TAA-specific TILs is a favorable prognostic factor in melanoma [6] and other solid cancers [7]. However, it has been reported that most malignant tumors can escape from the immune system and even induce immunosuppressive activity [1]. Several recent studies have focused on overcoming this issue.

Therapeutic cancer vaccine immunotherapy induces antitumor TAA-specific CTL responses by the administration of adjuvant TAAs. We have previously shown the overexpression of Wilms' tumor 1 (WT1), a potent TAA [8], in glioma and developed a peptide-based cancer vaccine targeting WT1 (WT1 peptide vaccine) [9, 10]. Subsequently, we performed a Phase II clinical trial in patients with recurrent malignant glioma and demonstrated the safety and clinical efficacy of the WT1 peptide vaccine [11]. While some patients (approximately 10%) with recurrent glioblastoma have survived and remained disease-free with the WT1 peptide vaccine alone for more than 10 years, a population of patients experienced rapid tumor recurrence with the same vaccine and died within a few months. This supports the rationale that the treatment-resistant tumors have a mechanism by which the tumor cells escape WT1-targeting and antitumor immune responses induced by the WT1 peptide vaccine. The present study aimed to understand the tumor immunological microenvironment of malignant gliomas treated with the WT1 peptide vaccine and identify the immune-histological and clinical factors associated with the prognosis of patients receiving immunotherapy. We successfully analyzed the density and populations of TILs and the expression of molecules that may affect immune responses pre- and post-vaccination. We used paired tumor specimens from 20 patients with malignant glioma, who had been treated with the WT1 peptide vaccine as a monotherapy for > 3 months and experienced disease progression, which was confirmed radiographically and/or clinically, during the WT1 vaccination.

Material and methods

Patients and specimens

Twenty patients from phase I and II clinical trials of WT1 [11, 12] were included in this study. The inclusion criteria were: (1) a histopathological diagnosis of malignant glioma according to the WHO 2007 criteria [2] before vaccination; (2) the completion of at least 12 doses of the WT1 vaccine; (3) the performance of a second surgery within one month after the last vaccination, due to disease progression that was deemed necessary and safe by the attending neurosurgeon; and (4) the availability of both pre- and post-vaccination surgical specimens (Fig. 1). Currently, the WHO 2016 classification is commonly used; however, the WHO 2007 classification was used in this study because all patients participated in the clinical study and were histopathologically diagnosed between 2004 and 2011. The clinical characteristics of the patients are shown in Table 1. Tumor samples for analysis were collected during resections performed pre- and post-WT1 vaccination. If more than two surgeries were performed before the WT1 vaccination, tumor samples from the last surgery before the WT1 vaccination were used for analysis. All pre- and post-vaccination specimens analyzed in this study contained varying amounts of glioma cells and were not pathologically diagnosed as pseudoprogression.

WT1 vaccination

WT1 peptide vaccine immunotherapy was performed with the approval of the Ethical Review Board of Osaka University Faculty of Medicine, as described previously [11, 12]. The inclusion criteria of the Phase II clinical trial for WT1

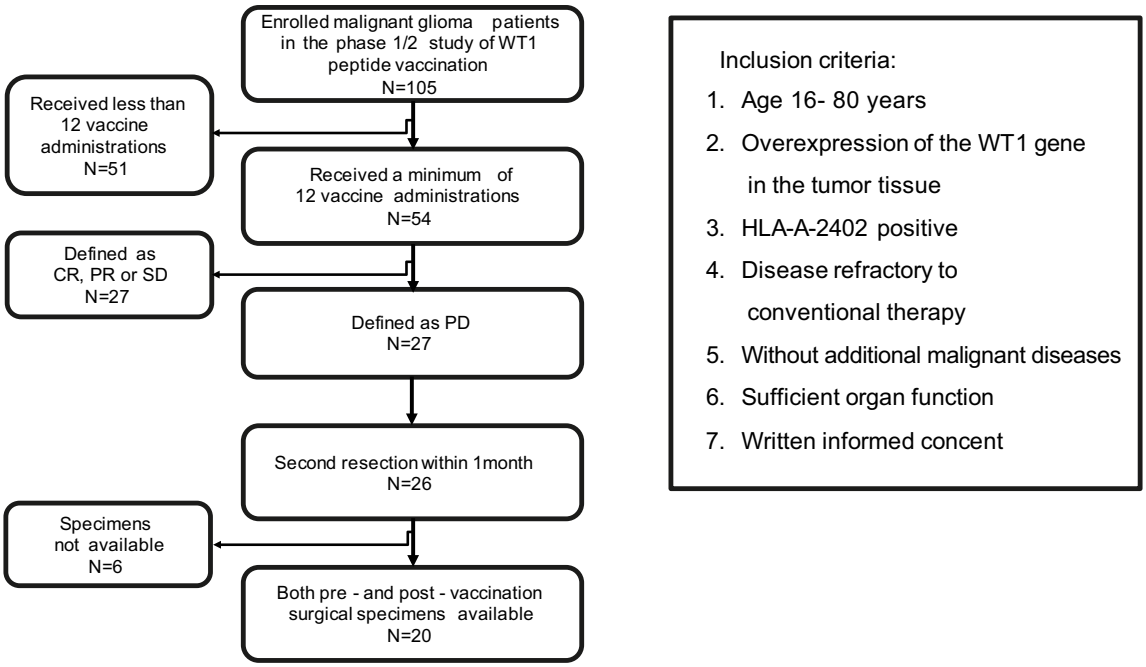

Fig. 1 Flow chart depicting the patient selection and inclusion criteria

Table 1 Patient characteristics and clinical information

	Factors	Values (%)
Patient background	Case number	20
	Age [median (range)]	43 (29—64)
	Gender (Male)	14 (70%)
	KPS[a] [median (range)]	90(70—100)
Histopathlogical diagnosis[b]		
(pre-vaccination)	Glioblastoma	14 (70%)
	Anaplastic astrocytoma	2 (10%)
	Anaplastic oligoastrocytoma	3 (15%)
	Anaplastic ependymoma	1 (5%)
(post-vaccination)	Glioblastoma	18 (90%)
	Anaplastic oligoastrocytoma	2 (10%)
IDH-1 R132H mutation[c]	Mutant type	3 (15%)
Treatment before vaccination	RT > 50 Gy	20 (100%)
	Interferon β	8 (40%)
	Temozolomide	16 (80%)
	Others[d]	5 (25%)
WT1 vaccination	Time from first operation [median (range)] (weeks)	23 (3.3—231.0)
	Vaccination period [median (range)] (weeks)	14 (12—49)

[a]KPS: Karnofsky Performance Status

[b]All specimens were histologically classified according to WHO 2007 criteria

[c]IDH-1 R132H mutation status was assesed by IDH-R132H immunohistochemistry

[d]PAV (procarbazine, nimustine, vincristine), CARE (carboplatin, etoposide), ICE (ifosfamide, carboplatin, etoposide), ACNU (nimustine), CE (cisplatin, etoposide) respectively

peptide vaccination of patients with recurrent glioblastoma multiforme are shown in Fig. 1. Briefly, all patients received intradermal injections of 3 mg of human leukocyte antigen (HLA)-A*2402-restricted modified 9-mer WT1 peptide emulsified with Montanide ISA51 adjuvant weekly for 12 consecutive weeks. HLA-A*2402 is the most common HLA class I type in the Japanese population, carried by approximately 60% of the Japanese population. The HLA-A*2402-restricted modified 9-mer WT1 peptide elicits potent antitumor immunity, which has been shown to induce a much stronger CTL response against WT1-expressing tumor cells [13]. A magnetic resonance imaging (MRI) scan was performed after 12 vaccinations to determine the response of the target lesion according to the Response Evaluation Criteria in Solid Tumors (RECIST) [14]. The results were reported as complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD). If a good response was observed and defined as CR, PR, or SD, vaccination was continued at 1-week intervals, until disease progression was noted. In contrast, if progression was observed and defined as PD and/or clinical deterioration, vaccination was continued at the patient's request. The local internal review board approved this treatment, and written informed consent was obtained from all patients. This trial was registered at the UMIN Clinical Trials Registry as Umin000000933 and Umin000002001. This clinical study was approved by the Ethical Review Board of Osaka University (approval number 07099 and 18,193).

Immunohistochemistry

Sections from the resected tumors were formalin-fixed, deparaffinized, and boiled with 10 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA) buffer (pH: 9.0, for CD4) or 10 mM citrate buffer (pH: 6.0, for others) in an autoclave (120 °C for 30 min for PD-L1 and 15 min for others) for antigen retrieval. The sections were then incubated overnight at 4 °C with anti-human WT1 mouse monoclonal antibody 6F-H2 (DAKO, Carpinteria, CA, USA; diluted 1:50), anti-human Transforming growth factor-β (TGF-β) rabbit polyclonal antibody (Abcam, Cambridge, MA, USA; diluted 1:200), anti–human Ki-67 mouse monoclonal antibody MIB-1 (DAKO; diluted 1:40), anti-human HLA class I-ABC mouse monoclonal antibody EMR8-5 (Hokudo, Sapporo, Japan; diluted 1:100), anti-human programmed death-ligand 1 (PD-L1) rabbit monoclonal antibody ab205921 clone 28–8, (Abcam; diluted 1:100), anti-human CD3 mouse monoclonal antibody F7.2.38 (DAKO; diluted 1:5), anti–human CD4 mouse monoclonal antibody 4B12 (DAKO; diluted 1:80), anti–human CD8 mouse monoclonal antibody C8/14B (Nichirei Biosciences, Tokyo, Japan; undiluted), anti-human CD79α mouse monoclonal antibody JCB117 (DAKO; diluted 1:200), anti-human PD-1 mouse monoclonal antibody clone NAT105 (Abcam; diluted 1:100), anti-human Foxp3 mouse monoclonal antibody 236A/E7 (Abcam; diluted 1:100), or anti IDH1R132H mouse monoclonal Antibody (FUJIFILM Wako Chemicals, Osaka, Japan; diluted 1:100). The stains were visualized with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) and diaminobenzidine (WAKO, Osaka, Japan). The sections were subsequently counterstained with hematoxylin.

WT1, TGF-β, IL-10, and PD-L1 scores

The WT1 expression level was classified based on the scale proposed by Hashiba and Izumoto [10, 11] as follows: 0, negative staining; 1, slightly increased staining in some tumor cells compared to normal glial cells; 2, staining of intermediate intensity in some tumor cells; 3, strong staining in some tumor cells and intermediate staining in almost all tumor cells; and 4, greatly increased staining in almost all tumor cells compared to normal glial cells (Fig. 2a). Wilm's tumor tissues were used as a positive control and normal brain tissues were used as a negative control. The levels of TGF-β and interleukin 10 (IL-10) expression in tumor cells were classified from 0 to 4 based on the same criteria as the WT1 score (Fig. 2a). Expression of PD-L1 in tumor cells was graded as follows: 0, absence of staining; 1, up to 25% of cells stained; 2, 25–50% of cells stained; and 3, more than 50% of cells stained (Fig. 2a) [15]. Samples were scored independently by three authors (CY, KT, and YC). A score agreed upon by at least two of them was deemed acceptable.

Biomarkers of glioma

IDH mutation status was assessed by IDH-R132H immunohistochemistry. Pyrosequencing of IDH mutations along with MGMT promoter and testing for 1p/19q codeletion were considered; however, insufficient tissue specimens discouraged this assessment.

MIB-1 and HLA class I staining indices

The MIB-1 staining index, reflecting each tumor's proliferation activity, was determined by calculating the percentage of positively stained tumor cell nuclei out of 1,000 tumor cell nuclei. All assessments were made in areas with the greatest degree of immunostaining. The HLA class I staining index was determined by calculating the percentage of positively stained tumor cells out of 1,000 tumor cells. All assessments were made in areas with the highest cell density. Lymphocytes and red blood cells were used as positive and negative controls, respectively.

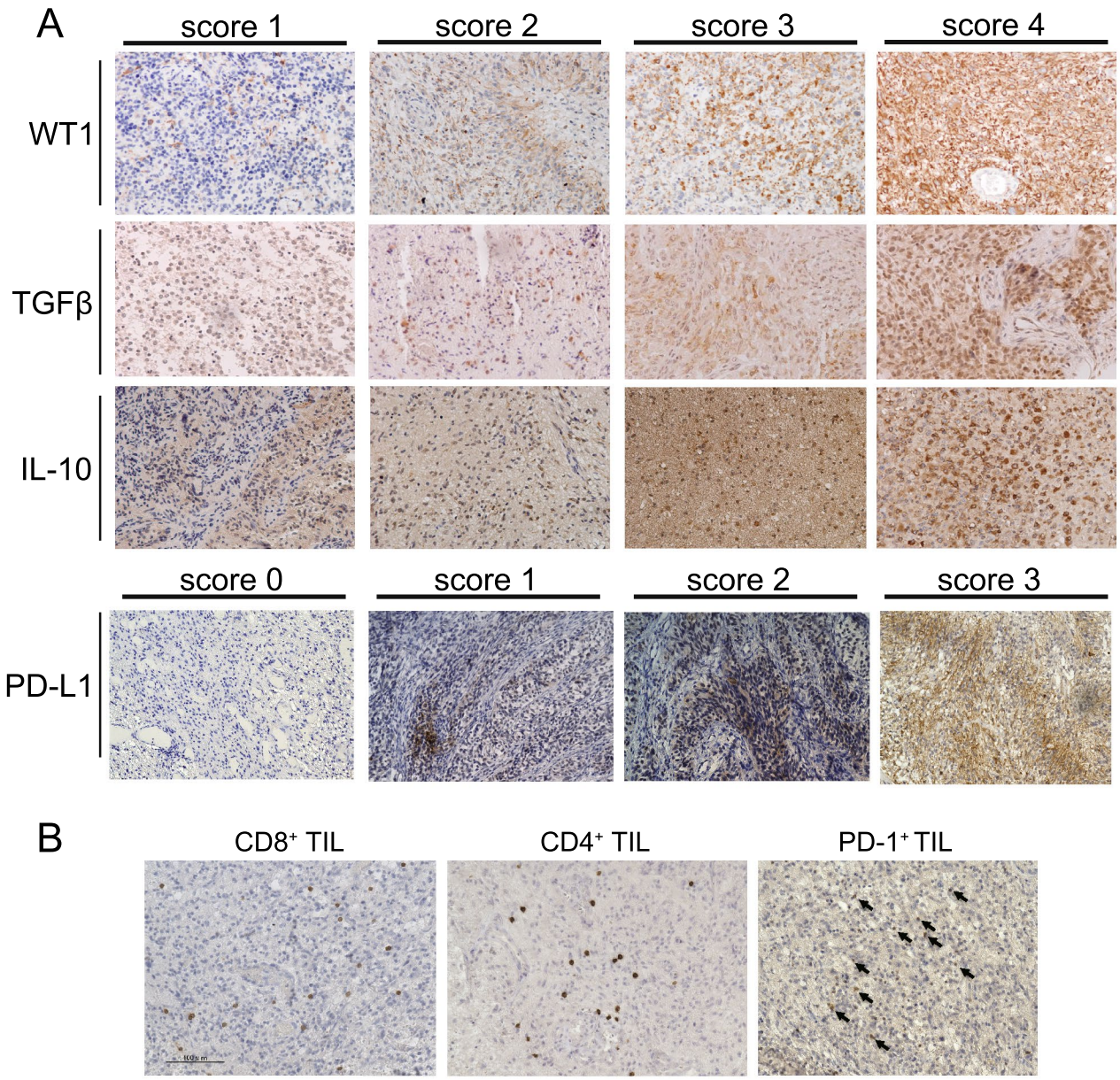

Fig. 2 Level of WT1 (**a**), TGFβ (**b**), and IL-10 (**c**) expression was classified as follows: 0, negative staining; 1, slightly increased staining in some tumors cells compared to that in normal glial cells; 2, staining of intermediate intensity in some tumor cells; 3, strong staining in some tumor cells and intermediate staining in almost all tumor cells; and 4, greatly increased staining in almost all tumor cells compared to that in normal glial cells. Expression of PD-L1 on tumor cells was graded as follows: 0, absence of staining; 1, up to 25% of cells stained; 2, 25–50% of cells stained; and 4, more than 50% of cells stained. Immunohistochemistry of tumor infiltrating lymphocytes (TILs). $CD8^+$, $CD4^+$, and $PD\text{-}1^+$ TILs were present diffusely throughout the tumor tissue in most positive cases

Quantification of TILs

For $CD3^+$, $CD4^+$, $CD8^+$, $CD79\alpha^+$, PD-1, or $Foxp3^+$ lymphocytes within the tumor specimens, we counted the number of each immunoreactive cell using a microscope (BZ-9000 Keyence, Osaka, Japan) with a magnification of ×200. Five non-overlapping fields with identical areas (×200) were selected, and the average numbers were converted into numbers per mm2 (Fig. 2b).

Statistical analysis

All results are presented as the mean or median (range) or absolute number (%). Statistical analyses for comparison of

the IHC staining results were carried out using the paired t-test or Wilcoxon signed-rank test. The Cox proportional hazard model was used to analyze the hazard ratio of each variable. Overall survival (OS-WT1) and progression-free survival (PFS-WT1) from the start of the WT1 vaccinations were calculated using the Kaplan–Meier method. The log-rank test was used to evaluate differences between groups. All probability values (p values) of < 0.05 were considered statistically significant and all statistical computations were performed using the JMP statistical software (JMP Pro, version 13.0; SAS Institute Inc., Cary, NC).

Results

Patient characteristics

A total of 20 patients were included in this study. Patient characteristics are presented in Table 1. Histopathological diagnosis was based on the WHO 2007 classification [2]. Histopathological analysis of tumor samples pre- and post-WT1 vaccination revealed that four patients developed tumors with a malignant transformation from WHO grade III (two with AA, one with AOA, and one with AE) to WHO grade IV (GBM).

All 20 patients included in this study experienced PD after WT1 vaccination (100%). This rate was higher than the PD rate of the patients in a previous clinical trial (54%; data not shown) [11]. Additionally, all patients received at least 50 Gy of postoperative radiotherapy, and 16 patients (80%; 13 patients with GBM, 2 with AA, and 1 with AE) received postoperative chemotherapy with temozolomide before WT1 vaccination. None of the patients received any of these treatments during vaccination and all of them received vaccination alone. The median number of vaccine injections was 14.5. Seven patients (35%) fulfilled the minimum criteria of 12 vaccinations, while the rest received extended vaccine injections, the highest being up to 49 doses.

Expression of TAAs and immunological factors in pre- and post-WT1 vaccination specimens

To examine the differences in the expression of TAAs and immunological factors between pre- and post-WT1 vaccination specimens, we analyzed WT1, TGF-β, IL-10, and PD-L1 scores, and MIB-1 / HLA class I staining indices by immunohistochemistry. A summary of the pathological examinations performed pre- and post-WT1 vaccination is presented in Table 2. The median WT1 expression score was significantly reduced from 3 to 2.5 following WT1 vaccination ($P = 0.012$; Fig. 3). The average HLA class I staining index was also significantly reduced from 33.9% to 16% ($P < 0.01$; Fig. 3). However, the median PD-L1 score was not significantly different between the pre- and post-vaccination specimens ($P = 0.85$; Fig. 3). The individual results of all patients in Fig. 3 are shown in the supplementary Fig. 1. These changes in the staining index and expression scores in tumor cells did not clearly correlate with one another or with the density of TILs (data not shown). Additionally, there was no significant correlation between the WT1 expression levels and that of HLA class I in both pre- and post-vaccination specimens ($P = 0.89$ and $P = 0.11$, respectively). These results suggested that these changes might have occurred due to the immune escape mechanism.

Table 2 Summary of IHC staining results of both pre- and post- WT1 vaccination specimens

	Pre-WT1	Post-WT1	P value[c]
Case number	20	20	
MIB-1 index (mean, %)	21.6	20.9	0.79
WT-1 score (median, 1–4)	3	2.5	0.012*
HLA class I (mean, %)	33.9	16	0.008**
HLA class II (mean, %)[a]	11.4	12.1	0.89
TGF-β score (median,1–4)	2	3	0.014*
IL-10 score (median, 1–4)	2	2	0.19
PD-L1 score (median, 0–3)[a]	1	1	0.85
$CD3^+$ cells (median, /mm^2)	137.9	94.1	0.83
$CD4^+$ cells (median, /mm^2)	126.8	51.6	0.018*
$CD8^+$ cells (median, /mm^2)	135.3	64.1	0.36
$CD79\alpha^+$ cells (median, /mm^2)	10.5	7.8	0.40
$Foxp3^+$ cells (median, /mm^2)[b]	18.0	14.9	0.44
$PD\text{-}1^+$ cells (median, /mm^2)[a]	3.8	3.8	0.61
$CD4^+/CD8^+$ ratio (median)	1.27	0.90	0.12
$PD\text{-}1^+/CD8^+$ ratio (median)	1.5	7.5	0.52

[a]Three patients were unavailable

[b]One patient was unavailable

[c]Paired t test (for MIB-1 index, HLA class 1 and HLA class 2) and Wilcoxon (for others) were used for comparison of IHC staining score between paired specimens

*$P < 0.05$

**$P < 0.01$

Density of TILs in pre- and post-WT1 vaccination specimens

We analyzed the changes in TIL densities between pre- and post-vaccination specimens to examine immune responses to WT1 vaccine therapy. The median $CD3^+$, $CD8^+$, $CD79\alpha^+$, $PD\text{-}1^+$, and $FOXP3^+$ lymphocyte densities in the tumors were stable (Table 2 and Fig. 3), however, the median $CD4^+$ lymphocyte density significantly reduced from 126.8 cells/mm^2 to 51.6 cells/mm^2 ($P < 0.018$; Fig. 3). The median $CD4^+/CD8^+$ ratio tended to decrease from 1.27 to 0.90 ($P = 0.12$; Table 2), while the $PD\text{-}1^+/CD8^+$ ratio remained stable ($P = 0.52$; Table 2). These results did not significantly

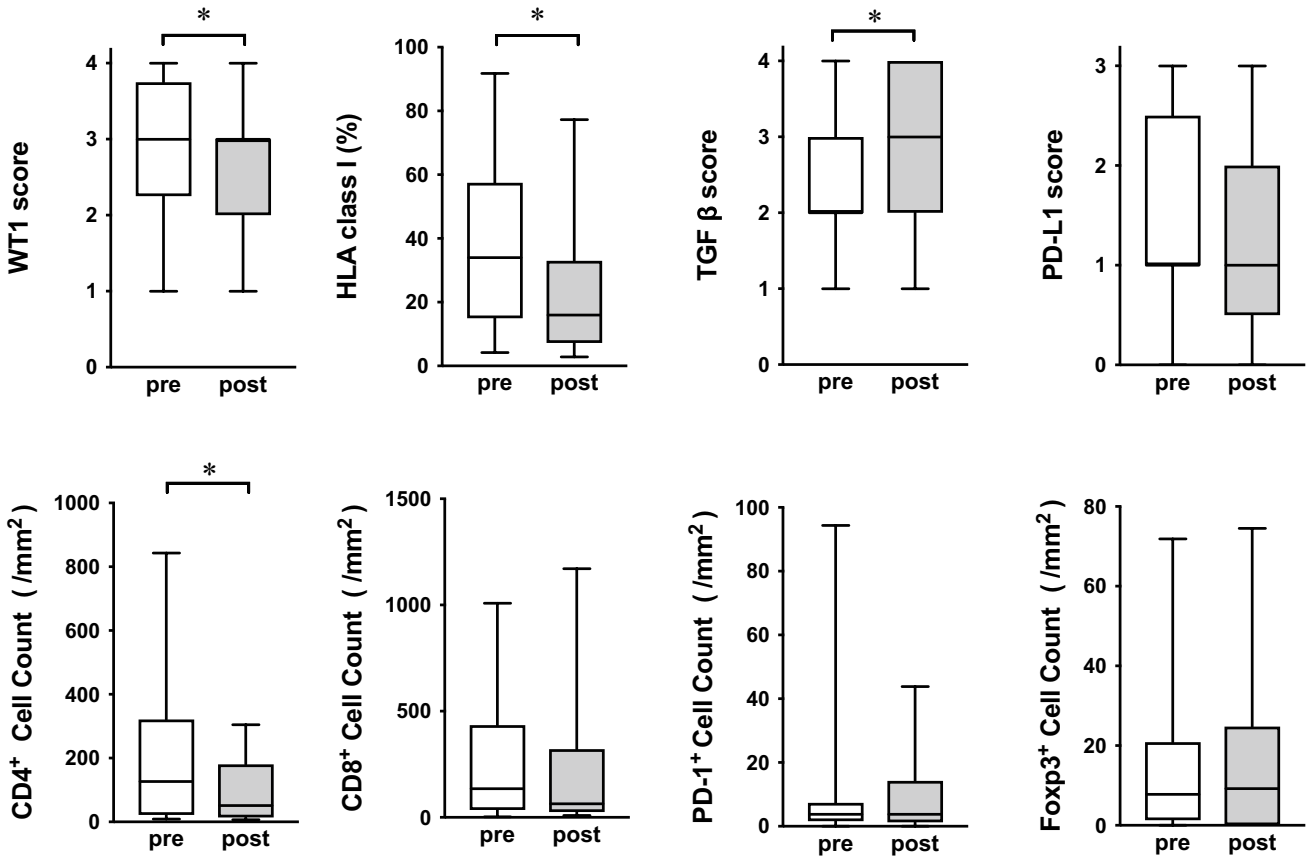

Fig. 3 Changes in the expression of WT1, HLA class I, TGF-β, PD-L1, and TIL count pre- and post- WT1 vaccination. The median WT1 score, TGF-β score, HLA class I staining index, and PD-L1 score were calculated. The median number of $CD4^+$, $CD8^+$, PD-1^+, and $Foxp3^+$ TILs per square millimeter were counted. These data were compared between tumor samples resected before and after WT1 vaccination. In this figure, the distribution of WT1 score, HLA class I staining index, TGF-β score, PD-L1 score, and TIL count are represented by the box plot, and the limits of the whiskers indicate the extremes and those of the box the 25 and 75% values. WT1 score, HLA class I staining index, and $CD4^+$ TILs were significantly reduced in tumor specimens post- WT1 vaccination. In contrast, the TGF-β score was increased, while the PD-L1 score, $CD8^+$ TILs, PD-1^+ TILs, and $Foxp3^+$ TILs were not significantly different (*$P < 0.05$)

correlate with each other or with the frequencies of TILs (data not shown).

Hazard ratio between immune-related proteins and TILs in pre- and post-vaccination tumor specimens and prognostic factors

To investigate the relationship between TILs, the expression of immune-related proteins in tumor tissues, and clinical prognostic factors in patients with GBM and AA, we characterized the $CD3^+$, $CD4^+$, $CD8^+$, PD-1^+, CD79α, $Foxp3^+$ TIL counts and the $CD4^+/CD8^+$ ratio as high or low, using either the median count or median ratio as the cutoff point, as appropriate. The hazard ratio (HR) for each variable was calculated using the Cox proportional hazard model (Table 3).

A high $CD4^+/CD8^+$ ratio (> median ratio) before WT1 vaccination was associated with an increased relative risk of death when compared to a low $CD4^+/CD8^+$ ratio (≤ median ratio, HR = 4.138; 95% confidence interval [CI] = 1.213–14.726; $P = 0.024$). Moreover, in patients with GBM or AA, univariate analysis showed that a low WT1 expression (score of 1 or 2) in tumor cells, in the pre- and post-vaccination specimens, was associated with an increased relative risk of death when compared to a high WT1 expression (score of 3 or 4) on performing univariate analysis (HR = 4.050; 95% CI = 1.012–14.558; $P = 0.048$ and HR = 5.552; 95% CI = 1.531–22.080; $P < 0.01$, respectively; Table 3). Low HLA class I expression (<) before WT1 vaccination was also associated with an increased relative risk of death when compared to high HLA class I expression (> median) (HR = 3.671; 95% CI = 1.086–16.756; $P = 0.035$). High $CD4^+$ TIL counts in the post-vaccination specimens had borderline associated with a decreased relative risk of death, whereas high $CD4^+$ TIL counts in the pre-vaccination specimens was not (HR = 0.335; 95% CI = 0.106–1.005;

Table 3 Univariate analysis using a Cox proportional hazards model for overall survival and progression free survival from the start of WT1 vaccination

Variable	Overall Survival			Progression-free Survival		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Gender (male)	1.160	0.252–4.052	0.84	0.993	0.218–3.376	0.99
Time interval from first operation to WT1 vaccination (> 23 weeks)	1.340	0.462–4.106	0.59	1.293	0.459–3.722	0.62
Age (> 50)	1.340	0.436–3.909	0.60	0.921	0.307–2.578	0.88
With Temozolomide	0.638	0.188–2.891	0.52	0.629	0.179–2.900	0.51
Presence of IDH1 mutation	0.591	0.089–2.291	0.48	0.602	0.091–2.284	0.49
Variable (Pre-vaccination)	Overall Survival			Progression-free Survival		
	HR	95% CI	*P* value	HR	95% CI	*P* value
High $CD3^+$	1.527	0.517–4.517	0.44	2.496	0.666–7.483	0.20
High $CD4^+$	1.043	0.372–3.313	0.94	1.310	0.464–3.974	0.61
High $CD8^+$	1.320	0.465–4.262	0.61	1.736	0.605–5.668	0.31
High PD-1^+	1.923	0.614–6.227	0.26	1.920	0.661–7.104	0.26
High $CD4^+/CD8^+$ ratio	4.138	1.213–14.726	0.024*	18.894	2.996–368.989	< 0.01**
High PD-$1^+/CD8^+$ ratio	1.923	0.641–6.227	0.257	2.088	0.661–7.104	0.20
High $CD79\alpha^+$	1.043	0.406–3.634	0.76	1.737	0.560–5.930	0.34
High $Foxp3^+$	0.920	0.280–2.688	0.88	1.130	0.340–3.410	0.83
High Ki-67	0.885	0.308–2.487	0.82	1.060	0.369–2.974	0.91
WT1 score (1.2)	4.050	1.012–14.558	0.048*	3.049	0.771–10.822	0.11
IL-10 score (1.2)	2.673	0.731–9.771	0.13	3.001	0.958–12.886	0.051
TGF-β score (1.2)	1.976	0.625–7.452	0.25	1.676	0.535–6.276	0.38
PD-L1 score (1.2)	0.553	0.164–1.692	0.30	1.677	0.521–6.365	0.39
High HLA class I	1.856	0.609–5.813	0.27	1.991	0.665–5.965	0.21
High HLA class II	2.887	0.688–14.449	0.15	2.630	0.701–7.291	0.17
Variable (Post-vaccination)	Overall Survival			Progression-free Survival		
	HR	95% CI	*P* value	HR	95% CI	*P* value
High $CD3^+$	1.177	0.415–3.421	0.75	2.675	0.857–9.203	0.090
High $CD4^+$	0.335	0.106–1.005	0.052	0.679	0.235–1.910	0.45
High $CD8^+$	0.845	0.297–2.458	0.75	1.415	0.485–4.354	0.52
High PD-1^+	0.385	0.119–1.207	0.10	1.891	0.598–6.434	0.27
High $CD4^+/CD8^+$ ratio	0.469	0.138–1.419	0.18	0.369	0.079–1.283	0.12
High PD-$1^+/CD8^+$ ratio	0.538	0.171–1.642	0.27	0.658	0.193–2.081	0.47
High $CD79\alpha^+$	0.837	0.283–2.618	0.75	1.466	0.473–5.018	0.51
High $Foxp3^+$	0.495	0.159–1.460	0.20	0.429	0.113–1.386	0.16
High Ki-67	1.408	0.496–4.085	0.51	1.373	0.488–3.946	0.54
WT1 score (1.2)	5.552	1.531–22.080	< 0.01**	5.340	1.458–21.712	0.012*
IL-10 score (1.2)	1.148	0.378–3.843	0.80	1.414	0.476–4.701	0.53
TGF-β score (1.2)	0.442	0.116–1.430	0.17	0.592	0.175–1.817	0.36
PD-L1 score (0.1)	0.555	0.141–1.888	0.34	0.555	0.142–1.883	0.35
High HLA class I	1.359	0.464–4.193	0.57	1.106	0.363–3.478	0.85
High HLA class II	0.660	0.197–2.307	0.50	0.730	0.22—2.500	0.59

HR hazard ratio; *CI* confidence interval; *P* value, *P* value at Wald test

**P*<0.05

***P*<0.01

$P = 0.052$ and HR = 1.043; 95% CI = 0.372–3.313; $P = 0.94$, respectively; Table 3). Other independent prognostic factors, including age, temozolomide therapy, and tumor cells with *IDH1* mutations, were not found to be significant in this study. These well-known prognostic factors in malignant gliomas were not statistically associated with the prognosis following WT1 vaccination in this study.

Correlation between the expression of immune-related proteins and TILs in pre- and post-WT1 vaccination tumor specimens, with survival outcomes of patients with GBM or AA

Survival outcomes of patients with high and low expressions of WT1, HLA class I, and $CD4^+/CD8^+$ ratio in both the pre- and post-WT1 vaccination specimens were estimated using the Kaplan–Meier method and compared between groups using the log-rank test, to examine the effects of the expression of immune-related proteins and TILs on survival. The median survival time in each group is shown in Supplementary Table. In 16 patients with AA or GBM, the median overall survival (OS)-WT1 and progression-free survival (PFS)-WT1 was 51.9 (95% CI = 35.0–72.3) and 12.7 (95% CI = 5.3–32.9) weeks, respectively. High WT1 (a score of 3 or 4) expression in the pre-WT1 vaccination specimens was significantly associated with a longer OS-WT1 (median, 60.5 vs. 29.5 weeks, high vs. low group; $P = 0.02$; Supplementary Table and Fig. 4). Additionally, there was a significant correlation between a high HLA class I staining index (≥ median) in the pre-WT1 vaccination specimens and longer OS-WT1 (median, 103.1 vs. 35.1 weeks, $P = 0.040$; Fig. 4) and PFS-WT1 (median, 47 vs. 8.8 weeks, $P = 0.035$; Table 3). A low $CD4^+/CD8^+$ ratio (< median) in the pre-vaccination specimens was significantly associated with an improved OS-WT1 (median, 66.5 vs. 35.3 weeks, low vs. high group; $P = 0.016$, Fig. 4) and PFS-WT1 (median, 28.4 vs. 5.4 weeks, $P < 0.01$, Supplementary Table). In the post-WT1 vaccination specimens, a high WT1 expression was significantly associated with longer OS-WT1 (median, 68.6 vs. 29.5 weeks, high vs. low group; $P < 0.01$; Fig. 4), whereas the expression of HLA class I and $CD4^+/CD8^+$ ratio was not significantly associated with survival ($P = 0.72$ and 0.36, respectively; Fig. 4). Conversely, no difference in the overall survival of patients who maintained a high WT1 score, high HLA class expression, and low $CD4^+/CD8^+$ ratio post-vaccination compared to patients who did not (Supplementary Fig. 2). However, this contradiction might be because of the limited number of patients. Further analysis with a larger sample is required to reach a definite conclusion. To conclude, the expression of WT1 was the only factor associated with survival in both pre- and post-vaccination specimens.

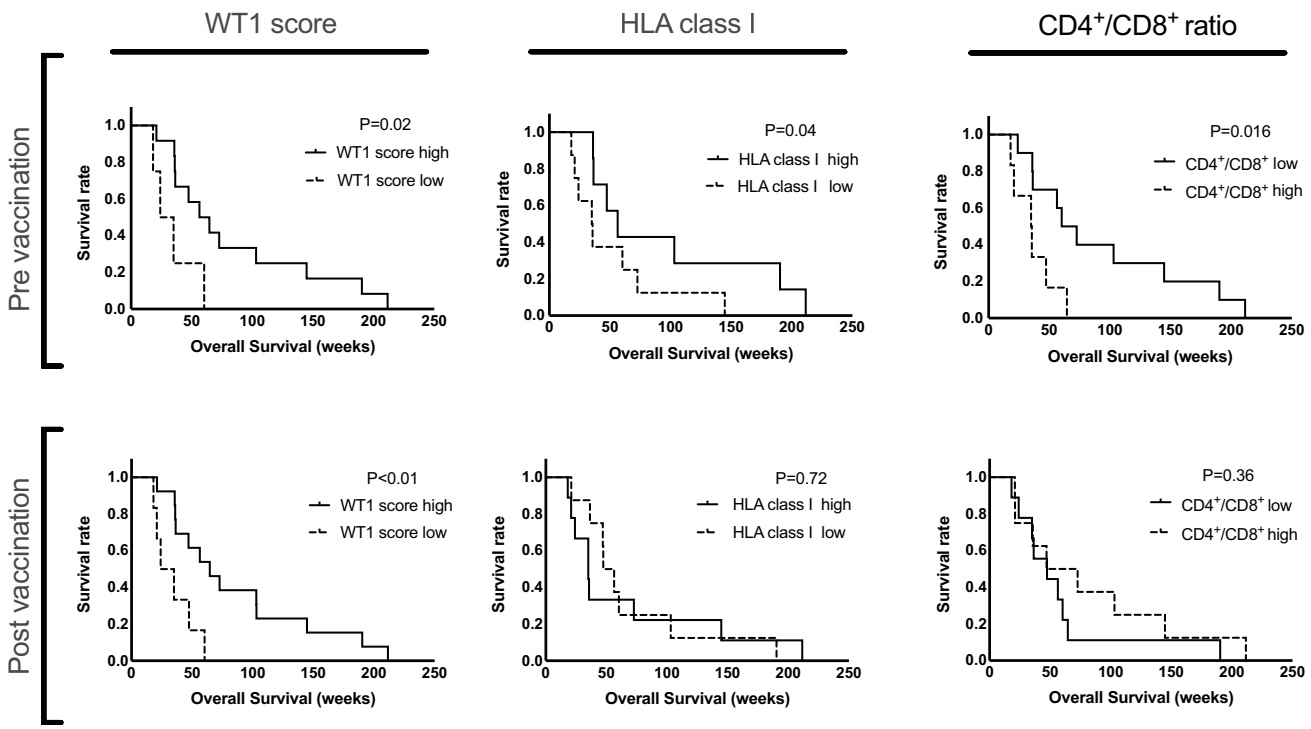

Fig. 4 Association between the prognostic markers and overall survival from the start of the WT1 vaccination (OS-WT1) is shown using Kaplan–Meier survival curves. The log-rank test was used to compare the differences

Discussion

In the present study, we analyzed the tumor immunological microenvironment in 20 patients with malignant astrocytic tumors who received the WT1 CTL peptide vaccine for 3 months or more, and experienced radiographical and/or clinical tumor progression. Notably, immunohistochemical studies on paired pre- and post-vaccine tumor tissues showed that tumor cells expressed the target molecule WT1 and the antigen-presenting molecule HLA class I at various levels, with lower levels of infiltrating $CD4^+$ cells being found in the post-vaccine tumor specimens. Furthermore, correlation analysis with clinical outcomes revealed that the maintenance of WT1, but not HLA class I, expression in tumor cells, in the post-vaccine tumor specimens was positively associated with both OS and PFS.

CTLs that recognize target antigens present on tumor cells and attack them are the effectors of antigen-specific cancer immunotherapies. In the present study, immunohistochemical analysis of paired tumor samples, obtained before and after vaccination, showed that the tumor cells exhibited decreased expression of WT1 and HLA class I in the post-vaccine tumor samples, in approximately half of the patients examined. The association between decreased expression of WT1 and HLA class I and OS-WT1 and PFS-WT1 in the post-vaccine tumors raises the possibility of an escape from the host antitumor immune response, which contributed to the progression of these tumors after treatment with the WT1 peptide vaccine. Loss or downregulation of the antigen-presenting molecule, HLA class I, is a well-known immune escape mechanism in antigen-specific immunotherapies and has been reported in various cancers[16], including lung cancer [17], prostate cancer [18], and GBM [19]**.** Previous studies have demonstrated that loss or downregulation of HLA class I antigen was found in 16%–50% of different malignant tumors and was clinically associated with a poor prognosis and reduced PFS [20]. Loss or downregulation of target TAAs is also an important immune escape mechanism [21–24]. In the present study, high expression of HLA class I in the pre-vaccine tumor samples was a positive prognostic factor, whereas that in the post-vaccine tumor samples was not. This contradiction can be explained by relatively high expression level of HLA class I in the post-vaccine tumor samples. The lowest HLA class I index among the post-vaccine samples in this study was 3.7% and there were no loss cases. Since HLA class I expression was maintained more than necessary in most patients in this study, significant survival benefit may not have been observed by high expression of HLA class I in the post-vaccine tumor samples. However, high expression of WT1 in both pre-and post-vaccine tumor samples was significantly associated with better clinical outcomes. These findings indicate that the expression of the target molecule, WT1, in tumor cells is essential for WT1 peptide vaccine cancer therapy, consistent with our previously reported findings [25]. WT1 transcriptionally regulates the expression of various target genes [26–30], and the decreased expression of the WT1 gene in tumor cells may have contributed towards the immune escape mechanism of tumors. Our findings suggest that the loss or downregulation of WT1 expression functions as a major immune escape mechanism, owing to the selection pressure exerted by WT1-specific immune responses, in glioblastoma patients treated with the WT1 peptide vaccine. Moreover, findings stating that the WT1 expression in tumor cells is positively associated with favorable clinical outcomes, supports the therapeutic concept, "Cytotoxic T lymphocytes that recognize target WT1 antigen present on the tumor cells and attack them are the effectors of WT1 peptide vaccine cancer immunotherapy."

Cellular and humoral factors in the tumor microenvironment can critically affect local antitumor immune responses at the tumor sites. In the present study, we analyzed the tumor infiltration of lymphocytes, which are important cellular constituents in tumors. We found that a low $CD4^+/CD8^+$ ratio in pre-vaccination tumors was significantly associated with survival time and high levels of infiltrating $CD4^+$ T cells in post-vaccination tumors also tended to be associated with survival time. These findings validate the important role of $CD4^+$ T cells in tumors, particularly in the maintenance of antitumor immune responses induced by the WT1 peptide cancer vaccine. The important role of $CD4^+$ T cells in antitumor immune responses is supported by our previous findings which demonstrated that the production of immunoglobulin G (IgG) antibodies against WT1 peptide was significantly and positively correlated with both PFS and OS, in patients with recurrent glioblastoma treated with the WT1 peptide vaccine [31]. This was because the class switch from immunoglobulin M (IgM) to IgG in WT1-specific B cells requires help from $CD4^+$ helper T cells. In recent years, tissue-resident memory T (TRM) cells have attracted the attention of researchers. These newly identified T cells are a population of non-recirculating $CD8^+$ T cells that reside permanently within peripheral tissues and are conveniently positioned to mediate regional tumor surveillance [32]. Reportedly, memory $CD4^+$ T cells can share immunosurveillance strategies with $CD8^+$ TRM cells. Mucosal $CD4^+$ TRM cells have been shown to fulfill a sensing and alarm function in an animal re-infection model [33]. Furthermore, our previous in vitro study revealed that WT1-specific $CD4^+$ T cells could exhibit cytotoxic functions [34]. Future studies are needed to identify the functions of $CD4^+$ T cells in the tumor microenvironment in glioblastoma patients and subsequently develop strategies to maintain and enhance the antitumor functions of $CD4^+$ T cells.

The criteria for patient selection in WT1 peptide vaccine cancer therapy remain unestablished. Many early clinical trials have adopted simple clinical indices, such as the serum levels of albumin and lactate dehydrogenase (LDH), hemoglobin level, and patient performance status to select patients for enrollment. In addition to these indices, optimization of patient selection for cancer vaccine therapies requires immunological biomarkers that are associated with patient survival. Our immunochemical data demonstrated an association between high expressions of WT1 and HLA class I along with a low $CD4^+/CD8^+$ ratio in pre-vaccination tumors and survival time in patients with malignant glioma, who were treated with the WT1 peptide vaccine. This indicates that these may be useful predictive biomarkers for selecting patients for the WT1 peptide cancer vaccine. Further studies are required to establish their efficacy as biomarkers for optimizing patient selection for WT1 peptide cancer vaccination in malignant glioma.

The present study has several limitations. First, only the patients who experienced disease progression with WT1 vaccination were enrolled in this study, resulting in a shorter median OS-WT1 and PFS-WT1 compared to those treated with the WT1 peptide vaccine in our previous study [11]. However, resection of the progressed tumors allowed us to examine paired tumor tissues, obtained before and after WT1 vaccination, in order to analyze the changes in the immunological tumor microenvironment during the vaccination period. Second, there was heterogeneity in the pre-vaccine treatment. Four patients in the study started WT1 vaccination before the approval of temozolomide as a drug for malignant glioma in our country. Not using the current standard drug, temozolomide [3, 35], may have affected the OS-WT1 and PFS-WT1 in these patients. Moreover, information on the genetic characteristics of the tumors, such as MGMT promoter methylation status, was unavailable in this study. Since these factors are known to be associated with the prognosis of malignant gliomas treated with temozolomide [36], the prognostic values of the indicators found in this study may be limited. However, since all patients in this study were refractory to the standard treatment and had not received any other treatment from the start of WT1 vaccination, we believe that the MGMT status had little effect on the overall survival from the start of the WT1 vaccinations.

Our study findings may have several theoretical and practical implications for cancer vaccine immunotherapies. Recent studies have reported that the expression of TAAs and HLA molecules may be regulated by epigenetic mechanisms in both normal and cancerous tissues [37]. Pharmaceutical agents targeting epigenetic modifications are being developed, and several clinical trials are in progress [37, 38]. A combination of these agents with WT1 vaccination may be beneficial for the maintenance or restoration of TAA or HLA expression to prevent cancer progression.

In conclusion, the present study provides new insights into the mechanisms of tumor immune escape after WT1 vaccination and further suggests research directions and potential clinical strategies for further cancer immunotherapy.

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Author contributions CY, KT, YC, NK, and NH participated in the conception and design of this study. CY, KT, and YC performed the experiments. NK, AT, YO, YO, SI, HS and NH contributed to patient recruitment, treatment, and clinical data collection. CY, KT, YC, and YO contributed to the statistical analyses. MK, NK, AT, YO, HS, HK, NH, and NK joined the discussion, following which, CY, KT, YO, NH, and NK collectively wrote the manuscript. All authors had contributed towards manuscript revisions and have approved the final manuscript.

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Declarations

Conflicts of interest The authors have no conflicts of interest to declare.

Ethical approval The study was performed in accordance with the Declaration of Helsinki, Japanese Ethical Guidelines for Clinical Research, and Japanese Guidelines for Medical and Health Research. This trial was registered at the UMIN Clinical Trials Registry as Umin000000933 and Umin000002001. This clinical study was approved by the Ethical Review Board of Osaka University (approval number 07099 and 18193).

Consent for publication The authors declare that this manuscript is original, has not been published before, and is not currently under consideration for publication elsewhere.

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