Use of $^{11}$C-methionine PET parametric response map for monitoring WT1 immunotherapy response in recurrent malignant glioma

Clinical article

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Object. Immunotherapy targeting the Wilms tumor 1 (WT1) gene product is a promising treatment modality for patients with malignant gliomas, and there have been reports of encouraging results. It has become clear, however, that Gd-enhanced MR imaging does not reflect prognosis, thereby necessitating a more robust imaging evaluation system for monitoring response to WT1 immunotherapy. To meet this demand, the authors performed a voxel-wise parametric response map (PRM) analysis of $^{11}$C-methionine PET (MET-PET) in WT1 immunotherapy and compared the data with the overall survival after initiation of WT1 immunotherapy ($OS_{WT1}$).

Methods. Fourteen patients with recurrent malignant glioma were included in the study, and $OS_{WT1}$ was compared with: 1) volume and length change in the contrast area of the tumor on Gd-enhanced MR images; 2) change in maximum uptake of $^{11}$C-methionine; and 3) a more detailed voxel-wise PRM analysis of MET-PET pre- and post-WT1 immunotherapy.

Results. The PRM analysis was able to identify the following 3 areas within the tumor core: 1) area with no change in $^{11}$C-methionine uptake pre- and posttreatment; 2) area with increased $^{11}$C-methionine uptake posttreatment (PRM$_{MRT}$); and 3) area with decreased $^{11}$C-methionine uptake posttreatment. While the results of Gd-enhanced MR imaging volumetric and conventional MET-PET analysis did not correlate with $OS_{WT1}$ ($p = 0.270$ for Gd-enhanced MR imaging volume, and $p = 0.110$ for MET-PET), the percentage of PRM$_{MRT}$ area showed excellent correlation ($p = 0.008$) with $OS_{WT1}$.

Conclusions. This study describes the limited value of Gd-enhanced MR imaging and highlights the potential of voxel-wise PRM analysis of MET-PET for monitoring treatment response in immunotherapy for malignant gliomas.


Key words: glioma • $^{11}$C-methionine PET • WT1 immunotherapy • parametric response map • oncology

Malignant glioma remains a devastating intracranial neoplasm. In particular, patients with newly diagnosed GBM have a median overall survival of only 14.6 months, even when treated with chemotherapeutic agents such as temozolomide. On the other hand, the products of the WT1 gene have been shown to be overexpressed in malignant gliomas, and this makes the WT1 antigen an attractive target for immunotherapy against malignant glioma.

The results of WT1 immunotherapy have been previously reported for the initial 21 patients participating in an ongoing Phase II clinical trial of WT1 vaccination for patients with recurrent malignant glioma, and the safety and efficacy of WT1 vaccination have been described (Phase I/II clinical trial of WT1 peptide-based vaccine for the patients with malignant tumors. UMIN000002001).6

This article contains some figures that are displayed in color online but in black and white in the print edition.
The median overall survival time after initiating WT1 immunotherapy was 36.7 weeks. In that report, the anti-tumor effect of the treatment was assessed by determining the response of the target lesions using MR imaging 12 weeks after initiating WT1 vaccination. The tumor length, corresponding to the contrast-enhanced area on Gd-enhanced MR images, was measured and analyzed according to RECIST version 1.0,18 with results reported as complete response, partial response, stable disease, and progressive disease.

In that analysis, however, the long-term survivors were assessed as having progressive disease at 12 weeks after WT1 vaccination initiation, suggesting that evaluation by contrast-enhanced T1-weighted MR imaging is not suitable for assessing the treatment response to WT1 immunotherapy. The fact that morphological imaging often does not adequately reflect the underlying tumor biology imposes a considerable demand to develop alternative biological markers for therapeutic response. Recently, a voxel-wise PRM has been developed to overcome the above-mentioned issue in other treatment modalities for malignant glioma.6-8

The present report focuses on the results in 14 patients who were enrolled in the same trial but were not included in the previous report. In this study, we have attempted to apply the voxel-wise PRM method to MET-PET in the setting of WT1 immunotherapy against recurrent malignant glioma and compare its clinical value with conventional analytical methods based on MR imaging and PET.

Methods

WT1 Immunotherapy

Patients received intradermal injections of 3.0 mg of modified 9-mer WT1 peptide emulsified with Montanide ISA51 adjuvant. The WT1 vaccinations were given weekly for 12 consecutive weeks. Twelve weeks after the initial vaccination, the response was evaluated by means of both MR imaging and MET-PET. Our local internal review board approved this treatment and written informed consent was obtained from all patients. Details of the procedures and protocol have been reported elsewhere.9,14

Patient Selection

Between 2004 and 2010, 66 patients with recurrent malignant glioma were treated with WT1 immunotherapy as described above as part of an ongoing clinical trial (UMIN000002001). Nineteen of these 66 patients underwent evaluation by means of MET-PET. These patients were not included in our previous report.9 Five of these 19 patients—2 patients with intratumoral hematoma and 3 patients whose tumor volume was 2 cm³ or less as measured by MET-PET—were excluded from the current analysis. All 14 patients whose data were analyzed for this study underwent MR imaging and MET-PET before (pre-WT1) and 12 weeks after (post-WT1) WT1 vaccination. Detailed information pertaining to these 14 patients is listed in Table 1. The overall survival was measured from WT1 immunotherapy initiation, denoted as OSWT1.

Magnetic Resonance Imaging

All MR images were obtained using a 3.0-T whole-body MR scanner (Signa, GE Medical Systems) with an acquisition time of approximately 3 minutes. After intravenous administration of Gd–diethylenetriamine penta-acetic acid (Gd-DTPA; 0.1 mmol/kg body weight), axial T1-weighted images were obtained using standard procedures. Those images were stored in 512 × 512 × 23 or 216 anisotropic voxels, with each voxel being 0.43 × 0.43 × 6.0 or 1.0 mm.

MET-PET Scans

All PET studies were performed using the Eminence PET system (Shimadzu Corp). 11C-methionine (111–222 MBq, 3–6 mCi), synthesized according to the method of Berger et al.,1 was injected intravenously. Tracer accumulation was recorded over 15 minutes in 99 transaxial slices from the entire brain. Total activity from 20 to 35 minutes after tracer injection was used for image reconstruction. The images were stored in 256 × 256 × 99 anisotropic voxels, with each voxel being 1 × 1 × 2.6 mm.

Tumor Length and Volume Measurement

Tumor length, corresponding to the contrast-enhanced area on T1-weighted MR images, was measured and analyzed according to RECIST version 1.0,18 using the ImageJ software from the National Institutes of Health (http://rsb.info.nih.gov/ij/).

Tumor volume was measured by performing a 3D threshold-based volume-of-interest analysis in all patients for contrast-enhanced lesions on Gd-enhanced MR images, using the ImageJ software. The contrast-enhanced area in each slice image was measured by manual tracking of the tumor boundaries, and the sum of the enhanced areas or high-uptake areas was multiplied by the slice interval.

Image Fusion and Registration

The MET-PET data were registered onto pre-WT1 contrast-enhanced T1-weighted standard anatomical images using normalized mutual information with the VINCI image analyzing software from the Max Planck Institute for Neurological Research in Cologne (http://www.nf.mpg.de/vinci/). Registration of the images was confirmed visually. The reported registration error for normalized mutual information is less than 1 mm.19 After image registration was completed, all image sets, including the standard anatomical MR images (pre-WT1) and MET-PET data (pre- and post-WT1), were converted into 256 × 256 × 256 isotropic, 1 × 1 × 1 mm images enabling further voxel-wise analysis of the images (Fig. 1).

Data Processing and ROI Selection

Three data sets (standard anatomical images and MET-PET data) were exported to in-house software written in MATLAB 7.6 (MathWorks) for further analysis. Regions of interest were selected as follows: for normal brain tissue, the contralateral hemisphere of the tumor was selected, including both the gray and white matter; for tumor, contrast-enhanced lesions were selected.
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### TABLE 1: Summary of clinical and demographic characteristics of 14 patients*

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age (yrs)†</th>
<th>Sex</th>
<th>ECOG PS</th>
<th>Diagnosis</th>
<th>Response per RECIST</th>
<th>OS&lt;sub&gt;WTT&lt;/sub&gt; (wks)‡</th>
<th>Tumor Vol by MET-PET (cm³)§</th>
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<td>2</td>
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<td>SD</td>
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<td>SD</td>
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<tr>
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<td>27.6</td>
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</table>

* AA = anaplastic astrocytoma; ECOG PS = Eastern Cooperative Oncology Group Performance Status; PD = progressive disease; PR = partial response; SD = stable disease.
† Mean 48.4 years.
‡ Median 59.0 weeks.
§ Median 26.5 cm³.
† The patient in Case 9 was alive as of this writing.

### Parametric Response Map Calculation Algorithm

As in Fig. 1, post-WT1 <sup>11</sup>C-methionine uptake was plotted as a function of pre-WT1 <sup>11</sup>C-methionine uptake in both normal brain and Gd-enhancing lesions. A linear regression fitting was applied to the data obtained by the ROI placed at the normal brain (Fig. 1, blue line), which can be expressed as follows: post-WT1 MET-PET = pre-WT1 MET-PET, where “post-WT1 MET-PET” and “pre-WT1 MET-PET” are the tumor/normal tissue (T/N) ratio of pre- and post-WT1 <sup>11</sup>C-methionine PET.

Next, the magnitude of deviation of each data point from the expected linear regression fitting was calculated as follows:

\[
\text{deviation}_{i} = \left[ \left( \text{post-WT1 MET-PET}_{i} - \text{pre-WT1 MET-PET}_{i} \right) \right] / \sqrt{2}
\]

The parametric response map (PRM) of each data point was defined as follows:

\[
\text{PRM}_{i} = \text{deviation}_{i} - \frac{\mu}{\rho}
\]

where \( \mu \) and \( \rho \) are the mean and standard deviation of deviation, within the ROI placed at the normal brain. In other words, PRM is identical to the z-score of each data point in the lesion from the expected linear regression line calculated for normal brain.

### Statistical Analysis

Statistical analyses were carried out using a Kaplan-Meier survival analysis with the log-rank test if not specified otherwise. A \( p \) value < 0.05 was considered statistically significant, and all statistical computation was performed using Prism 5 (GraphPad Software, Inc.) or JMP 9.0 (SAS Institute, Inc.).

### Results

#### Applying the PRM Calculation to WT1 Immunotherapy Patients

The PRM calculation, described above and in Fig. 1, was successfully performed in all 14 cases. The actual process that was performed is described below by presenting 2 representative cases, one (Case 2) in which the patient had a relatively long OS<sub>WTT</sub> of 144.7 weeks and was considered a treatment responder, and another (Case 7) in which the patient had a relatively short OS<sub>WTT</sub> of 20.9 weeks and was considered a treatment nonresponder.

#### Representative Treatment Responder

A representative case involving a treatment responder (Case 2) is illustrated in Fig. 2. First, a voxel-wise analysis was performed in normal brain tissue (Figs. 1 and 2). As shown in Fig. 2, pre- and post-WT1 <sup>11</sup>C-methionine uptake showed good positive linear correlation in normal brain tissue. A linear regression line and the ± 2 SD distribution range were calculated. Subsequently, the same analysis was performed in a tumor lesion. A contrast-enhanced area was selected as the ROI for analysis. In this particular case, most voxels were distributed in the −2 SD area, suggesting that <sup>11</sup>C-methionine uptake decreased after WT1 immunotherapy (Fig. 2). This area is presented as PRM<sup>MET</sup> (PRM with reduced methionine uptake).

This patient survived for 144.7 weeks after initiation of WT1 immunotherapy, although the contrast-enhanced area increased after WT1 immunotherapy, categorizing this patient as having progressive disease in the Gd-enhanced MR imaging–based RECIST analysis.
Representative Treatment Nonresponder. A representative case in which the patient had only a short OS\text{WTI} (Case 7) is illustrated in Fig. 3. The same analysis as described above was performed. In this particular case, most voxels were distributed in the $+2\sigma$ area (PRM with increased methionine uptake (PRM$^{\text{MET}}$)), suggesting that $^{11}$C-methionine uptake increased after WT1 immunotherapy. This patient survived for 20.9 weeks after initiation of WT1 immunotherapy.

Correlation of Treatment Response Assessment and OS\text{WTI}

Magnetic Resonance Imaging–Based Assessment. To assess the validity of evaluating the response to WT1 immunotherapy using contrast-enhanced MR imaging, the changes in length and volume of the tumor before and 12 weeks after initiating WT1 immunotherapy were calculated. As in Fig. 4A and B, both methods using Gd-enhanced MR imaging failed to show positive correlation with OS\text{WTI} (p = 0.270 and 0.960, respectively).

Conventional MET-PET Analysis. To assess the validity of evaluating the response to WT1 immunotherapy using MET-PET, the changes in maximum $^{11}$C-methionine uptake assessed using the tumor/normal tissue ratio (T/N max) before and 12 weeks after initiating WT1 immunotherapy were calculated. Change of T/N max failed to show any statistically significant correlation with OS\text{WTI} (p = 0.110) (Fig. 4C).

Parametric Response Map Analysis. Finally, correlation of the proposed voxel-wise PRM of MET-PET with OS\text{WTI} was investigated. Each voxel of contrast-enhanced area on the pretreatment MR images were categorized as a no-change area, PRM$^{\text{MET}}$, or PRM$^{\text{MET}}$, according to no change, increase, or decrease, respectively, in methionine uptake 12 weeks after initiation of WT1 immunotherapy. The percentage of the 3 categories was calculated 3-dimensionally and correlated with OS\text{WTI} (Fig. 5). While the percentage of the PRM$^{\text{MET}}$ area showed moderate correlation with OS\text{WTI} (p = 0.100) (Fig. 5 left), the percentage of the PRM$^{\text{MET}}$ area showed excellent correlation with OS\text{WTI} (p = 0.008) (Fig. 5 right). A threshold of 5% for PRM$^{\text{MET}}$ yielded the best performance for discriminating WT1 immunotherapy responders from non-responders (Fig. 5 right). When a Cox proportional hazard model was applied, adjusted by age (cutoff 50 years of age) and performance status (0 or 1 and 2), a threshold of 5% for PRM$^{\text{MET}}$ still remained as the only statistically significant factor (p = 0.01).
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![Image of brain scans and graphs](image)

**Fig. 2.** Case 2. A representative treatment responder with recurrent GBM (OS_{pre} 144.7 weeks). Images were analyzed as in Fig. 1. Voxel-wise PRM analysis revealed that most of the contrast-enhanced lesion was within the PRM_{PET} area. Although the OS_{pre} was 144.7 weeks, conventional MR imaging evaluated the response as progressive disease. Gd-MRI = Gd-enhanced MR imaging; T/Nr = T/N max.

**Discussion**

Conventionally, MR imaging is used to evaluate response to treatment in glioma patients. The maximum length of the contrast-enhanced area is measured and the effect of treatment is analyzed according to RECIST. This method is based on previous reports showing RE-

CIST to be useful in determining objective responses of contrast-enhancing brain tumors to therapy. Moreover, those reports showed that use of RECIST was comparable to volumetric methods.\(^5,6\) On the other hand, problems with using MR imaging–based tumor measurement as an indicator of treatment response have been reported. For example, temozolomide-based chemoradiotherapy for
Fig. 4. Correlation of OS_{WT1} with changes in tumor length and volume using contrast-enhanced MR imaging and the T/N max of MET-PET. Correlations between OS_{WT1} and changes (from before WT1 immunotherapy to 12 weeks after immunotherapy initiation) on Gd-enhanced MR imaging–measured tumor length (A), volume (B), and T/N max of MET-PET (C) are presented. The correlations were not statistically significant (p = 0.267, 0.956, and 0.111, respectively; 14 cases).

newly diagnosed GBM results in a transient increase in tumor enhancement on MR imaging in 20%–30% of patients (pseudoprogression), which is difficult to differentiate from true tumor progression. Similarly, in the present study, changes in tumor length and volume measured by contrast-enhanced MR imaging after WT1 immunotherapy did not correlate with OS_{WT1} (Fig. 4), suggesting that contrast-enhanced MR imaging is inappropriate for evaluating the clinical outcome of WT1 immunotherapy. Unlike chemotherapy or radiotherapy, immunotherapy causes an inflammatory reaction in the tumor, which results in infiltration of inflammatory cells, dilation of capillary vessels, and increased capillary permeability. Thus, it is possible that contrast enhancement does not reflect the tumor activity but rather represents the immune reaction in situ.

On the other hand, MET-PET provides high-resolution metabolic information about the tumor in vivo, information that is impossible to obtain using MR imaging. Previous studies have shown that the ratio of the maximum \(^{11}C\)-methionine uptake in tumor compared with the contralateral normal brain (T/N max) reflects prognosis. However, gliomas are heterogeneous in nature and have heterogeneous uptake of \(^{11}C\)-methionine. In fact, we have previously demonstrated that \(^{11}C\)-methionine uptake correlates with tumor cell density by comparing MET-PET images with stereotactically sampled tissue. Thus, instead of analyzing T/N max, which could result in comparisons between different locations within the tumor, a better method is to analyze the change in \(^{11}C\)-methionine uptake in each anatomical location to elucidate the global change in \(^{11}C\)-methionine uptake within the tumor. To satisfy this need, a voxel-wise PRM analysis was used in the present study and produced excellent correlation between OS_{WT1} and the percentage of PRM^{MET} (Fig. 5). This method showed far better correlation with OS_{WT1} than changes in T/N max by MET-PET, suggesting that the voxel-wise PRM is the most suitable method for assessing the treatment response of gliomas. Moreover, although the number of cases analyzed was small, a threshold of 5% for PRM^{MET} was the best indicator for discriminating WT1 immunotherapy responders from nonresponders in terms of survival time (Fig. 5 right). A similar method has already been applied for diffusion or perfusion MR im-

Fig. 5. Correlation of OS_{WT1} with PRM^{MET} and PRM^{MET}. Correlations between OS_{WT1} and percentage areas of PRM^{MET} (left) and PRM^{MET} (right) are presented. The percentage of PRM^{MET} within the contrast-enhanced lesion before WT1 immunotherapy initiation correlated best with OS_{WT1} (p = 0.008; 14 cases).
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aging analysis in glioma treatment using temozolomide and radiation therapy and has been suggested as an early biomarker for treatment response. The main difference between voxel-wise PRM analysis and conventional imaging analysis is that voxel-wise PRM analysis allows us to identify the location and extent of areas that responded to therapy, rather than comparing the maximum values of the pre- and posttreatment evaluation modality, which could be comparing different locations.

There are, however, limitations that should be noted. Because pre- and posttreatment 11C-methionine uptake is registered and compared, this method cannot be used when the shape or size dramatically change during therapy due to cyst formation or intratumoral hemorrhage. A more advanced method that could correct for tissue deformation is required to compensate for these changes. As the images compared were obtained 12 weeks apart, it is necessary to investigate the possibility of comparing images obtained in shorter intervals. Another limitation of this study is the retrospective nature of the data analysis and the limited sample size. Although a 5% cutoff of PRM^MET yields the best result for the survival analysis, a prospective study with a much larger sample size will be necessary to obtain the most suitable cutoff value. Moreover, other modalities, such as perfusion or diffusion MR images should also be investigated in a similar manner to elucidate whether these modalities could also be used for evaluating immunotherapy for malignant gliomas.

Conclusions

We performed a voxel-wise PRM analysis of MET-PET before and 12 weeks after WT1 immunotherapy initiation to evaluate the clinical responses to WT1 immunotherapy in recurrent malignant glioma patients. This method holds promise for evaluating the dynamics of immunotherapy, which can be difficult to assess using conventional Gd-enhanced MR imaging.

Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper. This work was supported in part by grants to Dr. Kinoshita from the Osaka Cancer Research Foundation, the Konica Minolta Imaging Science Foundation, the Osaka Cancer Researcher Training Fund, the Takeda Science Foundation, the Sagawa Foundation for Promotion of Cancer Research, and the Ministry of Education, Science and Culture of Japan, and by grants to Drs. Chiba and Hashimoto from the Ministry of Education, Science and Culture of Japan.

Author contributions to the study and manuscript preparation include the following. Concept and design: Kinoshita, Chiba, Tsuibo, Hashimoto, Yoshimine. Acquisition of data: Kinoshita, Chiba, Okita, Tsuibo, Isoshishi, Kagawa, Fujimoto, Oji, Oka, Shimosegawa, Hatazawa, Hashimoto, Yoshimine. Analysis and interpretation of data: all authors. Drafting the article: Kinoshita, Chiba, Okita, Morita, Hashimoto, Yoshimine. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Kinoshita. Statistical analysis: Kinoshita. Administrative/technical/material support: Tsuibo, Hashimoto, Yoshimine. Study supervision: Tsuibo, Oji, Oka, Hatazawa, Hashimoto, Yoshimine.

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Phase II clinical trial of Wilms tumor 1 peptide vaccination for patients with recurrent glioblastoma multiforme

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Object. The object of this study was to investigate the safety and clinical responses of immunotherapy targeting the WTI (Wilms tumor 1) gene product in patients with recurrent glioblastoma multiforme (GBM).

Methods. Twenty-one patients with WTI/HLA-A*2402–positive recurrent GBM were included in a Phase II clinical study of WTI vaccine therapy. In all patients, the tumors were resistant to standard therapy. Patients received intradermal injections of an HLA-A*2402-restricted, modified 9-mer WTI peptide every week for 12 weeks. Tumor size, which was obtained by measuring the contrast-enhanced area on magnetic resonance images, was determined every 4 weeks. The responses were analyzed according to Response Evaluation Criteria in Solid Tumors (RECIST) 12 weeks after the initial vaccination. Patients who achieved an effective response continued to be vaccinated until tumor progression occurred. Progression-free survival and overall survival after initial WTI treatment were estimated.

Results. The protocol was well tolerated; only local erythema occurred at the WTI vaccine injection site. The clinical responses were as follows: partial response in 2 patients, stable disease in 10 patients, and progressive disease in 9 patients. No patient had a complete response. The overall response rate (cases with complete or partial response) was 9.5%, and the disease control rate (cases with complete or partial response as well as those in which disease was stable) was 57.1%. The median progression-free survival (PFS) period was 20.0 weeks, and the 6-month (26-week) PFS rate was 33.3%.

Conclusions. Although a small uncontrolled nonrandomized trial, this study showed that WTI vaccine therapy for patients with WTI/HLA-A*2402–positive recurrent GBM was safe and produced a clinical response. Based on these results, further clinical studies of WTI vaccine therapy in patients with malignant glioma are warranted.

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Key Words: cancer vaccine • glioblastoma multiforme • glioma • immunotherapy • Wilms tumor 1

Currently, the standard treatment for gliomas is surgery, followed by external radiation and chemotherapy. In patients with newly diagnosed GBM, however, concurrent irradiation and temozolomide therapy, followed by adjuvant temozolomide therapy for at least 6 months, offered a modest benefit, with a median survival of 14.6 months and a 2-year survival rate of 26.5%. To date, therapeutic options for patients with malignant glioma have been limited, and extensive research is ongoing.

Immunotherapy against malignant gliomas includes several therapeutic approaches that involve dendritic cell–based immunotherapy and antibody-mediated immunotherapy. Cancer vaccination is another novel form of therapy. Recent advances in molecular biology and tumor immunology have resulted in the identification of a large number of tumor-associated antigens that could be used for cancer vaccination, since their epitopes associated with HLA Class I molecules were recognized by CTLs. One of the identified tumor-associated antigens was the product of the Wilms tumor gene, WTI.
The WT1 gene was isolated as a gene responsible for Wilms tumor. It encodes a zinc finger transcription factor, which is involved in cell proliferation and differentiation, apoptosis, and organ development. Although the WT1 gene was first categorized as a tumor suppressor gene, it was later proposed that the wild-type WT1 gene functions as an oncogene rather than as a tumor-suppressor gene. In response to granulocyte colony-stimulation factor, growth promotion and differentiation inhibition were identified in wild-type WT1 gene-transfected myeloid progenitor cells. In many reports, the wild-type WT1 gene was shown to be overexpressed in various types of solid tumors. The WT1 protein was found to be an attractive target antigen for immunotherapy against these malignancies.

Recently, we performed a Phase I clinical trial to examine the safety of a WT1-based vaccine, as well as the clinical and immunological responses of patients with a variety of cancer types, including leukemia, lung cancer, and breast cancer. The authors demonstrated that WT1 peptide vaccine emulsified with Montanide ISA51 adjuvant and administered at a dosage of 0.3, 1.0, or 3.0 mg at 2-week intervals was safe for patients other than those with myelodysplastic syndromes. Furthermore, the vaccination induced peptide-specific CTLs and was associated with clinical response. Very recently, it was confirmed that the potential toxicities of the weekly WT1 vaccination treatment schedule (3 mg per week) with the same adjuvant were also acceptable.

An increasing number of central nervous system studies have reported that systemic immunotherapy is capable of inducing an antitumor response within the immunologically privileged brain. These advances suggest the possibility of the development of a new peptide-based cancer immunotherapy. The blood–brain barrier, which was thought to be one of the hurdles hindering the development of therapeutically effective immunotherapy for gliomas, does not always function effectively in cases involving recurrent gliomas.

Like many other solid tumors, gliomas have been found to express WT1 protein. A definite correlation has been observed between WT1 expression and cellular proliferation activity, as indicated by WHO grade. In the present study, we investigated the clinical responses to peptide-based immunotherapy targeting the WT1 gene product in patients with recurrent GBMs. We also evaluated the correlation between the clinical response and the WT1 expression level in tumor tissues using immunohistochemical staining, as well as WT1-specific CTL frequencies (tetramer assay) in patients' PBMCs.

**Clinical Materials and Methods**

**The WT1 Peptide**

The immunization consisted of an HLA-A*2402-restricted, modified 9-mer WT1 peptide (amino acids 235–243 CYTWNQMNL), in which Y was substituted for M at amino acid position 2 (the anchor position) of the natural WT1 peptide. About 60% of Japanese have HLA-A*2402 which is the most common HLA Class I type in the Japanese population. The modified 9-mer WT1 peptide was shown to induce much stronger CTL activity against WT1-expressing tumor cells than the natural peptide. The WT1 peptide (GMP grade) was purchased from Multiple Peptide Systems as the lyophilized peptide.

**Patient Population**

Twenty-one patients were enrolled in this study. All patients seen in our unit who had recurrent or progressive GBM were eligible to be enrolled if their disease was resistant to conventional chemotherapy and radiotherapy. Patients who had refused chemotherapy but wanted to receive WT1 vaccine therapy under the auspices of this clinical trial were also eligible. In patients who received stereotactic radiosurgery as part of their initial therapy, true recurrence or progression was distinguished from radiation necrosis by metabolic imaging or histological confirmation.

Additional inclusion criteria were: 1) age between 16 and 80 years, 2) expression of WT1 in the glioma cells determined by immunohistochemical analysis, 3) HLA-A*2402– positivity, 4) estimated survival of more than 3 months, 5) ECOG Performance Status Grade 0–2, 6) no severe organ function impairment, and 7) the written informed consent of the patient. All enrolled patients had histologically proven GBM (Grade 4) based on the WHO criteria. After initial resection of the tumor, patients underwent a course of external radiation therapy and chemotherapy. Magnetic resonance imaging was used to monitor patients for recurrence or progression of their tumor during initial therapy and during maintenance therapy. No patient was treated with chemotherapy or radiotherapy during the 4 weeks prior to WT1 immunotherapy. Registered patients had methionine-PET, FDG-PET, thallium-SPECT, and MR imaging to confirm recurrence or progression and to exclude radiation necrosis. All patients underwent electrocardiography, and blood samples were obtained to confirm that there were no abnormalities.

After informed consent was obtained, it took at least 2 weeks for the immunohistochemical analysis, HLA-typing analysis, image analysis, and other tests to be completed. Therefore, the presence of tumor recurrence or progression was again assessed > 2 weeks after registration for WT1 treatment. The DSMC independently reviewed the eligibility of each enrolled patient. Protocol compliance, safety, and on-schedule study progress were also monitored by the DSMC. The WT1 peptide-based Phase II study was approved by the ethical review boards of the Osaka University Faculty of Medicine.

**Vaccine Preparation and Vaccination**

Patients received intradermal injections of 3.0 mg of HLA-A*2402-restricted modified 9-mer WT1 peptide emulsified with Montanide ISA51 adjuvant. The WT1 vaccinations were scheduled to be given weekly for 12 consecutive weeks. Twelve weeks after the initial vaccination, the response was evaluated on MR imaging. If an effect was observed after the 12 vaccinations, WT1 vaccination was continued at 1-week intervals (with the patients' informed consent) until tumor progression was again noted.

**Immunohistochemical Analysis**

Immunohistochemical analysis was performed to confirm WT1 protein expression in malignant glioma tissue using a procedure that has been previously described. Brief-
Wilms tumor 1 peptide vaccination for recurrent GBM

ly, formalin-fixed tissue sections were prepared from the resected tumors. Sections were microwaved in citrate buffer for antigen retrieval and incubated with anti-human WT1 mouse monoclonal antibody 6F-H2 (DAKO; diluted 1:50). The WT1 reaction was visualized with the Vectastain ABC kit (Vector Laboratories) and dianobenzidine (WAKO). The sections were then counterstained with hematoxylin. Control positive staining was evaluated with Wilms tumor, and control negative staining was evaluated with normal brain. Expression of WT1 seen in the sections was classified on a scale from 0 to 4 based on the staining density and the pattern of the gliona cells according to the following criteria: 0, negative staining; 1, slightly increased staining in some tumor cells compared with that in normal glial cells; 2, staining at 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 with that in normal glial cells. Three investigators scored every sample independently; scores agreed upon by at least 2 investigators were accepted.

For MIB-1 immunostaining, antibody against the Ki 67 antigen (DAKO) was diluted 1:50 and used as previously described.11 In each case, MIB-1 immunostaining was performed on the same serial sections used for WT1 immunohistochemical evaluation. The staining index reflecting each tumor’s proliferation activity was determined by calculating the percentage of positively stained tumor cell nuclei out of 1000 evaluated tumor cell nuclei. All assessments were made in areas with the greatest degree of immunostaining.

The WT1 peptide/HLA-A*2402 Tetramer Assay of WT1-Specific CTLs

The WT1 (a natural, HLA-A*2402–restricted, 9-mer WT1 peptide)/HLA-A*2402 tetramer was kindly provided by M. Gotoh of Sumitomo Pharmaceuticals. This tetramer stained > 90% of the TAK-1 cells, which were WT1-specific CTLs that could recognize the complex of the natural 9-mer WT1 peptide and HLA-A*2402 molecules. The PBMCs from HLA-A*2402–positive patients were double-stained with PerCP-Cy5.5 antibody (BD Pharmingen) and phycoerythrin tetramer. The cells were analyzed by fluorescence-activated cell sorting. A double-positive fraction was considered to represent WT1-specific CD8-positive CTLs.

Evaluation of Toxicity

Blood samples were evaluated every week. Toxicities were evaluated according to the US National Cancer Institute Common Toxicity Criteria and independently reviewed by the DSMC.

Evaluation of MR Images

Magnetic resonance imaging was performed every 4 weeks. After the WT1 vaccine was administered 12 times, the antitumor effect of the treatment was assessed by determining the response of the target lesions on MR images. The tumor size, corresponding to the contrast-enhanced area on T1-weighted MR images, was measured and analyzed according to RECIST,23 with results reported as complete response, partial response, stable disease, and progressive disease. The response rate was calculated as the percentage of the number of cases in which there was a complete or partial response divided by the total number of cases. The effective rate was calculated as the percentage of the number of cases in which there was a complete or partial response or stable disease divided by the total number of cases.

Additional Vaccinations and Calculation of the Survival Period

If an effect was observed after 12 vaccinations, further WT1 vaccination at 1-week intervals was given only with the patients’ informed consent. The PFS period was calculated from the day of the first WT1 vaccination to the day of the last image prior to the detection of disease progression; this was used as the principal end point. The overall survival period after WT1 vaccination was also calculated, as was the overall survival period after tumor recurrence for WT1-vaccinated patients.

Statistical Analysis

Our main objectives were to evaluate the duration of PFS, the 6-month PFS rate, the overall response rate, the disease control rate, and toxicity based on the WHO criteria. The objective assessments of tumor response were reported using RECIST and were based on major changes in tumor size seen on Gd-enhanced MR images in comparison with the baseline images. Hematological and nonhematological toxicities were assessed using the US National Cancer Institute Common Toxicity Criteria, and the safety and tolerability of the treatment were estimated. Statistical evaluation was performed using Stat View version 4.5 (Abacus Concepts, Inc.). Probability values < 0.05 were considered statistically significant. The Kaplan–Meier method was used to analyze overall survival and PFS. The log-rank test was used to assess the strength of the association between survival time and single variables corresponding to factors that were considered prognostic for survival.

The required sample size for this Phase II trial was estimated to be 20 at 5% Type I and 20% Type II errors, under the assumption of 10 and 30% 6-month PFS rates for the null and alternative hypotheses, respectively. Allowing for the possibility that we might not be able to obtain complete data in all cases, the sample size was set at 21.

Results

Patient Characteristics

During the trial period, 37 patients were assessed for inclusion in the trial. All 37 had WT1-positive GBM, as determined by immunohistochemical analysis. Because we use HLA-A*2402–restricted WT1 peptide, 16 patients with HLA-A*2402–negative type were excluded, and 21 patients (7 women and 14 men) with HLA-A*2402–positive type were enrolled in the study (Table 1). In all the cases involving HLA-A*2402–negative excluded patients, the survival time from recurrence or progression to death was investigated. The median survival time after tumor recurrence in the HLA-A*2402–negative patients was 21 weeks, which was almost the same as that of the historical
control patients at Osaka University Hospital (20 weeks, data not shown). The mean age of the 21 enrolled HLA-A*2402-positive patients was 51.4 years (range 20–76 years). Of the 21 patients, 15 had recurrent disease and 6 had disease progression after initial therapy. All patients had radiotherapy with or without chemotherapy or interferon treatment. All enrolled patients had an ECOG performance status of 0–2 (Karnofsky Performance Scale score ≥ 50), and 10 of them were receiving a maintenance dose (1–4 mg/day betamethasone) of glucocorticoid therapy at the time of vaccination due to local symptoms or symptoms of increased intracranial pressure caused by edema in the area around the tumor. Eight patients underwent surgery after recurrence for mass reduction and confirmed recurrence, and methionine-PET, thallium-SPECT, and FDG-PET were performed in all cases to confirm tumor recurrence.

**Clinical Response to Vaccination**

All treated patients had a local inflammatory response with erythema at the WT1 vaccine injection site. No Grade 3 or 4 toxicities were observed. Liver dysfunction was detected in Case 9, but improved after the patient’s anticonvulsant therapy was changed. This event was considered by the DSMC and was judged to have had no relationship to the WT1 treatment.

A summary of patient responses to WT1 immunotherapy is shown in Table 1. Clinical responses included partial response in 2 patients; stable disease in 10 patients; and progressive disease in 9 patients, including 2 who dropped out of the trial due to tumor progression and poor general condition (Cases 10 and 13). Patients who had an effective response continued to receive vaccinations until tumor progression was demonstrated. All responses were assessed by the DSMC.

The overall response rate was 9.5%. The disease control rate, calculated from the number of patients with complete response, partial response, or stable disease in the initial 3 months (the clinical trial period) was 57.1%. The Kaplan–Meier survival probability curves are shown in Fig. 1. Median PFS in the 21 patients with GBM who were included in the study was 20.0 weeks, and the PFS rate at 6 months (26 weeks) was 33.3%. Median overall survival after initial vaccination was 36.7 weeks. Median overall survival after tumor recurrence in WT1-vaccinated patients was 46 weeks.

Two patients (Case 2 and Case 16) experienced partial response. In both cases, immunohistochemical analysis of the tumor specimens showed high WT1 expression levels, but neither patient survived for a long period (PFS of 23.4 weeks in Case 2 and 20.0 weeks in Case 16). Both patients had disease progression after the 12-week trial period, with leptomeningeal dissemination of the glioma cells and formation of a mass at a different site.

In contrast, in the stable disease group 4 patients (Cases 8, 11, 15, and 18) experienced gradual tumor stabilization; that is, they had a response during the late period of the 3-month WT1 vaccination course. These patients survived for a long time without progression (PFS > 96.0 weeks in Case 8, 51.3 weeks in Case 11, 42.4 weeks in Case 15, and > 43.6 weeks in Case 18).

**Relationship Between PFS and WT1-Immunostaining Intensity**

In all 21 patients, immunostaining was positive for WT1. The WT1 expression score was 4 in 7 cases, 3 in 8 cases, 2

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<th>Sex</th>
<th>RT Dose (Gy)</th>
<th>Chemo</th>
<th>Add’l Tx</th>
<th>Steroid Tx</th>
<th>KPS Score</th>
<th>Response</th>
<th>PFS (wks)</th>
<th>OS (wks)</th>
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* ACNU = nimustine hydrochloride; Add’l = additional; CE = carmustine and etoposide; Chemo = chemotherapy; IFN = β-interferon; KPS = Karnofsky Performance Scale; OS = overall survival; PD = progressive disease; PR = partial response; RT = radiotherapy; SD = stable disease; SRS = stereotactic radiosurgery; Tx = therapy; — = not administered.
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in 4 cases, and 1 in 2 cases (Table 1). Figure 2 shows representative photomicrographs of Score 2 (Fig. 2A), Score 3 (Fig. 2B), and Score 4 (Fig. 2C) WT1 immunostaining, and Fig. 2D shows MIB-1 immunostaining of a section from the same lesion as Fig. 2C. Both of the patients who had a partial response to vaccination had Score 4 immunostaining. The patients were grouped according to WT1 expression scores, and PFS curves were estimated for each group and then compared. The patients with Score 3 immunostaining tended to have the longest PFS time. The patients with Score 3 or 4 had a statistically longer PFS time than the patients with Score 1 or 2 (p = 0.0020, Fig. 3 right). Among the patients with high WT1-immunostaining scores (3 and 4), the patients with Score 4 had a shorter PFS time than those with Score 3, although partial response was achieved in 2 patients with Score 4. This might reflect the fact that the patients with Score 4 had high proliferation activity of the GBM cells that was recognized by the high MIB-1 staining index, although they also had the highest amount of target WT1 protein recognized by the induced WT1-specific CTLs.

Relationship Between PFS and MIB-1 Staining Index

The MIB-1 staining index, which reflects each tumor’s proliferation activity, was determined by calculating the percentage of positively stained tumor cell nuclei. No statistical difference in PFS was observed between the 2 groups (Fig. 3 left). The proliferation activity was found not to directly affect PFS after WT1 vaccination.

Evaluation of WT1-Specific CTL Frequencies in PBMCs

The frequencies of WT1-specific CTLs before WT1 vaccination were significantly higher in patients with GBM than in healthy controls (p = 0.0019, Fig. 4). These results indicate that the immune system in patients with WT1-expressing GBM cells responded to the WT1 protein derived from the tumor cells and elicited WT1-specific CTLs that were present before WT1 vaccination; this suggests that the WT1 protein in GBM cells is naturally immunogenic. The existence of the high frequencies of WT1-specific CTLs before WT1 vaccination may have contributed to the favorable clinical responses in patients with GBM. There was no correlation between the induction of a clinical response and WT1-specific CTL frequencies in the PBMCs of the patients prior to vaccination (Fig. 4). Furthermore, the CTL frequencies did not increase after vaccination, even in the patients who responded.

Discussion

The WT1 gene is physiologically expressed in some organs, such as the kidneys, bone marrow, and pleura. Experimental evidence shows that WT1-specific CTLs kill WT1-expressing tumor cells without killing normal cells.24 Consistent with these data, in the present study, patients with a clinical response had adverse effects of the WT1 vaccination that were limited to local erythema at the injection sites of the WT1 vaccine.

The primary end points of this study were PFS and the PFS rate at 6 months. The objective response rate and the disease control rate with WT1 vaccination, as well as its safety and tolerability, were also estimated.

Fig. 1. Kaplan–Meier curves for PFS (solid line) and overall survival (dotted line) after initial WT1 vaccination for patients with recurrent GBM.

A review of the literature suggested that an agent demonstrating a 6-month progression-free survival rate \( \geq 10\% \) would be considered active.25 A retrospective analysis of 8 Phase II chemotherapy trials conducted from 1986 to 1995 and involving a total of 225 patients with GBM was performed at the M. D. Anderson Cancer Center; a median PFS of 9 weeks and a 6-month PFS rate of 15% were reported.26 Temozolomide, the most recent drug to be introduced for the treatment of GBM, has been shown to produce results that were not very different from those achieved with carmustine (BCNU). A study that included a series of 112 patients with GBM demonstrated a response rate of 6% with a 6-month PFS rate of 21%;27 another study, which included a series of 138 patients with GBM, demonstrated a response rate of 8% and a 6-month PFS rate of 18%.28 The use of BCNU chemotherapy in recurrent GBM was also recently studied; the median time to progression was 13.3 weeks, and the 6-month PFS rate was 17.5%.29 Following these reports, 6-month PFS rates for the null and alternative hypotheses were assumed to be 10 and 30%, respectively, in this trial, and the sample size was set at 21.

In our study, the median duration of PFS was 20.0 weeks, and the PFS rate at 6 months was 33.3%. The response rate was 9.5%, whereas the disease control rate was 57.1%. The 6-month PFS rate was 33.3% in our patients with GBM—which was higher than the 10% that was set as indicating an active level—and, moreover, was higher than the 30% that was set as the alternative hypothesis before the study was started. Thus, this result suggested that WT1 vaccination was active. The median PFS and median overall survival after WT1 vaccination were 20.0 weeks and 36.7 weeks, respectively; these results are comparable to those reported in the literature for various combination regimens of chemotherapy and/or radiotherapy.

All the treated patients had an inflammatory response with erythema at the WT1 vaccine injection site, but no systemic toxicities were observed. Taken together, these findings allow one to conclude that WT1 vaccination had an anti-GBM effect, it was safe, and the patients tolerated it well.

Although the response rate in our study (9.5%) was not
very high compared with findings reported in chemotherapy studies, the disease control rate of 57.1% was favorable. The ability of WT1 vaccination to stabilize tumor growth might explain a good PFS of the patients treated with the vaccine. It should be emphasized that WT1 immunotherapy is less toxic than all of the chemotherapy treatments reported. Taken together, the patients in our study had a median PFS, 6-month PFS rate, and disease control rate that were comparable to those achieved using other chemotherapy regimens but with much less toxicity. These findings indicate that WT1 vaccination may be useful for the treatment of GBM.

In our study, WT1-specific CTL frequencies were higher in the PBMCs of patients with GBM than in those of healthy controls; this same phenomenon has been seen in other solid cancers. The results, including good PFS and 6-month PFS rate and high stable disease rate, might be at least partly due to the high frequency of WT1-specific CTLs in the PBMCs of the patients prior to vaccination. Even in the responders, however, the CTL frequencies did not increase after vaccination. In our recent report, we found a correlation between the clinical response and an increase in WT1-specific CTL frequencies in the PBMCs of cancer patients after vaccination. The correlation was clear in patients with leukemia, but it was not that clear in those with solid tumors (lung and breast cancer; unpublished data). Several cancer immunotherapy trials have shown a poor correlation between clinical response and an increase in antigen-specific CTL frequencies. Germeau et al. reported that high frequencies of the antigen-specific CTL were observed before vaccination and did not correlate the clinical response in solid cancers. They suggest that a spontaneous antitumor T-cell response that has become ineffective can be awakened by vaccination and contribute to tumor rejection. After the vaccination, CTLs in the responders might change qualitatively, but not quantitatively. The successfully activated CTLs could have more migratory ability, which would lead to the accumulation of CTLs in the brain. These issues should be addressed by an intense analysis of the CTLs in WT1 vaccine-treated patients with GBM.

Immunohistochemical analysis showed that the patients with a high expression of WT1 protein in tumor specimens tended to respond well to WT1 vaccination. This finding suggests that the presence of high target antigen levels in the tumor cells plays an important role in the clinical responses. Taken together, both a high frequency of WT1-specific CTLs and a high WT1 protein expression level in tumor tissues may be needed for good clinical response to WT1 vaccination.

Under normal conditions, no lymphocytes are present in the brain parenchyma. However, tumor-infiltrating lym-
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Fig. 3. Left: Kaplan–Meier curves for PFS after initial WT1 vaccination for patients with recurrent GBM classified by MIB-1 staining index (S.I.) determined by means of immunohistochemical analysis. The solid line indicates cases with an MIB-1 staining index of < 20%, and the dotted line indicates cases with an MIB-1 staining index of ≥ 20%. No statistical difference in PFS was observed between the 2 groups. Right: Kaplan–Meier curves for PFS after initial WT1 vaccination of patients with recurrent GBM classified by WT1 expression level. The solid line indicates cases with low WT1 expression on tumor cells (Score 1 or 2), and the dotted line indicates cases with high WT1 expression on tumor cells (Score 3 or 4). Cases with scores of 3 or 4 were associated with better PFS than cases with scores of 1 or 2 (p = 0.002).

phocytes are found in and around the tumors in 35–80% of patients with malignant glioma; this may indicate that tumor-specific CTLs would be available to attack the tumor. It has also been reported that immunosuppressive mechanisms, such as the existence of regulatory T cells,6 hamper CTL function. Thus, the combination of a cancer vaccine with other modalities to inhibit immunosuppressive mechanisms may be useful for improving the efficacy of the vaccine.

It is probable that some cancer patients treated with cancer vaccines can survive long-term without remarkable tumor regression. On the other hand, their tumors could be stabilized or could regress following a temporary increase in size after vaccination since, in general, immunotherapy does not act as quickly as chemotherapy. In fact, some patients in the stable disease group in this study survived for a long time without the treatments achieving partial response. In Case 8, a decrease in tumor size, although it did not reach the partial response level, was observed 7 months after the initial WT1 vaccination. Furthermore, in some of the patients whose clinical response was classified as progressive disease (Cases 3 and 9), tumor stabilization was induced by WT1 vaccination at a later time during the trial. Therefore, one has to consider whether RECIST, which is the gold standard for evaluating the response of solid tumors to cancer chemotherapy, is suitable for evaluating the clinical response to cancer vaccine treatment.8

The mechanisms of tumor escape from immune recognition/ destruction are thought to be multifactorial. They include: downregulation of major histocompatibility complex Class I molecules, loss of tumor antigens, defective death receptor signaling, lack of costimulation, and the production of immunosuppressive cytokines and suppressive cells.1

Given the many different potential mechanisms, combination therapy strategies that use several treatment modalities could include sequential chemotherapy, radiotherapy, and immunotherapy protocols; these will need to be considered.27

Conclusions

In HLA-A2402–positive patients with GBM, immune therapy strategies that use several treatment modalities could include sequential chemotherapy, radiotherapy, and immunotherapy protocols; these will need to be considered.

Fig. 4. Graph showing the frequencies of WT1–specific CTLs before WT1 vaccination, 4 and 12 weeks after WT1 vaccination, and in healthy controls. Patients with controlled disease (partial response or stable disease, closed circles) as well as those with uncontrolled disease (progressive disease, open circles) had a higher frequency of WT1–specific CTLs during the entire evaluation period than healthy controls (diamonds). The horizontal bars indicate mean frequencies.
notherapy with HLA-A*2402–restricted, modified 9-mer WT1 peptide vaccination had disease-stabilizing, as well as disease progression–inhibiting, effects that were equal or superior to those of chemotherapy, with systemic toxicity that was much less than that of chemotherapy and thus allowed the vaccinations to be given for a long time. The WT1 protein is considered to be one of the most promising tumor antigens, since injection of a single WT1 peptide type can induce a clinical response. This is another advantage of the vaccine—one does not need to choose a suitable combination of peptides in the laboratory before vaccination. Compared with dendritic cell therapy, WT1 vaccination is simple. The use of a more suitable adjuvant, such as Mycobacterium bovis bacillus Calmette–Guérin cell wall skeleton (BCG-CWS),10 or combination therapy involving vaccination10 and other modalities may further enhance the clinical usefulness of this treatment for patients with GBM.

Disclaimer
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