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Immunohistochemical Detection of WT1 Protein in Endometrial Cancer

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Abstract. *Background: The Wilms' tumor gene WT1 is overexpressed in various kinds of solid tumors. However, it remains unclear whether WT1 plays a pathophysiological role in endometrial cancer. Patients and Methods: A series of 70 endometrial cancer patients who had undergone a curative resection was studied to determine the correlation between WT1 expression, clinicopathological characteristics and prognosis. Tissue specimens were evaluated for WT1 expression by immunohistochemistry. Results: The expression of WT1 was strong in 31 patients (44%) and weak in 39 patients (56%). WT1 overexpression was associated with advanced FIGO stage (p=0.0266), myometrial invasion (p=0.0477) and high-grade histological differentiation (p=0.0049). The expression level of WT1 was found to be a significant predictor of disease relapse in univariate analysis (p=0.0233), but not in multivariate analysis (p=0.4757). Conclusion: These results suggested that tumor-produced WT1 provided additional prognostic information in endometrial cancer patients.*

Endometrial cancer is the most common gynecological malignancy in the United States. In Japan, it is the second most common gynecological cancer, but its frequency has dramatically increased in the last decade. Although there are well-established surgical and chemotherapeutic treatments for endometrial cancer, the need for molecular-target therapy has increased, especially for recurrent disease that has acquired radio- or chemoresistance, thus, there is a need for a better understanding of the molecular pathways of endometrial carcinogenesis.

The Wilms' tumor gene *WT1* was isolated as a gene responsible for a childhood renal neoplasm, Wilms' tumor (1, 2). This gene encodes a zinc finger transcription factor and play important roles in cell growth and differentiation (3, 4). Although *WT1* gene was categorized at first as a tumor-suppressor gene, it was recently demonstrated that the wild-type *WT1* gene performed an oncogenic rather than a tumor-suppressor function in many kinds of malignancies (5). *WT1* gene is highly expressed in hematological malignancies and solid tumors, including endometrial cancer (6). However, it remains unclear whether *WT1* plays a pathophysiological role in endometrial cancer.

Therefore, in the present study, we immunohistochemically analyzed the expression of WT1 protein in 70 cases of primary endometrial cancer to study the relationship between WT1 expression and clinicopathological characteristics as well as prognosis to clarify the prognostic significance of WT1 protein expression in endometrial cancer patients.

Patients and Methods

Patients. This study included 70 primary endometrial carcinoma patients who had been consecutively admitted, treated and followed-up at the Department of Obstetrics and Gynecology, Kanazawa University Hospital from January 1995 to December 2002. None of the patients had received any pre-surgical treatment and all had undergone a total abdominal or radical hysterectomy plus bilateral salphingo-oophorectomy. At the time of laparotomy, peritoneal fluid samples were obtained for cytological testing. Systemic pelvic lymphadenectomy was performed in 51 (72.9%) patients. Paraaortic lymph node sampling was performed in two patients because of visible or palpable enlarged lymph nodes. All the patients were classified by the International Federation of Gynecology and Obstetrics (FIGO) surgical staging system (1988). No patient had remaining macroscopic tumors or known distant metastasis immediately after surgery. The high-risk patients (*e.g.* these with deep myometrial invasion, cervical involvement, special histology,

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or peritoneal cytology) underwent external radiotherapy and/or six cycles of chemotherapy (paclitaxel: 180 mg/m^2, carboplatin: according to Chatelut's formula [AUC=5 mg min/ml]) as postoperative adjuvant therapy.

The treatment was followed by a gynecological examination, recording of laboratory data, transvaginal/abdominopelvic ultrasonography and a radiological investigation. The data from regular follow-up visits to the outpatient department were stored in a database specifically designed for endometrial carcinoma patients. A telephone inquiry to update the present status of all surviving patients was made in August 2006. The exact date of disease recurrence was obtained from the referring physicians or from the physicians who attended the patient for the initial diagnosis of the recurrence. All the treatments and clinical research were conducted with written informed consent.

Immunohistochemistry. Formalin-fixed and paraffin-embedded tissues from 70 tumors were retrieved with informed consent from archive sources at Kanazawa University Hospital. The histological diagnosis of each tumor was confirmed on the hematoxylin and eosin-stained sections. Representative sections containing both the normal endometrium and the invasive front of the tumor tissue were selected for immunohistochemical staining. The slides were deparaffinized and rehydrated in graded alcohols. Epitope retrieval was performed using enzymatic digestion with Proteinase K for 30 minutes at 37°C (Dako Cytomation. Carpinteria, CA, USA), and by microwave heating for 15 minutes using Target Retrieval Solution (Dako Cytomation). Endogenous peroxidase activity was quenched by dipping in 3% hydrogen peroxide for 30 minutes. The slides were incubated with mouse monoclonal antibodies (clone 6F-H2; Dako Cytomation) diluted 1:100 at 4°C overnight. The subsequent steps were carried out according to the manufacturer's instructions by the EnVision+ ® System horseradish peroxidase (HRP)-labelled polymer (Dako Cytomation). Color development was carried out with peroxidase substrate 3-amino-9-ethylcarbazole (AEC). All the slides were counterstained with Mayer's hematoxylin. Formalin-fixed, paraffin-embedded sections of human Wilms' tumor were used as positive controls for WT1.

Evaluation of staining. For evaluation of WT1 expression, staining intensity was scored as 0 (negative), 1 (weak), 2 (medium) and 3 (strong). The extent of staining was scored as 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%) and 4 (76-100%) according to the percentage of the positive staining area in relation to the whole carcinoma area. The sum of the intensity and extent score was used as the final staining score (0-7) for WT1. Tumors having a final staining score of ≥5 were considered to exhibit strong expression. All the histological slides were examined by two observers (S.O. and Y.O.) who were unaware of the clinical data or the disease outcome.

Statistical analysis. The Chi-square test for 2x2 tables was used to compare the categorical data. Mortality and probability of relapse after surgery were compared by Kaplan-Meier analysis and the log-rank statistic. In the analysis of relapse-free survival rates, those who died of causes unrelated to endometrial cancer and those who had no detected evidence of disease recurrence were considered to be relapse-free. A p-value of <0.05 was considered to indicate statistical significance. All the statistical analyses were performed using the statistical package StatView version 5.0 for Macintosh (Abacus Concepts, Berkeley, CA, USA).

Results

Characteristics of the patients. The patients' average age at the time of surgery was 57.3 years (range, 26-78 years), 22 had premenopausal status, 4 had perimenopausal status and 44 had postmenopausal status. The patients' mean preoperative body mass index (BMI) was 24.0 (range, 16.9-32.9). Among the 70 patients, 12 patients (17.1%) had relapses of endometrial cancer at the time of the last follow-up. The median follow-up time for all the patients was 5.12 years (range, 0.56-11.08 years).

WT1 expression in endometrial cancer. WT1 expression was positive exclusively in cancer cells in 64 cases (91%). The expression of WT1 was strong (final staining score of 5-7) in 31 patients (44%) and weak (final staining score of 0-4) in 39 patients (56%). The typical WT1 expression in endometrial cancer cells is shown in Figure 1. A majority of the positive cases showed diffuse or granular staining in the cytoplasm. The staining of WT1 was heterogeneous in advanced tumors and WT1 was frequently located at the invasion front of the tumor. The association between WT1 expression and clinico-pathological variables is shown in Table I. WT1 overexpression was associated with advanced FIGO stage (p=0.0266), myometrial invasion (p=0.0477) and high-grade histological differentiation (p=0.0049), indicating up-regulation of WT1 expression with tumor progression in this study.

Prognostic impact of WT1 expression in endometrial cancer. Strong expression of WT1 was associated with reduced relapse-free survival in endometrial cancer (Figure 2A). Although there was no clear statistical significance, WT1 expression was a factor negatively influencing the overall survival rate (Figure 2B). Multivariate analysis indicated that WT1 expression had no independent significant effect (data not shown).

Discussion

With the use of anti-WT1 monoclonal (6F-H2) antibody, positive staining in the tumor cells was observed in 91% of the cases. The relatively high rate of positivity for WT1 in the present study contrasts with some previous reports. Acs *et al.* (7) reported that WT1 immunoreactivity was seen in ten of 16 serous, but in none of 35 endometrioid or 18 clear cell carcinomas among endometrial carcinomas. Egan *et al.* (8) also reported that two of 31 serous carcinomas and none of 39 endometrioid carcinomas were reactive for WT1. Meanwhile, Dupont *et al.* (9) confirmed that WT1 expression was found in twenty of 99 endometrioid carcinomas using polyclonal antibody against WT1 (Santa Cruz; clone C-19). The discrepancy between our findings and previous results could be explained by the different criteria employed to judge WT1 positivity: they regarded nuclear but not cytoplasmic

Figure 1. *Representative sections of endometrial cancer with immunohistochemical staining of WT1. Strong cytoplasmic staining is observed in the invasion front of the tumor (×40; inset, ×200).*

staining in the tumor cells as positive, because WT1 is principally a DNA-binding transcription factor mainly distributed in the nucleus. In the present study, granular or diffuse cytoplasmic staining in the tumor cells was judged as positive, for reasons explained below.

Nakatsuka *et al.* reported that Western blot analysis revealed the intracytoplasmic localization of WT1 protein in lung cancer cells (6). Oji *et al.* (10) and Drakos *et al.* (11) showed the cytoplasmic expression of WT1 protein in cell lines derived from glioblastoma and lymphoma. Moreover, Ye *et al.* (12) revealed that phosphorylation in the DNA-binding domain of WT1 alters the affinity for DNA and subcellular distribution of WT1. Post-translational phosphorylation at zinc fingers inhibits the ability to bind DNA, resulting in the cytoplasmic retention of WT1, and also inhibits transcriptional regulatory activity. As established by the interesting study of Niksic *et al.* (13), WT1 shuttles between the nucleus and cytoplasm and might be involved in the regulation of translation through its association with actively translating polysomes. Recent studies found that many types of tumor frequently showed strong cytoplasmic expression of WT1, suggesting that WT1 was involved in the development of tumors (6, 10, 14-16). In the present study, we also found that the majority of endometrial tumors showed strong cytoplasmic WT1 staining, which was associated with advanced FIGO stage, myometrial invasion and high-grade histological differentiation. These results suggest that up-regulation of WT1 expression is linked to tumor progression.

To date, few reports are available on the prognostic impact of WT1 expression in endometrial cancer patients. Miyoshi *et al.* (17) reported that the disease-free survival rate was significantly lower in breast cancer patients with high levels of *WT1* mRNA than those with low levels. Inoue *et al.* (18) showed that leukemia with strong *WT1* mRNA expression

Table I. *WT1 expression and clinicopathological characteristics.*

Variable	WT1 expression		*P*-value (χ^2 test)
	Strong (n=31)	Weak (n=39)	
Age (year)			
<60 (n=43)	16	27	
≥60 (n=27)	15	12	0.1325
FIGO stage			
I (n=52)	19	33	
II, III, IV (n=18)	12	6	0.0266
Lymph node metastasis			
Negative (n=65)	28	37	
Positive (n=5)	3	2	0.4629
Depth (myometrial invasion)			
a (n=17)	4	13	
b, c (b, n=36; c, n=17)	27	26	0.0477
Histopathology-degree of differentiation			
Grade 1 (n=38)	11	27	
Grade 2, 3 (n=32)	20	12	0.0049
Menopause			
Peri, pre (n=26)	8	18	
Post (n=44)	23	21	0.0801
Body mass index			
<25 (n=45)	19	26	
≥25 (n=25)	12	13	0.6410

showed a significantly lower rate of complete remission and significantly worse overall survival than that with weak expression. Moreover, Sera *et al.* (19) reported that overexpressed WT1 protein, which was confirmed by Western blotting and immunohistochemical staining, was an independent prognostic factor for disease-free survival in hepatocellular carcinoma patients. Høgdall *et al.* (20) demonstrated that univariate Kaplan-Meier survival analysis performed on 560 ovarian cancer patients showed a significantly shorter disease-specific survival in patients with positive WT1 protein expression in the tumor tissue. Netinatsunthorn *et al.* (21) also reported that immunohistochemical expression of WT1 was a prognostic predictor in patients with advanced serous epithelial ovarian carcinoma. In the present study, we found that strong expression of WT1 was associated with reduced relapse-free survival in endometrial cancer patients. Our results are congruent with previous reports of other types of cancer.

WT1 could be a novel tumor rejection antigen in immunotherapy for various kinds of WT1-expressing cancer. Clinical trials of WT1 peptide-based cancer immunotherapy showed that WT1 vaccination induced a reduction in tumor size or decrease in tumor marker levels in breast, lung cancer, leukemia and glioblastoma multiforme (22, 23). The results of the present study provide a rationale for immunotherapy targeting WT1 as a new treatment strategy for endometrial cancer.

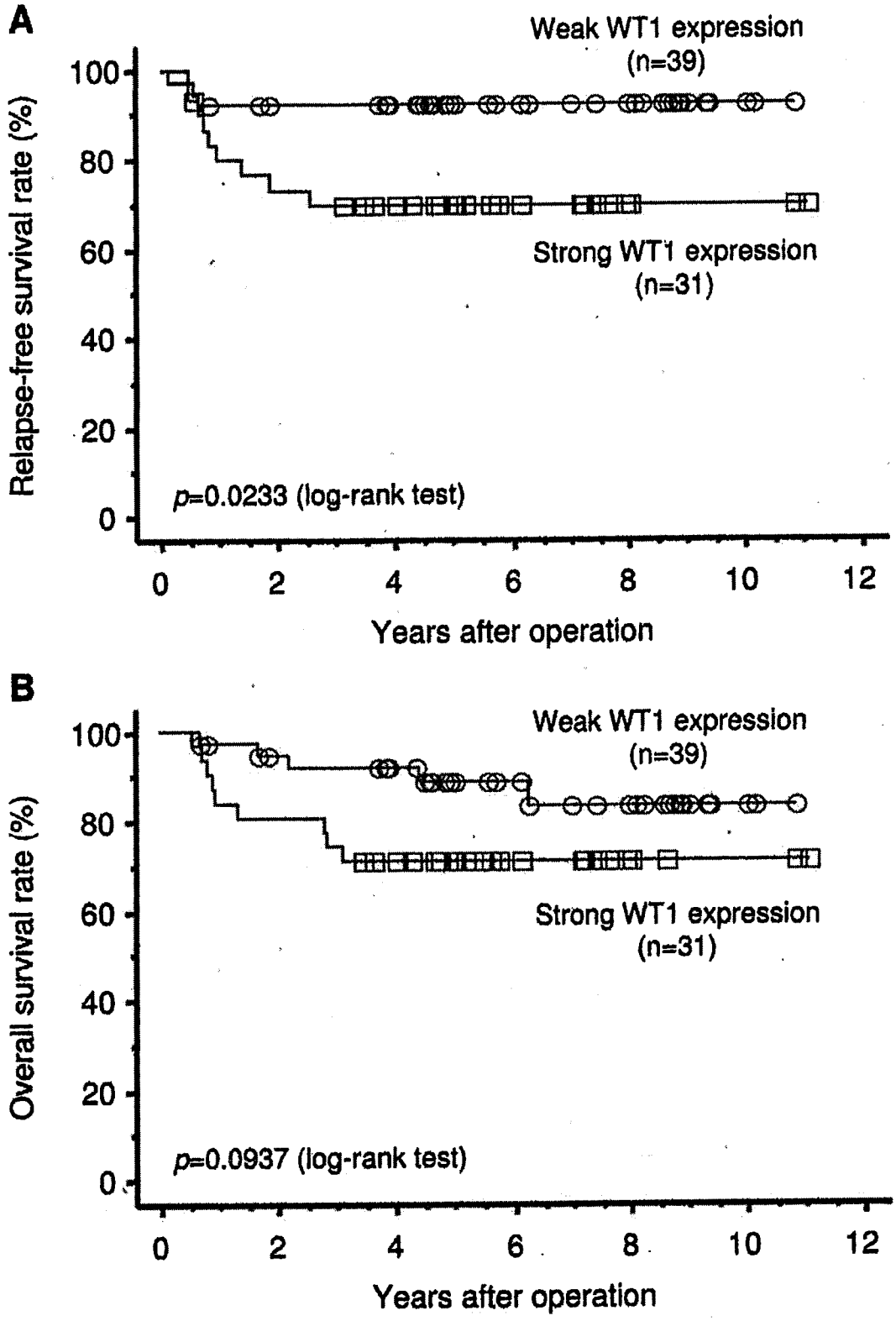

Figure 2. *The Kaplan-Meier survival curves of 70 patients with endometrial carcinoma in relation to WT1 expression are shown. A, Relapse-free survival rate; B, overall survival rate.*

In conclusion, our study now shows the cytoplasmic expression of WT1 might provide additional prognostic information for endometrial cancer patients.

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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 *WT1* (Wilms tumor 1) gene product in patients with recurrent glioblastoma multiforme (GBM).

Methods. Twenty-one patients with WT1/HLA-A*2402–positive recurrent GBM were included in a Phase II clinical study of WT1 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 WT1 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 WT1 treatment were estimated.

Results. The protocol was well tolerated; only local erythema occurred at the WT1 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 WT1 vaccine therapy for patients with WT1/HLA-A*2402–positive recurrent GBM was safe and produced a clinical response. Based on these results, further clinical studies of WT1 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%.[22] To date, therapeutic options for patients with malignant glioma have been limited, and extensive research is ongoing.

Immune therapy against malignant gliomas includes several therapeutic approaches that involve dendritic cell–based immunotherapy and antibody-mediated immunotherapy.[31] Cancer vaccination is another novel form of therapy.[30] 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, *WT1*.[17]

Abbreviations used in this paper: CTL = cytotoxic T-lymphocyte; DSMC = Data Safety Monitoring Committee; ECOG = Eastern Cooperative Oncology Group; FDG = fluorodeoxyglucose; GBM = glioblastoma multiforme; HLA = human leukocyte antigen; MR = magnetic resonance; PBMC = peripheral blood mononuclear cell; PET = positron emission tomography; PFS = progression-free survival; RECIST = Response Evaluation Criteria in Solid Tumors; SPECT = single-photon emission computed tomography; WHO = World Health Organization; WT1 = Wilms tumor 1.

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.[25] 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.[20]

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.[19] 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.[15]

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.[29–31] 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.[29]

Like many other solid tumors, gliomas have been found to express WT1 protein.[8] A definite correlation has been observed between WT1 expression and cellular proliferation activity, as indicated by WHO grade.[8] 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.[26] 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.[8] Brief-

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 diaminobenzidine (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 glioma 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-CD8 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

TABLE 1

*Characteristics of and clinical results in all enrolled patients**

Case No.	Age (yrs), Sex	RT Dosage (Gy)	Chemo	Add'l Tx	Steroid Tx	KPS Score	Response	PFS (wks)	OS (wks)	WT1 Score
1	63, M	60	CE × 3	IFN	yes	50	SD	28.1	36.1	4
2	33, M	60	—	—	yes	60	PR	23.4	32.4	4
3	45, M	60	CE × 3	IFN	yes	70	PD	5.1	32.6	1
4	29, F	60	CE × 3, ACNU × 2	IFN	—	90	SD	16.0	30.1	2
5	69, M	60	—	IFN	—	80	PD	8.0	36.7	3
6	69, M	60	CE × 3	IFN	—	80	SD	24.4	106.1	3
7	42, M	50	—	—	—	60	SD	32.0	87.1	4
8	46, F	56	—	SRS	yes	60	SD	>96.0	>96.0	3
9	63, M	60	ACNU × 3	SRS	yes	80	PD	0	>87.3	4
10	67, M	60	ACNU × 3	IFN	—	90	PD	4.0	15.0	3
11	40, F	60	ACNU × 3	—	—	80	SD	51.3	69.4	3
12	76, M	60	ACNU × 3	IFN	yes	70	SD	21.1	>79.4	1
13	54, M	50	CE × 3	IFN	yes	50	PD	4.0	18.4	2
14	55, M	60	CE × 3	IFN	—	90	PD	2.0	28.4	2
15	58, F	60	CE × 3	IFN	—	90	SD	42.4	61.7	3
16	20, F	60	ACNU × 2	—	—	90	PR	20.0	29.3	4
17	42, M	60	—	—	—	90	PD	4.3	35.6	3
18	41, M	60	CE × 3, ACNU × 2	SRS	yes	100	SD	>43.6	>43.6	3
19	54, M	60	ACNU × 3	IFN	yes	90	PD	8.0	>41.6	2
20	58, F	50	—	SRS	—	50	SD	>32.1	>32.1	4
21	55, F	60	—	—	yes	100	PD	0	>31.4	4

* ACNU = nimustine hydrochloride; Add'l = additional; CE = carboplatin 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.

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

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.

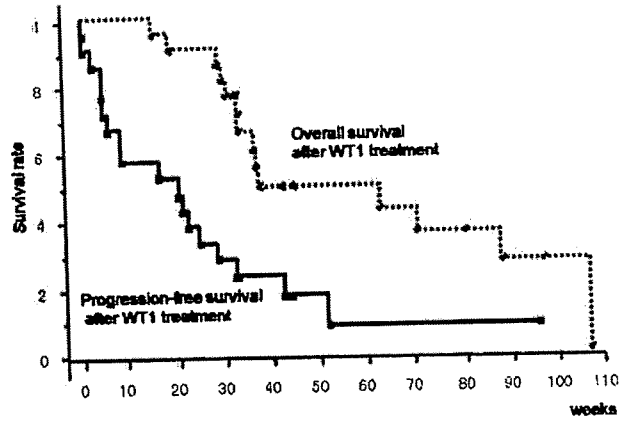

Fig. 1. Kaplan–Meier curves for PFS *(solid line)* and overall survival *(dotted line)* after initial WT1 vaccination for patients with recurrent GBM.

A review of the literature suggested that an agent demonstrating a 6-month progression-free survival rate ≥ 10% would be considered active.[9] 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.[28] 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%;[32] 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%.[3] 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%.[4] 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

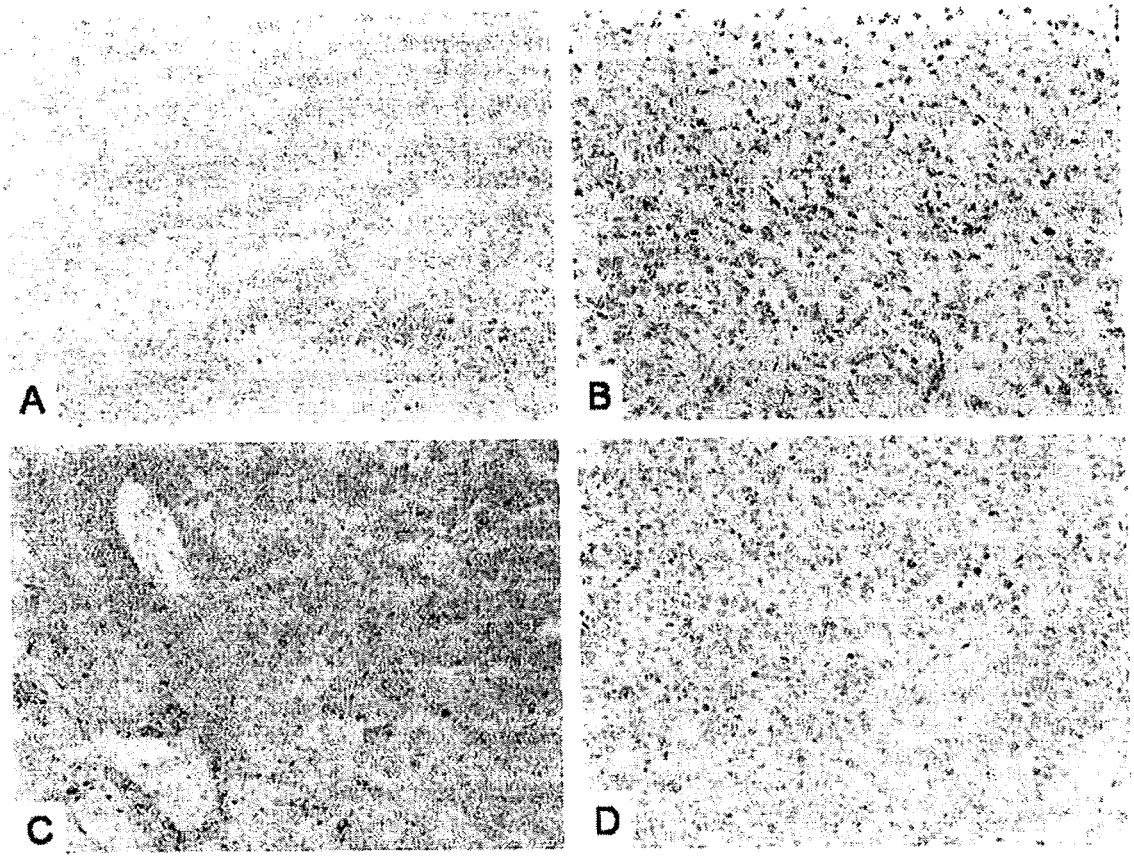

FIG. 2. Photomicrographs showing WT1 (A, B, and C) and MIB-1 (D) immunostaining. Representative WT1 immunostaining with scores of 2, 3, and 4 are shown in A, B, and C, respectively; MIB-1 immunostaining (D) in a specimen from the same case as panel C. Specimens were stained with antibody against WT1 protein (A, B, and C) or antibody against Ki 67 antigen (MIB-1) (D). Original magnification × 200.

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.[19] 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,[19] 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[2,13,14,21] have shown a poor correlation between clinical response and an increase in antigen-specific CTL frequencies. Germeau et al.[7] 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.[12] 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-

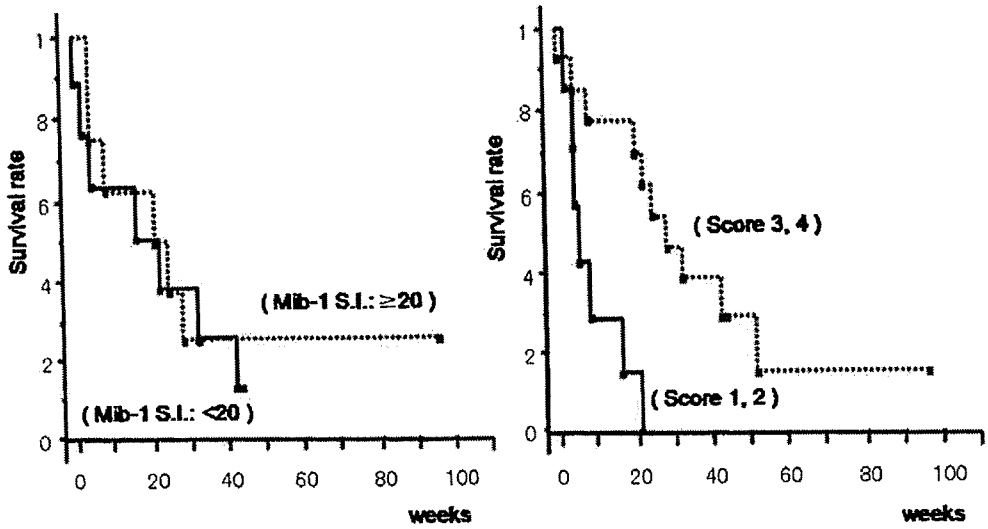

FIG. 3. *Left:* Kaplan–Meier curves for PFS after initial WT1 vaccination for patients with recurrent GBM classified by MIB-1 staining index (S.I.) determined by means of immunohistochemical analysis. The *solid line* indicates cases with an MIB-1 staining index of < 20%, and the *dotted line* indicates 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;[5] 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.[18]

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-A*2402–positive patients with GBM, immu-

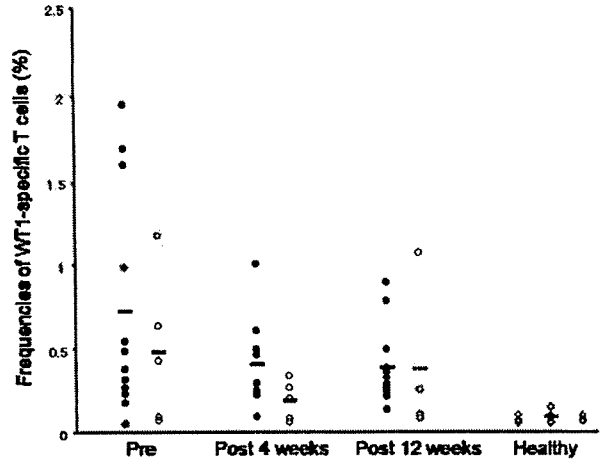

FIG. 4. Graph showing the frequencies of WT1-specific CTLs before WT1 vaccination, 4 and 12 weeks after WT1 vaccination, and in healthy controls. Patients with controlled disease (partial response or stable disease, *closed circles*) as well as those with uncontrolled disease (progressive disease, *open 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),[16] or combination therapy involving vaccination[10] and other modalities may further enhance the clinical usefulness of this treatment for patients with GBM.

Disclaimer

The authors have no conflicts of interest related to this paper.

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研究成果の刊行に関する一覧表

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