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ORIGINAL ARTICLE

Wilms tumor 1 peptide vaccination combined with temozolomide against newly diagnosed glioblastoma: safety and impact on immunological response

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Abstract To investigate the safety of combined Wilms tumor 1 peptide vaccination and temozolomide treatment of glioblastoma, a phase I clinical trial was designed. Seven patients with histological diagnosis of glioblastoma underwent concurrent radiotherapy and temozolomide therapy. Patients first received Wilms tumor 1 peptide vaccination 1 week after the end of combined concurrent radio/temozolomide therapy, and administration was continued once per week for 7 weeks. Temozolomide maintenance was started and performed for up to 24 cycles, and the observation period for safety encompassed 6 weeks from the first administration of maintenance temozolomide. All patients showed good tolerability during the observation period. Skin disorders, such as grade 1/2 injection-site reactions, were observed in all seven patients. Although grade 3 lymphocytopenia potentially due to concurrent radio/temozolomide therapy was observed in five patients (71.4 %), no other grade 3/4 hematological or neurological toxicities were observed. No autoimmune reactions were observed. All patients are still alive, and six are on Wilms tumor 1 peptide vaccination without progression, yielding a progression-free survival from histological diagnosis of 5.2–49.1 months. Wilms tumor 1 peptide vaccination was stopped in one patient after 12 injections by the patient's request. The safety profile of the combined Wilms tumor 1 peptide vaccination and temozolomide therapy approach for treating glioblastoma was confirmed.

Naoya Hashimoto and Akihiro Tsuboi have contributed equally to this work.

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Keywords WT1 peptide vaccination · Temozolomide · Newly diagnosed glioblastoma · Safe combination · Immunological response

Abbreviations list

CR	Complete response
CTCAE	Common Terminology Criteria for Adverse Events
DSMC	Data Safety Monitoring Committee
DTH	Delayed-type hypersensitivity
EORTC/NCIC	European Organization for Research and Treatment of Cancer/National Cancer Institute of Canada
FACS	Fluorescence-activated cell sorting
GBM	Glioblastoma
GTR	Gross total resection
mAbs	Monoclonal antibodies
NR	No recurrence
PR	Partial resection
Gy	Gray
IDH1	Isocitrate dehydrogenase 1
ORR	Objective response rate
OS	Overall survival
PBMCs	Peripheral blood mononuclear cells
PD	Progressive disease
PFS	Progression-free survival
PS	Performance status
RECIST	Response Evaluation Criteria in Solid Tumors
RT	Radiotherapy
RPA	Recursive partitioning analysis
TMZ	Temozolmide
TPS	Total prognostic score
WT1	Wilms tumor 1

Introduction

The standard treatment for glioma is surgery, followed by extended local irradiation and chemotherapy. In patients with newly diagnosed glioblastoma (GBM), however, combined radiotherapy (RT) and temozolomide (TMZ) treatment followed by adjuvant TMZ for at least 6 months offers a modest benefit, with a median survival of 14.6 months, a 2-year survival rate of 27.2 %, and a 5-year survival rate of 9.8 % [1]. Currently, therapeutic options with evidence confirming their efficacy in glioma patients are limited, although some new approaches, such as carmustine wafers and bevacizumab, are available for clinical use in many countries. Thus, surgical maximal resection followed by combined RT and TMZ is still recognized as the standard therapy for newly diagnosed GBM.

Recently, other novel immunological approaches for treating many cancers, as well as gliomas, have been developed, including dendritic cell-based immunotherapy, antibody-mediated immunotherapy [2], and cancer vaccination [3, 4]. A large number of tumor-associated antigens that could be used for vaccination against cancers have been identified, one of which is the product of the Wilms tumor 1 gene (*WT1*) [5]. Although *WT1* was first recognized as a tumor suppressor gene, wild-type *WT1* is now believed to function as an oncogene. Wild-type *WT1* is overexpressed in myelogenous and solid tumors [6, 7]. The WT1 protein is an attractive target antigen for immunotherapy, and in 2009, a pilot prioritization by researchers at the National Cancer Institute produced a list, ranking cancer antigens that can be used by the immunotherapy community. Of 75 antigens on the list, WT1 was indicated as the most promising [8]. In addition, we found that most high-grade glioma samples show overexpression of *WT1* both at the mRNA level and by immunohistochemistry analysis [9]. Furthermore, we found that the treatment with *WT1* antisense oligomer specifically inhibits the growth of several GBM cell lines and hypothesized that *WT1* may be a new molecular target for glioma therapy.

In 2008, we reported the results of a phase II clinical trial of WT1 peptide vaccination in patients with recurrent GBM. We showed that WT1 vaccination in patients with WT1/HLA-A*2402-positive recurrent GBM is safe and produces a clinical response comparable to that of previously reported new approaches for patients with recurrent GBM [10]. Although the appropriate dose and usage of TMZ alone in treating recurrent GBM remain controversial, several phase II studies reported an objective response rate (ORR) of 5–15 %, 6-month progression-free survival (PFS-6) of 19–44 %, and overall survival (OS) of 7–10 months [11–17]. Because our phase II study of WT1 vaccination alone yielded an ORR of 9.5 %, a PFS-6 of 33.3 %, and 8.4-month OS, we hypothesized that WT1 vaccination is comparable to TMZ in terms of response and survival, and particularly in its lack of severe adverse events.

Having obtained favorable results from the phase II study of TMZ for treating recurrent GBM, we began to consider a combination with TMZ targeting newly diagnosed GBM. Conventional thinking, however, has been that chemotherapy may suppress the immune system, and indeed, TMZ is myelosuppressive and does cause lymphocytopenia in a large proportion of patients [1]. In 2010, we conducted a preliminary study and found that the frequency of WT1-specific T cells in peripheral blood is maintained during the initial therapy with RT/TMZ in newly diagnosed GBM patients [18], although the number of those cells declines due to total lymphocytopenia, indicating that homeostatic proliferation of effector T cells could be expected during chemotherapy. As no published clinical studies were available at the time concerning the safe combination of chemotherapy (especially TMZ) and

immunotherapy, we conducted a phase I study of combined TMZ and WT1 immunotherapy and describe the results here.

Materials and methods

The protocol for this phase I clinical trial was designed to investigate the safety of combined WT1 peptide vaccination and TMZ in a small number of cases prior to a large-scale phase II efficacy study. WT1 peptide vaccination was simply added to the standard combined radiotherapy (RT)/TMZ regimen [1, 19]. After the surgical diagnosis was defined, fractionated conformal three-dimensional RT to a total dose of 60 gray (Gy) in 30 daily fractions of two Gy each was delivered. Concomitant chemotherapy consisted of oral TMZ at a daily dose of 75 mg/m^2, given 7 days per week from the first to the last day of RT, for a maximum of 49 days. After a 4-week break, patients received adjuvant oral TMZ (150–200 mg/m^2) for 5 days every 28 days as one cycle. In the original reports of Stupp et al. [1, 19], maintenance TMZ continued for up to six cycles, but in Japan, where there is a national health insurance system for all people and a lack of other effective therapeutic modalities, we usually continue up to 24 or 36 cycles in a standard neuro-oncological practice if no severe adverse events are observed. At our institution, we ordinarily set the maximum number of cycles at 24 due to the potential increased risk of secondary hematological malignancies associated with TMZ [20]. Prophylaxis against *Pneumocystis jirovecii* with either pentamidine or trimethoprim-sulfamethoxazole was mandatory during concomitant RT and TMZ, irrespective of lymphocyte count.

Three cases were registered as an initial cohort, starting WT1 peptide vaccination 1 week after the end of combined RT/TMZ, with continuing administration once per week for 7 weeks (Fig. 1). TMZ maintenance was started 4 weeks after the end of RT/TMZ, and the observation period for safety encompassed 6 weeks from the first administration of maintenance TMZ, for a total observation period of 9 weeks. The study was designed to recruit three more cases as the second cohort, after confirming the safety of the therapy over the observation period in the three patients of the initial cohort. In case there were severe adverse events in the initial cohort, we prepared a level 1 protocol with a prolonged vaccination interval of once per 2 weeks (Fig. 1). Patients received intradermal injections of 3.0 mg of the modified 9-mer WT1 peptide emulsified with Montanide ISA51 adjuvant. For this WT1 peptide vaccination, we previously reported the results of a phase I study of dose escalation from 0.3 to 3.0 mg in treating other solid and hematological cancers [21]. In that study, we defined an appropriate dose of 3.0 mg for treating solid cancers and used that dose in many clinical trials, including a phase II study examining treatment of recurrent GBM [10]. Thus, this was not a typical dose-escalation study.

The vaccination was the same as that used in the previous phase II clinical trial for recurrent GBM and consisted of an HLA-A*2402-restricted, modified 9-mer WT1 peptide (amino acids 235–243 CYTWNQMNL), in which the Y residue was substituted for a M residue at position 2 (the anchor position) of the natural WT1 peptide. This substitution was shown to induce much stronger cytotoxic T lymphocyte activity against WT1-expressing tumor cells than the natural peptide [22]. Lyophilized GMP-grade WT1 peptide was purchased from Multiple Peptide Systems (San Diego, CA). We chose the HLA-A*2402 allele because around 60 % of Japanese people are believed to have the HLA-A*2402 allele [23].

Patients who had newly diagnosed GBM (grade 4) were eligible for inclusion in the study. Additional inclusion

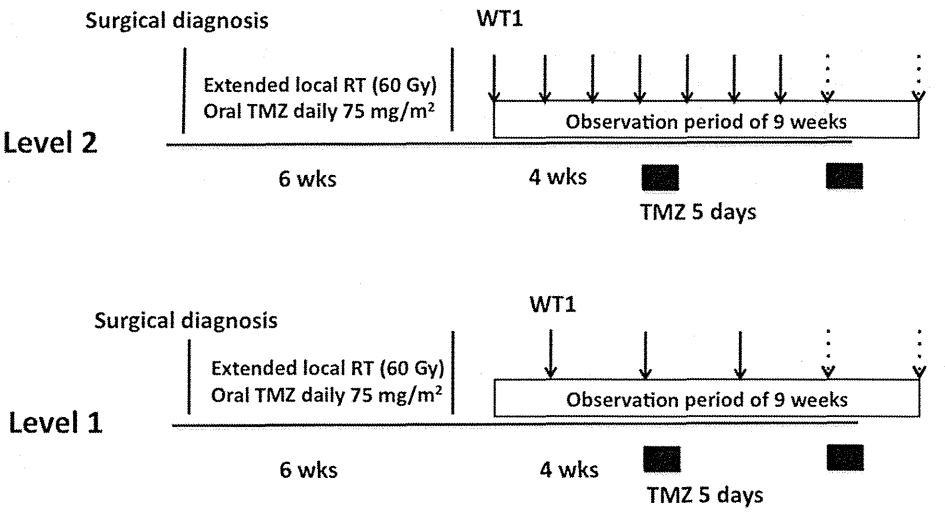

Fig. 1 Combined TMZ and WT1 peptide vaccination protocol. We started at level 2, in which the patients received weekly vaccination four times before TMZ maintenance. If safety was confirmed after seven vaccinations, the patient continued to receive WT1 vaccination biweekly (*dotted arrows*). We also prepared a level 1 protocol, which was not used in this study. See text for further explanation

criteria were: (1) age between 16 and 80 years, (2) expression of WT1 in glioma cells as determined by immunohistochemical analysis, (3) HLA-A*2402 positivity, (4) Eastern Cooperative Oncology Group (ECOG) performance status grade 0–2, (5) no severe organ function impairment, and (6) written informed consent of the patient. The Data Safety Monitoring Committee (DSMC) independently reviewed the eligibility of each enrolled patient. Protocol compliance, safety, and on-schedule study progress were also monitored by the DSMC. Blood samples were evaluated every week during the observation period, and toxicities were evaluated according to Common Terminology Criteria for Adverse Events (CTCAE), version 4.0.

For immunological monitoring, testing for delayed-type hypersensitivity (DTH) to WT1 peptide was performed using standard intradermal injection (200 μg/mL) in a volume of 0.05 mL. A positive test was defined as the presence of an area of induration >5 mm at 48 h. The WT1 peptide/HLA-A*2402 tetramer assay of WT1-specific T cells was performed to calculate the frequency of WT1-specific T cells in peripheral blood mononuclear cells (PBMCs), as described elsewhere [10]. Briefly, frozen PBMCs from patients were thawed and incubated for 1 h at 37 °C in X-VIVO 15 medium (Lonza, Walkersville, MD) supplemented with 10 % AB serum (Gemini Bio-Products, Woodland, CA). The cells were passed through a 40-mm nylon mesh to remove debris and were then incubated with Clear Back (MBL, Aichi, Japan) in phosphate-buffered saline containing 5 % fetal bovine serum and 0.02 % sodium azide (FACS buffer) at room temperature for 5 min. The cells were stained with phycoerythrin-labeled HLA-A*2402/$WT1_{235}$ wild-type and modified tetramer (MBL, Aichi, Japan) for 1 h at 4 °C. The cells were then stained with anti-CD3, anti-CD8, and anti-CD4 antibodies for 25 min at 4 °C in the dark, washed 3 times, resuspended in appropriate quantities of fluorescence-activated cell sorting (FACS) buffer, and incubated with 7-AAD (eBioscience, San Diego, CA) for 5 min before analysis. The cells were analyzed with FACSAria (BD Biosciences, San Jose, CA), and the resulting data were analyzed with FlowJo software (TreeStar, San Carlos, CA). The following monoclonal antibodies (mAbs) were used: anti-CD3-Pacific Blue, anti-CD3-V500, anti-CD4-V500, anti-CD4-APC-H7, anti-CD8-V450 (Life Technologies, Carlsbad, CA), and anti-CD8-FITC (Beckman Coulter, Brea, CA). The frequency of WT1-specific T cells was calculated as ($CD8^+$WT1-$tetramer^+$ T cells)/($CD8^+$ T cells). In addition, using anti-CD45RA-allophycocyanin (APC) (BioLegend, San Diego, USA) and anti-CCR7-PE-Cy7 (BD Pharmingen, San Diego, USA) mAbs, $CD8^+$WT1-$tetramer^+$ T cells were phenotypically classified into four differentiation stages: naïve (CD45RA+CCR7+), central memory (CD45RA−CCR7+), effector memory (CD45RA−CCR7−), and effector (CD45RA+CCR7−).

If safety was confirmed after seven vaccinations in each patient, further WT1 vaccination at 2-week intervals was given only with the patient's informed consent. The progression-free survival (PFS) period was calculated from the day of diagnosis (surgery) (PFS_d) and from the start of WT1 vaccination (PFS_v), based on Response Evaluation Criteria in Solid Tumors (RECIST) criteria, version 1.1. Predefined subgroups according to clinical prognostic factors were explored, and data were scored and regrouped with nomograms from the European Organization for Research and Treatment of Cancer/National Cancer Institute of Canada (EORTC/NCIC) trial [24] and a modification of the Radiation Therapy Oncology Group recursive partitioning analysis (RPA) classes [25].

Results

Although the number of patients to be enrolled was initially set at six, three patients of the initial cohort and four patients of the second cohort were registered to confirm the safety of the treatment regimen and to collect as much information as possible. The characteristics of the seven patients enrolled (four males and three females) are summarized in Table 1. The average age was 49 years (range 41–60 years), and all patients were thought to have primary GBM according to clinical and pathological review, as no isocitrate dehydrogenase 1 (*IDH1*) mutation was detected in any of the seven patient samples examined by immunohistochemistry [26]. The extent of surgery for diagnosis consisted of four gross total resections (GTRs), two partial resections (PRs), and one biopsy. The extent of resection was based on the surgeons' judgment, with no formal assessment required. Six of the seven patients showed a performance status (PS) of 0 at study entry, and only one patient had a PS of 1; this patient required steroid use during the clinical trial. Past history or complications were noted in only one patient (no. 3), who had been on oral anti-hypertensive agents. The total prognostic score (TPS), as defined by Gorlia et al. [24], ranged from 0 to 220; a total of three patients (nos. 1, 4, 5) had a TPS of 0. A total of four patients were in RPA class III, two were in class IV, and one was in class V [25].

All patients tolerated the treatment relatively well during the observation period. Adverse events are summarized in Table 2 according to CTCAE grade. Skin disorders, such as grade 1/2 injection-site reactions, were observed in all seven patients. Although grade 3 lymphocytopenia was observed in five patients (71.4 %), no other grade 3/4 hematological or neurological toxicity was observed. Grade 3 lymphocytopenia appeared early, at 3.6 weeks

Table 1 Patient characteristics

No.	Age	Sex	Dx.	Surg.	PS	STR	Sys.	TP	RPA
1	42	M	GBM	GTR	0	No	–	0	III
2	55	F	GBM	GTR	0	No	–	31	IV
3	48	M	GBM	B	0	No	HT	100	III
4	41	M	GBM	GTR	0	No	–	0	III
5	45	F	GBM	GTR	0	No	–	0	III
6	60	M	GBM	PR	0	No	–	81	IV
7	52	F	GBM	PR	1	Yes	–	220	V

No. patient number, *M* male, *F* female, *Dx.* histological diagnosis, *GBM* glioblastoma, *Surg.* extent of surgery, *GTR* gross total resection, *B* biopsy, *PR* partial resection, *PS* performance status, *STR* use of steroids, *Sys.* systemic complication or past history, *HT* hypertension, *TP* total points of prognostic score, *RPA* recursive partitioning analysis class

Table 2 Summary of adverse events

CTCAE category and symptom	CTCAE grade						Total	
	1		2		3			
	n	%	*n*	%	*n*	%	*n*	%
Blood/bone marrow								
Anemia	4	57.1					4	57.1
Leukocytopenia	2	28.6	2	28.6			4	57.1
Lymphopenia			2	28.6	5	71.4	7	100
Hyponatremia	3	42.9					3	42.9
Hypokalemia	3	42.9					3	42.9
Skin								
Injected site reaction	5	71.4	2	28.6			7	100
Neurology								
Seizure			1	14.3			1	14.3

CTCAE Common Terminology Criteria for Adverse Events

on average (range 0–7 weeks) from the start of vaccination and recovered to grade 2 or above by 5.8 weeks on average (range 4–9 weeks) in all five patients. The grade 2 and grade 3 lymphocytopenia observed in all seven patients also recovered to normal by 14.1 weeks (range 5–40 weeks) during the TMZ maintenance phase. All patients underwent the level 2 protocol without a step down to level 1 with a prolonged vaccination interval (Fig. 1). No symptomatic or asymptomatic autoimmune reactions were observed.

Notably, all seven patients were negative for DTH before vaccination, but six of the seven patients became positive during the observation period (Table 3). Representative FACS results for patient no. 3 are shown in Fig. 2a. A marked induction of $CD8^{+}$WT1-$tetramer^{+}$ T cells occurred 7 weeks after the start of vaccination. The threshold for positive or negative tetramer staining was determined as follows: PBMC samples were stained with appropriate sets of mAbs with or without tetramer, followed by the comparison of the two staining profiles. Further analysis was made in each patient by setting an arbitrary threshold seven times higher (Fig. 2b, red line) than an original threshold (Fig. 2b, red arrow) to define WT1-modified $tetramer^{high}$+ CD8 T cells (Fig. 2a, b). Figure 2c shows the frequency of WT1-specific T cells in PBMCs for each patient according to the wild-type and modified tetramers. In most patients, PBMCs were obtained before WT1 administration (Fig. 2, pre) in the early phase close to the start of WT1 vaccination (within 11 weeks) (Fig. 2, early), and in the late phase (beyond 1 year and 6 months) (Fig. 2, late), although some samples were missing. There is a tendency that the frequency increased in the early phase and decreased in the late phase, as shown in Fig. 2c. The analysis of WT1-modified $tetramer^{high}$+ CD8 T cells showed that in five of six patients, the frequency increased in the early phase and decreased in the late phase as depicted in Fig. 2d. In the late phase, all patients were on maintenance TMZ therapy. One patient (no. 7) was excluded in the analysis because the sample of early phase is missing. The actual data of those graphs are indicated in supplementary Table 1. Further

Table 3 Treatment, response, and survival summary

No.	n_vac	n_TMZ	DTH	Response	PFS_v	PFS_d	OS
1	89	24	+	NR	46.3	49.1	
2	60	20	+	NR	40.8	43.7	
3	81	24	+	CR	41.0	43.5	
4	65	24	+	NR	42.2	44.1	
5	65	20	−	NR	35.3	37.8	
6	12	23	+	PD	2.8	5.2	28.0
7	30	11	+	PR	23.8	26.0	

All survival data are indicated in months

No. patient number, *n_vac* number of vaccination, *n_TMZ* number of maintenance temozolomide cycle, *DTH* delayed-type hypersensitivity,+ became positive, − remained negative, *NR* no recurrence after gross total removal, *CR* complete response, *PD* progressive disease, *PR* partial response, *PFS_v* progression-free survival from the start of vaccination, *PFS_d* PFS from histological diagnosis, *OS* overall survival

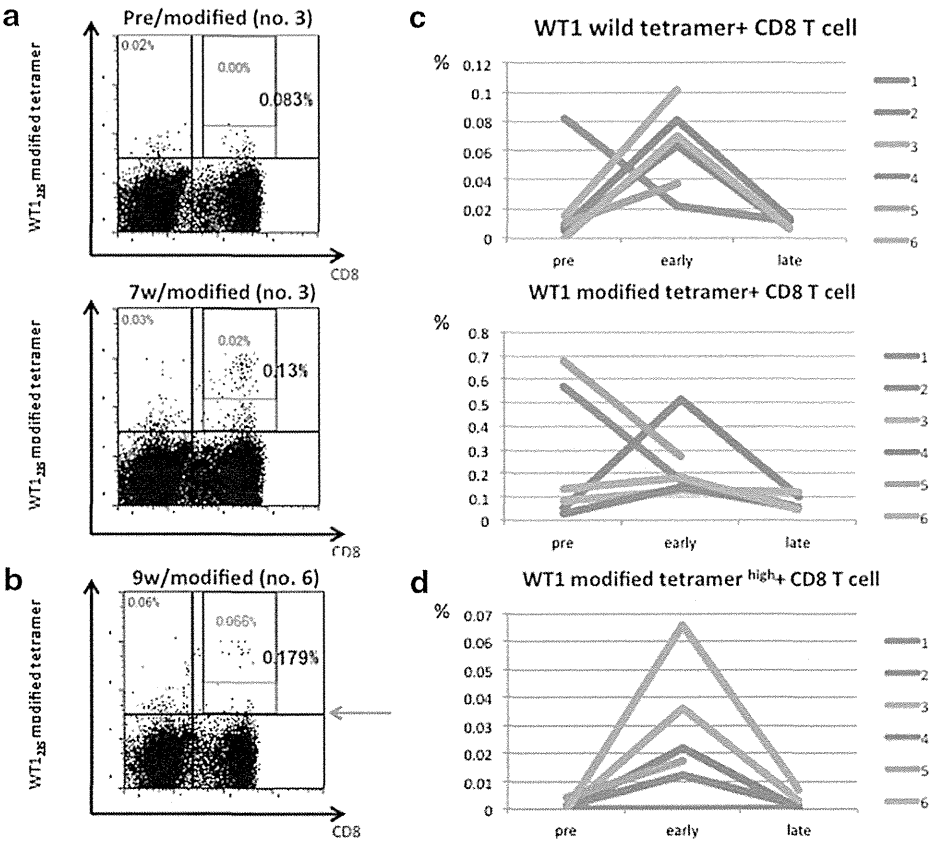

Fig. 2 Immunological responses of the seven patients enrolled in the study. **a** Actual fluorescence-activated cell sorting results for patient no. 3. *Upper* Raw data obtained using the HLA-A*2402/$WT1_{235}$-modified tetramer for peripheral blood mononuclear cells (PBMCs) obtained before vaccination (*upper*) and 7 weeks after the start of vaccination (*lower*). **b** A representative data for patient no. 6 showing WT1-modified tetramer ^{high}+ at 9 weeks, showing the seven times higher threshold (*red line*) than original threshold (*red arrow*). See text also. **c** Graphs showing the sequential frequency of WT1-specific T cells in PBMCs in each patient when using the wild-type (*upper*) and modified (*lower*) tetramers. PBMCs were obtained before WT1 vaccination (pre), in the early phase after the start of WT1 vaccination (within 11 weeks) (early), and in the late phase (beyond 1 year, 6 months) (late). The numbers next to the different colored line refer to patient nos. 1–6. **d** A graph showing the sequential frequency of WT1-modified tetramer ^{high}+ T cells in PBMCs in each patient when using seven times higher threshold

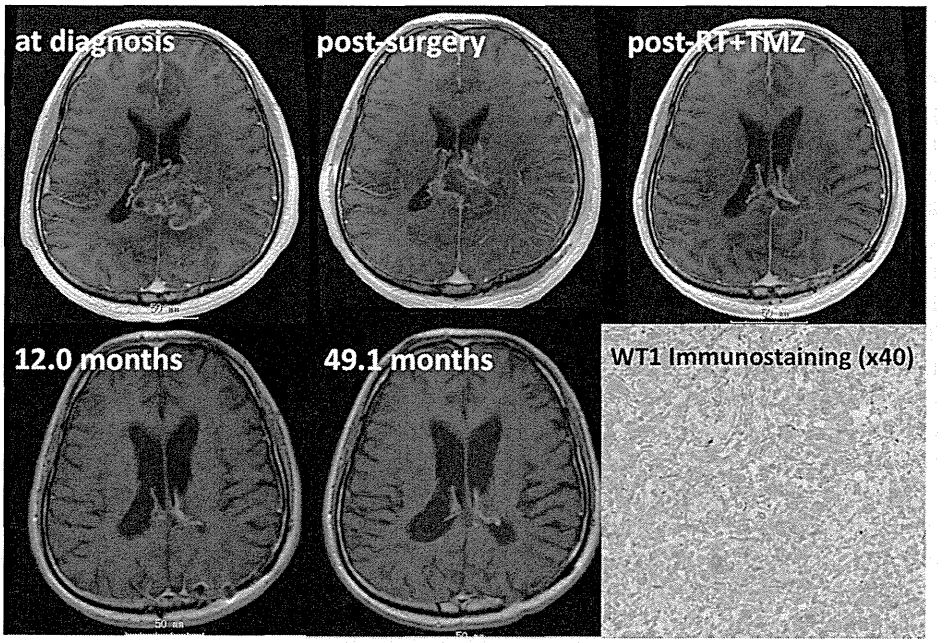

Fig. 3 Representative long-term treatment case. This 42-year-old male (no. 1) underwent gross total resection of the glioblastoma as well as combined radiotherapy/temozolomide treatment (RT/TMZ). The patient received a total of 89 WT1 vaccinations and 24 cycles of maintenance TMZ over 4 years. WT1 immunostaining at lower right shows high expression of WT1 protein in almost all cells, which is representative of most cases of glioblastoma

analysis to determine the phenotype including WT1-specific memory T cells was performed in three cases (nos. 2, 3, and 5), in whom we could obtain an enough amount of WT1-specific T cells. In all three cases, WT1-specific T cells in effector memory and effector subsets accounted for the dominant CTL populations both before and after vaccination (supplementary Fig. 1), as compared to peripheral blood of healthy donors, in whom the majority of subsets belonged to naïve phenotype (data not shown).

A representative long-term treatment case is shown in Fig. 3. This 42-year-old male (no. 1) underwent GTR for GBM and was also treated with RT/TMZ. He was then enrolled in this clinical trial, resulting in a total of 89 vaccinations and 24 cycles of maintenance TMZ over the past 4 years. As in most GBM cases, immunostaining revealed high expression of WT1 protein in almost all cells (Fig. 3, right lower).

Treatments, clinical responses, and survival data are summarized in Table 3. No patient received salvage or second-line therapies. Six of the seven patients remain on WT1 vaccination without progression at the end of 2013. PFS_v ranged from 2.8 to 46.3 months, whereas PFS_d ranged from 5.2 to 49.1 months. WT1 vaccination was stopped in one patient (no. 6) with progressive disease (PD) after 12 injections, as requested by the patient, although he was eligible to continue the vaccination according to the trial protocol. The patient is alive as of the end of 2013, yielding an OS of 28.0 months with continuing TMZ maintenance only. In terms of clinical response, four patients remained in a no recurrence (NR) status after GTR, two showed complete response (CR) according to RECIST, one showed partial response, and one showed PD, with the result that five of the seven patients remain with no measurable lesions.

Discussion

We report here the safety of combined chemo-immunotherapy featuring TMZ in patients with intractable newly diagnosed GBM. Although we encountered CTCAE grade 3 lymphocytopenia in 71.4 % of patients, this observation could be regarded as a consequence of the preceding RT/TMZ therapy, as some trials have demonstrated that this regimen results in a high frequency of grade 3/4 lymphocytopenia (79 % of patients) [19, 27]. In addition, lymphocyte counts recovered quickly in all patients during the TMZ maintenance phase of this study. As summarized in Table 2, aside from lymphopenia, we did not see any grade 3/4 adverse events, including autoimmune reactions, in any patient during the observation period through the date of the last magnetic resonance imaging evaluation. Because WT1 is expressed in certain cells in the kidney (podocytes), pleura, testis, and ovary, we carefully screened for autoimmune reactions such as nephritis and inflammation in these or other major organs [10, 21]. However, in this study, neither autoimmune reactions nor instances of severe toxicity were observed, indicating that combination therapy with TMZ is safe.

In addition to safety concerns, a central dogma regarding efficacy holds that chemotherapy and immunotherapy should not be combined because of possible

immunosuppressive effects of chemotherapeutic agents. As discussed above, TMZ may cause various myelosuppressive events as well as lymphocytopenia in a large proportion of patients, resulting in various opportunistic infections [28, 29]. To resolve the offsetting effects of combined TMZ/WT1 peptide vaccination, we conducted a preliminary study of 22 patients with newly diagnosed GBM, in which the frequency of WT1-specific T cells in peripheral blood was shown to be maintained during RT/TMZ therapy, as was the phenotype of the effector T cells [18]. Importantly, the total lymphocytic population showed a relatively quick increase after concomitant RT/TMZ therapy while maintaining the frequency of WT1-specific T cells, possibly indicative of recovery from myelosuppression. Thus, the protocol of this trial involved starting WT1 peptide vaccination just after the end of combined RT/TMZ therapy. We were also encouraged by the concept of homeostatic proliferation of immunocompetent or effector cells [30, 31].

Despite being addressed by some basic and clinical research studies, the question as to whether TMZ has an effect on the immune system remains controversial. Some studies have reported that experimental data indicate that TMZ enhances antitumor immunity [32–36] by inhibiting regulatory T cell (Treg) trafficking to the glioma microenvironment [34] or augmenting immunological responses nonspecifically with lymphodepletion, effects that have been described in both animal models [37] and human cancer patients [38]. In addition, a phase II clinical trial of an epidermal growth factor receptor variant III (EGFRvIII)-targeted vaccine against gliomas revealed that greater chemotherapy-induced lymphocytopenia enhances the tumor-specific immune response [39]. However, our clinical observations show that TMZ increases the frequency of circulatory Tregs, which may weaken antitumor immunity [18, 40]. Findings on this issue should be verified through further investigation.

This study also showed that WT1-specific immune responses are induced in a majority of patients soon after vaccination, as evidenced by calculation of the frequency of WT1-specific T cells in PBMCs (Fig. 2). It is still unclear whether WT1 peptide vaccination was capable of inducing those cells, because it is possible that WT1 antigens from the resolving tumor after RT/TMZ might have led to the conversion of T cells to WT1-specific T cells [18]. A decrease in the frequency of WT1-specific T cells in the late phase was seen in a majority of patients in whom WT1-specific T cells were induced in the early phase. The reason for the decrease in those cells is not known, and further studies are needed.

In terms of immunotherapy against gliomas, in a famous study from the USA, the EGFRvIII-targeted vaccine was successfully administered to 18 GBM patients [41]. The vaccine was used alone after standard concurrent RT/TMZ therapy and yielded a median PFS_d of 14.2 months and median OS from histological diagnosis (OS_d) of 26.0 months, giving quite good survival benefits. The same research group then performed a phase II trial of the same vaccination concurrent with maintenance TMZ therapy [39]. In the 12 patients who received a standard dose of maintenance TMZ as well as the vaccine, the median PFS_d and OS_d were reportedly 15.9 and 21 months, respectively. Although the present study involved an extremely small sample size, five of the seven patients given the WT1 peptide vaccine showed a PFS_d of over 36 months. PFS and OS treated with maintenance TMZ therapy of up to 24 cycles without WT1 vaccine are 10.7 and 21.0 months, respectively, in 52 newly diagnosed GBM in our institution. At this time, we cannot accurately compare previous reported results of immunotherapy and our institutional control, but we plan to proceed with advanced clinical trial phases with concurrent TMZ therapy and WT1 vaccination.

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Conflict of interest The funding source has no involvement in the study design, the collection, analysis, and interpretation of data, and in the writing of the report.

Ethical standards This study was conducted according to the principles expressed in the Declaration of Helsinki and approved by the ethics review boards of the Osaka University Faculty of Medicine. Written informed consents were obtained from all patients enrolled.

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Chemoimmunotherapy targeting Wilms' tumor 1 (WT1)-specific cytotoxic T lymphocyte and helper T cell responses for patients with pancreatic cancer

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Abstract

We designed a phase 1 study using dendritic cells (DCs) pulsed with a mixture of 3 types of Wilms' tumor 1 (WT1) peptides, including MHC class I/II restricted epitopes (DC/WT1-I/II). Our recent work reveals that the combination of DC/WT1-I/II and chemotherapy induced long-term WT1-specific CD4+ and CD8+ T cell responses.

Tumor-associated antigens (TAAs)-specific CD8+ cytotoxic T lymphocytes (CTLs) can eradicate tumor cells expressing TAAs in the context of MHC class I (MHC-I) molecules. Whereas, CD4+ T cells recognize antigenic peptides in association with MHC class II (MHC-II) molecules on antigen presenting cells (APCs) and required for priming and maintenance of TAAs-specific CD8+ CTLs. Dendritic cells (DCs) have been pulsed with various MHC-I-restricted TAAs-derived peptides to induce antigen-specific CD8+ CTLs in preclinical studies because most tumor cells are positive for MHC-I, but negative for MHC-II. However, the clinical responses using MHC-I-restricted peptide pulsed DCs were not as vigorous as in preclinical settings.

It is well known that CD4+ T cells are essential for the priming phase and help CD8+ CTLs to develop by activating APCs through CD40-CD40L interaction and/or production of cytokines such as interleukin (IL)-2 and interferon (IFN)-γ [1]. Moreover, CD4+ helper T cells also required for maintenance of CD8+ CTLs and the infiltration of CD8+ CTLs at the tumor site [2]. A recent study has demonstrated that a single infusion of a clonal population of NY-ESO-1-specific CD4+ T cells resulted in durable complete regression of the tumor [3]. We have reported that adoptive transfer of

MUC1-specific CD4+ T cells to tumor bearing Rag2-/- mice resulted in prevention of lung metastasis [4]. Therefore, it might be essential for designing cancer vaccine targeting both CD4+ helper and CD8+ T cells.

The Wilms' tumor 1 (WT1) has been reported one of the excellent TAAs for the target of immunotherapy, resulting from a ranking based on specificity, oncogenicity, immunogenicity, and therapeutic function [5]. Therefore, WT1 has been used for the target of immunotherapy. Interestingly, gemcitabine, standard chemotherapy used most often to treat patients with pancreatic cancer, sensitized the human pancreatic cancer cell lines with WT1-specific CTL responses [6], supporting the significance of the chemoimmunotherapy. We and other groups have shown that WT1-specific immune responses could be induced by WT1-specific and MHC-I restricted peptide combined with chemotherapeutic agents, such as gemcitabine and S-1 for patients with pancreatic cancer [7, 8]. In the clinical trial, we found the significant association between longer survival and positive delayed-type hypersensitivity (DTH) to WT1 peptide [7]. Patients with advanced pancreatic cancer have an especially poor prognosis, with a median survival of 4-6 months. Therefore, there is great need for a

novel therapeutic approach. We recently conducted a phase 1 clinical trial of a combination with chemotherapy and DCs pulsed with a mixture of 3 types of WT1 peptides, including MHC-I or -II (HLA type of A*02:01, A*02:06, A*24:02, DRB1*04:05, DRB1*08:03, DRB1*15:01, DRB1*15:02, DPB1*05:01, or DPB1*09:01) restricted epitopes (DC/WT1-I/II) to assess whether chemoimmunotherapy targeting WT1-specific CD4+ and CD8+ T cell responses can induce efficient clinical responses in patients with advanced pancreatic cancer (Figure 1) [9]. We detected WT1-specific DTH positive reactions in 4 of 7 patients received DC/WT1-I/II; however, 0 of 4 patients received DC/WT1-I or -II showed the DTH. Moreover, upon vaccination with DC/WT1-I/II, all patients developed circulating highly functional WT1-specific CD4+ and CD8+ T cells that maintained their IFN-γ-secreting potential. Moreover, WT1-specific CTLs induced by the DC/WT1-I vaccine were not maintained for the entire duration of the treatment protocol. In contrast, all DTH-positive patients vaccinated with DC/WT1-I/II maintained WT1-specific CTLs during the entire treatment period. Importantly, DC/WT1-I/II vaccinations not only stimulated WT1-specific CTLs but also maintained long-term WT1-specific memory

CD8+ T cells. The maintenance of the WT1-specific CTLs may be associated with prolonged survival of patients with pancreatic cancer. On the other hand, the WT1-specific CTLs generated by vaccination with DC/WT1-I may be functionally impaired, resulting in short-lived WT1-specific immune responses. When we compared the clinical outcome of pancreatic cancer patients vaccinated with DC/WT1-I/II with control patients treated with DC/WT1-I or -II, WT1-specific DTH-positive patients who received DC/WT1-I/II exhibited a significant increase in progression-free time (PFT) and overall survival (OS). These findings support the hypothesis that the co-activation of WT1-specific helper T cells upon DC/WT1-I/II stimulates the proliferation and maintenance of functional WT1-specific CTLs, resulted in stable disease (SD) of patients with metastatic pancreatic cancer. In our clinical trial, no complete response (CR) or partial response (PR), but long-term SD was observed. More stable stimulation by DC/WT1-I/II may elicit effector, effector memory, and central memory T cells, which are capable of long-lived WT1 recognition and therefore associate with long-term SD. The response ratio of gemcitabine in advanced pancreatic cancer patients is

approximately 10%. Therefore, the long-term SD might be a unique characteristic of DC/WT1-I/II.

The best-characterized signal for programmed death-ligand 1 (PD-L1) induction is IFN-γ, which is predominantly produced by CD4+ helper type 1 cells [10]. We observed strong PD-L1 expression of tumor cells in all 2 DTH-strong-positive patients received DC/WT1-I/II examined. The overexpression of PD-L1 on tumor cells and the WT1-specific PD-1+ CD8+ T cells at a little lower frequency that was observed in the super-responders received DC/WT1-I/II may be associated with long-term survival. Chemotherapy, such as gemcitabine treatment, can augment the antitumor effects of cancer vaccines by depleting Tregs and MDSCs, which can potentially enhance the antitumor immune responses. Moreover, an immune checkpoint blockade with antibodies targeting inhibitory immune receptors, such as PD-1 and PD-L1, was used to successfully treat patients with advanced melanoma [10]. Therefore, the blockade of multiple immune regulatory checkpoints by antibodies such as PD-1/PD-L1 combined with chemoimmunotherapy targeting WT1 using MHC-I/II restricted peptides may be effective in treating patients with advanced pancreatic cancer.