Table 2 Frequency of WT1-specific CTLs and subpopulation analysis

Case	Peripheral blood (%)										Bone marrow (%)									
	Pre-vaccination					Post-vaccination					Pre-vaccination					Post-vaccination				
	Total	Subpopulation				Total (day)[a]	Subpopulation				Total	Subpopulation				Total (day)[a]	Subpopulation			
		N	CM	EM	E		N	CM	EM	E		N	CM	EM	E		N	CM	EM	E
1	0.13	ND	ND	ND	ND	0.50 (+224)	7.4	2.1	42.9	47.6	0	0	0	0	0	0.19 (+350)	20.4	6.1	44.9	28.6
2	0.14	ND	ND	ND	ND	1.26 (+110)	52.8	0	0	47.2	0	0	0	0	0					
						2.10 (+306)	78	2.4	2.4	17						0.48 (+306)	87.5	0	0	12.5
3	0	0	0	0	0	0.26 (+118)	42.3	0	34.6	23.1	0	0	0	0	0	0.26 (+118)	16.2	2.7	24.3	56.8
						0.85 (+154)	35.2	1.4	28.2	35.2						0.09 (+154)	ND	ND	ND	ND

Abbreviations: CM, central memory; CTL, cytotoxic T lymphocytes; E, effector; EM, effector memory; N, naïve; ND, not done (unable to acquire a sufficient cells for analysis).
[a]Day after the start of vaccination.

0.5–2.1% in peripheral blood (PB) and from 0% to 0.19–0.29% in BM (Tables 1 and 2). WT1-specific CTLs were detected in BM of all cases after vaccination, but in none before vaccination. In Case 2, who had the chimeric marker *AML-MTG8* as MRD, the decrease of WT1 mRNA correlated with the decrease of *AML-MTG8* after vaccination. Cases 1 and 2 have subsequently received WT1 peptide vaccination once monthly and are still in CR for 33.5 and 40.3 months from SCT (Table 2). In Case 3, disease recurred again on day +201 after 23 doses of vaccination, whereas WT1-specific CTL frequency increased further to 0.85% (Table 2). Surface HLA expression on leukemic cells disappeared (data not shown), which implies occurrence of immunological escape of the leukemic cells. T-cells have been phenotypically classified into four differentiation stages: the naïve (N), the central memory (CM), the effector memory (EM) and the terminal differentiated effector (E) stage.[5] The frequency of WT1-specific CTLs and differentiation subpopulation analysis in our cases are shown in Table 2. In healthy subjects WT1-specific CTL frequencies in PB are usually $<0.1\%$.[4,6] The proportion of E or EM subset in the CTLs in PB of Cases 1 and 3 after vaccination was higher than that of healthy donors. In healthy donors, $80.0 \pm 8.4\%$ (mean ± s.d.) of the CTLs were reported to be N-type cells.[7,8] The origin of WT1-specific CTLs was donor type. There were no systemic vaccination-related adverse effects or exacerbation of GVHD.

WT1 has recently been recognized as having a high potential for immunotherapy by the National Cancer Institute Pilot Project.[9] However, the trials for childhood cancer are currently limited to our previous study where we found that the effect of WT1 peptide vaccination might be insufficient in the presence of gross residual disease.[10] Taking this into consideration, we conducted this study that was designed to prevent recurrence at MRD after SCT.

One ALL patient (Case 1) had chemo-resistant relapse and received SCT without remission. In the other two AML patients, one patient had primary induction failure and the other had AML M7 without Down syndrome, which has a poor prognosis. Considering the disease history of each patient before vaccination, all three met the criteria for high risk of relapse.

Monitoring of MRD by WT1 mRNA measurement is applicable to almost all leukemias including pediatric leukemias. Ogawa *et al.*[11] reported that WT1 measurement is highly useful for the prediction and management of relapse following allogeneic SCT; the probability of relapse was significantly increased according to the increase in WT1 mRNA levels. In our cases, WT1 transcripts in BM were $>10^3$ copies/μg/RNA before allogeneic SCT, which is above the normal upper limit,[4,12] and therefore, predicted the patients' poor prognosis.

WT1 transcripts in BM were elevated in all cases before WT1 peptide vaccination. However, levels decreased after vaccination to $<10^3$ copies/μg RNA, indicating its effectiveness. Case 2 had the chimeric marker *AML-MTG8* as MRD. Tobal *et al.*[13] have reported that patients in durable remission had levels of AML/MTG8 transcripts $<1 \times 10^3$ molecules/μg RNA in BM, whereas those having $\geqslant 2.27 \times 10^3$ molecules/μg RNA were at high risk of relapse. AML/MTG8 transcript levels in Case 2 were higher than this threshold. WT1 peptide vaccination induced reduction of transcript levels of AML/MTG8, as well as WT1, followed by a long-term CR, which indicates strong evidence for the vaccine's therapeutic potential.

WT1-specific CTLs in PB were detected and increased after vaccination in all cases (Table 2). Case 3 subsequently had recurrence during WT1 peptide vaccination despite increased frequencies of WT1-specific CTLs in PB. HLA expression disappeared on the surface of leukemic cells, suggesting a possibility that the cells had escaped from immunoreaction by WT1 peptide. Combined usage of interferons, which upregulate HLA expression on leukemic cells, may enhance the sensitivity of the cells to WT1-specific CTLs. We also evaluated WT1-specific CTL subpopulations. In Cases 1 and 2, the frequency of CM and EM cells increased. The enhanced ability of CM and EM T-cells to confer antitumor effects has been reported to be correlated with their greater proliferative capacity.[14]

WT1-specific CTLs originated from donor cells that are considered to be activated and differentiated by stimulation with tumor cell-derived WT1 protein. Rezvani *et al.*[15] have reported that the loss of these CTLs is associated with relapse. On the contrary, we found that the emergence of WT1-specific CTLs was associated with a decrease in WT1 mRNA, suggesting a WT1-driven GVL effect.

In conclusion, we report the first three cases in a phase II trial of WT1 peptide immunotherapy after SCT for pediatric patients. Our results highlight the potential of WT1 vaccination to boost the GVL effect. Larger studies are needed on the application of WT1 vaccination to prevent recurrence after pediatric SCT.

Conflict of interest

The authors declare no conflict of interest.

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References

1 Sugiyama H. Wilms tumor gene WT1 as a tumor marker for leukemic blast cells and its role in leukemogenesis. *Methods Mol Med* 2002; **68**: 223–237.
2 Oka Y, Tsuboi A, Taguchi T, Osaki T, Kyo T, Nakajima H *et al.* Induction of WT1 (Wilms' tumor gene)-specific cytotoxic T lymphocytes by WT1 peptide vaccine and the resultant cancer regression. *Proc Natl Acad Sci USA* 2004; **101**: 13885–13890.
3 Keilholz U, Letsch A, Busse A, Asemissen AM, Bauer S, Blau IW *et al.* A clinical and immunologic phase 2 trial of Wilms tumor gene product 1 (WT1) peptide vaccination in patients with AML and MDS. *Blood* 2009; **113**: 6541–6548.
4 Inoue K, Sugiyama H, Ogawa H, Nakagawa M, Yamagami T, Miwa H *et al.* WT1 as a new prognostic factor and a new marker for the detection of minimal residual disease in acute leukemia. *Blood* 1994; **84**: 3071–3079.
5 Murao A, Oka Y, Tsuboi A, Elisseeva OA, Tanaka-Harada Y, Fujiki F *et al.* High frequencies of less differentiated and more proliferative WT1-specific CD8+ T cells in bone marrow in tumor-bearing patients: an important role of bone marrow as a secondary lymphoid organ. *Cancer Sci* 2010; **101**: 848–854.
6 Jacobsohn DA, Tse WT, Chaleff S, Rademaker A, Duerst R, Olszewski M *et al.* High WT1 gene expression before haematopoietic stem cell transplant in children with acute myeloid leukaemia predicts poor event-free survival. *Br J Haematol* 2009; **146**: 669–674.
7 Ohta H, Hashii Y, Yoneda A, Takizawa S, Kusuki S, Tokimasa S *et al.* WT1 (Wilms tumor 1) peptide immunotherapy for childhood rhabdomyosarcoma: a case report. *Pediatr Hematol Oncol* 2009; **26**: 74–83.
8 Kawakami M, Oka Y, Tsuboi A, Harada Y, Elisseeva OA, Furukawa Y *et al.* Clinical and immunologic responses to very low-dose vaccination with WT1 peptide (5 microg/body) in a patient with chronic myelomonocytic leukemia. *Int J Hematol* 2007; **85**: 426–429.
9 Cheever MA, Allison JP, Ferris AS, Finn OJ, Hastings BM, Hecht TT *et al.* The prioritization of cancer antigens: a national cancer institute pilot project for the acceleration of translational research. *Clin Cancer Res* 2009; **15**: 5323–5337.
10 Hashii Y, Sato E, Ohta H, Oka Y, Sugiyama H, Ozono K. WT1 peptide immunotherapy for cancer in children and young adults. *Pediatr Blood Cancer* 2010; **55**: 352–355.
11 Ogawa H, Tamaki H, Ikegame K, Soma T, Kawakami M, Tsuboi A *et al.* The usefulness of monitoring WT1 gene transcripts for the prediction and management of relapse following allogeneic stem cell transplantation in acute type leukemia. *Blood* 2003; **101**: 1698–1704.
12 Tamaki H, Mishima M, Kawakami M, Tsuboi A, Kim EH, Hosen N *et al.* Monitoring minimal residual disease in leukemia using real-time quantitative polymerase chain reaction for Wilms tumor gene (WT1). *Int J Hematol* 2003; **78**: 349–356.
13 Tobal K, Newton J, Macheta M, Chang J, Morgenstern G, Evans PA *et al.* Molecular quantitation of minimal residual disease in acute myeloid leukemia with t(8;21) can identify patients in durable remission and predict clinical relapse. *Blood* 2000; **95**: 815–819.
14 Klebanoff CA, Gattinoni L, Restifo NP. CD8+ T-cell memory in tumor immunology and immunotherapy. *Immunol Rev* 2006; **211**: 214–224.
15 Rezvani K, Yong AS, Mielke S, Savani BN, Musse L, Superata J *et al.* Leukemia-associated antigen-specific T-cell responses following combined PR1 and WT1 peptide vaccination in patients with myeloid malignancies. *Blood* 2008; **111**: 236–242.

Functional role of BAALC in leukemogenesis

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High expression of *BAALC* in cytogenetically normal-acute myeloid leukemia (AML) patients is associated with primary resistant disease, shorter relapse-free, disease-free and overall survival.[1–4] However, there is no published data evaluating the role of BAALC in the hematopoietic system. *BAALC* is located on human chromosome 8q22.3 and is highly conserved in mammals. High *BAALC* expression levels were first identified in a study of AML patients with trisomy 8 as a sole abnormality,[5] and was shown to correlate highly with MN1 expression,[6,7] a potent oncogene in leukemogenesis.[8] Several isoforms of *BAALC* have been described with isoform 2 (also known as 1-6-8) being the most abundant in the brain and in AML samples.[5]

Using retroviral gene transfer, bone marrow (BM) transplantation and expression analysis of *BAALC* and *MN1* in cytogenetically normal AML patients, we evaluated the role of BAALC in hematopoiesis. Details of materials and methods can be found in the Supplementary information. In total, 140 newly diagnosed adult AML patients with cytogenetically normal AML were evaluated by real-time RT-PCR for *BAALC* and compared with *MN1* transcript expression.[2,3] *BAALC* expression was found to correlate highly with *MN1* expression ($R=0.71$, Pearson's correlation, Figure 1a).

This finding stimulated us to further investigate whether *BAALC* was upregulated as a consequence of *MN1* upregulation or *vice versa*. Retroviral overexpression of human full-length *MN1* in a murine model system induces rapid-onset AML.[8,9] We used murine BM cell lines immortalized by retroviral expression of Hoxa9 or NUP98HOXD13, and co-transduced these cells with full-length *MN1* or a control vector as described before.[10] *Baalc* expression was not increased but rather decreased in MN1-expressing NUP98HOXD13 cells as compared with control transduced NUP98HOXD13 cells (Figure 1b). *Baalc* expression decreased in BM cells that were freshly transduced with MN1 or Hoxa9 compared with control transduced BM cells

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Biased usage of T cell receptor β-chain variable region genes of Wilms' tumor gene (WT1)-specific CD8$^+$ T cells in patients with solid tumors and healthy donors

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Wilms' tumor gene 1 (WT1) protein is a promising tumor-associated antigen. In patients with WT1-expressing malignancies, WT1-specific CTLs are spontaneously induced as a result of an immune response to the WT1 protein. In the present study, we performed single cell-level comparative analysis of T cell receptor β-chain variable region (TCR-BV) gene families of a total of 750 spontaneously induced $WT1_{126}$ peptide (amino acids 126–134, $WT1_{126}$)-specific CTLs in both HLA-A*0201$^+$ patients with solid tumors and healthy donors (HDs). This is the first report of direct usage analysis of 24 kinds of TCR-BV gene families of $WT1_{126}$-specific CTLs at the single cell level. Usage analysis with single-cell RT-PCR of TCR-BV gene families of individual FACS-sorted $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells showed, for the first time, that: (i) BVs 3, 6, 7, 20, 27, and 28 were commonly biased in patients and HDs; (ii) BVs 2, 11, and 15 were biased only in patients; and (iii) BVs 4, 5, 9, and 19 were biased only in HDs. However, statistical analysis of similarity of individual usage frequencies of 24 kinds of TCR-BV gene families between patients and HDs indicated that the usage frequencies of TCR-BV gene families in patients reflected those in HDs. These results should provide us with a novel insight for a better understanding of WT1-specific immune responses. (*Cancer Sci* 2012; 103: 408–414)

Wilms' tumor gene (*WT1*) encodes a zinc-finger transcription factor and plays important roles in the regulation of cell proliferation, differentiation, and apoptosis.[1–3] The *WT1* gene was originally isolated as the gene responsible for a childhood renal neoplasm, namely Wilms' tumor, and was first categorized as a tumor-suppressor gene.[4,5] However, based on the result of a series of studies,[6–8] we proposed that the wild-type *WT1* gene had an oncogenic rather than a tumor-suppressor function in various kinds of hematological malignancies and solid tumors. Indeed, the *WT1* gene is expressed at high levels in acute myeloid leukemia (AML), acute lymphocytic leukemia, chronic myelogenous leukemia, and myelodysplastic syndromes (MDS), as well as in various types of solid tumors.[9–14] Because a correlation has been shown between *WT1* mRNA transcript levels and the amount of minimal residual disease (MRD) in the peripheral blood (PB) or bone marrow of leukemia patients,[15–17] measurement of *WT1* mRNA transcripts is now being used to monitor MRD in leukemia patients.

Previous studies have reported that WT1-specific CTLs can be generated from human PBMC in a human leukocyte antigen (HLA) Class I-restricted manner and can lyse WT1-expressing tumor cells as well as WT1 peptide-pulsed target cells.[18,19] Mice immunized with WT1 peptide or WT1 plasmid DNA elicit WT1-specific CTLs and reject challenges by WT1-expressing tumor cells.[20,21] Furthermore, WT1-specific CTLs and antibodies are induced spontaneously in WT1-expressing tumor-bearing patients.[22–24] These results indicate that the WT1 protein is highly immunogenic and a promising target antigen for cancer immunotherapy. In fact, WT1 has been rated as the most promising cancer antigen of 75 tumor-associated antigens.[25]

On the basis of the results of these preclinical studies, clinical studies of WT1 peptide vaccination were undertaken,[26–29] with promising clinical effects, including a reduction in leukemic blast cells and tumor size, as well as long-term stable disease, being seen in association with an increase in the frequency of WT1-specific CD8$^+$ T cells in PB.[26,27] In this context, analysis of the clonality of the WT1-specific CTLs is important to gain a better understanding of the WT1-specific CTL response in WT1-expressing tumor-bearing patients and, further, to obtain clues as to how to enhance WT1-specific CTL responses in WT1 immunotherapy.

Recently, using single-cell RT-PCR analysis of the T cell receptor β-chain variable region (TCR-BV) genes of individual FACS-sorted WT1 tetramer$^+$ CD8$^+$ T cells, we demonstrated biased usage of TCR-BV gene families of $WT1_{235}$ peptide (amino acids 235–243)-specific CTLs in HLA-A*2402$^+$ patients with AML or MDS, which reflected the biased usage in healthy donors (HDs).[30]

In the present study, we examined usage frequencies of TCR-BV gene families of CTLs specific for $WT1_{126}$, an HLA-A*0201-restricted CTL epitope, in both patients with solid tumors and HDs and found biased usage for these TCR-BV gene families in both the patients and HDs and that the patterns of biased usage were very similar between the two groups.

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Table 1. Characteristics of the patients and healthy donors

	Gender	Age (years)	Disease	Clinical stage	Prior therapy
Patients					
PT-1	M	33	GBM	N/A	Ope/RT
PT-2	F	56	GBM	N/A	Ope/RT
PT-3	M	28	GBM	N/A	Ope/RT/Chemo
PT-4	M	18	PNET	IV	Ope/RT/Chemo/ auto-PBSCT
PT-5	F	53	Ovarian cancer	IIIc	Ope/Chemo
PT-6	F	73	Cecal cancer	IV	Ope/Chemo
Healthy donors					
HD-1	F	23			
HD-2	M	45			
HD-3	F	24			
HD-4	F	25			
HD-5	M	37			

auto-PBSCT, autologous peripheral blood stem cell transplantation; Chemo, chemotherapy; GBM, glioblastoma multiforme; N/A, not available; Ope, operation; PNET, primitive neuroectodermal tumor; RT, radiation therapy.

Materials and Methods

Samples of PB from patients with solid tumors and HDs. Analysis of $WT1_{126}$-specific CTLs in PBMC was approved by the Institutional Review Board for Clinical Research, Osaka University Hospital. After written informed consent had been obtained, PB samples were obtained from six HLA-A*0201$^+$ patients with a solid tumor (patient (PT)-1, -2, -3, -4, -5, and -6) and five HLA-A*0201$^+$ HDs. Expression of WT1 protein in tumor cells was determined by immunohistochemical analysis, as described elsewhere.[31] The PBMC were separated by density gradient centrifugation using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use. Table 1 summarizes the characteristics of both the patients and HDs.

Flow cytometric analysis and single-cell sorting of WT1 tetramer$^+$ CD8$^+$ T cells. Thawed PBMC were rested at 37°C for 1.5 h in RPMI 1640 containing 10% FBS before being stained with phycoerythrin (PE)-labeled HLA-A*0201/$WT1_{126}$ tetramer ($WT1_{126}$ tetramer; MBL, Tokyo, Japan) in FACS buffer composed of PBS containing 5% FBS at 37°C for 30 min. The PBMC were then stained with a panel of mAbs at 4°C for 25 min in the dark, washed three times with FACS buffer, and finally resuspended in appropriate quantities of FACS buffer. The following mAbs were used: anti-CD4-FITC, anti-CD16-FITC, anti-CD45RA-allophycocyanin (APC) (BioLegend, San

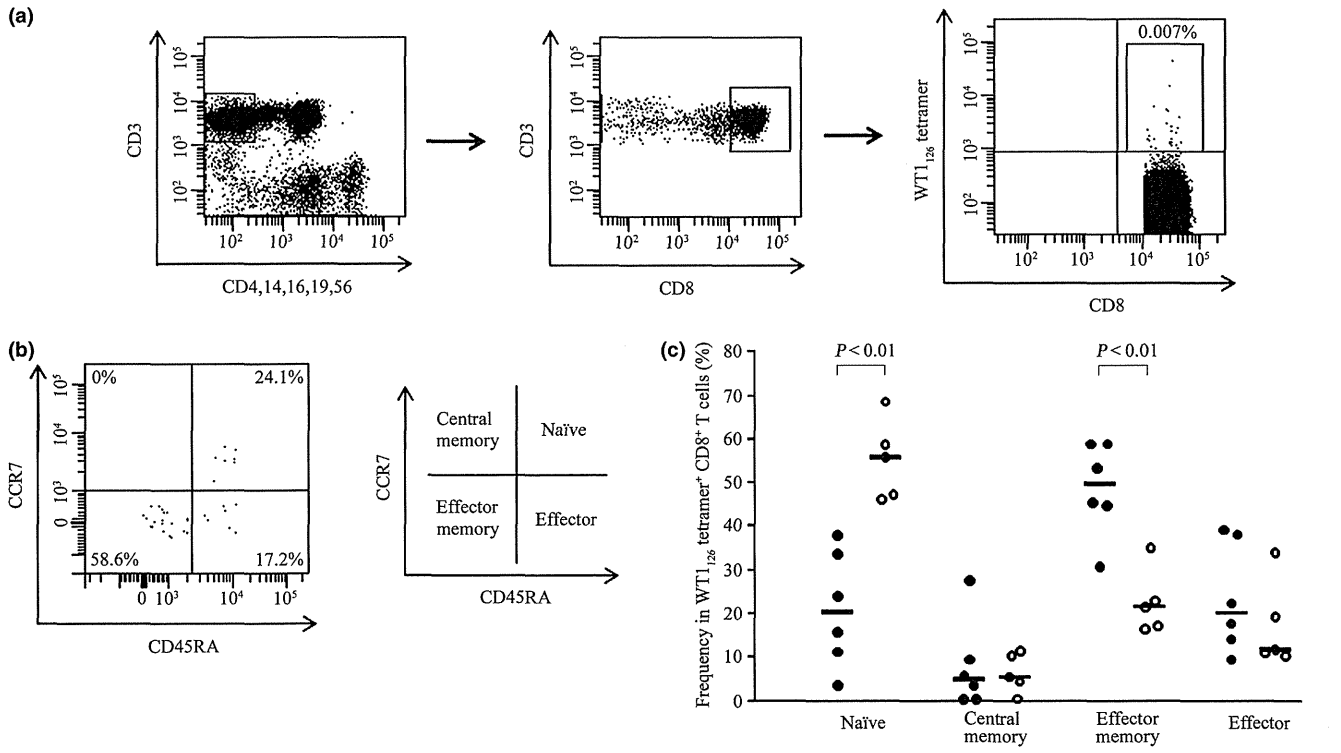

Fig. 1. Frequencies of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells in peripheral blood of patients with a solid tumor and healthy donors and phenotypic analysis of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells. (a) Representative data of flow cytometric analysis using $WT1_{126}$ tetramer. CD4$^-$, CD14$^-$, CD16$^-$, CD19$^-$, CD56$^-$, and $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells were defined as $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells. The percentages shown represent the frequencies of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells among total CD3$^+$ CD8$^+$ T cells. (b) $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells were classified into four distinct differentiation stages according to the cell surface expression of CCR7 and CD45RA as follows: (i) CCR7$^+$ CD45RA$^+$ (naïve) cells; (ii) CCR7$^+$ CD45RA$^-$ (central memory) cells; (iii) CCR7$^-$ CD45RA$^-$ (effector memory) cells; and (iv) CCR7$^-$ CD45RA$^+$ (effector) cells. Representative data from Patient 3 are shown. (c) Frequencies of each subset of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells. Closed and open circles represent patients and healthy donors, respectively. Bars indicate the median values of the frequencies.

Diego, CA, USA); anti-CD19-FITC, anti-CCR7-PE-Cy7 (BD Pharmingen, San Diego, CA, USA); anti-CD3-peridinin chlorophyll protein (PerCP), anti-CD8-APC-Cy7, anti-CD14-FITC (BD Biosciences, San Jose, CA, USA); and anti-CD56-FITC (eBioscience, San Diego, CA, USA). In the present study, lineage antigen (CD4, CD14, CD16, CD19, and CD56)-negative, CD3-, CD8-, and $WT1_{126}$ tetramer-positive lymphocytes were defined as $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells. The $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells were single-cell sorted using a FACSAria instrument (BD Biosciences), and data were analyzed using FACSDiva software (BD Biosciences).

Synthesis of cDNA from a single cell-sorted $WT1_{126}$ tetramer$^+$ CD8$^+$ T cell and determination of TCR-BV gene families. The $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells were directly single-cell sorted into PCR tubes containing cDNA reaction mix, and cDNA synthesis was performed as described previously.[30] The cDNA was amplified using 24 kinds of TCR-BV gene family-specific forward primers and a constant region-specific reverse primer.[30] Next, the PCR products were amplified by semi-nested PCR for the screening of the BV gene family as follows: the first PCR product was put into eight separate tubes, each of which was filled with a reaction mix containing the reagents, one of eight kinds of screening sets of BV gene family-specific forward primers and the reverse primer. The eight kinds of screening sets used in the present study were the same as those used in a previous study.[30] Each screening PCR product was run on a 2% agarose gel to identify the positive reaction among the eight kinds of screening sets. Finally, the TCR-BV gene family was identified by the second round of PCR using an individual TCR-BV gene family-specific forward primer, which was contained in the positive screening set, and the reverse primer. As a negative control, three PCR tubes without sorted cells were prepared in each experiment and were subjected to the same RT-PCR procedures.

A total of 750 $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells were analyzed in six patients (i.e. 59, 66, 46, 67, 88, and 73 cells from PT-1, -2, -3, -4, -5, and -6, respectively) and five HDs (i.e. 53, 57, 77, 79, and 85 cells from HD-1, -2, -3, -4, and -5, respectively). The International Immunogenetics Information System (IMGT) database site (http://www.imgt.org/IMGT_vquest/vquest?livret=0&Option=humanTcR, accessed 15 Nov 2011) was used to identify the human TCR-BV gene family.

Statistical analysis. The Mann–Whitney *U*-test was used to evaluate differences in frequencies and subset compositions of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells and CD3$^+$ CD8$^+$ T cells between patients and HDs. The significance of differences in usage frequencies of the 24 kinds of BV gene families between patients and HDs was also assessed using the Mann–Whitney *U*-test. Analyses were performed with the Stat Flex statistical software package (Artech, Osaka, Japan).

Results

Increase in $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells with effector memory phenotype in HLA-A*0201$^+$ patients with solid tumors. The CTL responses to an HLA-A*0201-restricted epitope $WT1_{126}$ of the WT1 protein were examined in HLA-A*0201$^+$ patients with solid tumors. The PBMC were FACS analyzed by using $WT1_{126}$ tetramer (Fig. 1), with Figure 1(a) showing representative profiles of the FACS analysis of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells. The frequencies of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells in patients and HDs were 0.007–0.122% (median 0.039%) and 0.009–0.079% (median 0.016%), respectively. Although there was a tendency for higher frequencies in patients than in HDs, the differences failed to reach statistical significance (data not shown).

Human CD3$^+$ CD8$^+$ T cells can be divided into four distinct differentiation stages according to the cell surface expression of CCR7 and CD45RA as follows: (i) CCR7$^+$ CD45RA$^+$ (naïve) cells; (ii) CCR7$^+$ CD45RA$^-$ (central memory) cells; (iii) CCR7$^-$ CD45RA$^-$ (effector memory) cells; and (iv) CCR7$^-$ CD45RA$^+$ (effector) cells.[32,33] These cell surface markers were used to classify the differentiation stages of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells and a representative pattern from PT-3 is shown in Figure 1(b). The frequency of the naïve phenotype of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells was significantly higher in HDs than in patients (45.8–68.4% [median 55.6%] *vs* 3.4–37.9% [median 19.9%], respectively; $P < 0.01$), while the frequency of the effector memory phenotype of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells was significantly higher in patients than in HDs (30.3–58.6% [median 49.0%] *vs* 15.8–34.4% [median 20.7%], respectively; $P < 0.01$; Fig. 1c). In contrast, there were no significant differences in frequencies of the four subsets of the whole CD3$^+$ CD8$^+$ T cells between patients and HDs (data not shown),

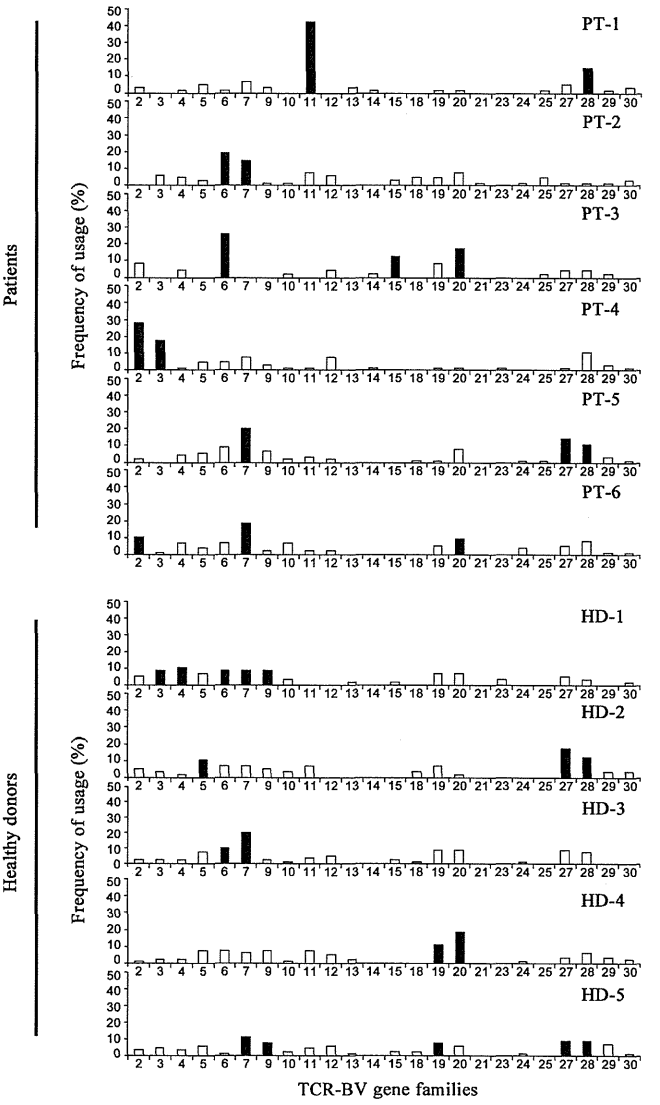

Fig. 2. Frequencies of T cell receptor β-chain variable region (TCR-BV) gene families used by T cell receptors (TCRs) in $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells. The usage frequencies were defined as the ratio of (the number of a given TCR-BV gene family used)/(the total number of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells analyzed). Closed columns indicate that the usage frequency is higher than the mean value + 1SD.

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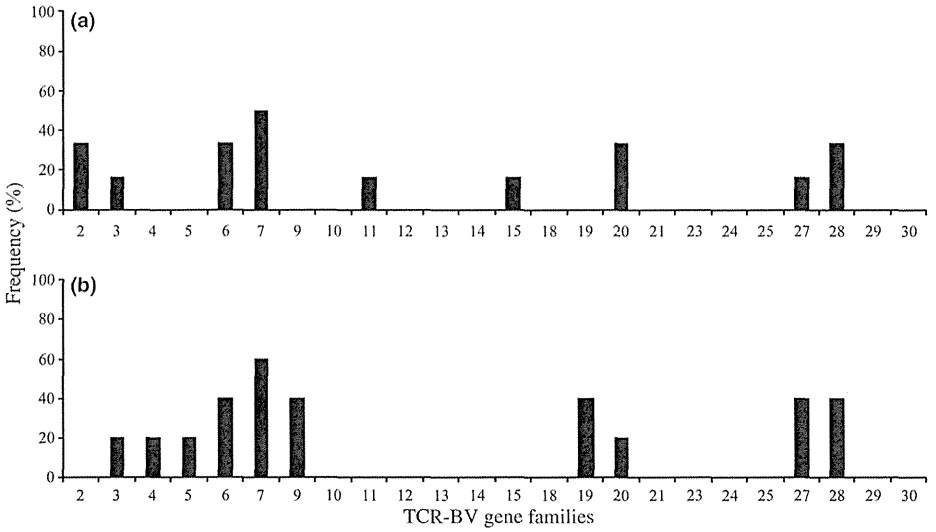

Fig. 3. *Usage frequencies of T cell receptor β-chain variable region (TCR-BV) gene families with biased usage in (a) patients and (b) healthy donors. The ratios show the number of patients or healthy donors with biased usage of the specific TCR-BV gene families to the total number of patients or healthy donors examined, respectively.*

indicating that the phenotypic difference in $CD3^+$ $CD8^+$ T cells between patients and HDs was restricted to $WT1_{126}$ $tetramer^+$ $CD8^+$ T cells. These results demonstrate that $WT1_{126}$ $tetramer^+$ $CD8^+$ T cells exhibit more differentiated/activated phenotypes in patients than in HDs.

Biased usage of TCR-BV gene families in $WT1_{126}$ $tetramer^+$ $CD8^+$ T cells. In the present study, TCR-BV gene families in $WT1_{126}$ $tetramer^+$ $CD8^+$ T cells were investigated by using the single cell-based RT-PCR technique for the six patients and five HDs. Usage frequencies for a given BV gene family were defined as the ratio of the number of $WT1_{126}$ $tetramer^+$ $CD8^+$ T cells with the usage of the BV gene family to the total number of $WT1_{126}$ $tetramer^+$ $CD8^+$ T cells analyzed. When the usage frequency of a given BV gene family was more than the mean value + 1SD for the usage of 24 different kinds of BV gene families, the usage was defined as biased, as described previously.[30] As shown in Figure 2, the biased usage of the TCR-BV gene families was as follows: BV2, in two of six patients; BV3, in one of six patients and one of five HDs; BV4, in one of five HDs; BV5, in one of five HDs; BV6, in two of six patients and two of five HDs; BV7, in three of six patients and three of five HDs; BV9, in two of five HDs; BV11, in one of six patients; BV15, in one of six patients; BV19, in two of five HDs; BV20, in two of six patients and one of five HDs; BV27, in one of six patients and two of five HDs; and BV28, in two of six patients and two of five HDs.

The ratios of the number of patients or HDs with biased usage of individual TCR-BV gene families in $WT1_{126}$ $tetramer^+$ $CD8^+$ T cells to the number of patients or HDs studied are shown in Figure 3. Nine TCR-BV gene families with biased usage were detected in patients and 10 were detected in HDs. These results show that: (i) BVs 3, 6, 7, 20, 27, and 28 are commonly biased in patients and HDs; (ii) BVs 2, 11, and 15 are biased only in patients; and (iii) BVs 4, 5, 9, and 19 are biased only in HDs.

The usage frequencies of TCR-BV gene families in patients reflect those in HDs. The frequencies of 24 TCR-BV gene families used by T cell receptors (TCRs) of $WT1_{126}$ $tetramer^+$ $CD8^+$ T cells were compared statistically between HLA-A*0201$^+$ patients and HDs (Fig. 4). In all BV gene families, except BVs 5 and 19, the usage frequencies did not differ significantly between patients and HDs, although the subset compositions of $WT1_{126}$ $tetramer^+$ $CD8^+$ T cells were significantly different between the two groups (see Fig. 1c). These results strongly indicate that the frequencies of TCR-BV families used by the TCR of $WT1_{126}$ $tetramer^+$ $CD8^+$ T cells in patients with solid tumors reflect those in HDs.

Discussion

Ratios of $WT1_{126}$ $tetramer^+$ $CD8^+$ T cells with the effector memory phenotype were significantly higher in HLA-A*0201$^+$ patients with solid tumors than in HLA-A*0201$^+$ HDs, while those with the naïve phenotype were significantly lower in patients than in HDs, indicating that $WT1_{126}$ $tetramer^+$ $CD8^+$ T cells were more activated and mature in patients than in HDs. These results are basically compatible with those of our previous study of $WT1_{235}$ $tetramer^+$ $CD8^+$ T cells in HLA-A*2402$^+$ patients with myeloid malignancies and HLA-A*2402$^+$ HDs, where the frequencies of $WT1_{235}$ $tetramer^+$ $CD8^+$ T cells were higher in patients than in HDs and $WT1_{235}$ $tetramer^+$ $CD8^+$ T cells were more activated and mature in patients than in HDs.[30]

In order to analyze TCR-BV gene family usage of the TCRs of human tumor-associated antigen (TAA)-reactive T cells, two methods are routinely used: (i) bulky lymphocyte populations are FACS analyzed using a panel of mAbs directed against individual TCR-BV gene family products; or (ii) the populations are analyzed by PCR using a panel of TCR-BV gene family-specific primers.[34–40] However, the former method does not cover all the BV gene segments distributed in each BV gene family and the latter does not guarantee that all the TCR-BV gene families are amplified from the cDNA with equal efficiency. For example, TCR-BV gene families of T cells that exist at very low frequencies in lymphocytes are easily missed using this sort of PCR method.[40] In contrast, because the present study was performed at the single cell level and because the amplification efficiency of TCR-BV cDNA from a single $WT1_{126}$ $tetramer^+$ $CD8^+$ T cell was >80% (data not shown), our results are thought to directly reflect TCR-BV gene family usage in $WT1_{126}$ $tetramer^+$ $CD8^+$ T cells.

Regardless of a striking difference in WT1-specific CTL responses between patients and HDs, the usage patterns of TCR-BV gene families in patients were similar to those in HDs. That is, patients and HDs shared biased usage of TCR-BV families 3,

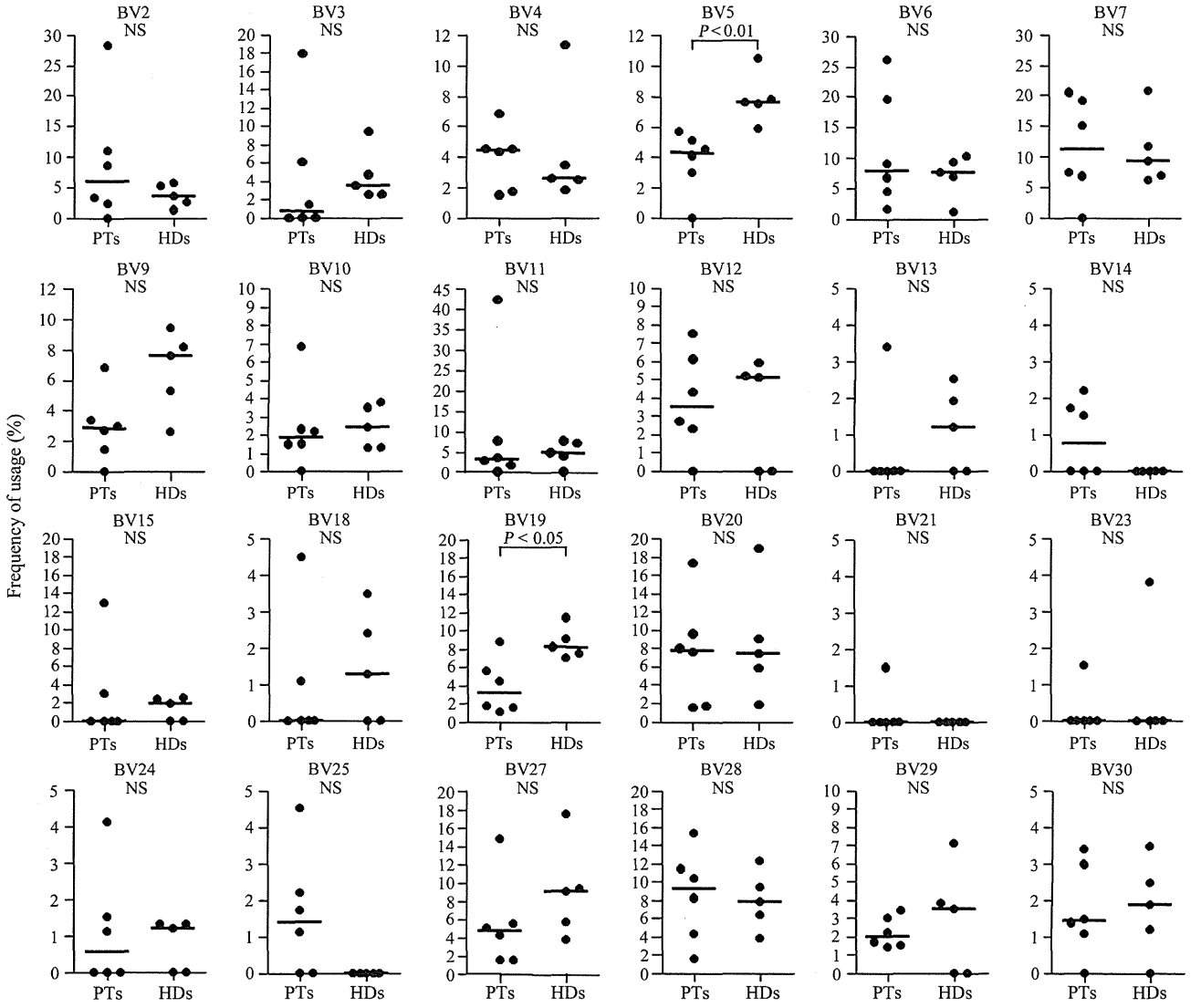

Fig. 4. Statistical comparison of usage frequencies of individual T cell receptor β-chain variable region (TCR-BV) gene families in $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells between patients (PTs) and healthy donors (HDs). The significance of differences was assessed using the Mann–Whitney *U*-test. NS, not significant.

6, 7, 20, 27, and 28, while TCR-BV families 2, 11, and 15 were specifically biased in patients and TCR-BV families 4, 5, 9, and 19 were specifically biased in HDs. In total, six (3, 6, 7, 20, 27, and 28) of ten TCR-BV families (3, 4, 5, 6, 7, 9, 19, 20, 27, and 28) with biased usage in HDs also exhibited biased usage in patients. Three TCR-BV families (2, 11, and 15) newly emerged as those with biased usage specific to patients. However, in all BV gene families, except BVs 5 and 19, the usage frequencies did not differ significantly between patients and HDs. Together, these results led us to speculate that WT1-specific CTLs that had existed predominantly prior to the onset of the solid tumor had expanded and differentiated to maintain their dominance in tumor-bearing patients, whereas a few WT1-specific CTL populations with distinct TCR-BV families expanded in a tumor-bearing patient-specific manner. Furthermore, it may be suggested that WT1-specific CTLs with a dominant set of TCR-BV families in HDs play an important role in immune surveillance against tumors, and that the dominant populations continue to expand due to stimulation of the tumor-derived WT1 protein in WT1-expressing tumor-bearing patients. The immune response to WT1 may be unique, compared with other tumor-associated antigens, in the sense that WT1-specific CTLs are retained in healthy people at relatively higher levels, suggesting that precursors of WT1-specific CTLs are not deleted by the thymus, pass through it, and flow into the periphery. In fact, Pospori *et al.*[41] demonstrated that after murine hematopoietic stem cells transducted with the TCR gene of human HLA-A*0201-restricted WT1-specific CTLs had been transplanted into HLA-A*0201 transgenic recipients, surprisingly WT1-specific CTLs were not impaired by central or peripheral tolerance and, instead, differentiated into memory phenotype T cells. This suggests that precursors of WT1-specific CTLs are not deleted by the thymus. Thus, WT1-specific CTLs are likely to have some role in immune surveillance against tumors in both healthy people and patients with solid tumors. It appears reasonable that TCR-BV families that were appropriately selected for immune surveillance against tumors under healthy conditions were also preferentially used for immune surveillance under tumor conditions.

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The question as to whether different TCR-BV families are used in distinct differentiation subsets of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells was addressed in the present study. To resolve this issue, we analyzed differences in the usage frequencies of individual TCR-BV families between naïve and effector memory phenotypes, which are major and important phenotypes of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells. However, only PBMC from HD-3 and -4 were available for this experiment because they were relatively abundant, while those from the other HDs and patients were too few in number to be analyzed. The $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells were divided into four cell populations of naïve, central memory, effector memory, and effector according to the cell surface expression of CCR7 and CD45RA, and both naïve and effector memory cell populations, which included more cells for the analysis, were provided for analysis of TCR-BV families. Eighteen naïve and nine effector memory cells from HD-3 and 26 naïve and 29 effector memory cells from HD-4 were FACS sorted and analyzed. As shown in Figure S1, available as Supplementary Material for this paper, usage frequencies of individual TCR-BV families were analyzed statistically between naïve and effector memory cell populations. In HD-3, no significant differences in usage frequencies of TCR-BV families were observed between naïve and effector memory cell populations. In addition, in HD-4, there were no significant differences in usage frequencies in most (13 of 15) of the TCR-BV families between the two cell populations, although the usage frequencies of only two TCR-BV families (i.e. BVs 12 and 19) were biased ($P = 0.0292$ and $P = 0.0019$, respectively). These results indicate that the usage pattern of TCR-BV families is similar between naïve- and effector memory-typed WT1-specific CTLs. These results also suggest that the patterns of biased usage of TCR-BV families does not change during the differentiation process from naïve to effector through central memory and effector memory.

In both patients and HDs, TCR-BV families 3, 6, 7, 20, 27 and 28 are preferentially used in $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells. As for TCR-BV families of CTLs for other TAAs, it has been reported that, in a melanoma patient, HLA-A2-restricted NY-ESO-1-specific CD8$^+$ T cells preferentially used TCR-BV families 6, 9, and 12.[35] Among these three TCR-BV families, TCR-BV family 6 was also preferentially used by TCRs of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells in patients and HDs in the present study, while TCR-BV family 9 was preferentially used by $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells in HDs. Thus, it is interesting to observe the phenomenon that a given set of TCR-BV families are preferentially used by certain TAA-specific CD8$^+$ T cells and that some of these families are shared by different TAA-specific CTLs. However, the reason why dominant CTLs for different TAAs (WT1 and NY-ESO-1) shared the same TCR-BV families 6 and 9 is difficult to explain at present. One explanation may be that TAA-specific CTLs with TCR-BV families 6 and 9 have an important role in tumor immunity in the context of HLA-A2 restriction. Further investigations are needed to address this issue.

Acknowledgments

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Disclosure Statement

The authors have no conflicts of interest.

References

1 Drummond IA, Madden SL, Rohwer-Nutter P, Bell GI, Sukhatme VP, Rauscher FJ 3rd. Repression of the insulin-like growth factor II gene by the Wilms tumor suppressor WT1. *Science* 1992; **257**: 674–8.
2 Hewitt SM, Hamada S, McDonnell TJ, Rauscher FJ 3rd, Saunders GF. Regulation of the proto-oncogenes *bcl-2* and *c-myc* by the Wilms' tumor suppressor gene WT1. *Cancer Res* 1995; **55**: 5386–9.
3 Kim J, Prawitt D, Bardeesy N *et al.* The Wilms' tumor suppressor gene (*wt1*) product regulates *Dax-1* gene expression during gonadal differentiation. *Mol Cell Biol* 1999; **19**: 2289–99.
4 Call KM, Glaser T, Ito CY *et al.* Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. *Cell* 1990; **60**: 509–20.
5 Gessler M, Poustka A, Cavenee W, Neve RL, Orkin SH, Bruns GA. Homozygous deletion in Wilms tumours of a zinc-finger gene identified by chromosome jumping. *Nature* 1990; **343**: 774–8.
6 Yamagami T, Sugiyama H, Inoue K *et al.* Growth inhibition of human leukemic cells by WT1 (Wilms tumor gene) antisense oligodeoxynucleotides: implications for the involvement of WT1 in leukemogenesis. *Blood* 1996; **87**: 2878–84.
7 Inoue K, Tamaki H, Ogawa H *et al.* Wilms' tumor gene (WT1) competes with differentiation-inducing signal in hematopoietic progenitor cells. *Blood* 1998; **91**: 2969–76.
8 Tsuboi A, Oka Y, Ogawa H *et al.* Constitutive expression of the Wilms' tumor gene WT1 inhibits the differentiation of myeloid progenitor cells but promotes their proliferation in response to granulocyte-colony stimulating factor (G-CSF). *Leuk Res* 1999; **23**: 499–505.
9 Inoue K, Sugiyama H, Ogawa H *et al.* WT1 as a new prognostic factor and a new marker for the detection of minimal residual disease in acute leukemia. *Blood* 1994; **84**: 3071–9.
10 Bergmann L, Miething C, Maurer U *et al.* High levels of Wilms' tumor gene (wt1) mRNA in acute myeloid leukemias are associated with a worse long-term outcome. *Blood* 1997; **90**: 1217–25.
11 Tamaki H, Ogawa H, Ohyashiki K *et al.* The Wilms' tumor gene WT1 is a good marker for diagnosis of disease progression of myelodysplastic syndromes. *Leukemia* 1999; **13**: 393–9.
12 Oji Y, Ogawa H, Tamaki H *et al.* Expression of the Wilms' tumor gene WT1 in solid tumors and its involvement in tumor cell growth. *Jpn J Cancer Res* 1999; **90**: 194–204.
13 Oji Y, Suzuki T, Nakano Y *et al.* Overexpression of the Wilms' tumor gene WT1 in primary astrocytic tumors. *Cancer Sci* 2004; **95**: 822–7.
14 Oji Y, Yano M, Nakano Y *et al.* Overexpression of the Wilms' tumor gene WT1 in esophageal cancer. *Anticancer Res* 2004; **24**: 3103–8.
15 Cilloni D, Gottardi E, De Micheli D *et al.* Quantitative assessment of WT1 expression by real time quantitative PCR may be a useful tool for monitoring minimal residual disease in acute leukemia patients. *Leukemia* 2002; **16**: 2115–21.
16 Tamaki H, Mishima M, Kawakami M *et al.* Monitoring minimal residual disease in leukemia using real-time quantitative polymerase chain reaction for Wilms tumor gene (WT1). *Int J Hematol* 2003; **78**: 349–56.
17 Ogawa H, Tamaki H, Ikegame K *et al.* The usefulness of monitoring WT1 gene transcripts for the prediction and management of relapse following allogeneic stem cell transplantation in acute type leukemia. *Blood* 2003; **101**: 1698–704.
18 Oka Y, Elisseeva OA, Tsuboi A *et al.* Human cytotoxic T-lymphocyte responses specific for peptides of the wild-type Wilms' tumor gene (WT1) product. *Immunogenetics* 2000; **51**: 99–107.
19 Tsuboi A, Oka Y, Udaka K *et al.* Enhanced induction of human WT1-specific cytotoxic T lymphocytes with a 9-mer WT1 peptide modified at HLA-A*2402-binding residues. *Cancer Immunol Immunother* 2002; **51**: 614–20.
20 Oka Y, Udaka K, Tsuboi A *et al.* Cancer immunotherapy targeting Wilms' tumor gene WT1 product. *J Immunol* 2000; **164**: 1873–80.
21 Tsuboi A, Oka Y, Ogawa H *et al.* Cytotoxic T-lymphocyte responses elicited to Wilms' tumor gene WT1 product by DNA vaccination. *J Clin Immunol* 2000; **20**: 195–202.
22 Elisseeva OA, Oka Y, Tsuboi A *et al.* Humoral immune responses against Wilms tumor gene WT1 product in patients with hematopoietic malignancies. *Blood* 2002; **99**: 3272–9.
23 Wu F, Oka Y, Tsuboi A *et al.* Th1-biased humoral immune responses against Wilms tumor gene WT1 product in the patients with hematopoietic malignancies. *Leukemia* 2005; **19**: 268–74.
24 Gaiger A, Carter L, Greinix H *et al.* WT1-specific serum antibodies in patients with leukemia. *Clin Cancer Res* 2001; **7**: 761s–5s.

25 Cheever MA, Allison JP, Ferris AS *et al.* The prioritization of cancer antigens: a national cancer institute pilot project for the acceleration of translational research. *Clin Cancer Res* 2009; **15**: 5323–37.
26 Oka Y, Tsuboi A, Taguchi T *et al.* Induction of WT1 (Wilms' tumor gene)-specific cytotoxic T lymphocytes by WT1 peptide vaccine and the resultant cancer regression. *Proc Natl Acad Sci U S A* 2004; **101**: 13885–90.
27 Kawakami M, Oka Y, Tsuboi A *et al.* Clinical and immunologic responses to very low-dose vaccination with WT1 peptide (5 microg/body) in a patient with chronic myelomonocytic leukemia. *Int J Hematol* 2007; **85**: 426–9.
28 Kaida M, Morita-Hoshi Y, Soeda A *et al.* Phase 1 trial of Wilms tumor 1 (WT1) peptide vaccine and gemcitabine combination therapy in patients with advanced pancreatic or biliary tract cancer. *J Immunother* 2011; **34**: 92–9.
29 Rezvani K, Yong AS, Mielke S *et al.* Repeated PR1 and WT1 peptide vaccination in montanide-adjuvant fails to induce sustained high-avidity, epitope-specific CD8+ T cells in myeloid malignancies. *Haematologica* 2011; **96**: 432–40.
30 Tanaka-Harada Y, Kawakami M, Oka Y *et al.* Biased usage of BV gene families of T-cell receptors of WT1 (Wilms' tumor gene)-specific CD8+ T cells in patients with myeloid malignancies. *Cancer Sci* 2010; **101**: 594–600.
31 Nakatsuka S, Oji Y, Horiuchi T *et al.* Immunohistochemical detection of WT1 protein in a variety of cancer cells. *Mod Pathol* 2006; **19**: 804–14.
32 Valmori D, Scheibenbogen C, Dutoit V *et al.* Circulating tumor-reactive CD8(+) T cells in melanoma patients contain a CD45RA(+)CCR7(−) effector subset exerting *ex vivo* tumor-specific cytolytic activity. *Cancer Res* 2002; **62**: 1743–50.
33 Sallusto F, Lenig D, Förster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 1999; **401**: 708–12.
34 Valmori D, Dutoit V, Liénard D *et al.* Tetramer-guided analysis of TCR beta-chain usage reveals a large repertoire of melan-A-specific CD8+ T cells in melanoma patients. *J Immunol* 2000; **165**: 533–8.
35 Derré L, Bruyninx M, Baumgaertner P *et al. In vivo* persistence of codominant human CD8+ T cell clonotypes is not limited by replicative senescence or functional alteration. *J Immunol* 2007; **179**: 2368–79.
36 Wadia P, Rao D, Pradhan T, Pathak A, Chiplunkar S. Impaired lymphocyte responses and their restoration in oral cancer patients expressing distinct TCR variable region. *Cancer Invest* 2008; **26**: 471–80.
37 Dietrich PY, Walker PR, Quiquerez AL *et al.* Melanoma patients respond to a cytotoxic T lymphocyte-defined self-peptide with diverse and nonoverlapping T-cell receptor repertoires. *Cancer Res* 2001; **61**: 2047–54.
38 Willhauck M, Möhler T, Scheibenbogen C *et al.* T-cell receptor beta variable region diversity in melanoma metastases after interleukin 2-based immunotherapy. *Clin Cancer Res* 1996; **2**: 767–72.
39 Mandruzzato S, Rossi E, Bernardi F *et al.* Large and dissimilar repertoire of Melan-A/MART-1-specific CTL in metastatic lesions and blood of a melanoma patient. *J Immunol* 2002; **169**: 4017–24.
40 Zhou J, Dudley ME, Rosenberg SA, Robbins PF. Selective growth, *in vitro* and *in vivo*, of individual T cell clones from tumor-infiltrating lymphocytes obtained from patients with melanoma. *J Immunol* 2004; **173**: 7622–9.
41 Pospori C, Xue SA, Holler A *et al.* Specificity for the tumor-associated self-antigen WT1 drives the development of fully functional memory T cells in the absence of vaccination. *Blood* 2011; **117**: 6813–24.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Statistical comparison of usage frequencies of T cell receptor β-chain variable region (TCR-BV) gene families in $WT1_{126}$ tetramer$^+$ $CD8^+$ T cells between naïve and effector memory fractions.

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WT1 Peptide Therapy for a Patient with Chemotherapy-resistant Salivary Gland Cancer

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Abstract. *Wilms' tumor (WT1) protein is one of the most promising target antigens for cancer immunotherapy. In fact, clinical responses, such as growth stabilization or shrinkage of tumor with immunological responses, have been reported in patients vaccinated with WT1 peptide. Here, we performed WT1 peptide-based immunotherapy for a patient with chemotherapy-resistant salivary gland cancer, whose histologic type was carcinoma ex pleomorphic adenoma. The patient with its pulmonary metastasis, refractory to chemotherapy, was intradermally injected with 3 mg of WT1 peptide emulsified with Montanide ISA51 adjuvant at one-week intervals for 12 weeks. The considerably rapid growth of tumor was inhibited after WT1 vaccination, and stable disease, lasting three months, was achieved. Concomitantly, immunological responses, i.e. an increase in frequencies of WT1 tetramer$^+$ CD8$^+$T cells and delayed type hypersensitivity response, were detected after the vaccination. These results indicate the potential of WT1 peptide-based immunotherapy for the treatment of chemotherapy-resistant salivery gland cancer.*

The Wilms' tumor gene *WT1* was first isolated and categorized as a tumor suppressor gene that was inactivated in Wilms' tumor and mutated in the germline of children with genetic predisposition to Wilms' tumor, a kidney neoplasm of childhood (1). The *WT1* gene encodes a zinc finger transcription factor, controls the expression of many genes associated with cell growth, cell differentiation, and apoptosis, and plays a role in mRNA splicing (1).

Our group and others have demonstrated high expression of the *WT1* gene and/or WT1 protein in leukemia and various kinds of solid cancers (2). Based on a series of experimental evidence, we proposed that the *WT1* gene has an oncogenic rather than a tumor-suppressive function in most malignant diseases (1). These results indicated that the wild-type *WT1* gene product could be the most promising target antigen for cancer immunotherapy (2). WT1 peptide or *WT1* cDNA-vaccinated mice rejected the challenge by WT1-expressing tumor cells without damage to normal tissues that physiologically expressed WT1 (2). Human WT1 protein-derived peptides that were able to elicit human leukocyte antigen (HLA)-class I-restricted WT1-specific cytotoxic T lymphocyte (CTL) response were also identified by us and other groups (2-5).

Based on these pre-clinical findings, we performed a phaseI clinical study of WT1 peptide vaccination for patients with acute myeloid leukemia (AML), myelodysplastic syndromes (MDS), lung cancer, and breast cancer (2, 6). In this study, 0.3-3.0 mg of natural or modified 9-mer WT1 peptide emulsified with Montanide ISA51 adjuvant were intradermally injected at biweekly intervals. This study demonstrated that WT1 vaccination was able to induce WT1-specific CTLs and cancer regression without damage to normal tissues in the clinical setting (6). In the present study, we report a case of chemotherapy- and radiotherapy-resistant salivary gland cancer, histologically diagnosed as carcinoma ex pleomorphic adenoma, in which WT1 peptide vaccination

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Key Words: Wilms' tumor protein, WT1, salivary gland cancer, cancer immunotherapy, cancer vaccine.

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considerably inhibited rapid growth of the tumor, leading to stable disease (SD) for three months.

Case Report

A 56-year-old man was diagnosed as having salivary gland cancer in February 2004. The histological diagnosis was carcinoma ex pleomorphic adenoma. The tumor was estimated as cT3N2bM1, and there was enlargement of the mediastinal lymph nodes. Left neck dissection, including submandibulectomy, was performed in February 2004, followed by postoperative radiotherapy, concurrent with S-1, targeted for the locoregional area and mediastinum. Since recurrence occurred with enlargement of mediastinal lymph nodes and elevation of tumor marker cytokeratin 19 fragment(CYFRA) in March 2005, chemotherapy with cisplatin and 5-fluorouracil was performed for three courses. However, since enlargement of mediastinal lymph nodes and lung tumor appeared after the three courses, chemotherapy with nedaplatin and docetaxel was performed for four courses. However, the disease eventually progressed with enlargement of mediastinal lymph nodes and lung tumor after the four courses. Metastasis in the right frontal lobe of brain was found in August 2006. The metastatic lesion in the brain was completely resected, followed by whole-brain radiotherapy. Histological examination of the brain tumor was compatible with metastasis of salivary gland cancer.

Since the patient was HLA-A*2402-positive, and WT1 expression of cancer tissue was proven by immuno-histochemical examination (Figure 1), he was enrolled into this clinical study of WT1 peptide vaccination (7,8). The clinical course and the immunological responses, including frequencies of WT1-Tetramer $(Tet)^+$ $CD8^+$ T-cells in peripheral blood (PB) (Figure 2) and the WT1 peptide-specific delayed type hypersensitivity (DTH), were evaluated (8). During the month before the start of vaccination (weeks -4 to 0), the tumor was considerably rapidly growing and a small amount of pleural effusion at the right side appeared. Representative data of the computed tomographic examination from weeks -4 to 12 (the end of the clinical study period) are shown in Figure 3. The CT examinations revealed that the sum of the longest diameter (SLD) of target lesions increased from 67% at week -4 to 100% at the vaccination start (week 0) (SLD at week 0 was defined as 100%) (Figure 4, upper panel).

The first injection of WT1 vaccine was performed on December 20, 2006. The WT1 vaccine was composed of 3 mg of a modified WT1 peptide (amino acids 235-243: CYTWNQMNL) for HLA-A*2402 type and Montanide ISA51 adjuvant, and the vaccination was scheduled to be performed 12 times at weekly intervals (8). After WT1 vaccination was begun, rapid growth of the tumor declined (Figure 4, upper panel). SLD slightly decreased from 100% at week 0 to approximately 90% at week 5, and was then stable for a further 7 weeks until the end of this clinical study period (week 12) (Figure 4, upper panel). Furthermore, necrotic lesion in the tumor was found on CT examination at week 12 (Figure 3, upper panel). The amount of pleural effusion, which appeared before the vaccination, did not increase during the 12 weeks. The patient's quality of life was also maintained (performance status: 0) and he was able to carry out his daily life without any limitation during the three months of this clinical study period. At the end of this clinical study (week 12), the clinical response was assessed as stable disease (SD). As for adverse effects, only local skin erythema at the injection sites of the WT1 vaccine was observed.

Delayed-type hypersensitivity (DTH) skin test for WT1 peptide was performed for the monitoring of immunological response (9, 10). The DTH test was negative at the beginning of WT1 vaccination, but turned positive at weeks 5, 9 and 13 (Figure 4). Frequencies of WT1-Tet^+ $CD8^+$ T-cells among $CD8^+$ T-cells was 0.098% before the vaccination, but increased to 0.16% at week 4, and the increased percentage was maintained at week 8 (Figure 4, lower panel).

Discussion

This report demonstrates the potential of WT1 vaccination for the treatment of salivary gland cancer, histologically diagnosed as carcinoma ex pleomorphic adenoma. WT1 peptide vaccination for a patient with recurrent, chemotherapy-resistant, considerably rapidly growing submandibular gland cancer induced a cessation of tumor growth, followed by a slight decrease in tumor size (10% decrease of SLD). The decrease in tumor size was revealed with CT examination at week 4 and remained unchanged until the end of this clinical trial (week 12) (Figure 4). Furthermore, necrotic lesion in the tumor was found on CT examination at week 12 (Figure 3, upper panel). Since the tumor growth was rapid before the beginning of WT1 vaccination, it is reasonable to consider that WT1 vaccination induced a clinical response to suppress tumor growth.

The frequency of WT1-Tet^+ $CD8^+$ T-cells in PB of the patient before the vaccination was 0.098%, while the mean value of those from healthy donors was 0.094%, indicating that the frequency of WT1-Tet^+ $CD8^+$ T-cells in the PB of the patient was at the same level as the ones of healthy donors. However, the frequency of WT1-Tet^+ $CD8^+$ T-cells increased after the vaccination from 0.098% at week 0 to 0.16% at week 4 (1.63-fold increase). We previously reported a clear correlation between clinical response and 1.5-fold increase in the frequency of WT1-Tet^+ $CD8^+$ T-cells after WT1 vaccination (6). The change of DTH from negative to positive reaction after the vaccination also supports the elicitation of a WT1-specific immunological response by the vaccination. Taken together, these data strongly suggest that

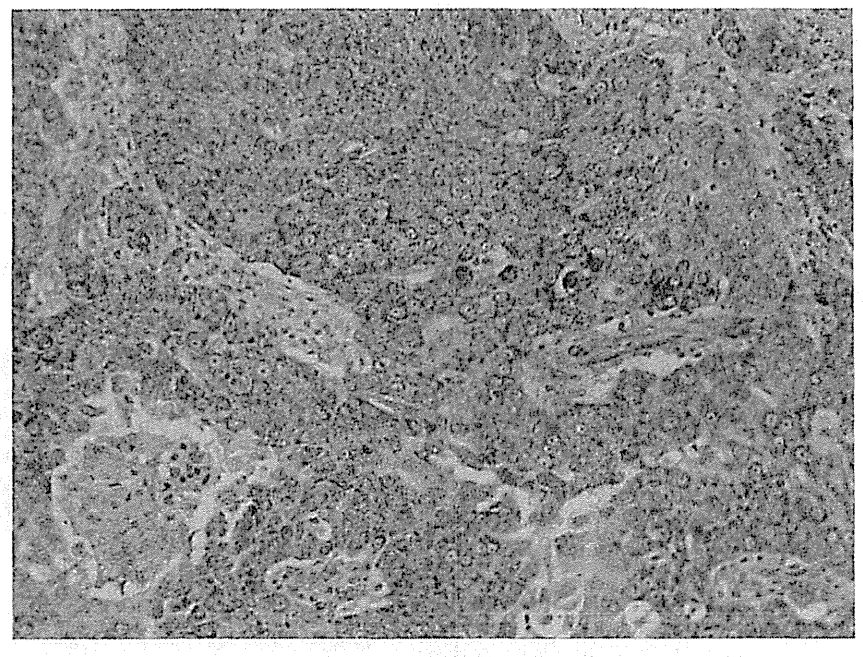

Figure 1. *Wilms' tumor (WT1) protein expression of tumor tissue. This tissue was stained with anti-WT1 protein antibody. The majority of cancer cells exhibit positive staining of WT1 protein, mainly in their cytoplasm.*

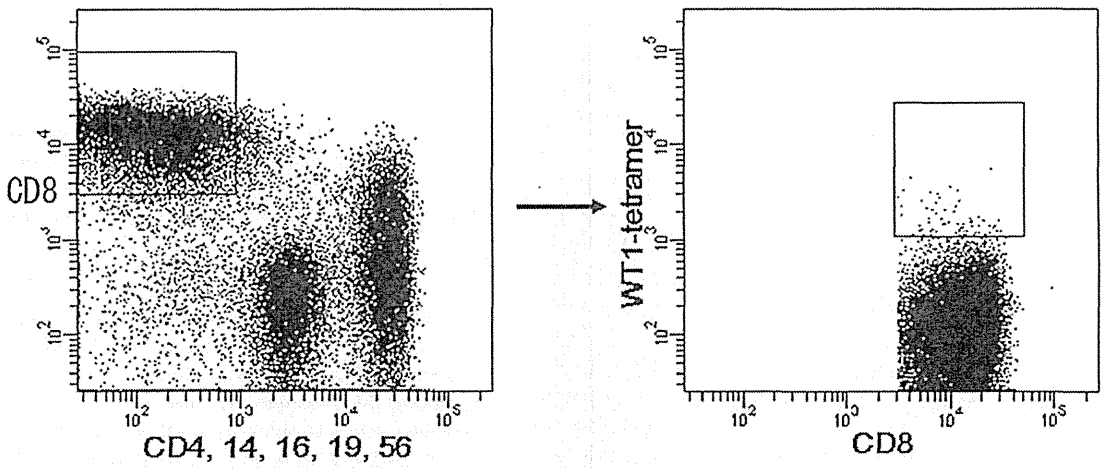

Figure 2. *Detection of WT1 tetramer$^+$ CD8$^+$ T-cells in the patient. CD4-, CD14-, CD16-, CD19- and CD56-positive cells were gated out from peripheral blood mononuclear cells, and these marker-negative, CD8- and WT1 tetramer-positive cells were defined as WT1-specific cytotoxic lymphocytes.*

a WT1 peptide vaccination-induced immunological response, detected by tetramer assay (*ex vivo* immune monitoring) and DTH reaction (*in vivo* immune monitoring), led to the clinical response, *i.e.* stabilization of the disease.

Surgical resection, followed by local radiotherapy, when needed, is a standard therapy for patients with stage I or II salivary gland cancer, and the prognosis is not so poor (11). However, the prognosis of patients with advanced stages of the disease, such as patients with its distant metastasis, is very poor (11). Carcinoma ex pleomorphic adenoma, presented in this report, accounts for about 12% of salivary malignancies and is a subtype of highly malignant tumor (12). Although novel therapies, including a combination therapy of trastuzumab and capecitabine, are being tested,

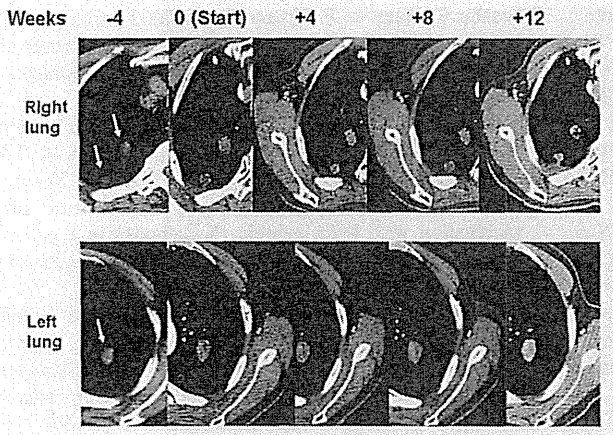

Figure 3. *Representative data of computed tomographic examination. Arrows indicate tumors in the chest. The growth of tumors was suppressed by WT1 vaccination.*

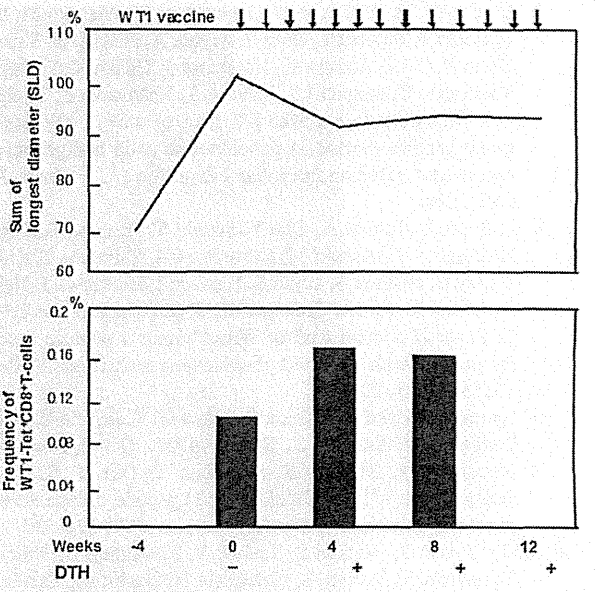

Figure 4. *The clinical course and immunological response of the patient to WT1 peptide vaccination. Upper panel: Sum of longest diameter (SLD); Lower panel: Frequencies of WT1-tetramer (Tet)$^+$ CD8$^+$ T-cells calculated as [(number of WT1-tetramer$^+$ CD8$^+$ T-cells/total number of CD8$^+$ T-cells)×100%] in peripheral blood. DTH: Delayed-type hypersensitivity.*

standard therapy for patients with advanced disease stages has not been established (12). In this context, immunotherapies, including WT1 peptide vaccine, may become alternatives for the treatment of this disease. In the course of the preparation of this manuscript, Sasabe *et al.* reported a case of pulmonary metastasis from adenoid cystic carcinoma of salivary gland that was successfully treated with WT1 peptide vaccination (13). Their report along with the current study strongly suggest that WT1 peptide vaccination has therapeutic potential for salivary gland cancer.

Besides salivary gland cancer, the favorable response of WT1 immunotherapy in various types of malignancies such as AML, MDS, multiple myeloma, glioblastoma multiforme, rhabdomyosarcoma, lung, breast, renal, ovarian cancers has been previously reported, which strongly suggests the superiority of WT1 protein as a target antigen for cancer immunotherapy (2-6, 8, 10, 14-16). In fact, WT1 protein was rated as the most promising target antigen in a recent review article (17). On this basis, WT1 peptide-based immunotherapy is expected to become a novel treatment for salivary gland cancer.

Conflict of Interest Statement

None declared.

Acknowledgements

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References

1 Sugiyama H: Wilms' tumor gene WT1: its oncogenic function and clinical application. Int J Hematol *73*: 177-187, 2001.

2 Oka Y, Tsuboi A, Oji Y, Kawase I and Sugiyama H: WT1 peptide vaccine for the treatment of cancer. Curr Opin Immunol *20*: 211-220, 2008.

3 Rezvani K, Yong ASM, Mielke S, Savani BN, Musse L, Superata J, Jafarpour B, Boss C and Barrett J: Leakemia-associated antigen-specific T-cell responses following combined PR1 and WT1 peptide vaccination in patients with myeloid malignancies. Blood *111*: 236-242, 2008.

4 Yasukawa M, Fujiwara H, Ochi T, Suemori K, Narumi H, Azuma T and Kuzushima K: Clinical efficacy of WT1 peptide vaccination in patients with acute myelogenous leukemia and myelodysplastic syndrome. Am J Hematol *84*: 314-315, 2009.

5 Keilholz U, Letsch A, Asemissen A, Bauer S, Blau IW, Hofmann WK, Uharek L, Thiel E and Schelbenbogen C: A clinical and immunologic phase 2 trial of Wilms tumor gene product 1(WT1) peptide vaccination in patients with AML and MDS. Blood *113*: 6541-6548, 2009.

6 Oka Y, Tsuboi A, Taguchi T, Osaki T, Kyo T, Nakajima H, Elisseeva OA, Oji Y, Kawakami M, Ikegame K, Hosen N, Yoshihara S, Wu F, Fujiki F, Murakami M, Masuda T, Nishida S, Shirakata T, Nakatsuka S, Sasaki A, Udaka K, Dohy H, Aozasa K, Noguchi S, Kawase I and Sugiyama H: Induction of WT1 (Wilms' tumor gene)-specific cytotoxic T lymphocytes by WT1 peptide vaccine and the resultant cancer regression. Proc Natl Acad Sci USA *101*: 13885-13890, 2004.

7 Morita S, Oka Y, Tsuboi A, Kawakami M, Maruno M, Izumoto S, Osaki T, Taguchi T, Ueda T, Myoui A, Nishida S, Shirakata T, Ohno S, Oji Y, Aozasa K, Hatazawa J, Udaka K, Yoshikawa H, Yoshimine T, Noguchi S, Kawase I, Nakatsuka S, Sugiyama H and Sakamoto J: A phase I/II trial of a WT1 (Wilms' tumor gene) peptide vaccine in patients with solid malignancy: safety assessment based on the phase I data. Jpn J Clin Oncol *36*: 231-236, 2006.

8 Izumoto S, Tsuboi A, Oka Y, Suzuki T, Hashiba T, Kagawa N, Hashimoto N, Maruno M, Elisseeva OA, Shirakata T, Kawakami M, Oji Y, Nishida S, Ohno S, Kawase I, Hatazawa J, Nakatsuka S, Aozasa K, Morita S, Sakamoto J, Sugiyama H and Yoshimine T: Phase II clinical trial of Wilms tumor 1 peptide vaccination for patients with recurrent glioblastoma multiforme. J Neurosurg *108*: 963-971, 2008.

9 Iiyama T, Udaka K, Takeda S, Takeuchi T, Adachi Y, Ohtsuki Y, Tsuboi A, Nakatsuka S, Elisseeva OA, Oji Y, Kawakami M, Nakajima H, Nishida S, Shirakata T, Oka Y, Shuin T and Sugiyama H: WT1 (Wilms' tumor 1) peptide immunotherapy for renal cell carcinoma. Microbiol Immunol *51*: 519-530, 2007.

10 Oji Y, Oka Y, Nishida S, Tsuboi A, Kawakami M, Shirakata T, Takahashi K, Murao A, Nakajima H, Narita M, Takahashi M, Morita S, Sakamoto J, Tanaka T, Kawase I, Hosen N and Sugiyama H: WT1 peptide vaccine induces reduction in minimal residual disease in an imatinib-treated CML patient. Eur J Haematology *85*: 358-360, 2010.

11 Lures JC, Wittekindt C, Streppel M and Guntinas O: Carcinoma ex pleomorphic adenoma of the parotid gland. Study and implications for diagnostics and therapy. Acta Oncologica *48*: 132-136, 2009.

12 Sharon E, Kelly RJ and Szabo E: Sustained response of carcinoma ex pleomorphic adenoma treated with trastuzumab and capecitabine. Head and Neck Oncol 2: 12, 2010.

13 Sasabe E, Hamada F, Iiyama T, Udaka K, Sugiyama H and Yamamoto T: Wilm's tumor gene WT1 peptide immunotherapy for pulmonary metastasis from adenoid cyctic carcinoma of the salivary gland. Oral Oncol *47*: 77-78, 2011.

14 Kawakami M, Oka Y, Tsuboi A, Harada Y, Elisseeva OA, Furukawa Y, Tsukaguchi M, Shirakata T, Nishida S, Nakajima H, Morita S, Sakamoto J, Kawase I, Oji Y and Sugiyama H: Clinical and immunologic responses to very low-dose vaccination with WT1 peptide (5 μg/body) in a patient with chronic myelomonocytic leukemia. Int J Hematol *85*: 426-429, 2007.

15 Ohta H, Hashii Y, Yoneda A, Takizawa S, Kusuki S, Tokimasa S, Fukuzawa M, Tsuboi A, Murao A, Oka Y, Oji Y, Aozasa K, Nakatsuka S, Sugiyama H and Ozono K: WT1 (Wilms' tumor 1) peptide immunotherapy for childhood rhabdomyosarcoma: a case report. Pediatr Hematol Oncol *26*: 74-83, 2009.

16 Ohno S, Kyo S, Myojo S, Dohi S, Ishizaki J, Miyamoto K, Morita S, Sakamoto J, Enomoto T, Kimura T, Oka Y, Tsuboi A, Sugiyama H and Inoue M: Wilms' tumor 1 (WT1) peptide immunotherapy for gynecological malignancy. Anticancer Res *29*: 4779-4784, 2009.

17 Cheever MA, Allison JP, Ferris JP, Finn OJ, Hastings BM, Hechet TT, Mellaman I, Prindiville SA, Viner JL, Weiner LM and Martrisian LM: The priorization of cancer antigens: A National Cancer Institute pilot project for the acceleration of translational research. Clinical Cancer Res *15*: 5323-5337, 2009.

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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^{+MET}); 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 length, $p = 0.960$ for Gd-enhanced MR imaging volume, and $p = 0.110$ for MET-PET), the percentage of PRM^{+MET} 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. Clinical trial registration no.: UMIN000002001.
(http://thejns.org/doi/abs/10.3171/2011.12.JNS111255)

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.[17] On the other hand, the products of the *WT1* gene have been shown to be overexpressed in malignant gliomas,[12,13] 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).[9]

Abbreviations used in this paper: GBM = glioblastoma multiforme; MET-PET = ^{11}C-methionine PET; OS_{WT1} = overall survival after initiation of Wilms tumor 1 immunotherapy; PRM = parametric response map; RECIST = Response Evaluation Criteria in Solid Tumors; ROI = region of interest; WT1 = Wilms tumor 1.

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 antitumor 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[3] 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 cm^3 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 OS_{WT1}.

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 pentaacetic 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.). ^{11}C-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.

TABLE 1: Summary of clinical and demographic characteristics of 14 patients*

Case No.	Age (yrs),† Sex	ECOG PS	Diagnosis	Response per RECIST	OS_{WT1} (wks)‡	Tumor Vol by MET-PET (cm^3)§
1	43, M	2	GBM	SD	87.1	31.2
2	64, M	1	GBM	PD	144.7	63.8
3	76, M	1	GBM	SD	144.6	29
4	60, F	0	GBM	SD	61.7	58.1
5	20, F	0	GBM	PR	29.3	24.9
6	64, F	1	AA	SD	65.0	51
7	29, M	2	GBM	PD	20.9	15.4
8	28, M	1	GBM	SD	57.7	9
9¶	62, M	0	gliosarcoma	SD	77.0	11.5
10	36, F	1	AA	SD	60.3	3.8
11	44, M	0	GBM	PD	48.1	13.2
12	62, F	1	GBM	PD	18.7	5
13	51, M	0	GBM	PD	35.0	39.3
14	39, F	1	GBM	PD	27.6	15.2

* 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 cm^3.
¶ The patient in Case 9 was alive as of this writing.

Parametric Response Map Calculation Algorithm

As in Fig. 1, post-WT1 ^{11}C-methionine uptake was plotted as a function of pre-WT1 ^{11}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 ^{11}C-methionine PET.

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

$$\text{deviation}_i = [(\text{post-WT1 MET-PET})_i - (\text{pre-WT1 MET-PET})_i] / \sqrt{2}$$

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

$$PRM_i = \text{deviation}_i - \mu / \rho$$

where μ and ρ are the mean and standard deviation of deviation_i 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-Meyer 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_{WT1} of 144.7 weeks and was considered a treatment responder, and another (Case 7) in which the patient had a relatively short OS_{WT1} 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 ^{11}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 ^{11}C-methionine uptake decreased after WT1 immunotherapy (Fig. 2). This area is presented as PRM^{-MET} (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.

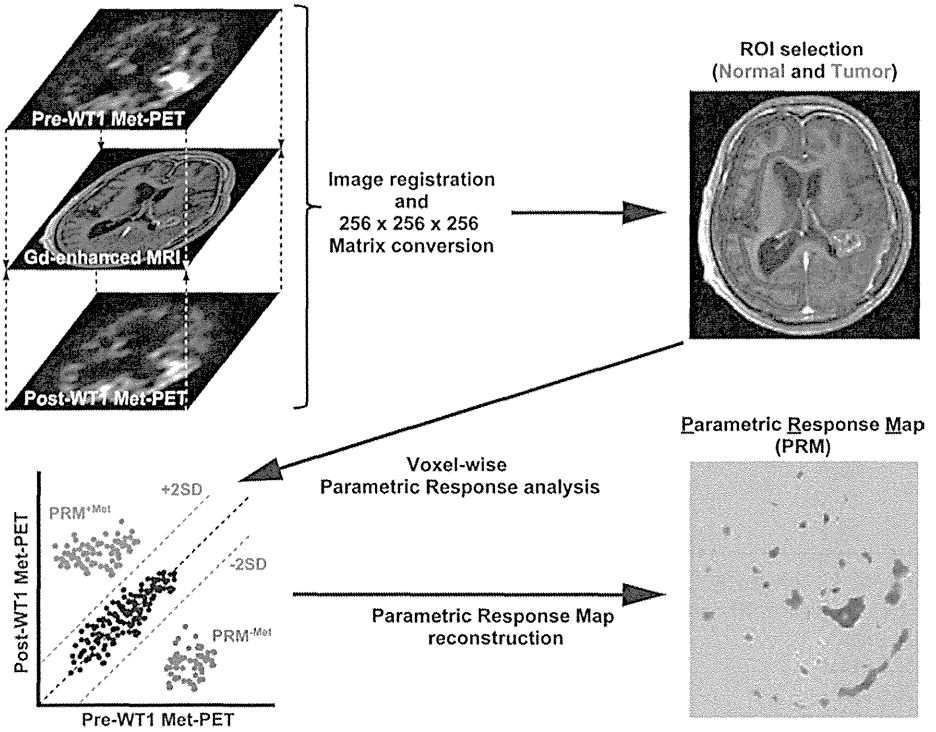

FIG. 1. Image processing procedures. ^{11}C-methionine PET data obtained before and 12 weeks after WT1 immunotherapy initiation were fused and registered onto conventional contrast-enhanced MR images. All 3 images were converted into a 256 × 256 × 256, 1-mm isotropic image matrix. Post-WT1 ^{11}C-methionine uptake was plotted as a function of pre-WT1 ^{11}C-methionine uptake. After calculating the linear regression line with the ± 2 SD distribution range in contralateral normal brain tissue, an ROI was set at the contrast-enhanced pre-WT1 immunotherapy lesion. The obtained plots were categorized into the following 3 areas: 1) area of no change in ^{11}C-methionine uptake pre- and posttreatment, 2) area with increased ^{11}C-methionine uptake posttreatment (PRM^{+MET}), and 3) area with decreased ^{11}C-methionine uptake posttreatment (PRM^{-MET}). These areas were reconstructed in images for visual inspection (PRM^{+MET} in *red* and PRM^{-MET} in *blue*).

Representative Treatment Nonresponder. A representative case in which the patient had only a short OS_{WT1} (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 SD area (PRM with increased methionine uptake [PRM^{+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_{WT1}

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_{WT1} ($p = 0.270$ 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_{WT1} ($p = 0.110$) (Fig. 4C).

Parametric Response Map Analysis. Finally, correlation of the proposed voxel-wise PRM of MET-PET with OS_{WT1} was investigated. Each voxel of contrast-enhanced area on the pretreatment MR images was categorized as a no-change area, PRM^{+MET}, or PRM^{-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_{WT1} (Fig. 5). While the percentage of the PRM^{-MET} area showed moderate correlation with OS_{WT1} ($p = 0.100$) (Fig. 5 left), the percentage of the PRM^{+MET} area showed excellent correlation with OS_{WT1} ($p = 0.008$) (Fig. 5 right). A threshold of 5% for PRM^{+MET} yielded the best performance for discriminating WT1 immunotherapy responders from nonresponders (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^{+MET} still remained as the only statistically significant factor ($p = 0.01$).

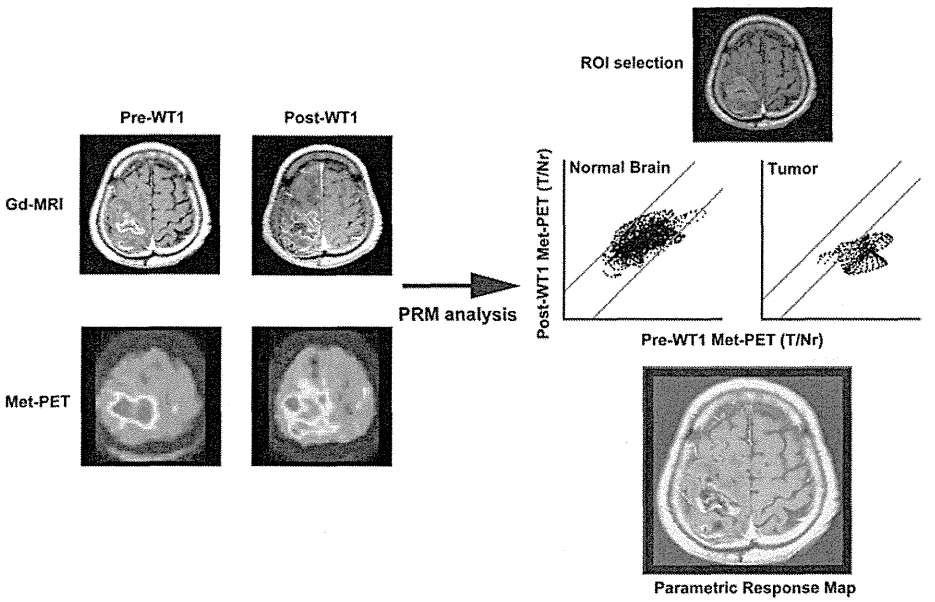

FIG. 2. Case 2. A representative treatment responder with recurrent GBM (OS_{WT1} 144.7 weeks). Images were analyzed as in Fig. 1. Voxel-wise PRM analysis revealed that most of the contrast-enhanced lesion was within the PRM^{-MET} area. Although the OS_{WT1} was 144.7 weeks, conventional MR imaging 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 RECIST 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.[5,16] 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

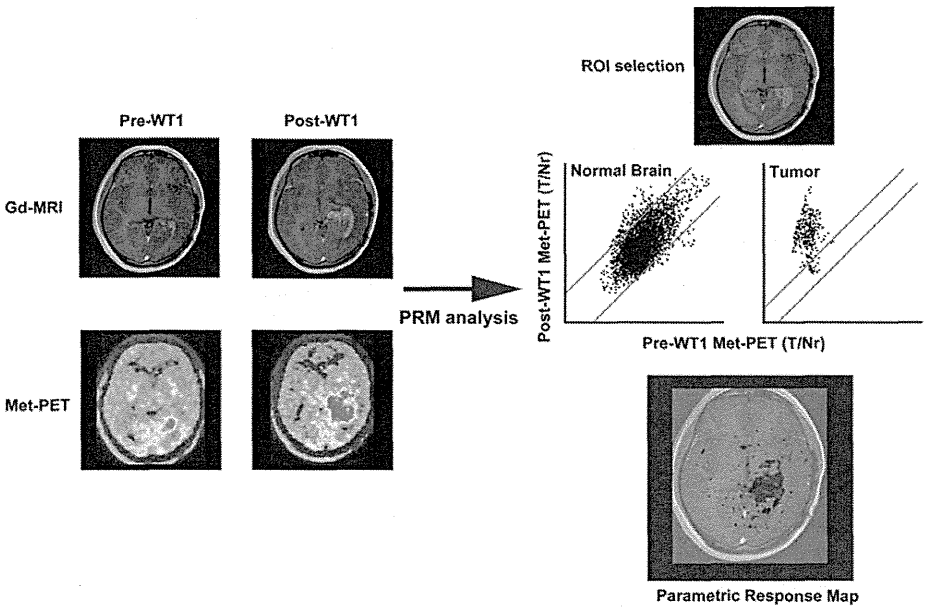

FIG. 3. Case 7. A representative treatment nonresponder with recurrent GBM (OS_{WT1} 20.9 weeks). Images were analyzed as in Fig. 1. Voxel-wise PRM analysis revealed that most of the contrast-enhanced lesion was within the PRM^{+MET} area, suggesting that the patient was not responsive to WT1 immunotherapy.

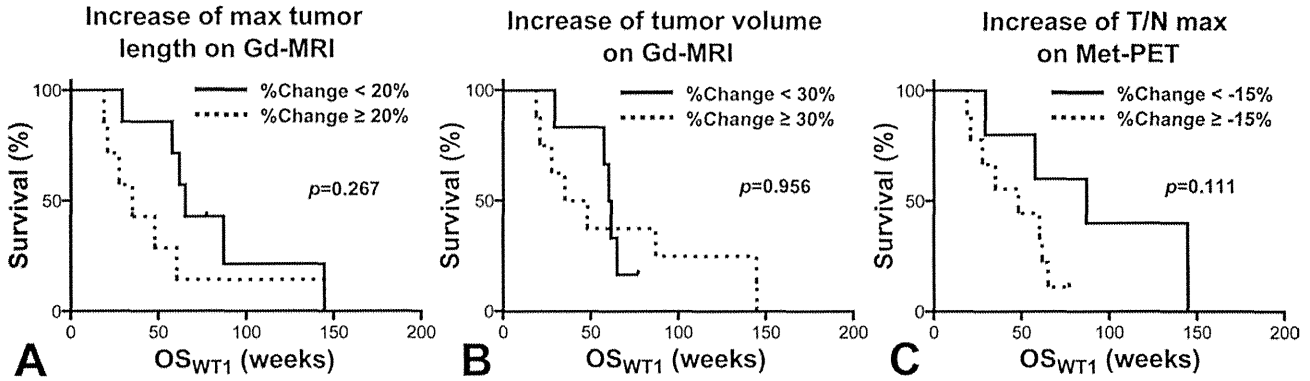

FIG. 4. Correlation of OS_{WT1} with changes in tumor length and volume using contrast-enhanced MR imaging and the T/N max of MET-PET. Correlations between OS_{WT1} and changes (from before WT1 immunotherapy to 12 weeks after immunotherapy initiation) on Gd-enhanced MR imaging–measured tumor length (**A**), volume (**B**), and T/N max of MET-PET (**C**) are presented. The correlations were not statistically significant (p = 0.270, 0.960, and 0.110, 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.[2] 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,[10] 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.[4,11] 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.[15] 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[6–8] 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-

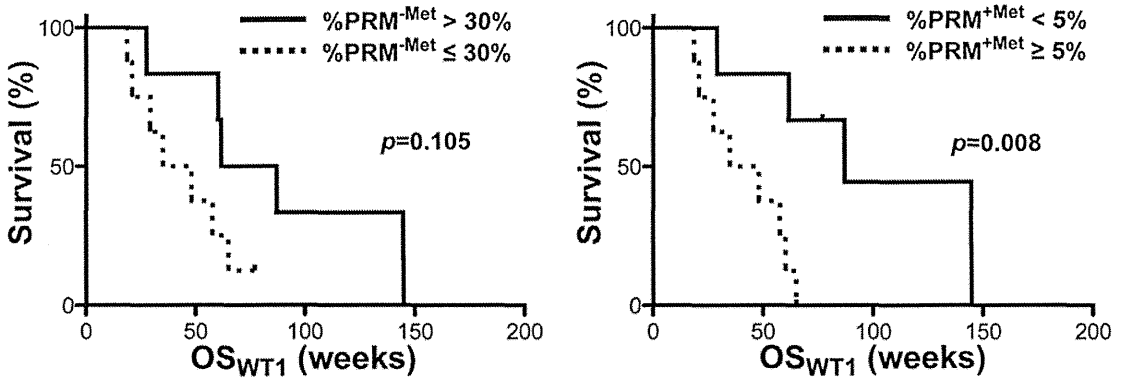

FIG. 5. Correlation of OS_{WT1} with PRM^{-MET} and PRM^{+MET}. Correlations between OS_{WT1} and percentage areas of PRM^{-MET} **(left)** and PRM^{+MET} **(right)** are presented. The percentage of PRM^{+MET} within the contrast-enhanced lesion before WT1 immunotherapy initiation correlated best with OS_{WT1} (p = 0.008; 14 cases).