aging analysis in glioma treatment using temozolomide and radiation therapy and has been suggested as an early biomarker for treatment response.[6–8] The main difference between voxel-wise PRM analysis and conventional imaging analysis is that voxel-wise PRM analysis allows us to identify the location and extent of areas that responded to therapy, rather than comparing the maximum values of the pre- and posttreatment evaluation modality, which could be comparing different locations.

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

Conclusions

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

Disclosure

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

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Vaccination with WT-1 (Wilms' Tumor gene-1) peptide and BCG-CWS in melanoma

The Wilms tumor gene, WT1, plays an important role in the regulation of cell proliferation, differentiation, etc. Wild-type WT1 is highly expressed in malignancies, including malignant melanoma, and seems to be important for maintaining the transformed phenotype and function of cancer cells [1, 2]. The Bacillus Calmette-Guerin cell wall skeleton (BCG-CWS) activates dendritic cells via toll like receptors and is expected to be a useful adjuvant for cancer immunotherapy [3, 4]. We present a metastatic malignant melanoma patient who received clinical benefits and showed immunological response in association with using WT1 peptide vaccination with BCG-CWS.

A 64-year-old male with Stage IV malignant melanoma originating from the left chorioid, which had metastasized to the lungs, was admitted to Osaka University Hospital for WT1 peptide-based immunotherapy in February, 2008. In 2007, a lung nodule was histopathologically diagnosed as metastasis of malignant melanoma. The remaining metastatic lesion increased in size in spite of administration of the standard chemotherapy. The patient met the inclusion criteria for the vaccine trials, including having the HLA-A*2402 genotype and WT1 protein expression, and so was enrolled in the phase I clinical trial of immunotherapy using the WT1 peptide and BCG-CWS. According to the trial protocol, we used a modified 9-mer WT1 peptide, CYTWNQMNL. The treatment schedule was as follows; on day 1, 100 μg BCG-CWS was intracutaneously injected in the upper arm, followed by an injection of WT1 peptide (0.25 mg intracutaneously/0.25 mg subcutaneously) at the same site on day 2. The administrations were performed in the 1st, 3rd, 6th weeks and sequentially every month thereafter. With regard to adverse events, only a grade 2 skin ulcer was observed, which occurred at the injection site a few days after the injection, and lasted less than 2 months. Although the size of the target lesion measured by computed tomography had been steadily increasing before treatment, stable disease (SD) was achieved according to the Response Evaluation Criteria in Solid Tumors guidelines *(figure 1A)*. Because no new metastatic lesions appeared for about 6 months after the beginning of vaccination, surgical resection of the right lower lobule, including the target lesion, was performed on day 188. Fluorescent immunostaining of the resected lung lesions before and after vaccination was performed.

The number of $CD8^+$ T cells was robustly increased after vaccination *(figures 1B-C)*. The delayed type hypersensitivity (DTH) reaction specific to the WT1 peptide shown by *in vivo* immuno-monitoring changed from negative to positive at one month after the first vaccination. For *ex vivo* immuno-monitoring, the frequencies

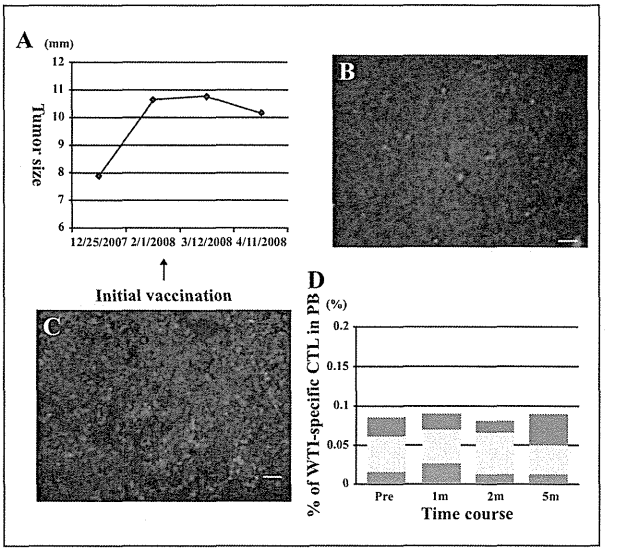

Figure 1. A) A graphical representation of the change in size of the target lesion in the lung. The tumor size was calculated by using computed tomography images, and the treatment response was evaluated according to the RECIST guidelines. The fluorescent immunostaining of the lung metastatic lesions before and after WT1 peptide vaccination. There were obviously more $CD8^+$ T cells (red) after the vaccination **(C)** than before the vaccination **(B)**. **D)** The frequencies of WT1-specific CTLs in peripheral blood and their subset compositions are shown. Based on CD45RA and CCR7 expression, the CTLs were phenotypically classified into four subsets; naïve (blue), central-memory (green), effector-memory (yellow), and effector (red).

of WT1-specific CTLs, determined by the percentages of WT1-tetramer$^+$$CD8^+$T cells among the total $CD8^+$T cells in the peripheral blood, were measured *(figure 1D)*. Furthermore, based on CD45RA and CCR7 expressions, a phenotype analysis of the CTLs was performed, in which they were classified into naïve (blue), central-memory (green), effector-memory (yellow), and effector (red) subsets. The frequency of WT1-specific CTLs remained at about 0.07% before and after vaccination. WT1-specific CTLs in effector-memory and effector subsets accounted for the dominant CTL populations both before and after the vaccination. The frequency of WT1-specific CTLs was not higher than that in healthy donors, however the subset was in sharp contrast to healthy donors, in whom WT1-specific CTLs in the naïve subset were dominant [5]. Such a high percentage of well-differentiated WT1-specific CTLs even before treatment might have contributed to the induction of a clinical response.Taken together, these findings suggest that WT1 peptide vaccination with a BCG-CWS adjuvant induced a stabilization of the disease, associated with induction of a WT1 peptide-specific immune-response and infiltration of $CD8^+$ T cells in the tumor tissue, offering evidence for the therapeutic potential of this treatment for malignant melanoma. ■

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Successful treatment of a folliculotropic mycosis fungoides with bexarotene and PUVA

According to the WHO-EORTC classification of primary cutaneous lymphomas, follicular mycosis fungoides (FMF) is a rare subtype of mycosis fungoides, the most common form of cutaneous T cell lymphoma. In comparison to classical MF, FMF often shows a more aggressive clinical course, with 5 year survival rates of only 64% [1].
We report a 69-year-old woman who presented with a 3-year history of a slowly-growing, well-demarcated, red tumor in the central and left face. The tumour was densly infiltrated with multilocular ulcerations and brownish crusts, leading to a facies leonina-like appearance *(figure 1A)*. There were no B-symptoms like fatigue, night sweats or weight loss. Despite previous histological investigations, the underlying cause of disease was still unclear. Under the suspected diagnosis of pyodermia, an antibiotic and steroid based therapy was initiated (sultamicillin 375 mg BID; erythromycin 500 mg BID; minocyclin 100 mg OD; prednicarbate locally BID). However, no response to therapy was observed, and the patient was transferred to our department.
Except for local symptoms on the face, physical examinations showed no other abnormal findings. Two new skin biopsies were taken. Histopathology revealed dense inflammatory infiltrates predominantly in the middle part of the dermis, clustering around destructed hair follicles *(figure 1C)*. Higher magnification unveiled a polymorphic folliculotropic lymphoid infiltrate with atypical features, containing limited numbers of eosinophils and plasma cells *(figure 1D)*. Alcian-PAS staining demonstrated deposits of mucin, especially in the areas of hair follicles. By immunohistochemical examination, lymphocytes stained positively for CD3, CD4 and CD45 RO. Approximately 10% of lymphoid cells were positive for the proliferation marker Mib-1. Multiplex-PCR verified clonality of the T cell receptor gamma chain.
Based on clinical appearance, histopathology and molecular findings, folliculotropic mycosis fungoides was diagnosed. Using imaging techniques (CT scans, ultrasound) and peripheral blood smear stainings, an extracutaneous involvement was ruled out. We initiated a combined therapy with oral psoralen (40 mg meladinine prior to irradiation)

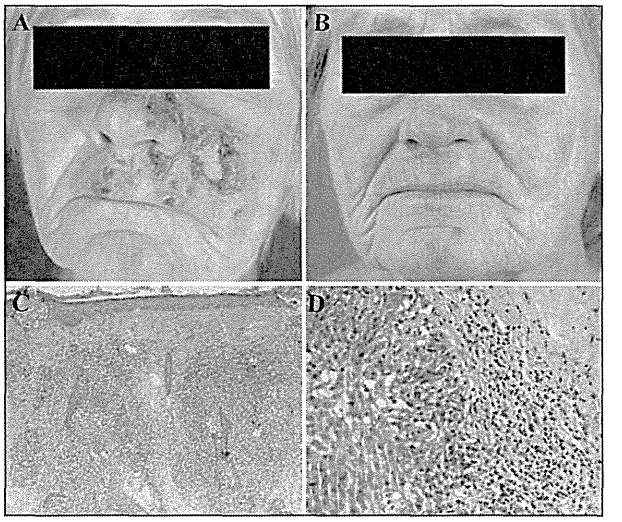

Figure 1. A) At presentation, densely infiltrated plaques with crusts and ulcerations were present on the central and left face, giving the patient a facies leonina-like appearance. **B)** 10 months after initiation of combined therapy with oral bexarotene and PUVA, lesions had cleared almost completely. **C)** Histopathology of skin biopsies showed dense inflammatory infiltrates in the dermis, especially around destructed hair follicles (H&E, ×50). **D)** Higher magnification (×200) reveals infiltration of hair follicles by lymphoid cells with atypical features.

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Diffuse large B cell lymphoma with an interfollicular pattern of proliferation shows a favourable prognosis: a study of the Osaka Lymphoma Study Group

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Diffuse large B cell lymphoma with an interfollicular pattern of proliferation shows a favourable prognosis: a study of the Osaka Lymphoma Study Group

Aims:]Diffuse large B cell lymphoma (DLBCL) occasionally shows an interfollicular pattern of proliferation (DLBCL-IF) preserving lymphoid follicles. In this study, clinicopathological findings in 31 cases of DLBCL-IF were analysed.

Methods and results: The study group comprised 20 males and 11 females, with ages ranging from 41 to 87 (median 69) years. The primary site was lymph node in 25 cases, and unknown in six due to advanced stage at diagnosis. Eight cases were clinical Stage I, 10 were Stage II, four Stage III, and nine Stage IV. A polymorphous pattern of proliferation containing large B cells and inflammatory cells was found in about 60% of cases. The overall survival rate of the DLBCL-IF patients was better than that of a DLBCL control group (log-rank test; $P < 0.05$). Multivariate analysis revealed that an interfollicular pattern of proliferation showed marginal significance for favourable prognosis ($P = 0.069$). Immunohistochemical double staining with antibodies for HLA-DR/CD68 (markers for M1-tumour-associated macrophage [M1-TAM]) or CD163/CD68 (M2-TAM) revealed that all DLBCL-IF patients with a low M2 count were alive at the end of observation.

Conclusions: These findings suggest that DLBCL-IF is a clinicopathological entity distinct from ordinary DLBCL. The possible origin of tumour cells in DLBCL-IF from marginal zone B cells is discussed.

Keywords: diffuse large B cell lymphoma, Epstein–Barr virus, interfollicular proliferation, macrophages, prognosis

Abbreviations: DLBCL, diffuse large B cell lymphoma; DLBCL-CG, DLBCL control group; DLBCL-IF, DLBCL with interfollicular pattern of proliferation; EBV, Epstein–Barr virus; EBER, EBV-encoded small RNA; GCB, germinal centre B cell; HPF, high-power field; Ig, immunoglobulin; IPI, international prognostic index; ISH, *in-situ* hybridization; LDH, lactate dehydrogenase; MZBCL, marginal zone B cell lymphoma; NHL, non-Hodgkin's lymphoma; OLSG, Osaka Lymphoma Study Group; PTCL-NOS, peripheral T cell lymphoma, not otherwise specified; PNA, peptide nucleic acid; TAM, tumour-associated macrophage; WHO, World Health Organization

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Introduction

Diffuse large B cell lymphoma (DLBCL), the most common type of malignant lymphoma in the World Health Organization (WHO) classification, is defined as diffuse proliferation of large neoplastic mature B cells.[1] When based on these criteria, DLBCL comprises a group of morphologically, immunohistochemically and clinically heterogeneous tumours rather than one single disease. Several diseases are included in the WHO classification (2008) such as DLBCL, not otherwise specified, DLBCL associated with chronic inflammation, and so on[1].

Several attempts have been undertaken to classify DLBCL into biologically and clinically relevant subtypes. On the basis of gene expression profiles, DLBCL could be categorized into the germinal centre B cell (GCB) type and non-GCB (activated B cell) type.[2,3] Generally, the GCB type shows a favourable prognosis compared to the non-GCB type. Recently, Lenz *et al.* reported that DLBCL could be categorized on the basis of gene expression signatures of stromal cells surrounding the neoplastic large B lymphoid cells: gene signatures of stromal cells found in cases with abundant histiocytes were correlated with a favourable prognosis.[4]

DLBCL usually shows a diffuse proliferation of large B cells effacing lymph nodal architectures including lymphoid follicles. Recently we reported a series of 12 cases of DLBCL with an interfollicular pattern of proliferation (DLBCL-IF) preserving lymphoid follicles.[5] These cases constituted 1.1% of all DLBCL, and frequently showed a polymorphous pattern of proliferation consisting of non-neoplastic inflammatory cells; immunophenotypes, as revealed by immunohistochemistry, were predominantly of non-GCB type. Clinically, DLBCL-IF showed a rather favourable prognosis compared to that of ordinary DLBCL, although this was not statistically significant, due possibly to the small number of cases examined. Further patients with DLBCL-IF have subsequently been identified and, in the present study, clinicopathological findings in 31 cases were analysed to evaluate whether this type of lymphoma represents a distinct clinicopathological entity of DLBCL.

Macrophages play an important role as one of the non-neoplastic, inflammatory cells in tumour tissues, where they are termed tumour-associated macrophages (TAMs). TAMs were reported to be associated with a worse prognosis in patients with follicular lymphoma[6] or classical Hodgkin's lymphoma,[7] whereas we found that a high number of epithelioid histiocytes in DLBCL was correlated with a better prognosis.[8] In contrast, Hasselblom *et al.* reported that the number of TAMs in DLBCL tissues was not correlated with patients' prognosis.[9] TAMs can be classified into two functionally distinct types, M1 and M2, and the balance of these cell types was reported to determine their effects against tumours, i.e. promotive (M2) or suppressive (M1).[10,11] In the present study, associations of M1 and M2 macrophage types with the prognosis of DLBCL-IF patients were evaluated using immunohistochemical double-staining.

Materials and methods

PATIENTS

From November 1999 to April 2010, 4800 cases were registered with the Osaka Lymphoma Study Group (OLSG), Japan. Histological samples in all cases were fixed in 10% formalin and processed routinely for paraffin embedding. Histological sections, cut at 4 μm, were stained with haematoxylin and eosin (H&E), and immunohistochemical procedure (Dako Autostainer; Dako, Glostrup, Denmark). All cases were reviewed by one of the authors (K.A.) and categorised according to the WHO classification. As a result, 3826 cases were diagnosed as malignant lymphoma, of which 3508 (91.7%) were non-Hodgkin's lymphoma (NHL) and 318 (8.3%) Hodgkin's lymphoma. There were 1708 cases of DLBCL, which constituted 48.7% of NHL. Of these, 28 cases (1.6%) showed the interfollicular pattern of proliferation. Three such cases diagnosed and treated at another institute were also included in this study. Therefore, a total of 31 cases of DLBCL-IF were analysed. Adequate clinical information was available in all patients. Ninety-six cases of ordinary-type DLBCL with adequate available clinical data and unstained sections registered with the OLSG during August 2000–May 2005 were used as a DLBCL control group (DLBCL-CG). Clinicopathological findings of the DLBCL-IF and DLBCL-CG groups are summarized in Table 1. To examine differences between DLBCL-IF and marginal zone B cell lymphoma (MZBCL), seven cases of nodal MZBCL registered with OLSG during the period June 2004–September 2008 were also examined. A brief summary comparing DLBCL-CG, DLBCL-IF and nodal MZBCL is shown in Table 2.

The study group comprised 20 males and 11 females, with ages ranging from 41 to 87 (median 69) years. The primary site was lymph node in 25 cases, and unknown in six due to advanced stage at diagnosis. Using physical examination records, surgical notes and the results of pathological examination, the Ann Arbor staging system was applied. Eight cases were disease Stage I, 10 were Stage II, four

Table 1. Brief clinicopathological findings

	Interfollicular group (n = 31)	Control group (n = 96)	P value
Age (years): range (mean/median)	41–87 (67.8/69)	24–86 (62.8/64)	<0.05
Age>60 years, n (%)	26/31 (83.9)	58/96 (60.4)	<0.05
Sex, male/female	20:11	56:40	NS
Primary site, nodal/extranodal/unknown	25/0/6	39/35/22	<0.01*
Serum LDH level > normal, n (%)	15/31 (48.4)	48/96 (50)	NS
Bulky mass, present/absent	3:27	21:75	NS
Stage 3 or 4, n (%)	13/31 (41.9)	43/96 (44.8)	NS
Involved extranodal organ > 1, n (%)	4/31 (12.9)	22/96 (22.9)	NS
IPI, HI or high, n (%)	11/30 (36.7)	39/96 (40.6)	NS
Proliferation pattern Monomorphous, n (%)	13 (41.9)	79 (82.3)	<0.01
Polymorphous, n (%)	18 (58.1)	17 (17.7)	
Fibrosis, present/absent	12:19	47:49	NS
Mitotic count (/high-power field) mean (range)	4.1 (0–10)	3.0 (0–10)	<0.05
MIB-1, %, mean (range)	62.9 (20–90)†	60 (20–90)‡	NS
GCB/non-GCB	4:25	42:49	<0.01
EBV positive, n (%)	5/29 (17.2)	2/44 (4.5)	NS

*Nodal/extranodal, †: n = 28, ‡n = 35.
IPI: International prognostic index; HI/H, high-intermediate/high; GCB, germinal center B-cell type; NS, not significant.

Stage III, and nine Stage IV. The international prognostic index (IPI) score was calculated with five adverse factors [age > 60 years, Ann Arbor Stages III and IV, Eastern Cooperative Oncology Group performance score 2–4, elevation of serum lactate dehydrogenase (LDH) and two or more extranodal lesions] present at the time of diagnosis.[12] For cases aged < 60 years an age-adjusted IPI score was applied, in which advanced stage, high performance score and elevation of LDH were considered as adverse factors.[12] Almost all patients received anthracycline-based chemotherapy, mainly a regimen including doxorubicin, cyclophosphamide, vincristine and prednisone. In the latter half of 2003, rituximab was included in the regimen for most patients with B cell lymphomas. Rituximab was used for 20 (66.7%) of 30 DLBCL-IF patients (data not available in one case) and 51 (53.1%) of 96 DLBCL-CG patients. The difference in frequency of patients receiving rituximab between DLBCL-IF and DLBCL-CG was not significant. Clinical outcome was evaluated according to the guidelines of the International Workshop to standardize response criteria for NHL.[13]

FOLLOW-UP

Follow-up periods for survivors in DLBCL-IF and DLBCL-CG cases ranged from 3.5 to 99 (average 32.4) and 11 to 99 (average 45) months, respectively. Follow-up data were not available in one DLBCL-IF case. Follow-up periods of nodal MZBCL cases ranged from 22 to 77.7 (average 38.6) months. Follow-up data were not available in two nodal MZBCL cases, but all but these two were alive at the end of observation.

Table 2. Brief summary comparing diffuse large B-cell lymphoma (DLBCL)-control group (CG), DLBCL with interfollicular pattern of proliferation (DLBCL-IF), and nodal marginal zone B-cell lymphoma (MZBCL)

	Sex male: female	Age (years) range/ median	Stage 3 or 4 *n* (%)	Size of tumor cells	Mean mitotic count (/high-power field)	Mean MIB-1 index (%)	p53 positive *n* (%)	EBV positive *n* (%)	5-year overall survival (%)
DLBCL-CG	56:40	24–86/64	43/96 (44.8)	Large	3.0	60	10/15 (66.7)	2/44 (4.5)	50.0
DLBCL-IF	20:11	41–87/69	13/31 (41.9)	Large	4.1	62.9	9/26 (34.6)	5/29 (17.2)	61.5
Nodal MZBCL	5:2	32–75/64	3/6 (50)	Small to medium	0.81	10.7	0/7 (0%)	1/7 (14.3%)	100

DLBCL-CG: Diffuse large B-cell lymphoma-control group; DLBCL-IF, diffuse large B-cell lymphoma with interfollicular pattern of proliferation; MZBCL, marginal zone B-cell lymphoma.

Kaplan–Meier estimated survival rates at 5 years were calculated, and overall survival rates were compared using the log-rank test.

IMMUNOHISTOCHEMISTRY

Antibodies used for immunohistochemistry were CD20, CD79a, CD3, CD8, Bcl-6, MUM1, MIB-1, CD68 (clone: PG-M1), and p53 (all Dako; dilution at 1:400, 1:100, 1:50, 1:100, 1:50, 1:100, 1:1, 1:50, 1:25, respectively); CD4 (1:40;Novocastra Laboratories, Newcastle, UK,USA); CD10 (used as a prediluted antibody; Nichirei Biosciences, Tokyo, Japan) HLA-DR (1:50; Medical & Biological Laboratories, Nagoya, Japan); and CD163 (1:50; Novocastra-Leica, Wetzlar, Germany). Immunohistochemistry was performed using an automated staining system (Dako Autostainer; Dako).

Immunohistochemical double staining of HLA-DR/CD68 or CD163/CD68 was performed using the EnVision™ G|2 Rabbit/Mouse Doublestain System (Dako). Primary antibody (HLA-DR or CD163) was stained brown using diaminobenzidine (DAB^+) and the secondary antibody (CD68) red using Permanent Red. The average number of double-positive cells ($HLA\text{-}DR^+/CD68^+$ or $CD163^+/CD68^+$) in three representative high-power field (HPF) (×400 magnification) tissue sections was counted by two investigators (N.W., M.A.A.Z.) in a blinded fashion. The cut-off value for $HLA\text{-}DR^+/CD68^+$ and $CD163^+/CD68^+$ cells was set at the median value, i.e. 23.8 and 20.65 double-positive cells/HPF, respectively.

IN-SITU HYBRIDIZATION (ISH)

ISH using the Epstein–Barr virus (EBV)-encoded small RNA (EBER) PNA (peptide nucleic acid) probe/fluorescein was performed to examine the presence of the EBV genome in formalin-fixed paraffin-embedded sections, employing the EBER DAB application (Dako), as described previously.[14] When EBER ISH yielded positive signals in the nuclei of more than 1% of the proliferating cells, such cases were defined as EBV-positive.

ISH was performed with κ and λ (mRNA) PNA probes/fluorescein to examine the restriction of κ/λ expression using a method similar to the EBER ISH (Dako).

CLONALITY ANALYSIS USING IMMUNOGLOBULIN (IG) GENE REARRANGEMENT (GENE SCAN ANALYSIS)

One to five sections of 4–10 μm thickness were cut from the paraffin-embedded samples, deparaffinized with xylene, washed with 70% and absolute ethanol and subsequently digested in lysis buffer [50 mM Tris–HCl, 10 mM ethylenediamine tetraacetic acid (EDTA), 150 mM NaCl, 0.5% sodium dodecyl sulphide (SDS) and 0.4 mg/l proteinase K] at 55°C overnight. DNA was extracted using a phenol–chloroform extraction-based protocol, and following ethanol precipitation was redissolved in TE buffer. Ig gene rearrangement was assessed according to modified BIOMED-2 protocols as described previously.[15,16]

STATISTICAL ANALYSIS

Differences in frequencies of various clinical and pathological factors between cases with DLBCL-IF and DLBCL-CG were compared with the Chi-square test or Fisher's exact probability test. Differences of mean values were compared using the *t*-test. Overall survival rates and survival curves were calculated using the

Kaplan–Meier method and were compared by the log-rank test. Multivariate analysis was performed with Cox's proportional hazard regression model.

Results

CLINICAL FINDINGS

Clinicopathological findings are summarized in Table 1. Sex ratio (M:F) and age (range and median) in DLBCL-IF and DLBCL-CG were 1.8:1 and 41–87 (median 69) years and 1.4:1 and 24–86 (median 64) years, respectively. The frequency of cases aged > 60 years in DLBCL-IF (83.9%) was higher than that in DLBCL-CG (60.4%) ($P < 0.05$). DLBCL-IF was principally nodal disease. There were no significant differences between the two groups with regard to sex ratio, LDH level, presence of a bulky mass, high stage, two or more extranodal lesions or high IPI score.

PATHOLOGICAL FINDINGS

In DLBCL-IF, there was a monomorphous pattern of proliferation of large lymphoid cells in 13 cases, and a polymorphous pattern containing macrophages, small lymphocytes, plasma cells and eosinophils together with large lymphoid cells in 18 cases. The frequency of cases showing a polymorphous pattern in DLBCL-IF, 18 (58.1%) of 31 cases, was higher than that in DLBCL-CG, 17 (17.7%) of 96 cases ($P < 0.01$). Varying degrees of fibrosis were relatively frequent in both groups. The mean mitotic count in the DLBCL-IF group (4.1/HPF) was significantly higher than that in the DLBCL-CG (3.0/HPF) ($P < 0.05$). There was no significant differences in MIB-1 index between the two groups (Table 1). The proliferating large B cells had a $CD20^+/CD79a^+/CD3^-$ phenotype (Figure 1). Intermingled small lymphoid cells among the large B cells in the polymorphous proliferation were predominantly (mixed $CD4^+$ and $CD8^+$ cell population) T lymphocytes.

Tumour cells showed a monocytoid-like appearance in nine of the 31 DLBCL-IF cases (Figure 2). Morphological features of DLBCL-IF were then compared with nodal MZBCL (Table 2). Tumour cells in DLBCL-IF were two to three times larger than those in nodal MZBCL, and nucleoli were evident. The mean mitotic count in DLBCL-IF (4.1/HPF) was significantly higher than that in nodal MZBCL (0.81/HPF) ($P < 0.01$). There was also a significant difference in MIB-1 index between DLBCL-IF (mean: 62.9%) and nodal MZBCL (mean: 10.7%) ($P < 0.01$). The positivity rate for p53 in DLBCL-IF (34.6%, nine of 26) was significantly lower than that of DLBCL-CG (66.7%, 10 of 15) ($P < 0.05$), but higher than that of nodal MZBCL (0%, none of seven) ($P = 0.068$).

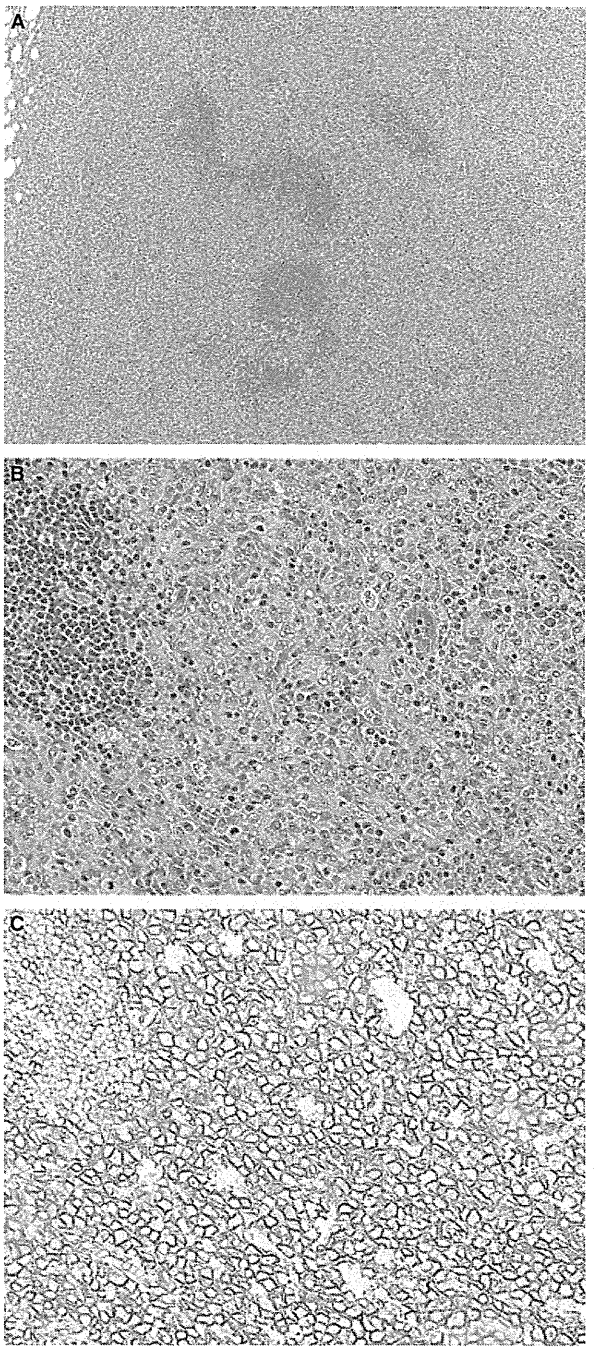

Figure 1. A, Large lymphoid cells show an interfollicular pattern of proliferation preserving lymph follicles (H&E). B, There are occasional mitotic figures (H&E). C, Large lymphoid cells in the interfollicular area are positive for CD20.

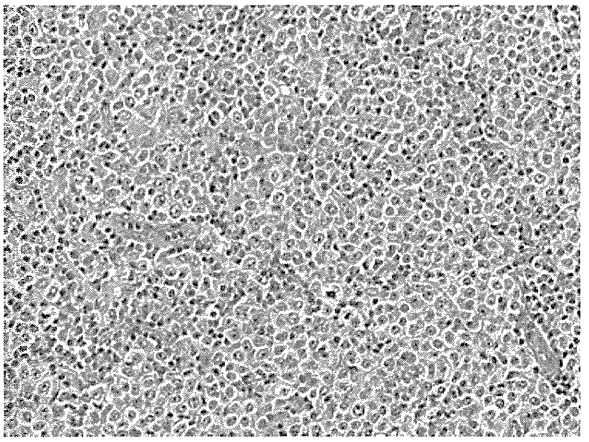

Figure 2. Lymphoid cells rich in pale cytoplasm resemble marginal zone B cells (H&E).

IMMUNOHISTOCHEMICAL ANALYSIS FOR GCB OR NON-GCB PHENOTYPE

Based on the expression of CD10, Bcl-6 and MUM-1, the DLBCL cases were categorized further into GCB and non-GCB subtypes, according to the decision tree proposed by Hans *et al.*[17] In brief, cases showing a $CD10^+$ or $CD10^-/Bcl\text{-}6^+/MUM\text{-}1^-$ phenotype were categorized as GCB and the remainder as non-GCB. DLBCL-IF cases were predominantly of non-GCB type, while the ratio of GCB to non-GCB types was equal in the DLBCL-CG group ($P < 0.01$) (Table 1).

IMMUNOHISTOCHEMICAL DOUBLE STAINING FOR HLA-$DR^+/CD68^+$ OR $CD163^+/CD68^+$ CELLS

Of 18 cases with DLBCL-IF showing a polymorphous pattern of proliferation, immunohistochemical double-staining and follow-up data were available in 16 cases. These 16 cases were classified into four groups based on the cut-off number of M1 and M2 macrophages: high M1, low M1, high M2 and low M2 groups. The cut-off value for HLA-$DR^+/CD68^+$. (M1) and $CD163^+/CD68^+$ (M2) cells was set at the median value of double-positive cells/HPF, i.e. 23.8 and 20.65, respectively. The estimated survival rate at 40 months in the high (eight cases) and low (eight cases) M1 groups, and high (eight cases) and low (eight cases) M2 groups, was 100% and 75.0%, and 66.7% and 100%, respectively. The differences were not statistically significant (log-rank test; $P > 0.1$). All patients with a low M2 count were alive at the end of observation (8–42.5 months, median; 22.3 months).

ISH

The frequency of the EBV^+ cases was higher in DLBCL-IF (17.2%) than in DLBCL-CG (4.5%), but the difference was not statistically significant. A few EBV-positive medium-to-large lymphoid cells were scattered in a background of small-to medium-sized monocytoid B cells in one of seven cases of nodal MZBCL.

Immunoglobulin light chain restriction could not be confirmed by *in-situ* hybridization in any of eight DLBCL-IF cases with a monocytoid appearance of tumour cells.

CLONALITY ANALYSIS USING IG GENE REARRANGEMENT

Informative data were obtained in 25 of 26 examined cases: all the 25 cases showed a monoclonal rearrangement of the Ig gene with at least one primer. In five DLBCL-IF cases, a large enough number of samples for DNA extraction was not available.

PROGNOSIS

The estimated survival rate at 5 years was 61.5% in DLBCL-IF and 50.0% in DLBCL-CG. Overall survival with DLBCL-IF was better than that with DLBCL-CG (log-rank test; $P < 0.05$) (Figure 3). Results of the univariate analysis are shown in Table 3. Elevated serum LDH level, advanced stage, more than one involved extranodal organ and high IPI score were unfavourable factors for prognosis ($P < 0.01$). Non-GCB type showed marginal significance as an unfavourable factor for prognosis ($P = 0.061$). Multivariate analysis revealed that non-GCB type and an interfol-

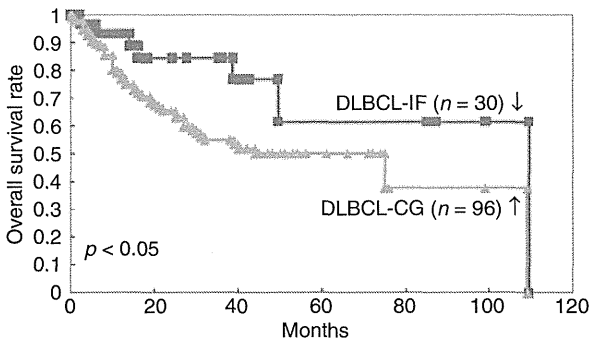

Figure 3. The estimated survival rates at 5 years for diffuse large B cell lymphoma with interfollicular pattern of proliferation (DLBCL-IF) and DLBCL control group (DLBCL-CG) were 61.5 and 50.0%, respectively (log-rank test; $P < 0.05$).

Table 3. Univariate analysis for overall survival of patients with DLBCL-IF and -CG

Characteristic	No. of patients	5-year OS (%)	*P*
Age, years			
age≤ 60	43	59.2	NS
age> 60	83	49.0	
Sex			
Male	75	45.7	NS
Female	51	62.0	
Primary site			
Nodal	63	51.9	NS
Extranodal	35	65.6	
Serum LDH level			
Normal or lower	64	66.4	<0.01
Higher than normal	62	38.4	
Bulky mass			
Present	24	62.8	NS
Absent	102	50.6	
Ann Arbor stage			
Stage 1 or 2	70	71.0	<0.01
Stage 3 or 4	56	24.8	
Extranodal involvement			
0–1 site	100	62.6	<0.01
>1 sites	26	16.8	
International prognostic index			
L/LI	76	72.8	<0.01
HI/H	49	20.2	
GCB/non-GCB			
GCB	46	64.0	0.061
Non-GCB	73	44.8	
Interfollicular pattern of proliferation			
Present	30	61.5	<0.05
Absent	96	50.0	

DLBCL-IF: Diffuse large B-cell lymphoma with interfollicular pattern of proliferation; DLBCL-CG, diffuse large B-cell lymphoma-control group; OS, overall survival; L/LI, low/low-intermediate; HI/H, high-intermediate/high; GCB, germinal center B-cell type; NS, not significant.

licular pattern of proliferation showed marginal significance for unfavourable ($P = 0.086$) and favourable ($P = 0.069$) prognosis, respectively (Table 4).

Table 4. Multivariate analysis of clinicopathologic factors for overall survival of patients with DLBCL–IF and –CG

Characteristic	Relative risk	95% CI	*P*
Serum LDH level > normal	1.29	0.64–2.60	NS
Stage 3 or 4	1.20	0.37–3.91	NS
Involved extranodal organ > 1	1.71	0.85–3.42	NS
IPI, HI/H	2.59	0.70–9.63	NS
Non-GCB	1.78	0.92–3.44	0.086
Interfollicular pattern of proliferation	0.42	0.16–1.07	0.069

DLBCL-IF: Diffuse large B-cell lymphoma with interfollicular pattern of proliferation; DLBCL-CG, diffuse large B-cell lymphoma-control group; CI, confidence interval; IPI, international prognostic index; HI/H, high-intermediate/high; GCB, germinal center B-cell type; NS, not significant.

Discussion

Diffuse large B cell lymphoma usually shows a diffuse pattern of proliferation effacing the nodal architecture, including lymphoid follicles. Conversely, tumour cells in peripheral T cell lymphoma, not otherwise specified (PTCL-NOS) usually proliferate in the paracortical region, frequently preserving lymphoid follicles. DLBCL-IF therefore resembles PTCL-NOS in its pattern of proliferation. In addition, in the current study about 60% of DLBCL-IF cases showed a polymorphous pattern of proliferation containing reactive inflammatory cells, further mimicking the histological picture of PTCL-NOS. DLBCL-IF showed a significantly more favourable prognosis than DLBCL-CG, whereas PTCL-NOS usually has a worse prognosis than DLBCL. Therefore, distinction between these two conditions is essential for making decisions on treatment modalities. Definite separation of DLBCL-IF from PTCL-NOS can be made only after immunohistochemical evaluation.

An interfollicular pattern of proliferation is the key feature differentiating DLBCL-IF from DLBCL-CG.[5] Clinically, DLBCL-IF contrasts with DLBCL-CG in later onset of disease and predominantly nodal involvement. Histologically, DLBCL-IF frequently shows a polymorphous pattern of proliferation and a marked predominance of non-GCB to GCB type tumours, as revealed by immunohistochemistry. The frequency of GCB and non-GCB types was about equal in a previous study of ordinary DLBCL,[2] and also in the present series

of DLBCL-CG. The follow-up study showed that the prognosis of patients with DLBCL-IF was favourable compared to that of DLBCL-CG; the difference was significant by univariate analysis ($P < 0.05$) and marginally significant by multivariate analysis ($P = 0.069$). These findings suggest that DLBCL-IF could be a clinicopathological entity.

The favourable prognosis and predominantly polymorphous pattern of proliferation observed in DLBCL-IF might argue against the neoplastic nature of this disease. The polymerase chain reaction (PCR)-based clonality analysis using paraffin-embedded samples revealed an Ig gene monoclonal rearrangement pattern, justifying the diagnosis of DLBCL. In addition, mitotic count in DLBCL-IF was higher than that in DLBCL-CG.

The majority of DLBCL-IF showed a non-GCB immunophenotype. Marafioti *et al.* reported that the interfollicular large B cells in normal lymph node show a non-GCB immunophenotype,[18] and share some characteristics with monocytoid B cells, i.e. they express CD20, CD75, CD79a, BOB-1, OCT-2 and caspase-3, but not IgD, CD5, CD23, CD10, BCL-6, VS38c(p63), CD138 or BCL-2. Indeed, tumour cells in nine of the present 31 DLBCL-IF cases showed a monocytoid appearance (Figure 2). Tumour cells in seven of these nine cases did not express CD10. Monocytoid B cells are derived from the marginal zone of lymph follicles. The clinicopathological features of DLBCL-IF were therefore compared to those of nodal MZBCL. Average mitotic count, MIB-1 index and p53 positivity in DLBCL-IF (4.1/HPF, 62.9%, 34.6%) were higher than in nodal MZBCL (0.81/HPF, 10.7%, 0%). p53 positivity in DLBCL-IF (34.6%) was lower than that in DLBCL-CG (66.7%). There were no significant differences in EBV positivity between DLBCL-IF (17.2%) and nodal MZBCL (14.3%). From these findings, it is postulated that DLBCL-IF is a transformed nodal MZBCL. Evaluation of gene expression profiling might provide further insight into the cell origin or cell characteristics of DLBCL-IF.

TAMs can be classified into two functionally distinct types, M1 and M2, and the balance of these cell types has been reported to determine their effects against tumours, i.e. promotive (M2) or suppressive (M1).[10,11] An association between $CD163^+$ M2-TAM and a worse prognosis has been reported for several tumours, including uveal melanoma,[19] intrahepatic cholangiocarcinoma,[20] pancreatic cancer[21] and glioblastoma.[22,23] Accurate estimation of the density of M2-TAM among all macrophages might provide more precise information for the role of M2-TAM in tumour development. CD68, HLA-DR and CD163 are commonly used for identification of macrophages; however, CD68 also labels immature CD1a-positive dendritic cells, HLA-DR peripheral lymphocytes and CD163 some dendritic cells. To resolve this point, double staining using pan-macrophage marker CD68 with HLA-DR (M1-TAM) or CD163 (M2-TAM) is useful.[24] We undertook immunohistochemical double staining to detect $HLA\text{-}DR^+/CD68^+$ or $CD163^+/CD68^+$ cells in the 16 DLBCL-IF cases showing a polymorphous pattern of proliferation, and found that all patients with a low M2 count were alive at the end of observation. A low content of M2-TAM may therefore be associated with a favourable prognosis for patients with DLBCL-IF.

Conflict of interest

We declare that we have no conflicts of interest.

Acknowledgements

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Long-term WT1 peptide vaccination for patients with acute myeloid leukemia with minimal residual disease

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Acute myeloid leukemia (AML) still has a rather poor prognosis, which is mainly ascribed to relapse in the majority of patients despite achievement of complete remission (CR). To prevent relapse, minimal residual disease (MRD) remaining in CR has to be completely eradicated. The graft-versus-leukemia effect induced by allogeneic hematopoietic stem cell transplantation is the most powerful mechanism for eradication of MRD, thus strongly suggesting the therapeutic potential of immunotherapy.

The Wilms' tumor gene *WT1* is highly expressed in various types of malignancies, and the WT1 protein is one of the most promising leukemia and tumor-associated antigens.[1–6] Based on findings of pre-clinical studies, we performed a phase I clinical study of WT1 peptide immunotherapy for patients with AML, myelodysplastic syndromes, breast or lung cancer, and were able to confirm the safety and clinical efficacy of this therapy.[7] In this report, we describe the long-term outcome for AML patients enrolled in this study.

The major inclusion criteria for vaccination were that patients had HLA-A*2402 and that *WT1* mRNA levels, which reflect the amount of MRD,[8,9] were $>1\times10^3$ or $>1\times10^2$ copies/μg RNA in bone marrow (BM) or peripheral blood (PB), respectively. A natural WT1 peptide (amino acid 235–243 CMTWNQMNL) or the modified WT1 peptide (CYTWNQMNL)[10] emulsified with Montanide ISA51 adjuvant (Freund Corporation, Tokyo, Japan) was i.d. injected, and the vaccination was performed three times at 2-week intervals.

The terms of both clinical and molecular responses were clearly defined as respectively, a decrease in leukemic blast cells in BM and a decrease in WT1 mRNA levels in BM and/or PB. Molecular CR was defined as undetectable levels of WT1 mRNA in PB (<50 copies/μg RNA). Molecular relapse was defined as abnormal levels of WT1 mRNA (⩾50 copies/μg RNA in PB and/or ⩾1000 copies/μg RNA in BM). Vaccination was extended for patients who showed clinical response or no progression within 2 weeks from the end of three WT1 vaccinations. As for immunological monitering, WT1 tetramer$^+$CD8$^+$T cells and IFN-γ$^+$WT1 tetramer$^+$CD8$^+$T cells were analyzed. Of the eight AML patients with hematological CR (leukemic blast <5%) but with MRD who were WT1-vaccinated, five patients who showed a decrease in *WT1* mRNA levels (molecular response) were repeatedly WT1-vaccinated, but two relapsed later. The remaining three patients received WT1 vaccine for ⩾8 years and have survived in molecular CR for ⩾8 years without significant adverse effects except for local erythema at the vaccine injection sites (Table 1).

CASE REPORTS

Case 1

A 32-year-old male was diagnosed as AML (M4Eo) in January 1997 and achieved hematological CR by induction chemotherapy. After

Table 1. Summary of characteristics and clinical courses of long-term survivors

Case no.	Age/sex	FAB	Karyotype	Prior to WT1 vaccination			WT1 peptide	No. of vaccination	Vaccination periods	Follow-up periods	Current[a] status	Adverse effects
				Status	MRD							
					WT1 mRNA	Other markers						
1	32/M	AML (M4Eo)	Normal	MR	(+)	(−)	Natural 3 mg ↓ modified 3 mg	110	8 yr.	8 yr. 5 m.	CR	Local skin eruption
2	49/F	AML (M1)	Normal	MR	(+)	CD34+ CD56+	Modified 3 mg	113+	8 yr. 3 m.	8 yr. 3 m.	CR	Local skin eruption
3	60/F	AML (Sec)	t(6;11) (q21;q23)	MR	(+)	(−)	Modified 3 mg	157	7 yr. 10 m.	8 yr. 3 m.	CR	Local skin eruption

Abbreviations: AML, acute myeloid leukemia; CR, complete remission; FAB, French American British classification; MR, molecular relapse; MRD, minimal residual disease; Sec, secondary AML. [a]The present data of white blood cell, hemoglobin and platelet (X104) : case 1: 5500, 15.1 and 10.2; case 2: 7200, 15.4 and 14.7; case 3: 4740, 13.0 and 28.0.

recurrence in November 1999, he received chemotherapy again and achieved the second hematological CR. BM examination in February 2003 showed hematological CR, but had MRD of 3.0×10^3 copies of *WT1* mRNA/μg RNA (Figure 1a). As his disease was considered to be at a high risk of hematological relapse, WT1 vaccination with 3 mg of natural WT1 peptide was begun in February 2003. *WT1* mRNA levels in BM decreased to normal levels during administration of three WT1 vaccinations (time point (1)) but increased to abnormal levels after discontinuation of the vaccination (time point (2)). WT1 vaccination was therefore started again, and again *WT1* mRNA levels decreased to normal levels (time point (3)). These findings demonstrated that WT1 vaccination was effective for eradication of MRD. As *WT1* mRNA levels increased after the second discontinuation of WT1 vaccination, the vaccination was initiated once more in June 2003 (time point (4)). The vaccine was changed from a natural to a modified WT1 peptide in September 2004 (Year 1.7) because a phase I/II clinical study of WT1 immunotherapy using a modified WT1 peptide for solid tumors showed the evidence of the modified WT1 peptides' effectiveness. *WT1* mRNA levels decreased to normal levels (<50 copies/μgRNA in PB) ~4 years after the start of WT1 vaccination and have been maintained at normal levels until now, although *WT1* mRNA levels remained at abnormal levels during the first 4 years. The WT1 vaccination was continued until February 2011, but stopped at that time point owing to patient's request with our agreement.

Case 2

A 49-year-old woman was diagnosed as AML (M1) in June 2000. Leukemic blast cells were characterized by $CD34^+CD56^+$. The patient achieved molecular CR (WT1 mRNA: <50 copies/μg RNA in PB) after the second induction chemotherapy followed by a multicourse consolidation and maintenance therapy. After molecular CR had been maintained for 2.5 years, however, BM examination showed molecular relapse with 4.5×10^3 copies of *WT1* mRNA/μg RNA in February 2003, and fluorescence-activated cell sorting analysis detected a low frequency of $CD34^+CD56^+$ blast cells in BM. The patient refused allogeneic hematopoietic stem cell transplantation and expressed a desire to be treated with WT1 peptide vaccination. The vaccination with 3 mg of modified WT1 peptide was begun in March 2003 (Figure 1b). *WT1* mRNA levels in PB decreased to normal during three WT1 vaccinations (time point (1)). Subsequently, *WT1* mRNA levels increased or decreased in parallel with the discontinuation or restart of WT1 vaccination, respectively. *WT1* mRNA levels remained abnormal from year 1.5 until year 4.0, but then became normalized and have remained normal until now, while $CD34^+CD56^+$ blast cells have become undetectable.

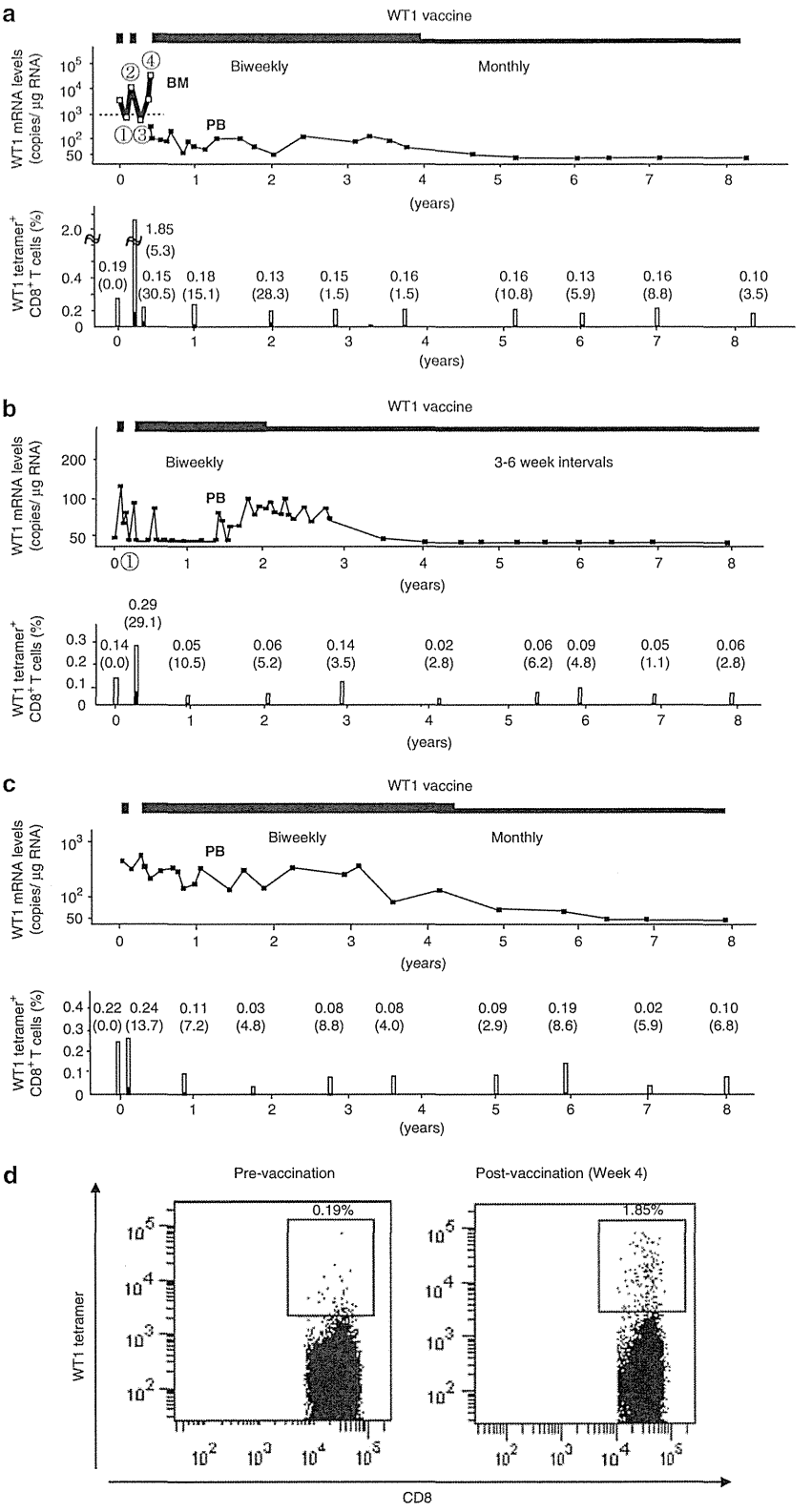
Case 3

A 60-year-old female was diagnosed as AML (M3) with t(15;17) in July 2000 and hematological CR was achieved by induction chemotherapy. The disease recurred in September 2002 in the form of secondary AML with t(6;11)(q21;q23). Induction chemotherapy was administered again and the patient achieved a second CR. BM examination in March 2003 showed hematological CR, but molecular relapse with 4.0×10^3 copies of *WT1* mRNA/μg RNA in BM (Figure 1c). As her disease was considered to be at a high risk of hematological relapse, WT1 vaccination with 3 mg of modified WT1 peptide was initiated in April 2004. *WT1* mRNA levels in PB gradually decreased from 880 copies/μg RNA before WT1 vaccination and reached undetectable levels (<50 copies/μg RNA) 6.5 years after the vaccination. Cells with t(6;11)(q21;q23) had also remained undetectable. WT1 vaccination was continued until February 2011, but stopped at that time point owing to the patient's request with our agreement.

In all three cases reported here, frequencies of WT1 $tetramer^+CD8^+$T cells that were already high before WT1 vaccination compared with those in healthy donors (HDs) transiently increased further after the vaccination and then decreased, but remained at higher levels during the vaccination. It should be noted that IFN-γ^+WT1 $tetramer^+CD8^+$T cells, which are considered to be functionally active, were not detected before WT1 vaccination, but appeared after the vaccination had been initiated and stayed at clearly detectable levels during the vaccination. In case 3, the increase to 0.19% in the frequency of WT1 $tetramer^+CD8^+$T cells including higher frequencies of IFN-γ^+ $CD8^+$T cells in PB around year 6 paralleled the decrease in *WT1* mRNA to undetectable levels.

DISCUSSION

In the three AML who have been treated with WT1 peptide vaccine for ⩾8 years, *WT1* mRNA levels that reflect the amount of MRD have remained at undetectable levels, suggesting that the vaccination may have resulted in a cure for these patients. On the basis of *WT1* mRNA levels, MRD before WT1 vaccination was



estimated at $\sim 10^{10}$ cells, which may be equivalent to $\sim 1.0\%$ leukemic blast cells in BM. The presence of such high levels of MRD in these patients suggested that clinical relapse was impending.[8,9] Conventionally, cure-oriented therapy for such patients comprises only allogeneic hematopoietic stem cell transplantation. As shown by these cases, WT1 immunotherapy may be the first choice of treatment for AML patients who have MRD after induction chemotherapy but cannot tolerate allogeneic

Figure 1. Clinical courses and immunomonitoring. (**a**) Case 1. (**b**) Case 2. (**c**) Case 3. Top: WT1 mRNA levels (copies of WT1 mRNA/μg RNA) in BM (white squares) and PB (black squares). Horizontal dotted lines represent upper limits of normal ranges of WT1 mRNA levels in BM. Bottom: Frequencies of WT1 tetramer$^+$ CD8$^+$ T cells in CD8$^+$ T cells in PB (white bars) and frequencies of IFN-γ$^+$ WT1 tetramer$^+$ CD8$^+$ T cells in the WT1 tetramer$^+$ CD8$^+$ T cells (black bars). Numbers represent the percentages of WT1 tetramer$^+$ CD8$^+$ T cells and numbers in parentheses show the percentages of IFN-γ$^+$ WT1 tetramer$^+$ CD8$^+$ T cells in WT1 tetramer$^+$ CD8$^+$ T cells. (**d**) Representative fluorescence-activated cell sorting data is shown for case 1. WT1 peptide (9-mer natural 235–243 peptide)/ HLA-A*2402 tetramer assay was performed as follows: frozen peripheral blood mononuclear cells (PBMCs) were thawed and stained with phycoerythrin (PE)-conjugated WT1 tetramer, subsequently with allophycocyanin (APC)-anti-CD3 antibody, fluorescein isothicynate (FITC)-anti-CD4, -14, -16, -19 and -56 antibodies, and APC-Cy7-anti CD8 antibody, followed by analysis with fluorescence-activated cell sorting instrument. WT1 tetramer-, CD3-, and CD8-positive, and CD4-14-, 16-, 19-, and 56-negative fractions were determined as WT1 tetramer$^+$ CD8$^+$ T cells. To quantify IFN-γ$^+$ WT1 tetramer$^+$ CD8$^+$ T cells, PBMCs were stimulated with WT1 peptide or irrelevant modified extracellular signal-regulated kinase (mERK) peptide, and then WT1 tetramer assay was performed for the PBMCs. Frequencies of IFN-γ$^+$ WT1 tetramer$^+$CD8$^+$ T cells were calculated as (number of IFN-γ$^+$ WT1 tetramer$^+$ CD3$^+$ CD8$^+$ T cells in WT1 peptide-stimulated PBMCs)−(number of IFN-γ$^+$ WT1 tetramer$^+$ CD3$^+$ CD8$^+$ T cells in mERK peptide-stimulated PBMCs)/number of WT1 tetramer$^+$CD3$^+$CD8$^+$ T cells in non-peptide-stimulated PBMCs.

hematopoietic stem cell transplantation. The findings of this study suggest that WT1 peptide vaccination merits consideration as an alternative cure-oriented therapy for AML patients showing hematological CR but also MRD, and are at a high risk of clinical relapse.

Frequency of WT1 tetramer$^+$CD8$^+$T cells in the three AML patients was already elevated before WT1 vaccination compared with that usually observed in HDs (<0.08%). These findings indicate that the immune system of these AML patients responded to the endogenous WT1 protein derived from leukemic cells. IFN-γ$^+$WT1 tetramer$^+$ CD8$^+$ T cells, an activated type of the WT1 tetramer$^+$ CD8$^+$ T cells, were not detected before WT1 vaccination, but appeared after WT1 vaccination in all three patients and remained at clearly detectable levels throughout the vaccination. The appearance and continuing presence of these IFN-γ$^+$WT1 tetramer$^+$ CD8$^+$ T cells are likely contributors to the eradication of MRD, thus resulting in molecular CR. Furthermore, it was shown that, upon stimulation with WT1 peptide, a fraction of WT1 tetramer$^+$ CD8$^+$ T cells expressed a CD107 antigen that appeared on surface of CTLs in direct association with secretion of cytotoxic granules that contained perforin and granzyme[11] (data not shown), strongly indicating the potential cytotoxicity of WT1 tetramer$^+$ CD8$^+$ T cells and the relevance of their frequency analysis during the clinical courses.

In conclusion, long-term WT1 peptide vaccination for ⩾8 years was found to be safe and effective for the eradication of MRD and WT1 immunotherapy merits consideration as a promising cure-oriented therapy for AML patients with MRD who are at high risk of relapse.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Phase I Trial of Wilms' Tumor 1 (WT1) Peptide Vaccine with GM-CSF or CpG in Patients with Solid Malignancy

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Abstract. *Background: The aim of this study was to investigate the safety and efficacy of combinatorial use of granulocyte-macrophage colony-stimulating factor (GM-CSF) and CpG oligodeoxynucleotides (CpG-ODN) as immunoenhancement adjuvants in Wilms' Tumor 1 (WT1) vaccine therapy for patients with solid malignancy. Patients and Methods: The patients were placed into treatment groups as follows: WT1 peptide alone, WT1 peptide with GM-CSF (100 μg) and WT1 peptide with CpG-ODN (100 μg). HLA-A *2402 or *0201/*0206-restricted, WT1 peptide emulsified with Montanide ISA51 was injected intradermally every week for eight weeks. Toxicities were evaluated according to the National Cancer Institute Common Terminology Criteria for Adverse Events ver. 3.0. Tumor size, which was measured by computed tomography, was determined every four weeks. The responses were analyzed according to Response Evaluation Criteria in Solid Tumors. Results: The protocol was well tolerated; only local erythema occurred at the WT1 vaccine injection site. The disease control rate of the groups treated with WT1 peptide alone (n=10), with combinatorial use of GM-CSF (n=8) and with combinatorial use of CpG-ODN (n=10), in the initial two months was 20%, 25% and 60%, respectively. Conclusion: Addition of GM-CSF or CpG-ODN to the WT1 peptide vaccine for patients with solid malignancy was safe and improved the effectiveness of clinical response.*

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Key Words: Wilms' tumor 1 (WT1), granulocyte-macrophage colony-stimulating factor (GM-CSF), CpG oligodeoxynucleotides (CpG-ODN), cancer vaccine, immunotherapy.

Recent advances in tumor immunology have resulted in the identification of a large number of tumor-associated antigens (TAAs) that might be used for cancer immunotherapy, since their epitopes, associated with human leukocyte antigen (HLA) class I molecules, are recognized by cytotoxic T-lymphocytes. One such identified TAA is the product of the Wilms' tumor gene, *WT1* (1, 2).

We performed a phase I clinical trial to examine the safety of a WT1-based vaccine, as well as the clinical and immunological response of patients with a variety of cancer types, including leukemia, lung cancer and breast cancer (3). The WT1 peptide vaccine, emulsified with Montanide ISA51 adjuvant and administered at a dosage of 0.3, 1.0, or 3.0 mg at two-week intervals, was safe for patients, other than those with myelodysplastic syndromes. Furthermore, it has been confirmed that the potential toxicities of the weekly WT1 vaccination treatment schedule (3.0 mg dose) with the same adjuvant agent were also acceptable (4). In the past, clinical response to weekly WT1 peptide-based immunotherapy in phase II trials has been reported for renal cell carcinoma (5), multiple myeloma (6), glioblastoma multiforme (7) and gynecological malignancies (8). In these studies, the activity of WT1 peptide alone was examined and no specific adjuvant, that would activate immune reactions, was included. As a result, the peptide vaccine had limited effectiveness against malignant tumors.

In clinical studies, the identification of predictive factors of treatment is extremely important for the improvement of clinical response. The most representative factor that predicts the outcome of cancer peptide vaccine therapy is the expansion and/or induction of TAA-specific cytotoxic T-lymphocytes (CTLs). Klebanoff *et al.* reported that not only the induction of effector CTLs, but also the maintenance of memory CTLs, are required for ideal antitumor immune response in tumor-bearing patients (9). Moreover, Fujiki *et al.* confirmed that occurrence of an antigen-specific helper T-cell (Th) response predicted good clinical response of CTL epitope

vaccination (10). We have demonstrated that the percentage of dendritic cells (DCs) in peripheral blood may represent a new interesting biological marker predicting therapeutic response in patients treated with WT1 peptide vaccination (11). The main function of DCs is to process antigen material and present it on their surface of other cells (*e.g.* Th and CTLs) of the immune system. In accordance with these results, we focused on the adjuvant agent used to activate antigen-presenting cells (*e.g.* DCs and macrophages), in order to enhance the therapeutic efficacy of cancer peptide vaccination.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine that functions as a white blood cell growth factor. GM-CSF stimulates stem cells to produce granulocytes and monocytes. The various cellular responses (*i.e.* division, maturation and activation) are induced through GM-CSF binding to specific receptors, expressed on the cell surface of target cells (12). GM-CSF increases the cytotoxicity of monocytes towards certain neoplastic cell lines (13).

CpG oligodeoxynucleotides (CpG-ODN) are short, single-stranded, synthetic DNA molecules that contain a cytosine "C" followed by a guanine "G". The "p" refers to the phosphodiester backbone of DNA, however some ODNs have a modified phosphorothioate backbone. When these CpG motifs are unmethlyated, they act as immunostimulants (14). CpG motifs are considered pathogen-associated molecular patterns (PAMPs) due to their abundance in microbial genomes and their rarity in vertebrate genomes (15). The CpG-ODN PAMP is recognized by the pattern recognition receptor toll-like receptor 9 (TLR9).

In the present study, we investigated the safety and efficacy of GM-CSF and CpG-ODN as immunoenhancement adjuvants in WT1 vaccine therapy for patients with solid malignancy

Patients and Methods

Trial protocol. A phase I clinical trial of the WT1 with immunostimulatory adjuvants was designed to evaluate the safety and tumor response. Patients with histologically confirmed solid malignancies were eligible if they exhibited a performance status of the Eastern Cooperative Oncology Group of 0–2 and had measurable disease. Additional inclusion criteria were: (i) age ranging from 16 to 80 years; (ii) overexpression of the WT1 gene in the cancerous tissue as determined by immunohistochemistry; (iii) HLA-A*2402, or A*0201, or A*0206 positivity; (iv) disease refractory to conventional chemotherapy, radiotherapy, and/or hormonal therapy; (v) no history of antitumor therapy within 4 weeks prior to enrolment; (vi) in patients not having primary brain tumor, absence of brain metastases should be confirmed by computed tomography or magnetic resonance imaging; (vii) sufficient organ function and (viii) written informed consent.

Following written informed consent, the patients received injections of 3.0 mg of WT1 peptide emulsified with Montanide ISA51 adjuvant (SEPPIC S.A., Paris, France). The emulsion was injected intradermally into four different regions (bilateral axillary and inguinal region). The WT1 vaccinations were scheduled to be administered weekly, for eight consecutive weeks. The initial group of patients (cohort 1) received WT1 emulsion alone. The subsequent group of patients (cohort 2) received WT1 emulsion with GM-CSF (sargramostim) (Bayer Health Care Pharmaceuticals, LLC, Seattle, WA, USA). GM-CSF was administered subcutaneously as four separate injections of 100 μg in the same region as each vaccine dose. The final group of patients (cohort 3) received WT1 emulsion admixed with 100 μg CpG-ODN (5'-TCGTCGTTTTGTCGTTTTG TCGTT-3') (Hokkaido System Science Co., Ltd, Hokkaido, Japan).

The Independent Safety Monitoring Committee (ISMC) monitored and reviewed the protocol compliance, safety and on-schedule study progress. The protocol was approved by the Institutional Review Board and the Ethical Committee at Tokyo Women's Medical University. The study was registered in the University Hospital Medical Information Network Clinical Trial Registry (UMIN-CTR) Clinical Trial (Unique trial number: UMIN 000002771) on November 11, 2009 (UMIN-CTR URL: http://www.umin.ac.jp/ctr/index.htm).

WT1 peptide. The WT1 peptide was manufactured by NeoMPS, Inc. (San Diego, CA, USA). For patients with HLA-A*2402, modified 9-mer WT1 peptide (amino acids 235-243 CYTWNQMNL) was synthesized, in which Y was substituted for M at amino acid position 2 (the anchor position) of the natural WT1 peptide. This variant induces stronger cytotoxic activity than the natural peptide (16). For patients with HLA-A*0201 or A*0206 an 9-mer WT1 peptide (amino acids 187-195 SLGEQQYSV), which is able to bind to both HLA-A*0201 and A*0206, was synthesized (17). Peptides were stored in dimethyl sulfoxide (DMSO) at –80°C and thawed on the day of injection. A water-in-oil emulsion vaccine was then prepared, consisting of the peptide (aqueous phase) and the adjuvant Montanide (oil phase), by combining equal volumes of the peptide and the adjuvant. All synthesis, production and formulation of the two different kinds of peptides were in accordance with applicable current Good Manufacturing Practices and met the applicable criteria for use in humans.

Immunohistochemical analysis. Positive immunostaining of WT1 protein in the patient's tumor was a mandatory requirement for entry into the trial. A standardized staining protocol was adopted from a preceding trial (18). Briefly, formalin-fixed and paraffin-embedded tissue sections were first autoclaved in order to expose antigenic epitopes and were then stained with polyclonal rabbit anti-WT1 IgG antibodies (C-19, sc-192; Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by a Vectastain abidin-biotin-peroxidase complex (ABC) kit (Vector Laboratories, Burlingame, CA, USA). Staining with a more specific monoclonal antibody, 6F-H2 (Dako, Glostrup, Denmark), was also performed and the results were consistent with those obtained with the polyclonal antibodies.

Evaluation of toxicity. Toxicities were evaluated according to the National Cancer Institute Common Terminology Criteria for Adverse Events ver. 3.0 (19). If an adverse event of grade 2 or 3 continued, further immunization was suspended until the problem was solved. An adverse event of more than grade 4 forced the immediate termination of the immunotherapy.

Evaluation of clinical response. After the WT1 vaccine was administered eight times, the antitumor effect of the treatment was assessed by determining the response of the target lesions on

Table I. *Characteristics of patients treated with WT1 peptide alone.*

No.	Diagnosis	Gender	Age (years)	HLA	Completion of therapy (times and duration of vaccination)	RECIST (at 8 weeks)	Adverse events (>G3)
Peptide 1	Colon cancer	Male	60	A2402	No: poor general condition (4 times)	PD	None
Peptide 2	Colon cancer	Male	71	A0201	No: disease progression (5 times)	PD	None
Peptide 3	Colon cancer	Female	59	A2402	No: disease progression (5 times)	PD	None
Peptide 4	Pancreatic cancer	Female	42	A2402	Yes (21 times: 21 weeks)	PD	None
Peptide 5	Colon cancer	Female	80	A0206	Yes (9 times: 9 weeks)	PD	None
Peptide 6	Pancreatic cancer	Female	45	A0206	No: disease progression (5 times)	PD	None
Peptide 7	Rectal cancer	Male	53	A2402	No: disease progression (8 times)	PD	None
Peptide 8	Lung cancer	Male	46	A2402	Yes (19 times: 30 weeks)	SD	None
Peptide 9	Lung cancer	Female	62	A0206	Yes* (25 times: 34 weeks)	SD	None
Peptide 10	Gastric cancer	Male	76	A0201	No: disease progression (7 times)	PD	None

PD: Progressive disease; SD: stable disease. *Continuous administration.

Table II. *Characteristics of patients treated with WT1 peptide with GM-CSF.*

No.	Diagnosis	Gender	Age (years)	HLA	Completion of therapy (times and duration of vaccination)	RECIST (at 8 weeks)	Adverse events (>G3)
GM-CSF 1	Biliary cancer	Female	63	A2402	Yes (22 times: 22 weeks)	PD	None
GM-CSF 2	Esophageal cancer	Male	68	A2402	Yes (9 times: 9 weeks)	PD	None
GM-CSF 3	Pancreatic cancer	Male	65	A2402	Yes (9 times: 9 weeks)	PD	None
GM-CSF 4	Pancreatic cancer	Male	65	A2402	Yes (23 times: 23 weeks)	SD	None
GM-CSF 5	Colon cancer	Female	61	A0206	No: poor general condition (3 times)	PD	None
GM-CSF 6	Colon cancer	Female	63	A0206	No: poor general condition (2 times)	PD	None
GM-CSF 7	Colon cancer	Male	74	A0201	Yes (9 times: 9 weeks)	PD	None
GM-CSF 8	Ovarian cancer	Female	50	A0201	Yes* (30 times: 33 weeks)	SD	None

PD: Progressive disease; SD: stable disease. *Continuous administration.

computed tomographic images. The tumor size was analyzed according to Response Evaluation Criteria in Solid Tumors (RECIST) (20), with results reported as complete response (CR), partial response (PR), stable disease (SD) or progressive disease (PD). The disease control rate was calculated as the percentage of the number of patients in which there was a CR, PR or SD divided by the total number of patients.

Results

Patients' characteristics. Between January 2010 and November 2010, a total of 28 patients were enrolled in this study. Their clinical characteristics are summarized in Tables I-III. The mean age of the 28 enrolled patients was 55.3 (cohort 1: 59.4, cohort 2: 63.6, cohort 3: 54.3) years. All the patients had been treated with surgery as initial therapy. For recurrent diseases and disease progression after initial therapy, all patients received chemotherapy with or without radiotherapy.

Administration protocol and toxicities. The median number of vaccination was nine (cohort 1: 7.5, cohort 2: 9, cohort 3: 15.5), with a range from 2 to 47 (cohort 1: 4-25, cohort 2: 2-30, cohort 3: 6-47), with four patients still on treatment at the end of September 2011. Nine patients received fewer than nine vaccinations due to disease progression and poor general condition. The patients who had an effective response continued to receive weekly or biweekly vaccinations after the period of the clinical trial, until tumor progression was demonstrated.

All patients developed an injection-site reaction (grade 1 or 2), such as erythema, itching or swelling. Patient CpG 5 (Table III) had multiple colonic liver metastases with hepatic portal infiltration at the time of enrollment in the study. Eight weeks after the initial vaccination, bleeding from esophageal varices, which occurs as a result of portal-systemic shunting, was observed. Endoscopic variceal ligation was performed and hemostasis was promptly achieved. The ISMC review of this adverse event confirmed that the gastrointestinal bleeding was not related to WT1 treatment.

No other toxicities (grade 1-5) were observed. These results indicate that repeated WT1 vaccination with GM-CSF and CpG-ODN is sufficiently tolerable.