B-cell lymphoma): a study of Osaka Lymphoma Study Group. *Virchows Arch.* 2009; **455**; 285–293.
9. Hasselblom S, Hansson U, Sigurdardottir M, Nilsson-Ehle H, Ridell B, Andersson PO. Expression of CD68+ tumor-associated macrophages in patients with diffuse large B-cell lymphoma and its relation to prognosis. *Pathol. Int.* 2008; **58**; 529–532.
10. Mantovani A, Bottazzi B, Colotta F, Sozzani S, Ruco L. The origin and function of tumor-associated macrophages. *Immunol. Today* 1992; **13**; 265–270.
11. Mantovani A, Sica A, Locati M. New vistas on macrophage differentiation and activation. *Eur. J. Immunol.* 2007; **37**; 14–16.
12. A predictive model for aggressive non-Hodgkin's lymphoma The International Non-Hodgkin's Lymphoma Prognostic Factors Project. *N. Engl. J. Med.* 1993; **329**; 987–994.
13. Cheson BD, Horning SJ, Coiffier B *et al.* Report of an international workshop to standardize response criteria for non-Hodgkin's lymphomas NCI Sponsored International Working Group. *J. Clin. Oncol.* 1999; **17**; 1244–1253.
14. Wada N, Ikeda J, Hori Y *et al.* Epstein–Barr virus in diffuse large B-cell lymphoma in immunocompetent patients in Japan is as low as in Western countries. *J. Med. Virol.* 2011; **83**; 317–321.
15. Van Dongen JJ, Langerak AW, Brüggemann M *et al.* Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia* 2003; **17**; 2257–2317.
16. Wada N, Kohara M, Ikeda J *et al.* Diffuse large B-cell lymphoma in the spinal epidural space: a study of the Osaka Lymphoma Study Group. *Pathol. Res. Pract.* 2010; **206**; 439–444.
17. Hans CP, Weisenburger DD, Greiner TC *et al.* Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. *Blood* 2004; **103**; 275–282.
18. Marafioti T, Jones M, Facchetti F *et al.* Phenotype and genotype of interfollicular large B cells, a subpopulation of lymphocytes often with dendritic morphology. *Blood* 2003; **102**; 2868–2876.
19. Bronkhorst IH, Ly LV, Jordanova ES*et al.* Detection of M2 macrophages in uveal melanoma and relation with survival. *Invest. Ophthalmol. Vis. Sci.* 2011; **52**; 643–650.
20. Hasita H, Komohara Y, Okabe H *et al.* Significance of alternatively activated macrophages in patients with intrahepatic cholangiocarcinoma. *Cancer Sci.* 2010; **101**; 1913–1919.
21. Kurahara H, Shinchi H, Mataki Y*et al.* Significance of M2-polarized tumor-associated macrophage in pancreatic cancer. *J. Surg. Res.* 2009; 16 June [Epub ahead of print].
22. Fujiwara Y, Komohara Y, Ikeda T, Takeya M. Corosolic acid inhibits glioblastoma cell proliferation by suppressing the activation of signal transducer and activator of transcription-3 and nuclear factor-kappa B in tumor cells and tumor-associated macrophages. *Cancer Sci.* 2011; **102**; 206–211.
23. Komohara Y, Ohnishi K, Kuratsu J, Takeya M. Possible involvement of the M2 anti-inflammatory macrophage phenotype in growth of human gliomas. *J. Pathol* 2008; **216**; 15–24.
24. Ma J, Liu L, Che G, Yu N, Dai F, You Z. The M1 form of tumour-associated macrophages in non-small cell lung cancer is positively associated with survival time. *BMC Cancer* 2010; **10**; 112. Available at: http://www.biomedcentral.com/1471-2407/10/112.

© 2012 Blackwell Publishing Ltd,

ANTICANCER RESEARCH 32: 2263-2270 (2012)

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

SATOSHI OHNO[1,2], RYUJI OKUYAMA[1], ATSUSHI ARUGA[3],
HARUO SUGIYAMA[4] and MASAKAZU YAMAMOTO[1]

[1]*Department of Surgery, Institute of Gastroenterology, Tokyo Women's Medical University, Tokyo, Japan;*
[2]*Consolidated Research Institute for Advanced Science and Medical Care, Waseda University, Tokyo, Japan;*
[3]*Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Tokyo, Japan;*
[4]*Department of Functional Diagnostic Science, Osaka University, Graduate School of Medicine, Osaka, Japan*

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.*

Correspondence to: Dr. Satoshi Ohno, Consolidated Research Institute for Advanced Science and Medical Care, Waseda University, 513, Wasedatsurumaki-cho, Shinjuku-ku, Tokyo 162-0041, Japan. Tel: +81 352721206 Fax: +81 352721208, e-mail: satoshi.ohno55@gmail.com

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.

Table III. *Characteristics of patients treated with WT1 peptide with CpG.*

No.	Diagnosis	Gender	Age (years)	HLA	Completion of therapy (times and duration of vaccination)	RECIST (at 8 weeks)	Adverse events (>G3)	Causality
CpG 1	Cervical cancer	Female	39	A2402	Yes (9 times: 9 weeks)	PD	None	
CpG 2	Epithelioid sarcoma	Female	54	A2402	Yes* (47 times: 65 weeks)	SD	None	
CpG 3	Rectal cancer	Male	55	A0206	Yes (9 times: 9 weeks)	PD	None	
CpG 4	Pancreatic cancer	Female	67	A2402	Yes (19 times: 19 weeks)	SD	None	
CpG 5	Colon cancer	Female	55	A2402	Yes (9 times: 9 weeks)	PD	Gastrointestinal bleeding	No
CpG 6	Lung cancer	Female	61	A0206	Yes (15 times: 19 weeks)	SD	None	
CpG 7	Lung cancer	Male	71	A2402	No: poor general condition (6 times)	PD	None	
CpG 8	Papilla cancer	Female	54	A2402	Yes (18 times: 21 weeks)	SD	None	
CpG 9	Ovarian cancer	Female	52	A2402	Yes (16 times: 17 weeks)	SD	None	
CpG 10	Pancreatic cancer	Male	35	A2402	Yes* (32 times: 43 weeks)	SD	None	

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

Clinical outcome. Clinical outcome data for all patients categorized by immunoenhancing adjuvants are summarized in Tables I-III. For primary analysis, clinical response was assessed according to the RECIST criteria. The disease control rate of cohort 1, 2 and 3 in the initial two months (the clinical trial period) was 20%, 25% and 60%, respectively.

Discussion

In this study, patients with HLA-A*2402, A-*0201 or A-*0206 were immunized by injecting the WT1 peptide, added with GM-CSF or CpG-ODN, intradermally once every week for eight weeks and evaluated the safety and efficacy. As vaccine-related adverse events, grade 1 and 2 injection-site reactions were observed within 24-72 h. The intensity of the skin reaction was augmented by repeated vaccinations, suggesting the reaction was a delayed-type hypersensitivity reaction towards WT1 peptide. It is reasonable to believe that the skin toxicity of vaccine therapy at the injection sites is due to the natural course of the immune activation. Therefore, the treatment was considered to be well-tolerated.

The potential of the WT1 protein as a cancer antigen is of considerable interest. Many cancer antigens are relatively easy to isolate because of advances in tumor and molecular immunology. Nevertheless, determination of the clinical efficacy of these cancer antigens can be achieved only by clinical studies that are very laborious, and moreover, only clinical studies can determine their potential as cancer antigens. It is therefore a laborious and time-consuming work to determine and confirm the clinical usefulness of a given cancer antigen. Recently, 75 representative cancer antigens including WT1 were prioritized (21). The selection and prioritization of these antigens were performed according to the following criteria: (i) therapeutic function, (ii) immunogenicity, (iii) role of the antigen in oncogenicity, (iv) specificity, (v) expression level and percentage of antigen-positive cells, (vi) stem cell expression, (vii) number of patients with antigen-positive cancer, (viii) number of antigenic epitopes, and (ix) cellular location of antigen expression. Although none of the 75 cancer antigens had all the characteristics of the ideal cancer antigen, WT1 was at the top of the ranking. This finding can be expected to promote the development of WT1-targeted cancer immunotherapy.

The cytokine GM-CSF is involved in the recruitment and maturation of antigen-presenting cells and has been incorporated into numerous clinical studies with cancer vaccines to enhance immune responses (22-24). Previous studies have revealed the safety of therapeutic application using WT1 peptides in Montanide adjuvant with GM-CSF in patients with myeloid malignancy (25-27) and mesothelioma (28). The present study also demonstrated that GM-CSF was safe as adjuvant in patients with various types of cancer. However, the disease control rate in the group of patients treated with the WT1 peptide vaccine with GM-CSF (cohort 2) (25%), was only slightly better than or comparable to that of the group treated with the WT1 peptide alone (cohort 1) (20%).

CpG-ODN can be synthesized for therapeutic use and has been evaluated as a vaccine adjuvant in several clinical studies. CpG-ODN acts as a very potent adjuvant in combination with Montanide, and has been shown to promote strong antigen-specific $CD8^+$ T-cell responses in patients with melanoma (29, 30). In addition, intradermal injections of CpG-ODN around the excision site of melanoma activate the plasmacytoid DCs and myeloid DCs, and reduce the number of regulatory T-cells in sentinel lymph nodes (31, 32). Vaccination with NY-ESO-1 peptide in combination with CpG-ODN was reported to successfully induce NY-ESO-1-specific immune responses and revealed clinical benefit by extending survival in patients with NY-ESO-1-positive cancer (33). As established by the seminal

study of Iwahashi *et al.* (34), immunization with two kinds of squamous cell carcinoma-specific peptides, LY6K-177 and TTK-567, in combination with CpG-ODN, successfully elicited antigen-specific $CD8^+$ T-cell responses in patients with advanced esophageal squamous cell carcinoma. In addition, expression of interferon (IFN)-α and its related chemokines were up-regulated and, correspondingly, natural killer (NK) cells were activated. These results suggest that not only tumor-specific acquired immunity, but also innate immunity were enhanced by this vaccination.

CpG-ODN can stimulate both innate immunity and adoptive immune responses through endosomal TLR9, which is expressed in plasmacytoid DCs in humans. Plasmacytoid DCs produce high levels of type I interferons, as well as a variety of other cytokines and chemokines to promote Th1-like immune responses involving other cell types, including additional DC subsets, monocytes, NK cells, and neutrophils (35-37). Therefore, CpG-ODN is considered to play important roles as an adjuvant for cancer vaccines using epitope peptides.

In our study, we have shown that the disease control rate in the group of patients treated with the WT1 peptide vaccine with CpG-ODN (cohort 3) (60%), was much higher than that of the other groups. Recently, Hong *et al.* (38) revealed that idiotypic vaccine combined with CpG-ODN or IFN-α, but not GM-CSF, not only efficiently protected mice from developing myeloma, but also eradicated the already established myeloma. The therapeutic responses were associated with an induction of strong humoral immune responses, including anti-idiotypic antibodies, and cellular immune responses, including idiotype- and myeloma-specific $CD8^+$ CTLs, $CD4^+$ Th1 cells and memory T-cells in mice receiving idiotypic vaccine combined with CpG or IFN-α. Furthermore, idiotypic vaccine, combined with CpG or IFN-α induced idiotype- and tumor-specific memory immune responses that protected surviving mice from tumor reocenitence. Thus, these results clearly show that CpG is a better immune adjuvant than GM-CSF. However, our study was still a phase I trial, and we will determine whether the immune response to WT1 can be induced by this vaccine protocol in the next phase II study.

For decades, investigators have relied on modified WHO criteria (39) or, more recently, RECIST (20) to assess the clinical activity of anticancer agents. These standard criteria were designed to capture effects of cytotoxic agents and depend on tumor shrinkage to demonstrate activity. However, the response patterns seen with immunotherapeutic agents extend beyond those of cytotoxic agents and can manifest, for example, after a period of stable disease in which there is no tumor shrinkage, or after initial tumor burden, an increase in, or the appearance of new lesions (*e.g.* tumor-infiltrating lymphocytes) (40-43). This potential delayed detection of clinical activity on radiographic assessment may reflect the dynamics of the immune system, the time required for T-cell expansion followed by infiltration of the tumor, and a subsequent measurable antitumor effect. For example, our previous trial (8, 44) and other studies (40-43) of clinical cancer vaccines demonstrated that patients with stable or progressive disease may have subsequent tumor regression, or initial mixed responses, with regression in some lesions, while other lesions remain stable or progress.

Such patterns have been noted by many investigators; however, they were inconsistently included in publications or were not systematically captured because of the absence of suitable response criteria, which, in turn, did not allow for their clinical significance to be adequately studied (45). It has become evident that RECIST and WHO criteria may not offer a complete description of the response to immunotherapeutic agents, and therefore either adjusted or new criteria are needed (45).

Cancer immunotherapy is considered to be the fourth cancer therapy after the three major cancer therapies of surgery, chemotherapy and radiotherapy. It is thought that complete eradication of cancer stem cells is essential for the cure of cancer and that only immunotherapy is capable of killing non-dividing, quiescent cancer stem cells. Therefore, ideal and future immunotherapy should be started as soon as possible after the diagnosis of cancer and continued as long as possible, so that surgery, chemotherapy and radiotherapy can be performed under conditions of enhanced cancer immunity.

In conclusion, the addition of GM-CSF or CpG-ODN to a WT1 peptide vaccine, for patients with solid malignancy, was safe and apparently improved the effectiveness of clinical response.

Acknowledgements

This work was supported by a Grant-in-Aid for Young Scientists (A) (No. 21689044) from the Ministry of Education, Culture, Sports, Science and Technology, of the Japanese Government. We would like to thank T. Umeda and H. Nakajima for their technical assistance and coordination of the clinical research.

References

1 Oka Y, Tsuboi A, Elisseeva OA, Udaka K and Sugiyama H: WT1 as a novel target antigen for cancer immunotherapy. Curr Cancer Drug Targets *2*: 45-54, 2002.

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 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.

4 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.

5 Iiyama T, Udaka K, Takeda S, Takeuchi T, Adachi YC, 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.

6 Tsuboi A, Oka Y, Nakajima H, Fukuda Y, Elisseeva OA, Yoshihara S, Hosen N, Ogata A, Kito K, Fujiki F, Nishida S, Shirakata T, Ohno S, Yasukawa M, Oji Y, Kawakami M, Morita S, Sakamoto J, Udaka K, Kawase I and Sugiyama H: Wilms' tumor gene *WT1* peptide-based immunotherapy induced a minimal response in a patient with advanced therapy-resistant multiple myeloma. Int J Hematol *86*: 414-417, 2007.

7 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.

8 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.

9 Klebanoff CA, Gattinoni L and Restifo NP: $CD8^+$ T-Cell memory in tumor immunology and immunotherapy. Immunol Rev *211*: 214-224, 2006.

10 Fujiki F, Oka Y, Kawakatsu M, Tsuboi A, Tanaka-Harada Y, Hosen N, Nishida S, Shirakata T, Nakajima H, Tatsumi N, Hashimoto N, Taguchi T, Ueda S, Nonomura N, Takeda Y, Ito T, Myoui A, Izumoto S, Maruno M, Yoshimine T, Noguchi S, Okuyama A, Kawase I, Oji Y and Sugiyama H: A clear correlation between WT1-specific Th response and clinical response in WT1 CTL epitope vaccination. Anticancer Res *30*: 2247-2254, 2010.

11 Ohno S, Takano F, Ohta Y, Kyo S, Myojo S, Dohi S, Sugiyama H, Ohta T and Inoue M: Frequency of myeloid dendritic cells can predict the efficacy of Wilms' tumor 1 peptide vaccination. Anticancer Res *31*: 2447-2452, 2011.

12 Park LS, Friend D, Gillis S and Urdal DL: Characterization of the cell surface receptor for human granulocyte/macrophage colony-stimulating factor. J Exp Med *164*: 251-262, 1986.

13 Grabstein KH, Urdal DL, Tushinski RJ, Mochizuki DY, Price VL, Cantrell MA, Gillis S and Conlon PJ: Induction of macrophage tumoricidal activity by granulocyte-macrophage colony-stimulating factor. Science *232*: 506-508, 1986.

14 Weiner GJ, Liu HM, Wooldridge JE, Dahle CE and Krieg AM: Immunostimulatory oligodeoxynucleotides containing the CpG motif are effective as immune adjuvants in tumor antigen immunization. Proc Natl Acad Sci USA *94*: 10833-10837, 1997.

15 Bauer S and Wagner H: Bacterial CpG-DNA licenses TLR9. Curr Top Microbiol Immunol *270*: 145-154, 2002.

16 Tsuboi A, Oka Y, Udaka K, Murakami M, Masuda T, Nakano A, Nakajima H, Yasukawa M, Hiraki A, Oji Y, Kawakami M, Hosen N, Fujioka T, Wu F, Taniguchi Y, Nishida S, Asada M, Ogawa H, Kawase I and Sugiyama H: 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 *51*: 614-620, 2002.

17 Li Z, Oka Y, Tsuboi A, Fujiki F, Harada Y, Nakajima H, Masuda T, Fukuda Y, Kawakatsu M, Morimoto S, Katagiri T, Tatsumi N, Hosen N, Shirakata T, Nishida S, Kawakami Y, Udaka K, Kawase I, Oji Y and Sugiyama H: Identification of a WT1 protein-derived peptide, WT1, as a HLA-A 0206-restricted, WT1-specific CTL epitope. Microbiol Immunol *52*: 551-558, 2008.

18 Nakatsuka S, Oji Y, Horiuchi T, Kanda T, Kitagawa M, Takeuchi T, Kawano K, Kuwae Y, Yamauchi A, Okumura M, Kitamura Y, Oka Y, Kawase I, Sugiyama H and Aozasa K: Immunohistochemical detection of WT1 protein in a variety of cancer cells. Mod Pathol *19*: 804-814, 2006.

19 the National Cancer Institute Common Terminology Criteria for Adverse Events ver. 3.0: http://ctep.cancer.gov/protocol Development/electronic_applications/docs/ctcaev3.pdf

20 Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom AT, Christian MC and Gwyther SG: New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst *92*: 205-216, 2000.

21 Cheever MA, Allison JP, Ferris AS, Finn OJ, Hastings BM, Hecht TT, Mellman I, Prindiville SA, Viner JL, Weiner LM and Matrisian LM: The prioritization of cancer antigens: a national cancer institute pilot project for the acceleration of translational research. Clin Cancer Res *15*: 5323-5337, 2009.

22 Borrello I and Pardoll D: GM-CSF-based cellular vaccines: a review of the clinical experience. Cytokine Growth Factor Rev *13*: 185-193, 2002.

23 Villinger F: Cytokines as clinical adjuvants: How far are we? Expert Rev Vaccines *2*: 317-326, 2003.

24 Chang DZ, Lomazow W, Joy Somberg C, Stan R and Perales MA: Granulocyte-macrophage colony stimulating factor: an adjuvant for cancer vaccines. Hematology *9*: 207-215, 2004.

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

26 Keilholz U, Letsch A, Busse A, Asemissen AM, Bauer S, Blau IW, Hofmann WK, Uharek L, Thiel E and Scheibenbogen 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.

27 Rezvani K, Yong AS, Mielke S, Jafarpour B, Savani BN, Le RQ, Eniafe R, Musse L, Boss C, Kurlander R and Barrett AJ: Repeated PR1 and WT1 peptide vaccination in Montanide-adjuvant fails to induce sustained high-avidity, epitope-specific $CD8^+$ T-cells in myeloid malignancies. Haematologica *96*: 432-440, 2011.

28 Krug LM, Dao T, Brown AB, Maslak P, Travis W, Bekele S, Korontsvit T, Zakhaleva V, Wolchok J, Yuan J, Li H, Tyson L and Scheinberg DA: WT1 peptide vaccinations induce CD4 and CD8 T-cell immune responses in patients with mesothelioma and non-small cell lung cancer. Cancer Immunol Immunother *59*: 1467-1479, 2010.

29 Speiser DE, Liénard D, Rufer N, Rubio-Godoy V, Rimoldi D, Lejeune F, Krieg AM, Cerottini JC and Romero P: Rapid and strong human CD8+ T-cell responses to vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909. J Clin Invest *115*: 739-746, 2005.

30 Valmori D, Souleimanian NE, Tosello V, Bhardwaj N, Adams S, O'Neill D, Pavlick A, Escalon JB, Cruz CM, Angiulli A, Angiulli F, Mears G, Vogel SM, Pan L, Jungbluth AA, Hoffmann EW, Venhaus R, Ritter G, Old LJ and Ayyoub M: Vaccination with NY-ESO-1 protein and CpG in Montanide induces integrated antibody/Th1 responses and CD8 T-cells through cross-priming. Proc Natl Acad Sci USA *104*: 8947-8952, 2007.

31 Molenkamp BG, Sluijter BJ, van Leeuwen PA, Santegoets SJ, Meijer S, Wijnands PG, Haanen JB, van den Eertwegh AJ, Scheper RJ and de Gruijl TD: Local administration of PF-3512676 CpG-B instigates tumor-specific CD8+ T-cell reactivity in melanoma patients. Clin Cancer Res *14*: 4532-4542, 2008.

32 Molenkamp BG, van Leeuwen PA, Meijer S, Sluijter BJ, Wijnands PG, Baars A, van den Eertwegh AJ, Scheper RJ and de Gruijl TD: Intradermal CpG-B activates both plasmacytoid and myeloid dendritic cells in the sentinel lymph node of melanoma patients. Clin Cancer Res *13*: 2961-2969, 2007.

33 Karbach J, Gnjatic S, Bender A, Neumann A, Weidmann E, Yuan J, Ferrara CA, Hoffmann E, Old LJ, Altorki NK and Jäger E: Tumor-reactive CD8+ T-cell responses after vaccination with NY-ESO-1 peptide, CpG 7909 and Montanide ISA-51: association with survival. Int J Cancer *126*: 909-918, 2010.

34 Iwahashi M, Katsuda M, Nakamori M, Nakamura M, Naka T, Ojima T, Iida T and Yamaue H: Vaccination with peptides derived from cancer-testis antigens in combination with CpG-7909 elicits strong specific CD8+ T-cell response in patients with metastatic esophageal squamous cell carcinoma. Cancer Sci *101*: 2510-2517, 2010.

35 Krieg AM: CpG motifs in bacterial DNA and their immune effects. Annu Rev Immunol *20*: 709-760, 2002.

36 Ballas ZK, Krieg AM, Warren T, Rasmussen W, Davis HL, Waldschmidt M and Weiner GJ: Divergent therapeutic and immunologic effects of oligodeoxynucleotides with distinct CpG motifs. J Immunol *167*: 4878-4886, 2001.

37 Weber JS, Zarour H, Redman B, Trefzer U, O'Day S, van den Eertwegh AJ, Marshall E and Wagner S: Randomized phase 2/3 trial of CpG oligodeoxynucleotide PF-3512676 alone or with dacarbazine for patients with unresectable stage III and IV melanoma. Cancer *115*: 3944-3954, 2009.

38 Hong S, Qian J, Li H, Yang J, Lu Y, Zheng Y and Yi Q: CpG or IFN-α are more potent adjuvants than GM-CSF to promote anti-tumor immunity following idiotype vaccine in multiple myeloma. Cancer Immunol Immunother *61*: 561-571, 2012.

39 WHO Handbook for Reporting Results of Cancer Treatment. Geneva, Switzerland: World Health Organization Offset Publication No. 48, 1979.

40 Berd D, Sato T, Cohn H, Maguire HC Jr and Mastrangelo MJ: Treatment of metastatic melanoma with autologous, hapten-modified melanoma vaccine: regression of pulmonary metastases. Int J Cancer *94*: 531-539, 2001.

41 Kruit WH, van Ojik HH, Brichard VG, Escudier B, Dorval T, Dréno B, Patel P, van Baren N, Avril MF, Piperno S, Khammari A, Stas M, Ritter G, Lethé B, Godelaine D, Brasseur F, Zhang Y, van der Bruggen P, Boon T, Eggermont AM and Marchand M: Phase 1/2 study of subcutaneous and intradermal immunization with a recombinant MAGE-3 protein in patients with detectable metastatic melanoma. Int J Cancer *117*: 596-604, 2005.

42 van Baren N, Bonnet MC, Dréno B, Khammari A, Dorval T, Piperno-Neumann S, Liénard D, Speiser D, Marchand M, Brichard VG, Escudier B, Négrier S, Dietrich PY, Maraninchi D, Osanto S, Meyer RG, Ritter G, Moingeon P, Tartaglia J, van der Bruggen P, Coulie PG and Boon T: Tumoral and immunologic response after vaccination of melanoma patients with an ALVAC virus encoding MAGE antigens recognized by T-cells. J Clin Oncol *23*: 9008-9021, 2005.

43 Hodi FS, Butler M, Oble DA, Seiden MV, Haluska FG, Kruse A, Macrae S, Nelson M, Canning C, Lowy I, Korman A, Lautz D, Russell S, Jaklitsch MT, Ramaiya N, Chen TC, Neuberg D, Allison JP, Mihm MC and Dranoff G: Immunologic and clinical effects of antibody blockade of cytotoxic T-lymphocyte-associated antigen 4 in previously vaccinated cancer patients. Proc Natl Acad Sci USA *105*: 3005-3010, 2008.

44 Dohi S, Ohno S, Ohno Y, Takakura M, Kyo S, Soma G, Sugiyama H and Inoue M: WT1 peptide vaccine stabilized intractable ovarian cancer patient for one year: a case report. Anticancer Res *31*: 2441-2445, 2011.

45 Hoos A, Parmiani G, Hege K, Sznol M, Loibner H, Eggermont A, Urba W, Blumenstein B, Sacks N, Keilholz U and Nichol G; Cancer Vaccine Clinical Trial Working Group: A clinical development paradigm for cancer vaccines and related biologics. J Immunother *30*: 1-15, 2007.

Received April 4, 2012
Revised May 12, 2012
Accepted May 14, 2012

6. Pasqualotto AC, Antunes AG, Severo LC. Candida guilliermondii as the aetiology of candidosis. *Rev Inst Med Trop* Sao Paulo, 2006,48 :123-127.

doi:10.1684/ejd.2011.1613

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 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. ■

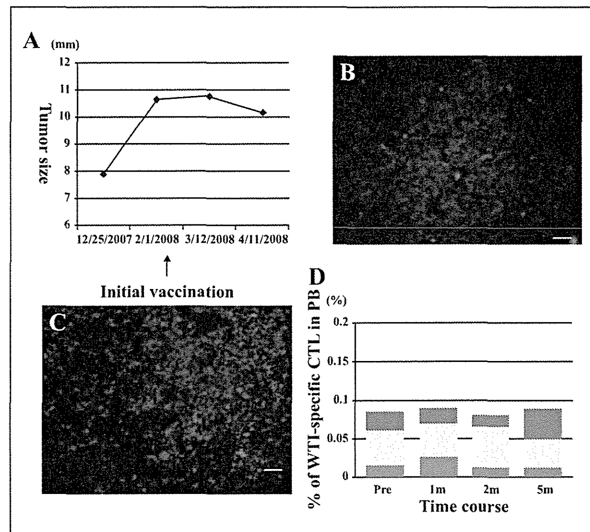

Figure 1. A) A graphical representation of the change in size of the target lesion in the lung. The tumor size was calculated by using computed tomography images, and the treatment response was evaluated according to the RECIST guidelines. The fluorescent immunostaining of the lung metastatic lesions 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).

Disclosure. *Acknowledgement: We thank Ms. Kana Hasegawa, Kazuko Takahashi and Tomoe Umeda for their excellent technical support and Masayoshi Inoue for surgical resection of the lung metastatic lesion. Financial*

support: This work was supported in part by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan and a grant from the Ministry of Health, Labor and Welfare. Conflict of Interest: none.

[1] Department of Dermatology,
[2] Department of functional Diagnostic Science,
[3] Department of Cancer Immunotherapy,
[4] Department of Cancer Stem Cell Biology
[5] Department of Respiratory Medicine, Allergy and Rheumatic Diseases,
Osaka University Graduate School of Medicine,
2-2, Yamadaoka Suita-shi,
Osaka 565-0871,
Japan
[6] Professor Emeritus of Hokkaido University,
Japan
[a]These authors contributed equally to this work.
<tanemura@derma.med.osaka-u.ac.jp>

Megumi NISHIOKA[1,a]
Atsushi TANEMURA[1,a]
Sumiyuki NISHIDA[3,a]
Akiko NAKANO[2]
Akihiro TSUBOI[3]
Yusuke OJI[4]
Yoshihiro OKA[5]
Ichiro AZUMA[6]
Haruo SUGIYAMA[2]
Ichiro KATAYAMA[1]

1. Nakatsuka S, Oji Y, Horiuchi T, *et al.* Immunohistochemical detection of WT1 protein in a variety of cancer cells. *Mod Pathol* 2006; 19:804-814.
2. Oka Y, Tsuboi A, Fujiki F, *et al.* "Cancer antigen WT1 protein-derived peptide"-based treatment of cancer -toward the further development. *Curr Med Chem* 2008; 15:3052-3061.
3. Tsuji S, Matsumoto M, Takeuchi O, *et al.* Maturation of human dendritic cells by cell wall skeleton of Mycobacterium bovis bacillus Calmette-Guérin: involvement of toll-like receptors. *Infect Immun* 2000; 68:6883-6890.
4. Nakajima H, Kawasaki K, Oka Y, *et al.* WT1 peptide vaccination combined with BCG-CWS is more efficient for tumor eradication than WT1 peptide vaccination alone. *Cancer Immunol Immunother* 2004; 53:617-624.
5. 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 ;85:426-429.

doi:10.1684/ejd.2011.1619

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)

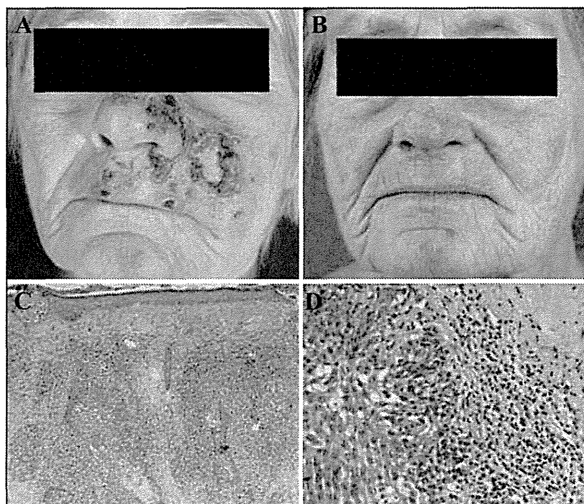

Figure 1. A) At presentation, densely infiltrated plaques with crusts and ulcerations were present on the central and left face, giving the patient a facies leonina-like appearance. **B)** 10 months after initiation of combined therapy with oral bexarotene and PUVA, lesions had cleared almost completely. **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.

ANTICANCER RESEARCH 32: 5201-5210 (2012)

Recognition of a Natural WT1 Epitope by a Modified WT1 Peptide-specific T-Cell Receptor

TAICHI TAMANAKA[1], YOSHIHIRO OKA[1,6], FUMIHIRO FUJIKI[2], AKIHIRO TSUBOI[3], AKIKO KATSUHARA[4], HIROKO NAKAJIMA[2], NAOKI HOSEN[4], SUMIYUKI NISHIDA[3], YU-HUNG LIN[1], SHO TACHINO[1], YOSHIKI AKATSUKA[7], KIYOTAKA KUZUSHIMA[8], YUSUKE OJI[5], ATSUSHI KUMANOGOH[1,6] and HARUO SUGIYAMA[4]

Departments of [1]Respiratory Medicine, Allergy and Rheumatic Disease, [2]Cancer Immunology, [3]Cancer Immunotherapy, [4]Functional Diagnostic Science, [5]Cancer Stem Cell Biology, Osaka University Graduate School of Medicine, Osaka, Japan; [6]Department of Immunopathology, WPI Immunology Frontier Research Center, Osaka University, Osaka, Japan; [7]Department of Hematology and Oncology, School of Medicine, Fujita Health University, Toyoake, Japan; [8]Division of Immunology, Aichi Cancer Center Research Institute, Nagoya, Japan

Abstract. *Wilms' tumor gene WT1 is highly expressed in leukemia and in various types of solid tumors and exerts an oncogenic function. Thus, WT1 protein is a most promising tumor-associated antigen. We have been successfully performing WT1 vaccination with a 9-mer modified $WT1_{235}$ peptide, which has one amino acid substitution (M→Y) at position 2 of 9-mer natural $WT1_{235}$ peptide (235-243 a.a.), for close to 700 HLA-A*24:02-positive patients with leukemia or solid tumors. Although vaccination of modified $WT1_{235}$ peptide induced natural $WT1_{235}$ peptide-recognizing cytotoxic T-lymphocytes (CTLs) and exerted cytotoxic activity towards leukemia and solid tumor cells that expressed the natural $WT1_{235}$ peptide (epitope) but not the vaccinated modified $WT1_{235}$ peptide (epitope), the molecular basis has remained unclear. In this study, we established a modified $WT1_{235}$ peptide-specific CTL clone, we isolated T-cell receptor (TCR) genes from it and transduced the TCR genes into $CD8^+$ T-cells. The TCR-transduced $CD8^+$ T-cells produced interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα) in response to stimulation not only with the modified $WT1_{235}$ peptide but also with the natural $WT1_{235}$ peptide and lysed modified or natural $WT1_{235}$ peptide-pulsed target cells and endogenously WT1-expressing leukemia cells in a HLA-A*24:02-restriction manner. These results provided us, for the first time at molecular basis, with a proof-of-concept of modified $WT1_{235}$ peptide-based immunotherapy for natural $WT1_{235}$ peptide-expressing malignancies.*

Correspondence to: Professor Haruo Sugiyama, MD, Ph.D., Department of Functional Diagnostic Science, Osaka University Graduate School of Medicine, 1-7, Yamada-Oka, Suita City, Osaka 565-0871, Japan. Tel/Fax: +81 668792593, e-mail: sugiyama@sahs.med.osaka-u.ac.jp

Key Words: Wilms' tumor gene (*WT1*), cytotoxic T-lymphocytes (CTLs), peptide vaccine, cancer immunotherapy.

It is evident that T-cell-mediated immunity plays a crucial role in tumor regression and eradication, and the main effector cells that attack tumor cells are $CD8^+$ cytotoxic T-lymphocytes (CTLs) (1, 2). These CTLs recognize tumor-associated antigen (TAA)-derived peptides presented on the surface of target cells in association with major histocompatibility complex (MHC) class I molecules. To enhance the activity of the TAA-specific CTLs, various types of immunotherapies, including cancer vaccines, are being performed (3, 4).

WT1, which was originally identified as a gene responsible for the pediatric neoplasm Wilms' tumor, encodes a zinc finger transcription factor involved in the regulation of cell proliferation and differentiation (5-8). Although the *WT1* gene was first categorized as a tumor suppressor gene, we showed that it had an oncogenic function and the WT1 protein was highly expressed in various kinds of malignant neoplasms, including hematopoietic malignancies and solid tumors, indicating that the WT1 protein is a most promising TAA (9-21).

Our group and others have identified WT1 protein-derived CTL epitope peptides with the restriction of several HLA class I types. Clinical trials using WT1 CTL epitopes, including HLA-A*0201-restricted $WT1_{126}$ and HLA-A*24:02-restricted $WT1_{235}$ peptides, were performed and showed successful results with clinical response (22, 23). However, we identified a modified $WT1_{235}$ peptide with much higher affinity for HLA-A*24:02 than the natural $WT1_{235}$ peptide. The modified $WT1_{235}$ peptide was found to have the ability to elicit robust induction of the peptide-

specific CTLs that also recognized the natural $WT1_{235}$ peptide (epitope) presented on the tumor cell surface (24). In fact, vaccination of the modified $WT1_{235}$ peptide, which was mainly conducted by our group, showed favorable clinical response, including tumor shrinkage and leukemia cell reduction, in association with immunological response, such as an increase in the frequency of natural $WT1_{235}$ peptide-specific $CD8^+$ T-cells in the peripheral blood (PB) of patients with various kinds of malignancies (3, 25-36). However, why the vaccination of modified $WT1_{235}$ peptide exerted clinical effect and killed tumor cells that expressed the natural $WT1_{235}$ peptide (epitope) but not the modified $WT1_{235}$ peptide (epitope) has not yet been explained on a molecular basis.

In the present study, we describe the establishment of a modified $WT1_{235}$ peptide-specific CTL clone, the isolation of the T-cell receptor (TCR) genes from it, and the molecular basis of clinical findings that the vaccination of modified $WT1_{235}$ peptides is effective for eradication of natural $WT1_{235}$ peptide (epitope)-expressing tumor cells.

Materials and Methods

Cells. Peripheral blood mononuclear cells (PBMCs) were obtained from a healthy donor with HLA-A*24:02 by density gradient using a lymphocyte separation solution (Nacalai Tesque, Kyoto, Japan), and $CD8^+$ T-cells were isolated from the PBMCs using the Human CD8 T-Lymphocyte Enrichment Set-DM (BD Biosicences, San Jose, CA, USA).

K562 is a cell line derived from a blast crisis of chronic myeloid leukemia (CML). K562 endogenously expresses *WT1*, but does not express HLA molecules on the cell surface. K562/24:02 is an HLA-A*24:02-expressing K562 cell line, which was generated by the transduction of HLA-A*24:02 cDNA into K562 (37). T2 is a cell line deficient in transporter-associated with antigen processing (TAP) protein that is essential for the transportation and presentation of peptides generated from endogenous proteins. T2/24:02 was made by the transduction of HLA-A*24:02 cDNA into T2 cells (38). K562, K562/A24:02, and T2/A24:02 cells were cultured in RPMI-1640 (Nacalai Tesque), supplemented with 10% fetal bovine serum (FBS; EuroClone, Pero, Italy).

Induction of the modified $WT1_{235}$ peptide-specific $CD8^+$ T-cell clones. Modified $WT1_{235}$ peptide (CYTWNQMNL)-specific $CD8^+$ T-cell clones were generated by a mixed lymphocyte peptide culture (MLPC) in a modification of the method described by Karanikas *et al.* (39). PBMCs from an HLA-A*24:02$^+$ healthy donor were cultured in X-VIVO 15 medium (Lonza, Walkersville, MD, USA), supplemented with 10% human AB type serum (GEMINI Bio-Products, West Sacramento, CA, USA) in the presence of the modified $WT1_{235}$ peptide (1 μg/ml) and recombinant interleukin-2 (IL-2) (40 U/ml, kindly donated by Shionogi & Co., Ltd., Osaka, Japan) in a 96-well U-bottom plate at a density of 2×10^5 cells/well so that cell expansion occurred in fewer than 10 wells among 96 wells (39).

After two weeks of culture, the expanded cloned cells were screened for positivity for the phycoerythrin (PE)-conjugated modified $WT1_{235}$ peptide tetramer (MBL, Nagoya, Japan) and positive clones were confirmed for the peptide specificity by peptide-specific interferon-γ (IFNγ) production.

Cloning of TCR cDNA and construction of a lentivirus vector. cDNA was obtained by reverse-transcription of total mRNA of the modified $WT1_{235}$ peptide-specific $CD8^+$ T-cell clone B10. cDNAs of TCR-α and -β chains were cloned, amplified by 5'RACE PCR using SMARTer™ RACE cDNA Amplification Kit (Clontech Laboratories, Inc., Mountain View, CA, USA) with gene-specific primers of *TRAC* (CTGTCTTACAATCTTGCAGATC) for TCR-α chain, and *TRBC1* (CACTTCCAGGGCTGCCTTC) and *TRBC2* (TGACCTGGGATGGTTTTGGAGCTA) for TCR-β chain, and sequenced.

To construct a vector that simultaneously expressed both the TCR-α and -β chains, cDNAs of the TCR-α and -β chains were linked *via* a viral P2A sequence (40), followed by cloning into a lentiviral SIN vector (CSII-EF-MCS-IRES2-Venus), with the Venus gene that expressed yellow fluorescent proteins (YFPs) (41).

Transduction of TCR construct into $CD8^+$ T-cells. HEK293T packaging cells were transfected with the TCR construct vector, pCAG-HIVgp and pCMV-VSV-G-RSV-Rev using linear polyethyleneimines (Polysciences, Inc., Warrington, UK) in low-serum media (Gibco, Grand Island, NY, USA). The original CSII-EF-MCS-IRES2-Venus mock vector (mock vector) was used as a negative control. After 12 h of incubation, the HEK293T cells were cultured for virus production in DMEM, containing 4.5 g/l glucose (Nacalai Tesque) supplemented with 10% FBS for 48 h. The virus particles were concentrated by precipitating the culture supernatant using polyethylene glycol (SBI, Mountain View, CA, USA).

The TCR genes were transduced into $CD8^+$ T-cells. In brief, $CD8^+$ T-cells were isolated from PBMCs of an HLA-A*24:02$^+$ healthy donor and activated in X-VIVO 15 medium containing a monoclonal antibody (mAb) against CD28 (eBiosicence Inc., San Diego, CA, USA) and 10% human AB type serum in a CD3 mAb (eBiosicence Inc.)-coated culture plate. After 3 days of activation, the cells were infected with the TCR-containing lentivirus vector using 8 μg/ml of polybrene in RetroNectin (TaKaRa, Tokyo, Japan)-coated plate for 12 h, washed, and cultured in X-VIVO 15 medium, supplemented with 10% human AB type serum.

Flow cytometric analysis. For multicolor staining of cells with tetramer and mAbs, the cells were suspended in phosphate-buffered saline (PBS) containing 2% FBS, followed by staining with the PE-conjugated natural or modified WT_{235} tetramer according to the manufacturer's protocol. The cells were then stained with mAbs on ice for 20 min, washed twice with PBS, containing 2% of FBS, and analyzed with a FACSAria instrument (BD Biosciences). mAbs used were Pacific Blue-conjugated anti-CD3 (BD BioScience), allophycocyanin (APC)-conjugated anti-CD8 (BD BioScience), and PE-conjugated anti-Vβ1 (TRBV9 in another family nomenclature) mAbs (Beckman Coulter Inc., Bera, CA, USA).

Cytokine production assay. For cytokine production assay, 2.5×10^4 of responder cells were stimulated by the appropriate stimulator cells pulsed with 10 μg/ml of a natural $WT1_{235}$ peptide (CMTWNQMNL), the modified $WT1_{235}$ peptide (CYTWNQMNL), or an irrelevant CMV pp65 peptide (QYDPVAALF) in culture medium containing anti-CD28/49d (BD Bioscience) and 10 μg/ml

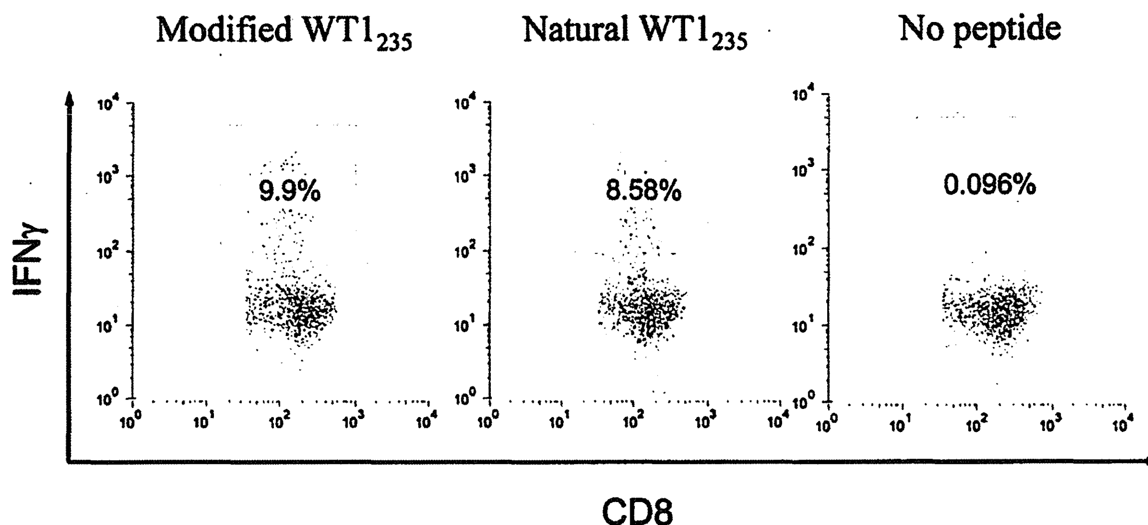

Figure 1. *Establishment of a modified WT1$_{235}$ peptide-specific CD8$^+$ T-cell clone, B10. B10 cells were stimulated by the modified WT1$_{235}$ peptide, the natural WT1$_{235}$ peptide, or not stimulated. Flow cytometory of interferon-γ (IFNγ) production by B10 cells is shown.*

Brefeldin A for 5 h. After the stimulation, the responder cells were stained with APC-Cy7-conjugated anti-CD8 mAb, washed twice, fixed, and permeabilized with Cytofix/Cytoperm (BD Bioscience). The cells were then stained by a PE-conjugated anti-IFNγ and APC-conjugated anti-TNFa mAbs (BD Bioscience), and analyzed using a FACSAria instrument.

For HLA blocking assay, an appropriately titrated blocking mAb for HLA class I (clone wb/32) or HLA-DR (clone L243) was added to cell culture for cytokine production assay.

Cytotoxicity assay. Target cells for cytotoxicity assay were labeled with ^{51}Cr in X-VIVO 15 medium, supplemented with 1% human AB type serum for 2 h, and washed with PBS. The target cells were incubated with appropriate concentrations of antigen peptides, if needed. TCR-transfected CD8+ T-cells were co-cultured with the ^{51}Cr-labeled target cells in X-VIVO 15 medium supplemented with 1% human AB type serum for 4 h. The supernatant was collected, and the radioactivity was counted using a MicroBeta2 plate counter. The percentage-specific lysis was calculated by the equation: (cpm experimental release – cpm spontaneous release)/(cpm maximum release – cpm spontaneous release).

Results

Establishment of a modified WT1$_{235}$ peptide-specific CD8$^+$ T-cell clone. PBMCs of an HLA-A*24:02$^+$ healthy donor were stimulated with modified WT1$_{235}$ peptide seeded at concentrations of 2×10^5 cells/well in a 96-well plate and then cultured in the presence of modified WT1$_{235}$ peptide (1 μg/ml) and IL-2 (40 IU/ml) for two weeks. Cell expansion was observed in only two of a total of 192 wells and finally only one clone, designated B10, was established. B10 cloned cells were positive for staining with HLA-A*24:02/modified WT1$_{235}$ tetramer and produced IFNγ on stimulation with not only modified WT1$_{235}$ but also natural WT1$_{235}$ peptides (Figure 1). These results show that B10 was a modified WT1$_{235}$ and natural WT1$_{235}$ peptide-specific CD8$^+$ T-cell clone.

Isolation of the TCRs from B10 and establishment of the TCR-transfected CD8$^+$ T-cells. cDNA of TCR-α and -β chains was made from mRNA of the B10 cells using each gene-specific primer, cloned, and sequenced. V- and J- regions of Vα were TRAV27*01 and TRAJ28*01, respectively, while V-, D-, and J-regions of Vβ were TRBV9*01, TRBD2*01, and TRBJ2-3*01, respectively. The TCRs isolated from B10 cells are referred to as B10-TCRs in the following text.

Next, the TCR-α and -β chain genes were linked *via* a viral P2A sequence for dual gene expression (40) and inserted into a lentiviral vector for transfection. Activated CD8$^+$ T-cells were transfected with a B10-TCR-containing lentiviral vector, stimulated by irradiated autologous PBMCs loaded with modified the WT1$_{235}$ peptide three days after transfection, cultured for two weeks, and stained with mAbs to CD3, CD8 and either of the anti-Vβ1 family mAb and the modified WT1$_{235}$-tetramer (Figure 2).

A considerable proportion (18.3%, 9.6/(9.6+42.9)) of YFP-positive cells in B-10-TCR-transfected CD8$^+$ T-cells were positive for staining with mAb to Vβ1 (=TRBV9), whereas 4.3% (2.9/(2.9+65.3)) of YFP-positive cells in mock-transfected CD8$^+$ T-cells were positive for staining with the mAb against Vβ1 mAb (Figure 2a). On the other hand, 4.1% (4.1/(4.1+95.9)) of the untransfected CD8$^+$ T-cells were stained with mAb to Vβ1, which suggested

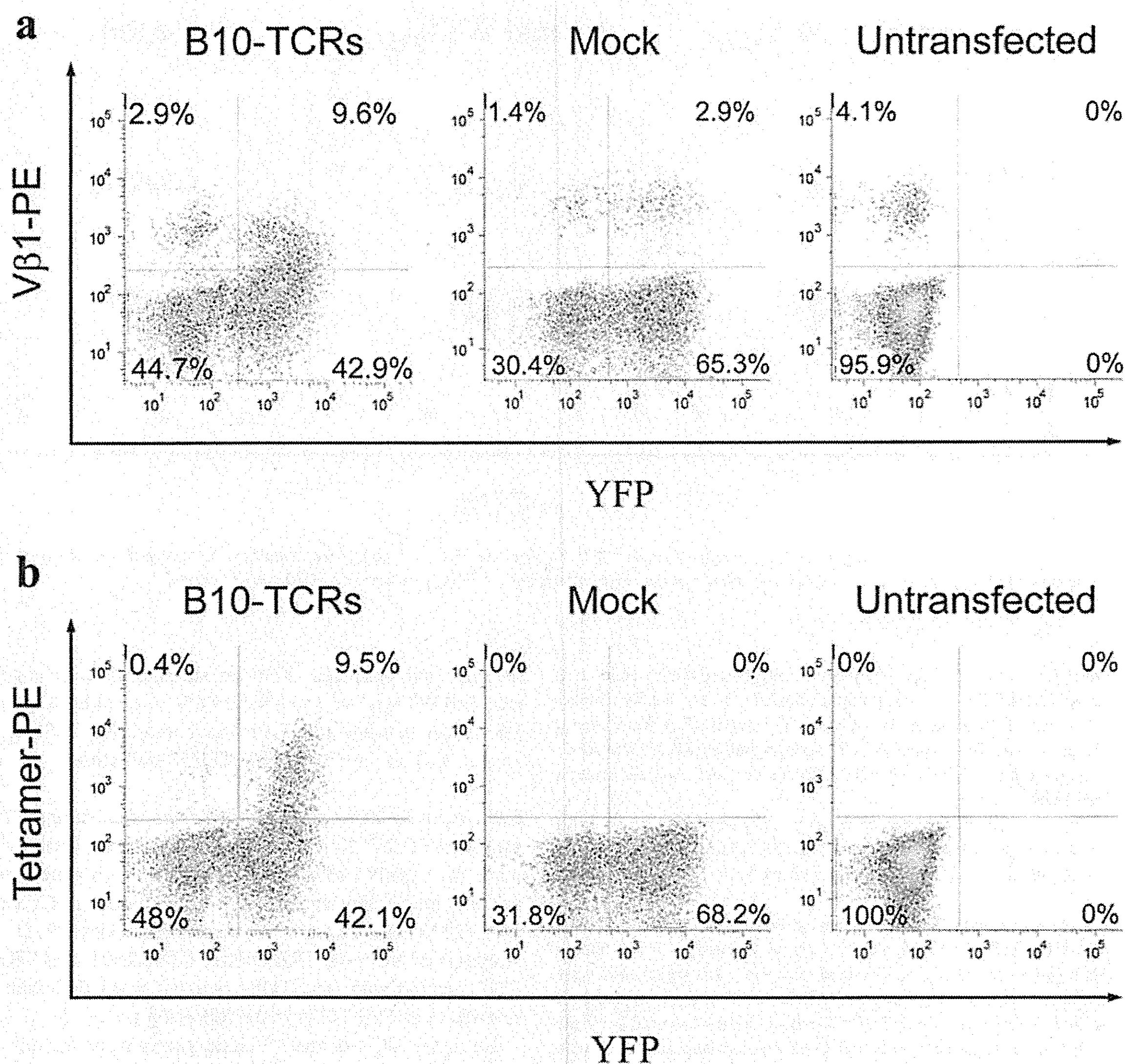

Figure 2. *Functional expression of B10-TCR genes in CD8⁺ T-cells. Activated CD8⁺ T-cells were transfected with B10- TCR-containing a lentivirus vector or a mock vector, and then stained with a monoclonal antibody to Vβ1 family (a) or modified $WT1_{235}$-tetramer (b). Representative data of three experiments are shown.*

endogenous expression of Vβ1 and/or artificial staining with mAb to Vβ1. Furthermore, importantly, modified $WT1_{235}$ tetramer-positive cells were detected only in B10-TCR-transfected $CD8^+$ T-cells at frequencies of 18.4% (9.5/(9.5+42.1)) in YFP-positive cells (Figure 2b). These results indicate that the TCRs from B10 were successfully transduced into $CD8^+$ T-cells and were functional.

To assess the function of B10-TCRs, the antigen-specific cytokine production from the $CD8^+$ T-cells transfected with B10-TCRs was examined (Figure 3a and b). B10-TCR-transfected $CD8^+$ T-cells were stimulated by irradiated autologous PBMCs loaded with the modified $WT1_{235}$ peptide for two weeks and then stimulated again with modified, natural $WT1_{235}$ peptide, or irrelevant CMV pp65 peptide for 5 h and examined for production of IFNγ and TNFα. Cells stimulated with the modified or natural $WT1_{235}$ peptide produced IFNγ and TNFα, whereas cells stimulated with the irrelevant peptide (CMV pp65) did not.

Next, HLA class I restriction of B10-TCR-transfected $CD8^+$ T-cells was examined (Figure 3c). The B10-TCR-

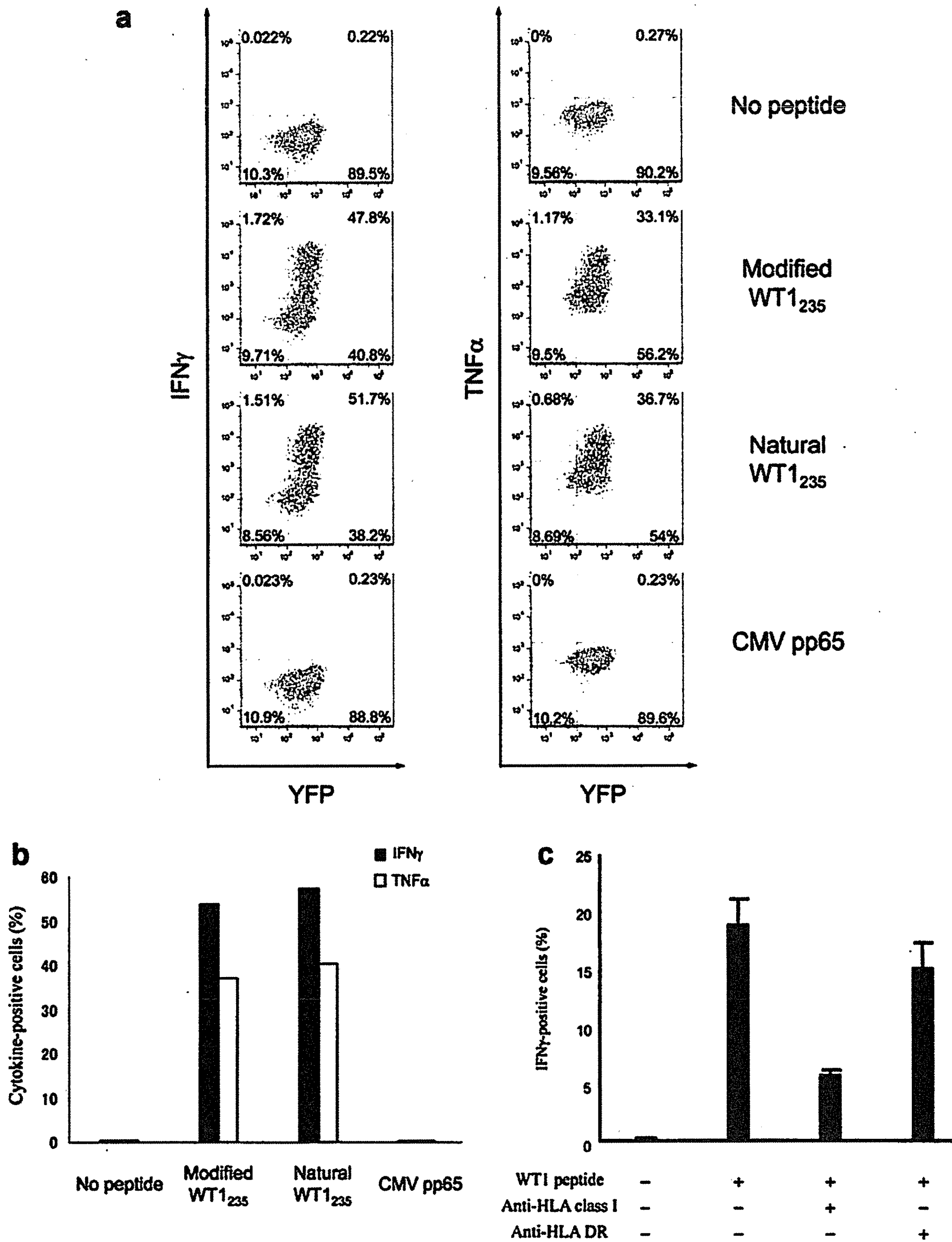

Figure 3. *Cytokine production by the stimulation of B10-TCR-transfected CD8+ T-cells. a: B10-TCR-transfected CD8+ T-cells were stimulated with the indicated antigen peptides and examined for IFNγ and TNFα production. Representative data of two experiments is shown. b: Frequencies of intracellular IFNγ- and TNFα-positive cells among YFP-positive cells in B10-TCR-transfected CD8+ T-cells, stimulated with the indicated antigen peptides. c: CD8+ T-cells transfected with the B10-TCRs were stimulated with the modified $WT1_{235}$ peptide-loaded T2/24:02 cells, and were assayed for IFNγ production in the presence of HLA class I- or HLA DR-blocking monoclonal antibody. Representative data of two experiments are shown. T2/24:02 cells, HLA-A*24:02-positive T2 cells.*

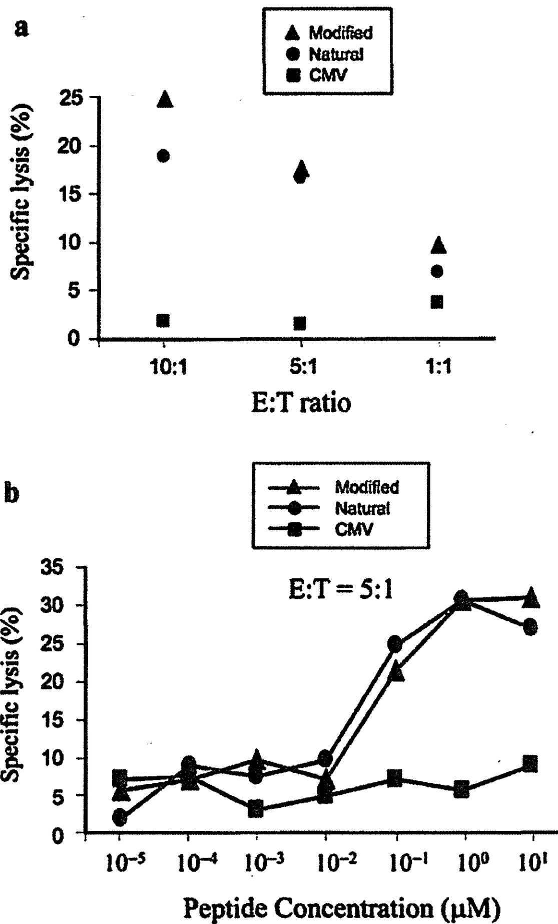

Figure 4. *$WT1_{235}$ peptide-specific cytotoxic activity of B10-TCR-transfected CD8+ T-cells. CD8+ T-cells transfected with B10-TCRs were assayed for their cytotoxic activity towards T2/24:02 cells loaded with modified, natural $WT1_{235}$ peptide, or with CMV pp65 peptide, at a concentration of 20 μM (a), or at different concentrations (b). Representative data of two experiments are shown. E:T, effector/target ratio.*

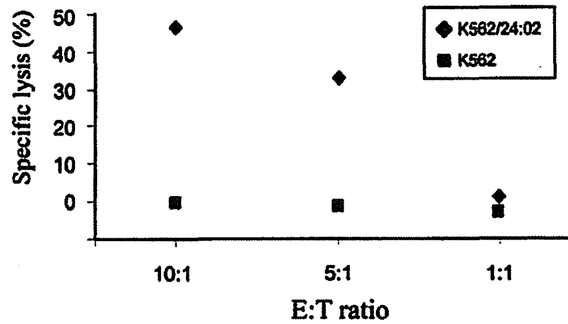

Figure 5. *Cytotoxic activity of B10-TCR-transfected CD8+ T-cells towards endogenously WT1-expressing leukemia cells. Cytotoxic activity of B10-TCR-transduced CD8+ T-cells towards endogenously WT1-expressing K562 leukemia cells with or without HLA-A*24:02 expression was examined. Representative data of three experiments are shown.*

transfected CD8+ T-cells were stimulated by T2/24:02 cells loaded with the modified $WT1_{235}$ peptide in the presence of an HLA class I or HLA DR blocking mAb and stained for intracellular IFNγ. The production of IFNγ was inhibited by anti-HLA class I mAb, but not by anti-HLA DR blocking mAb. These results indicate that the cytokine production of B10-TCR-transfected CD8+ T-cells by antigenic stimulation was restricted to HLA class I.

$WT1_{235}$ peptide-specific cytotoxic activity of B10-TCR-transfected CD8+ T-cells. To test the antigen-specific cytotoxicity of B10-TCR-transfected CD8+ T-cells, they were co-cultured with irradiated autologous PBMCs loaded with modified $WT1_{235}$ peptide for two weeks and assayed for cytotoxicity towards ^{51}Cr-treated HLA-A*24:02-transfected T2 (T2/24:02) cells, loaded with modified or natural $WT1_{235}$ peptide (Figure 4a). The B10-TCR-transfected CD8+ T-cells exhibited cytotoxicity towards the T2/24:02 cells loaded with modified or natural $WT1_{235}$ peptide in an effector/target (E/T) ratio-dependent manner, but not towards those loaded with an irrelevant peptide (CMV pp65 peptide). These results indicated that B10-TCRs recognized not only the modified $WT1_{235}$ peptide/HLA-A*24:02 complex but also the natural $WT1_{235}$ peptide/HLA-A*24:02 complex.

Next, specific lysis by B10-TCR-transfected CD8+ T-cells was assayed for the T2/24:02 target cells pulsed with different concentrations of modified or natural $WT1_{235}$ peptide (Figure 4b). The specific lysis increased in parallel with an increase in the peptide concentrations and reached a plateau at an E/T ratio of 5:1, at a concentration of 1 μM in both peptides. The half-maximal lysis for modified and natural $WT1_{235}$ peptide was obtained at a concentration of about 0.06 μM and 0.04 μM, respectively. These results indicate that the affinity of B10-TCRs for natural $WT1_{235}$ peptide/HLA-A*24:02 complex was high enough to expect that B10-TCRs would be able to recognize the endogenous WT1 protein-derived (natural) $WT1_{235}$ peptide that was presented on the cell surface in association with HLA-A*24:02 molecules.

*Lysis of endogenously WT1-expressing leukemia cells by B10-TCR-transfected CD8+ T-cells with an HLA-A*24:02 restriction.* Whether or not B10-TCR-transfected CD8+ T-cells had the ability to lyse endogenously WT1-expressing leukemia cells with a restriction of HLA-A*24:02 was

examined. The B10-TCR-transfected CD8+ T-cells were stimulated by irradiated autologous PBMCs loaded with the modified $WT1_{235}$ peptide. After two weeks of the stimulation, the B10-TCR-transfected CD8+ T-cells were assayed for the lysis of HLA-A*24:02-transfected K562 leukemia cells (K562/24:02) that endogenously expressed WT1. The B10-TCR-transfected CD8+ T-cells were cytotoxic towards the K562/24:02 cells, but not towards K562 cells without an HLA-A*24:02 expression (Figure 5). These results indicate that B10-TCR-transfected CD8+ T-cells were able to kill endogenously WT1-expressing leukemia cells in an HLA-A*24:02 restriction manner.

Discussion

In the present study, a modified $WT1_{235}$ peptide-specific CTL clone (B10) was established and its TCRs (B10-TCRs) were cloned. B10-TCR-transfected CD8+ T-cells were able to kill both modified $WT1_{235}$ peptide-pulsed and natural $WT1_{235}$ peptide-pulsed target cells and endogenously WT1-expressing leukemia cells.

An important finding presented here was that B10-TCRs, isolated from a modified $WT1_{235}$ peptide-specific CTL clone, was able to recognize and kill both natural $WT1_{235}$ peptide-pulsed target cells and endogenously WT1-expressing leukemia cells that were possibly expressing natural $WT1_{235}$ peptide (epitope) on their cell surface in complexes with HLA-A*24:02 molecules. The evidence, at the molecular level, showing that a modified $WT1_{235}$ peptide-specific TCR recognizes both its own modified and other natural $WT1_{235}$ peptides (epitopes) has been demonstrated here for the first time due to our successful cloning a modified $WT1_{235}$ peptide-specific TCR gene. This evidence provided us with a strong proof-of-concept of modified $WT1_{235}$ peptide-based immunotherapy, in which the modified (not natural) $WT1_{235}$ peptides were effectively vaccinated for the eradication of tumor cells that were possibly expressing natural (not modified) $WT1_{235}$ peptides in complexes with HLA-A*24:02 molecules. In fact, there are some clinical findings showing that vaccination with modified $WT1_{235}$ peptides induced modified $WT1_{235}$ peptide-specific CTLs and other CTLs that were able to recognize both the modified and natural $WT1_{235}$ peptides (epitopes). For example, Narita *et al.* successfully vaccinated a patient with CML with the modified $WT1_{235}$ peptides and showed that some CD8+ T-cells in PBMCs that were obtained after repeated WT1 vaccination were dually stained with the modified $WT1_{235}$ peptide-specific and natural $WT1_{235}$ peptide-specific tetramers. They also showed that the modified $WT1_{235}$ peptide-specific CTL clones established, exerted cytotoxic activity towards both the modified $WT1_{235}$ peptide-pulsed and natural $WT1_{235}$ peptide-pulsed target cells (42). However, since the cloning of TCRs from the modified $WT1_{235}$-specific CTLs was not done, it was not demonstrated, at the molecular level, that the TCRs of the modified $WT1_{235}$-specific CTLs recognized both the modified and natural $WT1_{235}$ peptides (epitopes). On the other hand, it was demonstrated that a natural $WT1_{235}$ peptide-specific CTL clone, TAK-1, recognized both the natural and modified $WT1_{235}$ peptides (24). However, the molecular basis of this finding has not yet been reported. Thus, detailed analysis at the molecular level for explaining how $WT1_{235}$ peptide-specific CTLs are able to recognize both natural and modified $WT1_{235}$ peptides (epitopes) has been reported here for the first time.

Results presented here suggest the possibility for adoptive transfer therapy of CD8+ T-cells transfected with the modified $WT1_{235}$ peptide-specific TCR genes. Half-maximal lysis by the CD8+ T-cells that were transfected with the TCRs from the modified $WT1_{235}$ peptide-specific CTLs was obtained against the natural $WT1_{235}$ peptide-pulsed target cells at concentrations of as low as 0.04 μM. This indicates the high affinity of the TCRs for the natural $WT1_{235}$ epitope on tumor cells. These results should allow us to expect a good clinical effect of adoptive cell therapy using the TCR genes isolated here.

Acknowledgements

This study was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports, Culture, and Technology, Japan.

References

1 Kast WM, Offringa R, Peters PJ, Voordouw AC, Meloen RH, van der Eb AJ and Melief CJ: Eradication of adenovirus E1-induced tumors by E1A-specific cytotoxic T lymphocytes. Cell *59*: 603-614, 1989.

2 Falkenburg JH, Smit WM and Willemze R: Cytotoxic T-lymphocyte (CTL) responses against acute or chronic myeloid leukemia. Immunol Rev *157*: 223-230, 1997.

3 Oka Y and Sugiyama H: WT1 peptide vaccine, one of the most promising cancer vaccines: its present status and the future prospects. Immunotherapy 2: 591-594, 2010.

4 Waldmann TA: Immunotherapy: past, present and future. Nat Med *9*: 269-277, 2003.

5 Drummond IA, Madden SL, Rohwer-Nutter P, Bell GI, Sukhatme VP and Rauscher FJ 3rd: Repression of the insulin-like growth factor II gene by the Wilms tumor suppressor WT1. Science *257*: 674-678, 1992.

6 Englert C, Hou X, Maheswaran S, Bennett P, Ngwu C, Re GG, Garvin AJ, Rosner MR and Haber DA: WT1 suppresses synthesis of the epidermal growth factor receptor and induces apoptosis. Embo J *14*: 4662-4675, 1995.

7 Goodyer P, Dehbi M, Torban E, Bruening W and Pelletier J: Repression of the retinoic acid receptor-alpha gene by the Wilms' tumor suppressor gene product, wt1. Oncogene *10*: 1125-1129, 1995.

8 Hewitt SM, Hamada S, McDonnell TJ, Rauscher FJ, 3rd and Saunders GF: Regulation of the proto-oncogenes bcl-2 and c-myc by the Wilms' tumor suppressor gene WT1. Cancer Res *55*: 5386-5389, 1995.

9 Inoue K, Sugiyama H, Ogawa H, Nakagawa M, Yamagami T, Miwa H, Kita K, Hiraoka A, Masaoka T, Nasu K *et al*: WT1 as a new prognostic factor and a new marker for the detection of minimal residual disease in acute leukemia. Blood *84*: 3071-3079, 1994.

10 Brieger J, Weidmann E, Fenchel K, Mitrou PS, Hoelzer D and Bergmann L: The expression of the Wilms' tumor gene in acute myelocytic leukemias as a possible marker for leukemic blast cells. Leukemia *8*: 2138-2143, 1994.

11 Menssen HD, Renkl HJ, Rodeck U, Maurer J, Notter M, Schwartz S, Reinhardt R and Thiel E: Presence of Wilms' tumor gene (wt1) transcripts and the WT1 nuclear protein in the majority of human acute leukemias. Leukemia *9*: 1060-1067, 1995.

12 Bergmann L, Miething C, Maurer U, Brieger J, Karakas T, Weidmann E and Hoelzer D: High levels of Wilms' tumor gene (wt1) mRNA in acute myeloid leukemias are associated with a worse long-term outcome. Blood *90*: 1217-1225, 1997.

13 Menssen HD, Renkl HJ, Rodeck U, Kari C, Schwartz S and Thiel E: Detection by monoclonal antibodies of the Wilms' tumor (WT1) nuclear protein in patients with acute leukemia. Int J Cancer *70*: 518-523, 1997.

14 Tamaki H, Ogawa H, Ohyashiki K, Ohyashiki JH, Iwama H, Inoue K, Soma T, Oka Y, Tatekawa T, Oji Y, Tsuboi A, Kim EH, Kawakami M, Fuchigami K, Tomonaga M, Toyama K, Aozasa K, Kishimoto T and Sugiyama H: The Wilms' tumor gene WT1 is a good marker for diagnosis of disease progression of myelodysplastic syndromes. Leukemia *13*: 393-399, 1999.

15 Ogawa H, Tamaki H, Ikegame K, Soma T, Kawakami M, Tsuboi A, Kim EH, Hosen N, Murakami M, Fujioka T, Masuda T, Taniguchi Y, Nishida S, Oji Y, Oka Y and Sugiyama H: The usefulness of monitoring WT1 gene transcripts for the prediction and management of relapse following allogeneic stem cell transplantation in acute type leukemia. Blood *101*: 1698-1704, 2003.

16 Oji Y, Ogawa H, Tamaki H, Oka Y, Tsuboi A, Kim EH, Soma T, Tatekawa T, Kawakami M, Asada M, Kishimoto T and Sugiyama H: Expression of the Wilms' tumor gene WT1 in solid tumors and its involvement in tumor cell growth. Jpn J Cancer Res *90*: 194-204, 1999.

17 Oji Y, Miyoshi S, Maeda H, Hayashi S, Tamaki H, Nakatsuka S, Yao M, Takahashi E, Nakano Y, Hirabayashi H, Shintani Y, Oka Y, Tsuboi A, Hosen N, Asada M, Fujioka T, Murakami M, Kanato K, Motomura M, Kim EH, Kawakami M, Ikegame K, Ogawa H, Aozasa K, Kawase I and Sugiyama H: Overexpression of the Wilms' tumor gene WT1 in *de novo* lung cancers. Int J Cancer *100*: 297-303, 2002.

18 Miyoshi Y, Ando A, Egawa C, Taguchi T, Tamaki Y, Tamaki H, Sugiyama H and Noguchi S: High expression of Wilms' tumor suppressor gene predicts poor prognosis in breast cancer patients. Clin Cancer Res *8*: 1167-1171, 2002.

19 Oji Y, Miyoshi Y, Koga S, Nakano Y, Ando A, Nakatsuka S, Ikeba A, Takahashi E, Sakaguchi N, Yokota A, Hosen N, Ikegame K, Kawakami M, Tsuboi A, Oka Y, Ogawa H, Aozasa K, Noguchi S and Sugiyama H: Overexpression of the Wilms' tumor gene WT1 in primary thyroid cancer. Cancer Sci *94*: 606-611, 2003.

20 Oji Y, Yamamoto H, Nomura M, Nakano Y, Ikeba A, Nakatsuka S, Abeno S, Kiyotoh E, Jomgeow T, Sekimoto M, Nezu R, Yoshikawa Y, Inoue Y, Hosen N, Kawakami M, Tsuboi A, Oka Y, Ogawa H, Souda S, Aozasa K, Monden M and Sugiyama H: Overexpression of the Wilms' tumor gene WT1 in colorectal adenocarcinoma. Cancer Sci *94*: 712-717, 2003.

21 Cheever MA, Allison JP, Ferris AS, Finn OJ, Hastings BM, Hecht TT, Mellman I, Prindiville SA, Viner JL, Weiner LM and Matrisian LM: The prioritization of cancer antigens: a national cancer institute pilot project for the acceleration of translational research. Clin Cancer Res *15*: 5323-5337, 2009.

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

23 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.

24 Tsuboi A, Oka Y, Udaka K, Murakami M, Masuda T, Nakano A, Nakajima H, Yasukawa M, Hiraki A, Oji Y, Kawakami M, Hosen N, Fujioka T, Wu F, Taniguchi Y, Nishida S, Asada M, Ogawa H, Kawase I and Sugiyama H: 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 *51*: 614-620, 2002.

25 Tsuboi A, Oka Y, Kyo T, Katayama Y, Elisseeva OA, Kawakami M, Nishida S, Morimoto S, Murao A, Nakajima H, Hosen N, Oji Y and Sugiyama H: Long-term WT1 peptide vaccination for patients with acute myeloid leukemia with minimal residual disease. Leukemia *26*: 1410-1413, 2012.

26 Hashii Y, Sato-Miyashita E, Matsumura R, Kusuki S, Yoshida H, Ohta H, Hosen N, Tsuboi A, Oji Y, Oka Y, Sugiyama H and Ozono K: WT1 peptide vaccination following allogeneic stem cell transplantation in pediatric leukemic patients with high risk for relapse: successful maintenance of durable remission. Leukemia *26*: 530-532, 2012.

27 Shirakata T, Oka Y, Nishida S, Hosen N, Tsuboi A, Oji Y, Murao A, Tanaka H, Nakatsuka S, Inohara H and Sugiyama H: WT1 peptide therapy for a patient with chemotherapy-resistant salivary gland cancer. Anticancer Res *32*: 1081-1085, 2012.

28 Oka Y, Tsuboi A, Murakami M, Hirai M, Tominaga N, Nakajima H, Elisseeva OA, Masuda T, Nakano A, Kawakami M, Oji Y, Ikegame K, Hosen N, Udaka K, Yasukawa M, Ogawa H, Kawase I and Sugiyama H: Wilms tumor gene peptide-based immunotherapy for patients with overt leukemia from myelodysplastic syndrome (MDS) or MDS with myelofibrosis. Int J Hematol *78*: 56-61, 2003.

29 Tsuboi A, Oka Y, Osaki T, Kumagai T, Tachibana I, Hayashi S, Murakami M, Nakajima H, Elisseeva OA, Fei W, Masuda T, Yasukawa M, Oji Y, Kawakami M, Hosen N, Ikegame K, Yoshihara S, Udaka K, Nakatsuka S, Aozasa K, Kawase I and Sugiyama H: WT1 peptide-based immunotherapy for patients with lung cancer: report of two cases. Microbiol Immunol *48*: 175-184, 2004.

30 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.

31 Sugiyama H: Cancer immunotherapy targeting Wilms' tumor gene WT1 product. Expert Rev Vaccines *4*: 503-512, 2005.

32 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 microg/body) in a patient with chronic myelomonocytic leukemia. Int J Hematol *85*: 426-429, 2007.

33 Iiyama T, Udaka K, Takeda S, Takeuchi T, Adachi YC, 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.

34 Tsuboi A, Oka Y, Nakajima H, Fukuda Y, Elisseeva OA, Yoshihara S, Hosen N, Ogata A, Kito K, Fujiki F, Nishida S, Shirakata T, Ohno S, Yasukawa M, Oji Y, Kawakami M, Morita S, Sakamoto J, Udaka K, Kawase I and Sugiyama H: Wilms tumor gene WT1 peptide-based immunotherapy induced a minimal response in a patient with advanced therapy-resistant multiple myeloma. Int J Hematol *86*: 414-417, 2007.

35 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.

36 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.

37 Akatsuka Y, Goldberg TA, Kondo E, Martin EG, Obata Y, Morishima Y, Takahashi T and Hansen JA: Efficient cloning and expression of HLA class I cDNA in human B-lymphoblastoid cell lines. Tissue Antigens *59*: 502-511, 2002.

38 Kuzushima K, Hayashi N, Kimura H and Tsurumi T: Efficient identification of HLA-A*2402-restricted cytomegalovirus-specific CD8(+) T-cell epitopes by a computer algorithm and an enzyme-linked immunospot assay. Blood *98*: 1872-1881, 2001.

39 Karanikas V, Lurquin C, Colau D, van Baren N, De Smet C, Lethe B, Connerotte T, Corbiere V, Demoitie MA, Lienard D, Dreno B, Velu T, Boon T and Coulie PG: Monoclonal anti-MAGE-3 CTL responses in melanoma patients displaying tumor regression after vaccination with a recombinant canarypox virus. J Immunol *171*: 4898-4904, 2003.

40 Szymczak AL, Workman CJ, Wang Y, Vignali KM, Dilioglou S, Vanin EF and Vignali DA: Correction of multi-gene deficiency *in vivo* using a single 'self-cleaving' 2A peptide-based retroviral vector. Nat Biotechnol *22*: 589-594, 2004.

41 Nagai T, Ibata K, Park ES, Kubota M, Mikoshiba K and Miyawaki A: A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. Nat Biotechnol *20*: 87-90, 2002.

42 Narita M, Masuko M, Kurasaki T, Kitajima T, Takenouchi S, Saitoh A, Watanabe N, Furukawa T, Toba K, Fuse I, Aizawa Y, Kawakami M, Oka Y, Sugiyama H and Takahashi M: WT1 peptide vaccination in combination with imatinib therapy for a patient with CML in the chronic phase. Int J Med Sci *7*: 72-81, 2010.

Received September 20, 2012
Revised October 22, 2012
Accepted October 23, 2012

Ann Thorac Cardiovasc Surg 2012; 18: 530–535 doi: 10.5761/atcs.oa.11.01811

Original Article

Pathological Status of Mediastinal Lymph Nodes after Preoperative Concurrent Chemoradiotherapy Determines Prognosis in Patients with Non-Small Cell Lung Cancer

Yasushi Shintani, MD, PhD,[1,2] Yasunobu Funakoshi, MD, PhD,[3] Masayoshi Inoue, MD, PhD,[1] Yukiyasu Takeuchi, MD, PhD,[3] Meinoshin Okumura, MD, PhD,[1] Hajime Maeda, MD, PhD,[3] and Mitsunori Ohta, MD, PhD[2]

Objectives: The benefits of preoperative chemoradiotherapy for advanced nonsmall cell lung cancer (NSCLC) remain controversial. To evaluate prognostic indicators of clinical N2 NSCLC patients treated with concurrent chemotherapy followed by pulmonary resection, we performed a retrospective study.
Methods: We retrospectively investigated 52 patients with pathologically proven N2 NSCLC who underwent concurrent chemoradiotherapy before pulmonary resection. Each received 2 cycles of cisplatin-vinca alkaloid-based chemotherapy every 4 weeks. Radiotherapy, directed at the tumor and mediastinal nodes, was started on day 2 at a median dose of 44 Gy. A thoracotomy was performed 6 to 8 weeks after completion of chemoradiotherapy.
Results: The overall 5-year survival rate for the 52 patients was 38%. Complete pathological response by the tumor was found in 11 (21%). Down-staging of nodal stage occurred in 29 patients, (56%) and overall survival was better in those with lower pathological N status. The 5-year survival rate was 58% for pathological N0-N1 disease and 0% for N2 disease. While the response to induction therapy by the primary tumor was correlated with postoperative nodal stage, multivariate analysis revealed postoperative nodal stage as an independent prognostic factor.
Conclusion: Pathological status of mediastinal lymph nodes in response to preoperative concurrent chemoradiotherapy determined prognosis in our patients.

Keywords: nonsmall cell lung cancer, preoperative concurrent chemoradiotherapy, prognostic indicator

[1]*Department of General Thoracic Surgery, Osaka University Graduate School of Medicine, Suita, Osaka, Japan*
[2]*Department of General Thoracic Surgery, Osaka Prefectural Medical Center for Respiratory and Allergic Disease, Habikino, Osaka, Japan*
[3]*Department of General Thoracic Surgery, National Hospital Organization Toneyama Hospital, Toyonaka, Osaka, Japan*

Received: October 13, 2011; Accepted: February 7, 2012
Corresponding author: Yasushi Shintani, MD, PhD. Department of General Thoracic Surgery, Osaka University Graduate School of Medicine, Suita, Osaka 565-0877, Japan
Email: yshintani@thoracic.med.osaka-u.ac.jp
©2012 The Editorial Committee of *Annals of Thoracic and Cardiovascular Surgery*. All rights reserved.

Introduction

The most effective treatment for patients with locally advanced nonsmall cell lung cancer (NSCLC) remains controversial. The results of surgical resection alone for locally advanced NSCLC are poor; thus the treatment option of induction chemoradiotherapy has been investigated.[1,2] Although there is consensus about the indication for a multimodality approach in most patients with locally advanced disease, there is no clear agreement about which local therapy should be applied in a given situation.[3] Some reports have demonstrated that induction chemoradiotherapy followed by surgical resection