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Epstein–Barr Virus in Diffuse Large B-Cell Lymphoma in Immunocompetent Patients in Japan is as Low as in Western Countries

Naoki Wada,[1] Junichiro Ikeda,[1] Yumiko Hori,[1] Shigeki Fujita,[1] Hiroyasu Ogawa,[2] Toshihiro Soma,[2] Haruo Sugiyama,[3] Shirou Fukuhara,[4] Akihisa Kanamaru,[5] Masayuki Hino,[6] Yuzuru Kanakura,[7] Eiichi Morii,[1] and Katsuyuki Aozasa[1]*

[1]*Department of Pathology, Osaka University Graduate School of Medicine, Suita, Osaka, Japan*
[2]*Department of Internal Medicine, Hyogo College of Medicine, Nishinomiya, Hyogo, Japan*
[3]*Department of Functional Diagnostic Science, Osaka University Graduate School of Medicine, Suita, Osaka, Japan*
[4]*First Department of Internal Medicine, Kansai Medical University, Moriguchi, Osaka, Japan*
[5]*Department of Hematology, Kinki University School of Medicine, Sayama, Osaka, Japan*
[6]*Department of Clinical Haematology and Diagnostics, Osaka City University Graduate School of Medicine, Osaka, Japan*
[7]*Department of Hematology and Oncology, Osaka University Graduate School of Medicine, Suita, Osaka, Japan*

According to previous reports, the frequency of Epstein–Barr virus (EBV) positivity in diffuse large B-cell lymphoma is higher in East Asia (approximately 9%) than in Western countries. The presence of the EBV genome was examined in diffuse large B-cell lymphoma patients registered with the Osaka Lymphoma Study Group (OLSG) in Osaka, Japan, situated in East Asia. The EBV-positive rate was examined with in situ hybridization (ISH) in 484 immunocompetent diffuse large B-cell lymphoma patients registered with OLSG. The male-to-female ratio was 1.29, with ages ranging from 16 to 95 (median, 68) years. ISH with EBV-encoded small RNAs (EBER) probes revealed positive signals in the nuclei of tumor cells: the frequency of positively stained cells among all tumor cells was almost none in 458 cases, 5–10% in 5, 10–20% in 5, 20–50% in 11, and >50% in 5. When the frequency was >20% or >50%, the EBV-positive rate in the present series (3.3% or 1.0%) was rather similar to that reported in Western cases. Careful evaluation of patient backgrounds, including age distribution, type of lymphomas, exclusion of immunocompromised patients, and establishment of definite criteria for EBV positivity (>20%, >50%, or almost all tumor cells) are essential in comparing geographical differences. ***J. Med. Virol. 83:317–321, 2011.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: Epstein–Barr virus; diffuse large B-cell lymphoma; in situ hybridization; immunocompetence; geographical differences

INTRODUCTION

The Epstein–Barr virus (EBV), a γ-herpesvirus, is transmitted by mucosal secretions among the human population. An association between EBV and human lymphomas, including endemic Burkitt's lymphoma, Hodgkin lymphoma, and non-Hodgkin lymphomas of B-, T-, or NK-cell immunophenotypes, has been reported [Shah and Young, 2009]. In the recent WHO classification (2008), the chapter for EBV-positive diffuse large B-cell lymphoma of the elderly states that the EBV-positive rate in diffuse large B-cell lymphoma in Asian countries is 8–10% [Nakamura et al., 2008]. Hematologists and hematopathologists in the Osaka Lymphoma Study Group (OLSG) in Osaka, Japan, situated in the East Asia, felt a sense of incongruity for this figure, although they had no data on this.

Abbreviations: EBV, Epstein–Barr virus; OLSG, Osaka Lymphoma Study Group; LMP, latent membrane protein; ISH, in situ hybridization, EBER, EBV-encoded small RNAs; MTX, methotrexate; PNA, peptide nucleic acid; FITC, fluorescein isothiocyanate; DAB, 3,3′-diaminobenzidine tetrahydrochloride; CTL, cytotoxic T-lymphocytes.

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*Correspondence to: Katsuyuki Aozasa, MD, PhD, Department of Pathology (C3), Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan.
E-mail: aozasa@molpath.med.osaka-u.ac.jp

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There have been two large-scale studies on the EBV positivity in diffuse large B-cell lymphoma from East Asian countries, one in Korea and one in Japan. Both studies showed a similar frequency of EBV positivity among diffuse large B-cell lymphoma, that is, 9% [Oyama et al., 2007; Park et al., 2007]. However, two points regarding these reports should be addressed. First, the age distribution was quite different between the Korean and Japanese cases: the percentage of cases older than 60 years was 36.6% in Korean and 60.6% in Japanese cases. It seems that the EBV-positive rate is higher in elder patients; thus, the difference in age distribution might affect EBV positivity. Second, the criteria for EBV positivity of diffuse large B-cell lymphoma was different: >20% of tumor cells with positive signals by in situ hybridization (ISH) in one report [Park et al., 2007] and >50% in another [Oyama et al., 2007]. Some Japanese investigators, adopting the criteria of almost all tumor cells with positive signals as EBV-positive, reported that 11.4% of their diffuse large B-cell lymphoma cases were EBV-positive [Kuze et al., 2000]. However, reports from Western countries apparently showed a much lower EBV-positive rate than did the East Asian ones [Gibson and Hsi, 2009; Hoeller et al., 2010]. Hoeller et al. [2010] reported that 10 (3.9%) of their 258 diffuse large B-cell lymphoma cases were EBV-positive: the percentage of positive cells was <20% in two cases, <50% in four, and >50% in four. Gibson and Hsi [2009] reported that none of their 90 diffuse large B-cell lymphoma, all older than 60 years, was EBV-positive. They stated that EBV-positive tumor cells were not identified in any of the 90 cases, and only three demonstrated very rare EBV-positive signals in small bystander lymphocytes (<1% of non-tumor cells).

In this study, we examined the presence of the EBV genome with ISH in the tumor cells in cases of diffuse large B-cell lymphoma registered with OLSG. The EBV-positive rate was then evaluated according to the criteria used in the previous and present studies, and the data were then compared with those reported from East Asia and Western countries in the light of clinical findings such as age, gender, and presence of immunodeficiencies.

MATERIALS AND METHODS

Patients

Between November 1999 and October 2009, in total, 4,490 cases were registered with the OLSG, Japan, in which 63 hospitals participate and register cases of malignant lymphomas and related conditions. All of the hematoxylin and eosin- and immunoperoxidase-stained sections were reviewed by one of the authors (K.A.) and classified according to the WHO classification. A diagnosis of malignant lymphoma was confirmed in 3,571 (79.5%) of 4,490 cases. Of these 3,571 cases, 3,273 (91.7%) were non-Hodgkin lymphoma and 298 (8.3%) were Hodgkin lymphoma. The number of diffuse large B-cell lymphoma cases was 1,590, which constituted 48.6% of all non-Hodgkin lymphoma. The latest 500 cases with diffuse large B-cell lymphoma registered during February 2007 to October 2009 were studied for the presence of the EBV genome. Most patients received anthracyclin-based chemotherapy and rituximab after the diagnosis. The numbers of patients who received excisional biopsy or surgical resection, punch biopsy, or needle biopsy were 384, 76, and 40, respectively. In cases receiving punch or needle biopsy, an adequate amount of material for histological analyses was obtained. The primary site was lymph node in 165 cases, extranodal organs in 204, and unknown in 131. Among them, two patients suffered from rheumatoid arthritis, one each from idiopathic thrombocytopenic purpura, interstitial pneumonia, Sjögren's syndrome, and uveitis, and were treated with prednisone, together with cyclosporine in one. These six cases were excluded for further analysis because of possible underlying immune abnormalities. During the same period, 14 cases of polymorphous lymphoproliferative disorders, 14 methotrexate (MTX)-associated lymphoproliferative disorders, five post-transplant lymphoproliferative disorders (liver, kidney, and cord blood in two, two, and one case, respectively), and one Hodgkin-like lymphoproliferative disorder were registered with the OLSG. Polymorphous lymphoproliferative disorders are defined as lymphoproliferative disorders showing a polymorphous pattern of proliferation, consisting of large lymphoid cells and various numbers of inflammatory cells [Nakamichi et al., 2010]. Polymorphous lymphoproliferative disorders usually affect individuals with a background of immunodeficiency. Clinical findings entered on the registration card for the OLSG included the following: name of hospital, age, gender, occupation, site of disease, clinical diagnosis, present illness, and peripheral blood and bone marrow findings, including differential counts, serum data, and chromosomal abnormalities of the tumor cells. History of medication (those receiving immunosuppressants) was also checked.

The Institutional Review Board for Clinical Research at Osaka University Hospital approved this study.

Immunohistochemistry

Antibodies used for immunohistochemistry were CD20, CD79a, CD3, CD30, and anti-Epstein–Barr virus/LMP/Clones CS. 1–4 (DakoCytomation, Glostrup, Denmark, diluted at 1:400, 1:100, 1:50, 1:25, and 1:100, respectively). Immunohistochemistry was performed using an automated staining system (Dako Autostainer, DakoCytomation).

In Situ Hybridization

ISH using the EBV-encoded small RNAs (EBER) probe was performed to examine the presence of the EBV genome in the formalin-fixed paraffin-embedded sections with the EBER DAB application kit (DakoCytomation). Briefly, sections were treated with proteinase K, diluted at 1:10 with TBS (50 mmol/L Tris–HCl buffered saline containing 150 mmol/L NaCl, pH 7.6), then hybridized with EBER PNA (peptide nucleic acid)

probe/fluorescein (DakoCytomation) at 55°C for 90 min. After blocking endogenous peroxidase activity, sections were incubated with rabbit anti-FITC (fluorescein isothiocyanate) antibody (1:50; Invitrogen, Carlsbad, CA) at room temperature for 30 min, incubated with ChemMate ENVISION/HRP polymer (DakoCytomation) at room temperature for 30 min, and colored with DAB (3,3′-diaminobenzidine tetrahydrochloride). This ISH method using an EBER probe was recently used in the author's laboratory. The EBV-positive rate in NK/T cell lymphoma by this method on paraffin-embedded sections was similar (80%) to that with the previously used method using an EBER-1 probe [Li et al., 2000].

The frequency of EBV positivity in the malignant large lymphoid cells was determined by averaging the number of positive cells from three high-power fields where the positive cells were larger in number. The tumors were then separated into five groups: tumors with almost no EBV positivity, tumors with 5–10%, 10–20%, 20–50%, and >50% of cells showing EBV positivity. According to the previous study, we adopted the criterion of >20% as EBV-positive.

Follow-Up

Adequate follow-up data was available in 16 EBV-positive and 204 EBV-negative diffuse large B-cell lymphoma cases. Follow-up periods for survivors in EBV-positive and -negative cases ranged from 5.2 to 38.5 (average 22.6) and 16.4–38.1 (average 25.5) months, respectively. Kaplan–Meier estimated survival rates at 2 and 3 years were calculated, and overall survival rates were compared by a log-rank test.

RESULTS

EBV-ISH was performed in 494 cases; however, informative data was not obtained in 10 cases because of specimen loss during staining procedures or severe non-specific staining of background/overstaining. As a result, staining results could be evaluated in only 484 cases. Positive signals were found in the nuclei of tumor cells: the frequency of positively stained cells among all tumor cells was almost none in 458 cases, 5–10% in 5, 10–20% in 5, 20–50% in 11, and >50% in 5 (Fig. 1).

Of 16 cases with >20% EBV-positive tumor cells (EBV-positive cases), large geographical necrosis was found in five cases. Angiotropism was not found. CD30 positivity was found in 6 of 15 EBV-positive cases. Tumor cells expressed latent membrane protein (LMP)-1 in 8 of 14 EBV-positive cases (Fig. 1).

In total, 40 cases with diffuse large B-cell lymphoma were excluded, due to the putative presence of immunocompromised conditions, including autoimmune diseases, receiving prednisone and cyclosporine, polymorphous lymphoproliferative disorders, MTX-associated lymphoproliferative disorders, post-transplant lymphoproliferative disorders, and Hodgkin-like lymphoproliferative disorders. The frequency of positively stained cells among all tumor cells was 20–50% in six cases and >50% in five.

The estimated survival rates at 2 and 3 years for the EBV-positive and -negative cases were 58.9% and 71.9% at 2 years and 58.9% and 69.0% at 3 years, respectively. The overall survival rates of the two groups were not statistically different (log-rank test; $P > 0.1$).

DISCUSSION

According to previous reports, frequency of EBV positivity in diffuse large B-cell lymphoma was higher in East Asia than in Western countries (Table I) [Kuze et al., 2000; Oyama et al., 2007; Park et al., 2007; Gibson and Hsi, 2009; Hoeller et al., 2010]. However, before accepting this, a careful evaluation of the data presented in the previous studies is necessary. Regarding clinical findings, the age distribution and gender ratio varied among these reports [Kuze et al., 2000; Oyama et al., 2007; Park et al., 2007; Gibson and Hsi, 2009; Hoeller et al., 2010]. A study referenced in the WHO classification adopting >50% of the criteria included cases of immunodeficiency-associated lymphomas, although the exact number was not shown. Lymphomas known to be closely associated with EBV infection such as pyothorax-associated lymphoma and Burkitt's lymphoma were also included [Oyama et al., 2007]. These procedures inevitably increased the EBV-positive rate of the study (13.6%). The routinely registered cases with the OLSG constitute the present series; thus, collection bias in the cases could be avoided. The age distribution and gender ratio in the present series are within the spectrum of the previously reported diffuse large B-cell lymphoma cases; thus, the present findings could be adopted as standard data.

The criteria for defining EBV-positive cases vary among the previous studies; >20% of the tumor cells in one study [Park et al., 2007] and >50% in another [Oyama et al., 2007]. In particular, a study from Japan adopting the criteria of almost all EBV-positive cells reported the EBV-positive rate to be 11.4% [Kuze et al., 2000]. When adopting >20% or >50% as EBV-positive, the EBV-positive rates among diffuse large B-cell lymphoma in immunocompetent hosts in the OLSG (3.3% or 1.0%) were rather similar to those in Western series [0–2.7%, 0–1.9%; Gibson and Hsi, 2009; Hoeller et al., 2010], but much lower than that in Korean (8.9%) [Park et al., 2007] and Japanese series (11.4–13.6%) [Kuze et al., 2000; Oyama et al., 2007].

The present and previous [Park et al., 2007; Gibson and Hsi, 2009; Hoeller et al., 2010] studies have analyzed the presence of the EBV genome in diffuse large B-cell lymphoma in immunocompetent patients. When a total of 40 cases with diffuse large B-cell lymphoma, which had been excluded from the calculation of the EBV-positive rate due to the putative presence of immunocompromised conditions (such as autoimmune disease, receiving prednisone and cyclosporine, polymorphous lymphoproliferative disorders, MTX-associated lymphoproliferative disorders, post-transplant lymphoproliferative disorders, and

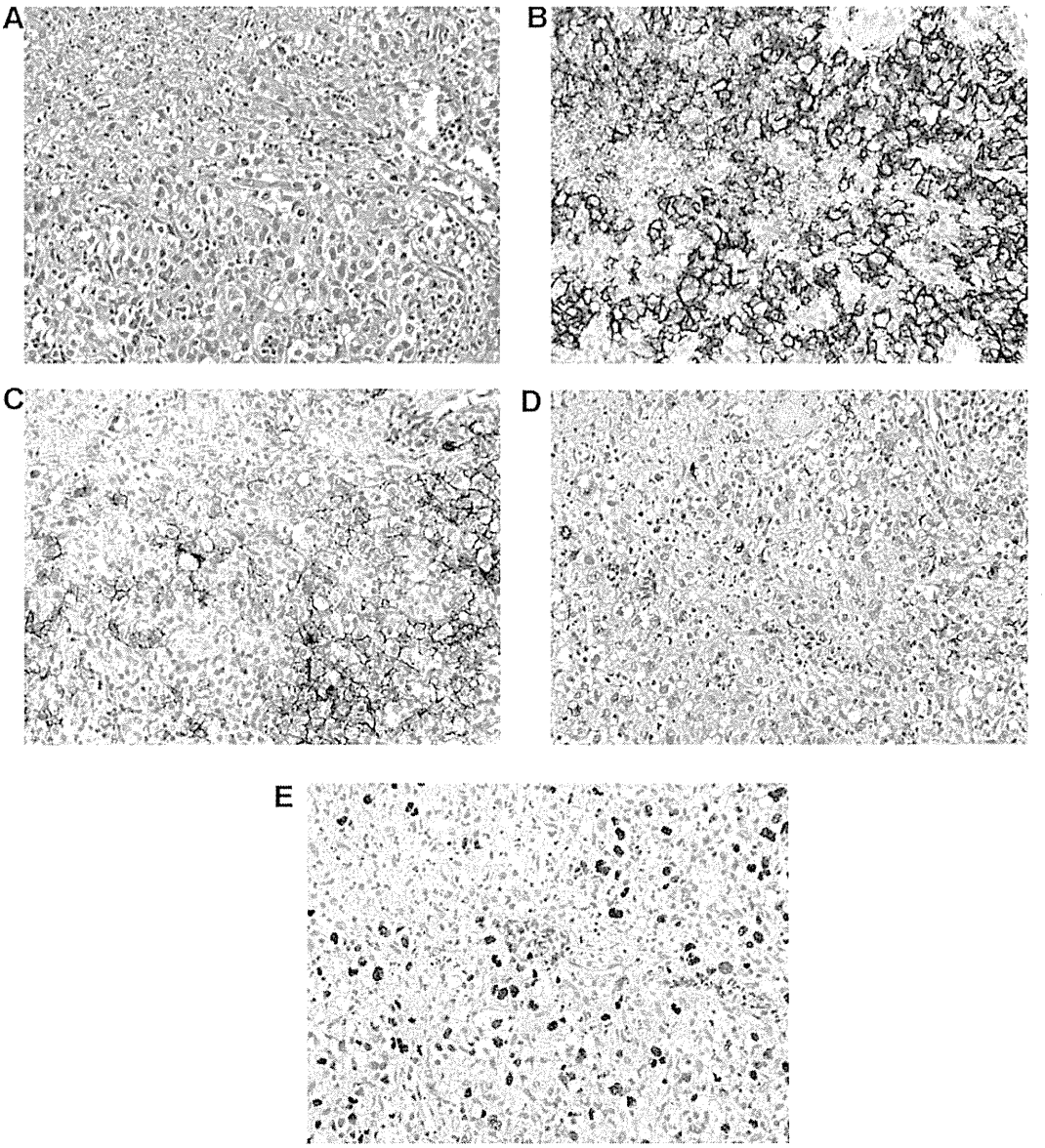

Fig. 1. **A**: Diffuse proliferation of large lymphoid cells with prominent nucleoli. H&E. Large lymphoid cells expressed CD20 (**B**) and less frequently CD30 (**C**), and LMP-1 (**D**). In situ hybridization with the EBER probe revealed positive signals in the nuclei of large lymphoid cells (**E**). All from the same case. Magnification: 400×.

Hodgkin-like lymphoproliferative disorders) were re-included in this study, the EBV-positive rate reached 5.2% or 1.9% using the >20% or >50% criteria, respectively.

The EBV genome has been detected in various kinds of malignant lymphomas, and latent infection genes of EBV, including LMP-1, show transforming activity in infected cells. LMP-1 serves as a target molecule for host cytotoxic T-lymphocytes (CTL); thus, B-lymphocytes expressing LMP-1 are recognized and eliminated by host CTL under normal immune conditions [Murray et al., 1992]. Of the present 14 EBV-positive cases, tumor cells expressed LMP-1 in eight.

Overall survival was reported to be significantly worse in EBV-positive diffuse large B-cell lymphoma cases than in EBV-negative cases [Oyama et al., 2007; Park et al., 2007]. In fact, this claim of a poor prognosis was one reason for incorporating the provisional entity, "EBV-positive diffuse large B-cell lymphoma of the elderly" (>50 years old), in the recently published textbook regarding the WHO classification (2008) [Nakamura et al., 2008]. In this study, however, overall survival rates of EBV-positive and -negative cases were not statistically different. In the present series from OLSG, about 90% of cases of diffuse large B-cell lymphoma were diagnosed at >50 years old. Two of 16 EBV-

TABLE I. Brief Clinical Findings and EBV-Positive Rate of Diffuse Large B-Cell Lymphoma in the Present and Reported Cases

		Western countries		East Asian countries		
	Present series (n = 484)	Switzerland, Italy, and Austria[a] (n = 341)	US[b] (n = 90)	Korea[c] (n = 380)	Japan[d] (n = 1,792)	Japan[e] (n = 114)
Age (years)						
Range (mean or median)	16–95 (mean: 67.0; median: 68)	M: 12–90 (mean: 64; median: 67) F: 18–93 (mean: 65; median: 67)	NA	18–95 (median: 56)	NA	11–89 (mean 64.1)
% of cases >50 years	89.7%	71.3%	NA	NA	80.5%	NA
% of cases >60 years	74.0%	NA	100%	36.6%	60.6%	NA
Sex ratio (M/F)	1.29	1.11	NA	1.39	NA	1.43
Immunologic abnormalities	Absent	Absent	Absent	Absent	Present in some cases[f]	NA
Criteria for EBV-positive rate (% among examined cases)						
≧5%	5.4%	NA	0%	NA	NA	NA
≧10%	4.3%	3.1%	0%	NA	NA	NA
≧20%	3.3%	2.7%	0%	8.9%	NA	NA
≧50%	1.0%	1.9%	0%	NA	13.6%	11.4%

NA, not available.
[a]Hoeller et al. [2010].
[b]Gibson and Hsi [2009].
[c]Park et al. [2007].
[d]Oyama et al. [2007].
[e]Kuze et al. [2000].
[f]Exact number was not shown.

positive cases were under 50 years old (38 and 27 years old).

In conclusion, this study revealed that EBV positivity in diffuse large B-cell lymphoma of immunocompetent patients in Japan is rather similar to that in Western countries. Careful evaluation of patient backgrounds and the adoption of common criteria for EBV positivity are essential in comparing geographical differences in EBV positivity in diffuse large B-cell lymphoma.

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Frequency of Myeloid Dendritic Cells Can Predict the Efficacy of Wilms' Tumor 1 Peptide Vaccination

SATOSHI OHNO[1,2,3*], FUMIHIDE TAKANO[4*], YASUYUKI OHTA[4], SATORU KYO[3], SUBARU MYOJO[3], SATOSHI DOHI[3], HARUO SUGIYAMA[5], TOMIHISA OHTA[4] and MASAKI INOUE[3]

[1]*Consolidated Research Institute for Advanced Science and Medical Care, Waseda University, Tokyo, Japan;*
[2]*Department of Surgery, Institute of Gastroenterology, Tokyo Women's Medical University, Tokyo, Japan;*
[3]*Department of Obstetrics and Gynaecology, Kanazawa University, Graduate School of Medical Science, Ishikawa, Japan;*
[4]*Department of Pharmacognosy and Chemistry of Natural Products, School of Pharmacy and Pharmaceutical Sciences, Kanazawa University, Ishikawa, Japan;*
[5]*Department of Functional Diagnostic Science, Osaka University, Graduate School of Medicine, Osaka, Japan*

Abstract. *Background: The object of this study was to investigate the clinical predictive capability of peripheral myeloid dendritic cells (DCs) in Wilms' tumor 1 (WT1) vaccine therapy for patients with gynaecological cancer. Patients and Methods: Six patients with WT1/human leukocyte antigen (HLA)-A*2402-positive gynaecological cancer were included in this study. The patients received intradermal injections of a modified 9-mer WT1 peptide every week for 12 weeks. Peripheral blood samples were obtained at 0, 4, 8 and 12 weeks after the initial vaccination. Circulating DCs were detected by flow cytometry. Results: The frequencies of $CD14^+CD16^+CD33^+CD85^+$ myeloid DCs were significantly higher in the therapeutically effective group than in therapeutically inert group ($p<0.05$). Conclusion: These results suggested that myeloid DCs, which should be associated with inducing cytotoxic T-cells, provided additional prognostic information in the use of cancer peptide vaccine.*

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

WT1 was isolated as a gene responsible for a childhood renal neoplasm, Wilms' tumor (3, 4). This gene encodes a zinc finger transcription factor and plays important roles in cell growth and differentiation (5, 6). Although the *WT1* gene was categorized at first as a tumor suppressor gene, it has recently been demonstrated that the wild-type *WT1* gene performed an oncogenic rather than a tumor-suppressor function in many kinds of malignancies (7). The *WT1* gene is highly expressed in various types of cancer, including gynaecological cancer (8, 9).

We have 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 (10). The WT1 peptide vaccine emulsified with Montanide ISA51 adjuvant and administered at a dosage of 0.3, 1.0, or 3.0 mg at 2-week intervals was safe for patients, other than for those with myelodysplastic syndromes. Furthermore, it has been confirmed that the potential toxicities of the weekly WT1 vaccination treatment schedule (3.0 mg per body) with the same adjuvant were also acceptable (11). In the past, clinical response to WT1 peptide-based immunotherapy in phase II trials with the weekly WT1 vaccinations has been reported for renal cell carcinoma (12), multiple myeloma (13), glioblastoma multiforme (14) and gynaecologic malignancy (15).

In clinical studies, the identification of predictive factor 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 tumor-associated antigen (TAA)-specific cytotoxic T lymphocytes (CTLs). Klebanoff *et al.* reported that not only the induction of effector CTLs but also maintenance of memory CTLs are required for ideal antitumor immune response in tumor-bearing patients (16).

*Both authors contributed equally to this work.

Correspondence to: 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), myeloid dendritic cell, cancer vaccine, immunotherapy.

Moreover, Fujiki *et al.* confirmed that occurrence of an antigen-specific helper T-cell (Th) response could predict good clinical response of CTL epitope vaccination (17). In animal models, Klages *et al.* showed that depletion of FOXP3 (+) regulatory T-cells (Tregs) had the potential to evoke efficient antitumor responses (18).

Dendritic cells (DCs) are immune cells forming part of the mammalian immune system. Their main function is to process antigen material and present it on their surface to other cells (*e.g.* Th and CTLs) of the immune system. To date, however, the role of DCs, which should be associated with inducing CTL in cancer immunotherapy, remains unclear.

In the present study, we investigated the clinical predictive capability of peripheral myeloid DCs in WT1 vaccine therapy for patients with gynaecological cancer.

Patients and Methods

The WT1 peptide. The immunization consisted of an HLA-A*2402-restricted, modified 9-mer WT1 peptide (amino acids 235-243 CYTWNQMNL), in which Y was substituted for M at amino acid position 2 (the anchor position) of the natural WT1 peptide. This variant induces stronger cytotoxic activity than the natural peptide (19). The WT1 peptide [Good Manufacturing Practice (GMP) grade] was purchased from Multiple Peptide Systems (San Diego, CA, USA) as lyophilized peptides.

Trial protocol. The entry criteria were as follows: 16-79 years of age; expression of WT1 in the cancer cells determined by immunohistochemical analysis; HLA-A*2402-positivity; estimated survival of more than 3 months; performance status 0-1; no severe organ function impairment and the written informed consent of the patient. At least 4 weeks prior to immunotherapy, the patients were free from antitumor treatments such as surgery, chemotherapy and radiation. Patients with brain metastasis were excluded. The protocol was approved by the Institutional Review Board and the Ethical Committee at Kanazawa University.

Vaccination. The patients received intradermal injections of 3.0 mg of HLA-A*2402-restricted modified 9-mer WT1 peptide emulsified with Montanide ISA51 adjuvant (SEPPIC S.A., Paris, France). The WT1 vaccinations were scheduled to be given weekly for 12 consecutive weeks.

Preparation of peripheral blood mononuclear cells (PBMCs). Peripheral blood samples from individual patients enrolled in the clinical trial were collected at 0, 4, 8 and 12 weeks. Collected blood in the vacutainer tube was transferred to a 50 ml conical tube (BD Falcon, Franklin Lakes, NJ, USA), diluted to a volume of 30 ml with HBSS (Gibco Invitrogen Corporation, Grand Island, NY, USA), and underlayed with 10 ml of Ficoll-Paque PLUS™ (GE Healthcare UK Ltd.). The 50 ml tubes were centrifuged at 400 × *g* for 30 min, after which the PBMCs were collected at the interface layer. PBMCs were collected by gently inverting the collection tube several times and drawing off the PBMCs containing plasma with a pipette. PBMCs from both sets of tubes were washed twice with HBSS and counted for recovery and viability using 0.4% Trypan Blue (Sigma, St. Louis, MO, USA).

Flow cytometric analysis. Flow cytometric analysis of stained DCs in PBMCs was performed on a flow cytometer (FACScalibar™; Becton Dickinson, San Diego, CA, USA). An acquisition gate was established based on a forward scatter and side scatter parameter that included only white blood cells, except for dead cells and debris as illustrated in Figure 1A.

Immunophenotyping of circulating DCs. To evaluate the phenotype of DCs in PBMCs isolated from the vaccinated patients, we used a panel of fluorescein isothiocyanate (FITC)- or phycoerytrin-conjugated monoclonal antibodies: mouse anti-human CD14/CD16 and mouse anti-CD33/CD85k, as well as FITC- or PE-conjugated isotype control antibodies (IgG2a and IgG1; Beckman Coulter, Hialeah, FL, USA). PBMCs (1×10^6 cells) were washed twice with ice-cold phosphate-buffered saline (PBS), and the resultant cells were counted and resuspended in PBS. Cells were stained directly with fluorochrome conjugated with specific antibodies or isotype control antibodies. After 30 min of incubation at 4°C in the dark, the cells were washed and resuspended in the same buffer. The DC population in the PBMCs was analyzed using flow cytometry as described below.

Data were acquired using CellQuest software (Becton Dickinson). Between 10,000 and 20,000 events were acquired per sample. All data are indicated as quadrant analysis in the PBMC gate, and were representative, being derived from triplicate analyses.

Evaluation of clinical response. After the WT1 vaccine was administered 12 times, the antitumor effect of the treatment was assessed by determining the response of the target lesions on 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 internationally approved RECIST guideline was originally developed for the evaluation of chemotherapy. However, peptide immunotherapy, especially if peptide is administered alone without adjuvant, may not lead to such drastic tumor regression as in chemotherapy. It is probable that some cancer patients treated with cancer vaccines can survive long-term without remarkable tumor regression (12-15). Their tumors could be stabilized or could regress following a temporary increase in size after vaccination since, in general, peptide-based immunotherapy does not act as quickly as chemotherapy due to the time needed to induce lymphoid activation. For this reason, it might be allowable to modify the RECIST guideline according to peptide-based immunotherapy. In this study, an assessment strategy in which the baseline of the sum of the longest diameters of the target lesions was shifted to 1 month after the initial WT1 vaccination was defined as 'modified RECIST'.

Statistical analysis. Differences between test groups were analyzed using Student's *t*-test. Calculations were performed using the statistical software package StatVeiw (Abacus Concepts, Berkeley, CA, USA).

Results

Patient characteristics. During the trial period, 6 patients were evaluated for frequencies of DCs at 0, 4, 8 and 12 weeks. The mean age of the 6 enrolled patients was 55.7 years (range 43-64 years). A summary of the patient's characteristics and response to WT1 immunotherapy is shown in Table I.

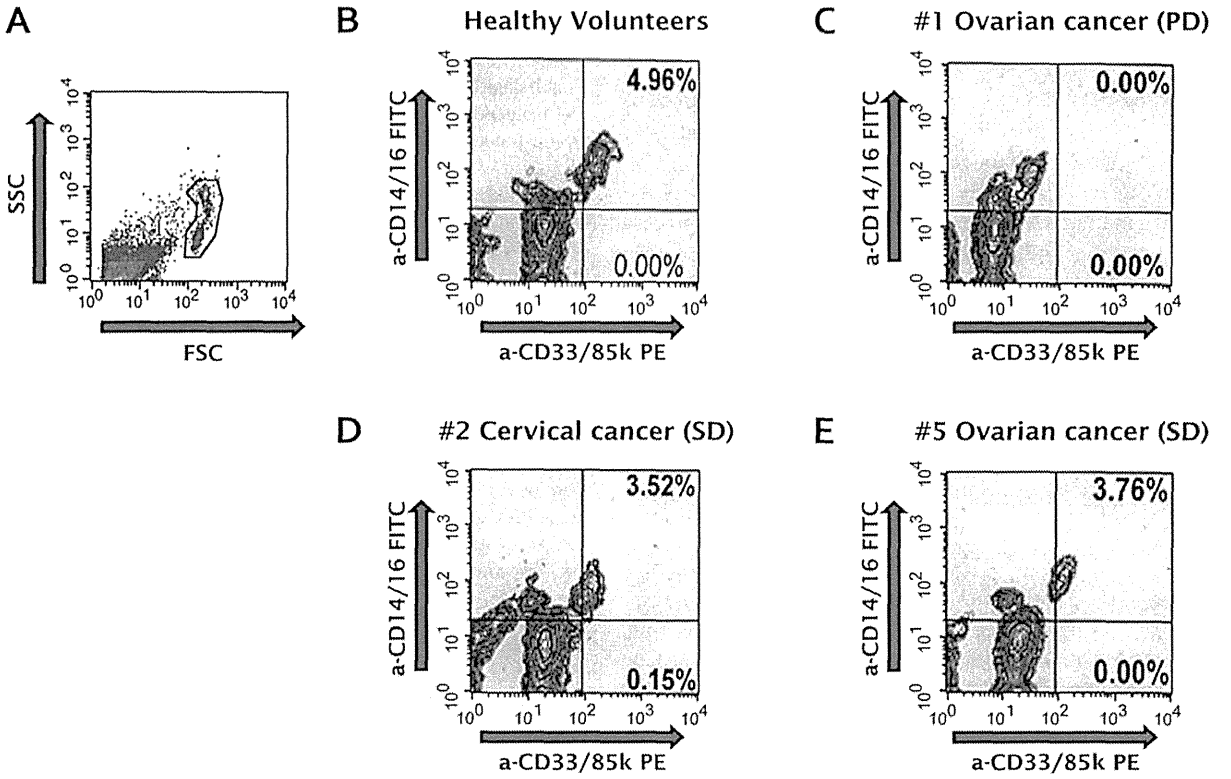

Figure 1. *Flow cytometric profiles of DCs in peripheral blood mononuclear cells (PBMCs). PBMCs collected from a healthy volunteer or cancer patients were stained with lineage-specific FITC- or PE-conjugated markers including anti-CD14-, anti-CD16-, anti-CD33- and anti-CD85 monoclonal antibodies. Gates to include viable PBMCs for analysis were set by forward and side scatter to delineate DCs. A: Dot-plot analysis of unlabeled PBMCs; B: quadrant analysis and population of lineage-specific markers for myeloid DC positive in PBMCs from a healthy volunteer; C: quadrant analysis and population of lineage-specific markers for myeloid DCs in PBMCs from a typical cancer patient treated with WT1 in the group with progressive disease; D and E: quadrant analysis and population of lineage-specific markers for mature DCs in PBMCs from two typical cancer patients-treated with WT1 in the group with stable disease.*

Analysis of DCs in cancer patients with WT1 vaccination. We evaluated the mature myeloid DCs ($CD14^+$-, $CD16^+$-, $CD33^+$- and $CD85^+$-positive cells) in PBMCs collected from healthy volunteers and the cancer patients with vaccination. As illustrated in Figure 1B, the population of myeloid DCs in PBMCs of healthy volunteers composed 4.96%. In contrast, the frequencies of myeloid DC in PBMCs from cancer patients divided into PD or SD groups were 0.0% (Figure 1C; in PD), 3.52% (Figure 1D; first case in SD) and 3.76% (Figure 1E; second case in SD), respectively.

Each population of peripheral myeloid DCs in the 6 cancer patients was compared according to the clinical response. The frequency of $CD14^+CD16^+CD33^+CD85^+$ PBMCs was significantly higher (p=0.0374) in the SD (3.206±0.543%) group than in PD group (2.026±1.443%) (Figure 2A). A significant difference (p=0.0027) between SD (3.182±0.520%) and PD (1.657±1.472%) groups was also observed using the 'modified RECIST' assessment (Figure 2B).

Table I. *Patient characteristics.*

No.	Age (years)	Gender	Diagnosis	RECIST	Modified RECIST
1	62	F	Ovarian cancer	PD	PD
2	57	F	Cervical cancer	SD	SD
3	43	F	Cervical cancer	PD	SD
4	55	F	Endometrial cancer	PD	PD
5	53	F	Ovarian cancer	SD	SD
6	64	F	Ovarian cancer	PD	PD

PD: Progressive disease; SD: stable disease.

Discussion

The present study demonstrated that the percentage of circulating myeloid DCs in patients with therapeutical effectiveness of cancer peptide vaccination were significantly

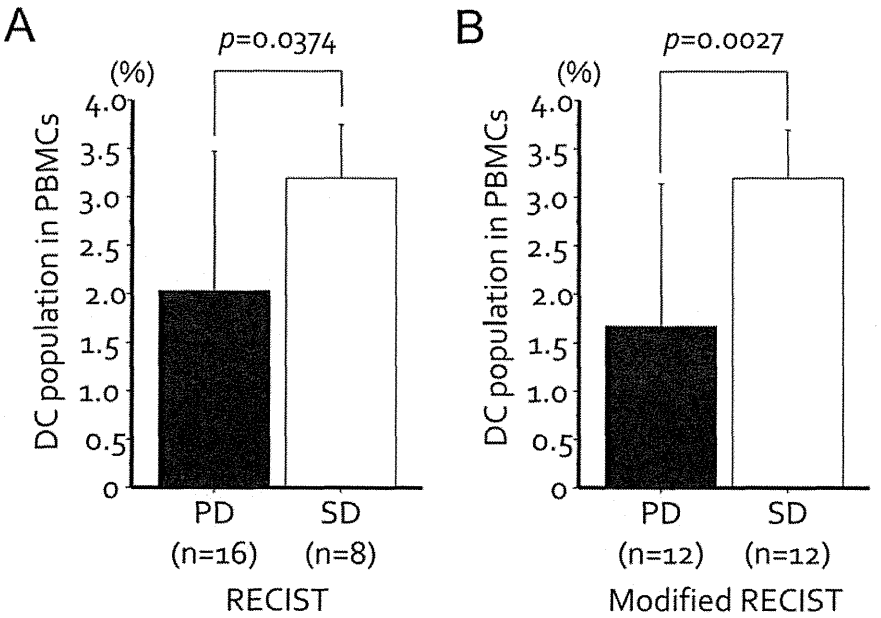

Figure 2. *Validation for circulating myeloid DCs in peripheral blood. Among the 6 gynaecological cancer patients with WT1 peptide vaccination, those in the progressive disease (PD) group (black histograms) showed a depletion of the myeloid DC subset, which was statistically significant compared with DC frequencies in those with stable disease (SD) (open histograms).*

higher than in those with therapeutical inertness. Recent studies point to a numerical decrease and sometimes even functional impairment of circulating DC subsets in various pathologies. In hematopoietic cancer patients, DC counts may be significantly reduced in lymphoid or myeloid leukemia (21, 22). A similar observation was made for certain solid cancers (23). Furthermore, numbers of circulating DCs are reduced in patients with metastatic cancer as compared to those with localized cancer (24). These findings suggest that DC deficiency may play a role in inducing cancer-related immunosuppression.

Moreover, chemotherapeutic techniques have a range of side-effects that depend on the type of medication used. The most common medications mainly affect the fast-dividing cells of the body, such as blood cells. Virtually all chemotherapeutic regimens can cause depression of the immune system, often by inactivating the bone marrow and leading to a decrease of white blood cells, red blood cells and platelets. In very severe myelosuppression, which occurs in some regimens, almost all the bone marrow stem cells (cells that produce white and red blood cells) are destroyed. Bone marrow has recently been shown to be an important site for T-cell priming and reactivation, generation of T-cell memory and recruitment of large amounts of circulating memory T-cells and antigen-loaded DCs (25-29). Therefore, myelosuppression associated with chemotherapy may block CTL activation in cancer patients.

In patients with advanced cancer, the basal metabolic rate declines and cachexia occurs. The pathophysiological pathway of cachexia is thought to be secondary to stimulation by enhanced levels of pro-inflammatory cytokines. Elevation of tumor necrosis factor-α and other plasma cytokines has been demonstrated in many conditions associated with cachexia (30). Cachexia is often associated with breakdowns in the host immune system and may result in reduced therapeutic response of peptide vaccine.

In tumor immunosurveillance, it is generally thought that $CD8^+$ CTLs are the main effector cells because they can effectively expand and kill malignant cells. Therefore, the most common approaches to combat tumors have centered on the induction of TAA-specific CTLs. In this study, the activity of WT1 peptide alone was examined and adjuvant that would activate DCs with subsequent induction of CTLs was not included. To enhance the therapeutic efficacy of cancer peptide vaccination, the use of a more suitable adjuvant, such as bacillus Calmette-Guerin cell-wall skeleton (31), granulocyte-macrophage colony-stimulating factor (32, 33), CpG (34), interferon-α (35) and interleukin-2 (36) should be allowed.

In conclusion, the demonstration of a diminished percentage of DCs in peripheral blood might represent a new interesting biological marker predicting a poor prognosis in patients treated with WT1 peptide vaccination. The reduced DC numbers may contribute to reduced therapeutic response and thus restoration of DCs may be a goal for cancer

peptide-based immunotherapy. The present study gives us an indication of enhancement of clinical response in WT1 protein-targeted immunotherapy.

Acknowledgements

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WT1 Peptide Vaccine Stabilized Intractable Ovarian Cancer Patient for One Year: A Case Report

SATOSHI DOHI[1], SATOSHI OHNO[1,2], YUMIKO OHNO[1], MASAHIRO TAKAKURA[1], SATORU KYO[1], GEN-ICHIRO SOMA[3,4], HARUO SUGIYAMA[5] and MASAKI INOUE[1]

[1]*Department of Obstetrics and Gynecology, Kanazawa University, Graduate School of Medical Science, Ishikawa, Japan;*
[2]*Consolidated Research Institute for Advanced Science and Medical Care, Waseda University, Tokyo, Japan;*
[3]*Institute for Health Sciences, Tokushima Bunri University, Tokushima, Japan;*
[4]*Center for Drug Delivery Research, Tokyo University of Science, Chiba, Japan;*
[5]*Department of Functional Diagnostic Science, Osaka University Graduate School of Medicine, Osaka, Japan*

Abstract. *We report on Wilms tumor (WT1) peptide immunotherapy in a patient with intractable ovarian cancer patient over an extended period. Case Report: Immunotherapy using WT1 peptide has been undergoing clinical trials for gynecological cancer. We used WT1 peptide vaccination to treat a 53-year-old woman suffering from ovarian cancer with peritoneal dissemination. After 2 months, her pleural and cardiac effusion had disappeared, and the sum of the longest diameter of the target lesion (in the pelvic mass) was reduced. There was a weak positive correlation between CA125 and mononuclear phagocyte/lymphocyte ratio (Spearman's ϱ=0.275, p=0.015). Intradermally administered WT1 peptide vaccination in a case of intractable ovarian cancer stabilized the disease over the course of one year. However, the immunotherapeutic mechanism of WT1 peptide and immunological escape mechanism for carcinoma cells remain to be elucidated.*

Ovarian cancer is one of the most common gynecological malignancies in Japan. Its frequency has dramatically increased in the last decade. Although there are well-established surgical and chemothera-peutic treatments, the need for molecular-target therapy has increased, especially for recurrent disease that has acquired radio- or chemoresistance.

Correspondence to: Satoshi Dohi, Department of Obstetrics and Gynecology, Kanazawa University, Graduate School of Medical Science, 13-1 Takaramachi, Kanazawa, Ishikawa, 920-8640 Japan. Tel: +81 762652425, Fax: +81 762344266, e-mail: satoshi.dohi1018@gmail.com

Key Words: WT1 peptide vaccine, immunotherapy, ovarian cancer.

The Wilms' tumor gene *WT1* has been isolated and identified as a gene responsible for a childhood renal neoplasm, Wilms' tumor (1-3). This gene encodes a zinc finger transcription factor and plays important roles in cell growth and differentiation (4, 5). Although *WT1* was first categorized as a tumor-suppressing gene, it was recently demonstrated that the wild-type *WT1* possessed an oncogenic rather than tumor-suppressing function in many kinds of malignancies (6). *WT1* is highly expressed in hematological malignancies and solid tumors, including ovarian cancer (7, 8).

WT1 is now regarded as a molecular target for immunotherapy in various malignant tumor types. Clinical trials of WT1 peptide-based cancer immunotherapy are ongoing: WT1 peptide vaccination has been shown to be safe and clearly effective against several kinds of malignancies (9-13).

Ohno *et al.* reported that twelve patients with WT1/human leukocyte antigen (HLA)-A*2402-positive gynecological cancer were included in a phase II clinical trial of WT1 vaccine therapy. This study evaluated clinical response after a WT1 vaccine was administered 12 times over three months and found that WT1 vaccine therapy for patients with gynecological cancer was safe and produced clinical responses: stable disease (SD) in 3 patients and progressive disease (PD) in 9 patients (13).

In the following study, we report a case of intractable ovarian cancer in which an intradermally administered WT1 peptide vaccination stabilized the disease over the course of a year.

Clinical study. This case report concerns a patient from our WT1 peptide vaccination study. Entry criteria for the study were as follows: 16-79 years of age, immunohistochemical expression of WT1 in cancer cells of more than 3 months, performance status 0-1, no severe organ function impairment and the written informed consent of the patient. At least 4

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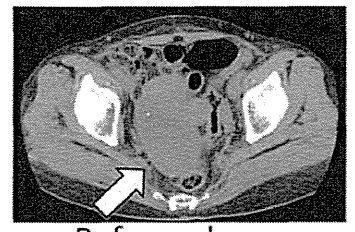


Month	0	1	2	3	5	8	12
SLD [mm]	96.6	97.8	84.2	82.7	80.0	80.0	112.9
Percentage change		+1.3	−12.8	−14.7	−17.2	−17.2	−16.9

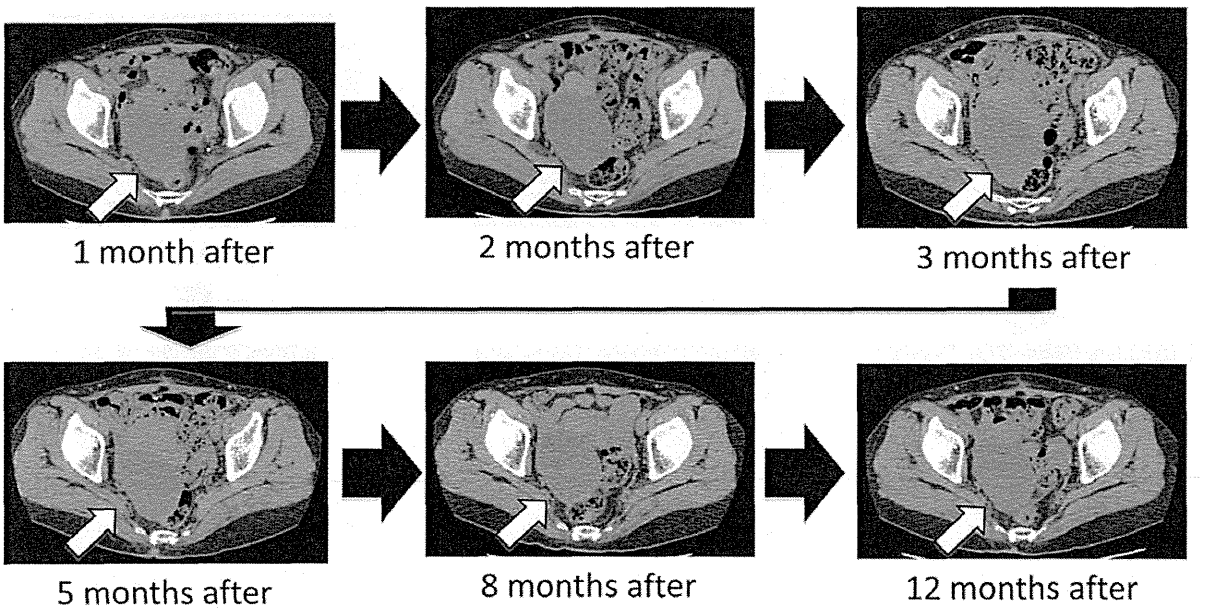


Figure 1. *Computed tomography of the patient before, during and after treatment. White arrows indicate primary tumor mass. SLD, Sum of the longest diameters.*

weeks prior to immunotherapy, the patient had to be free from antitumor treatments such as surgery, chemotherapy and radiotherapy. The protocol was approved by the Institutional Review Board and the Ethical Committee at Kanazawa University.

WT1 peptide treatment plan. The WT1 peptide vaccine consists of an HLA-A*2402-restricted, modified 9-mer WT1 peptide (amino acids 235-243 CYTWNQMNL), in which Y is substituted for M at amino acid position 2 (the anchor position) of the natural WT1 peptide. The WT1 peptide [Good Manufacturing Practice (GMP) grade] was purchased from Multiple Peptide Systems (San Diego, CA, USA) as lyophilized peptide.

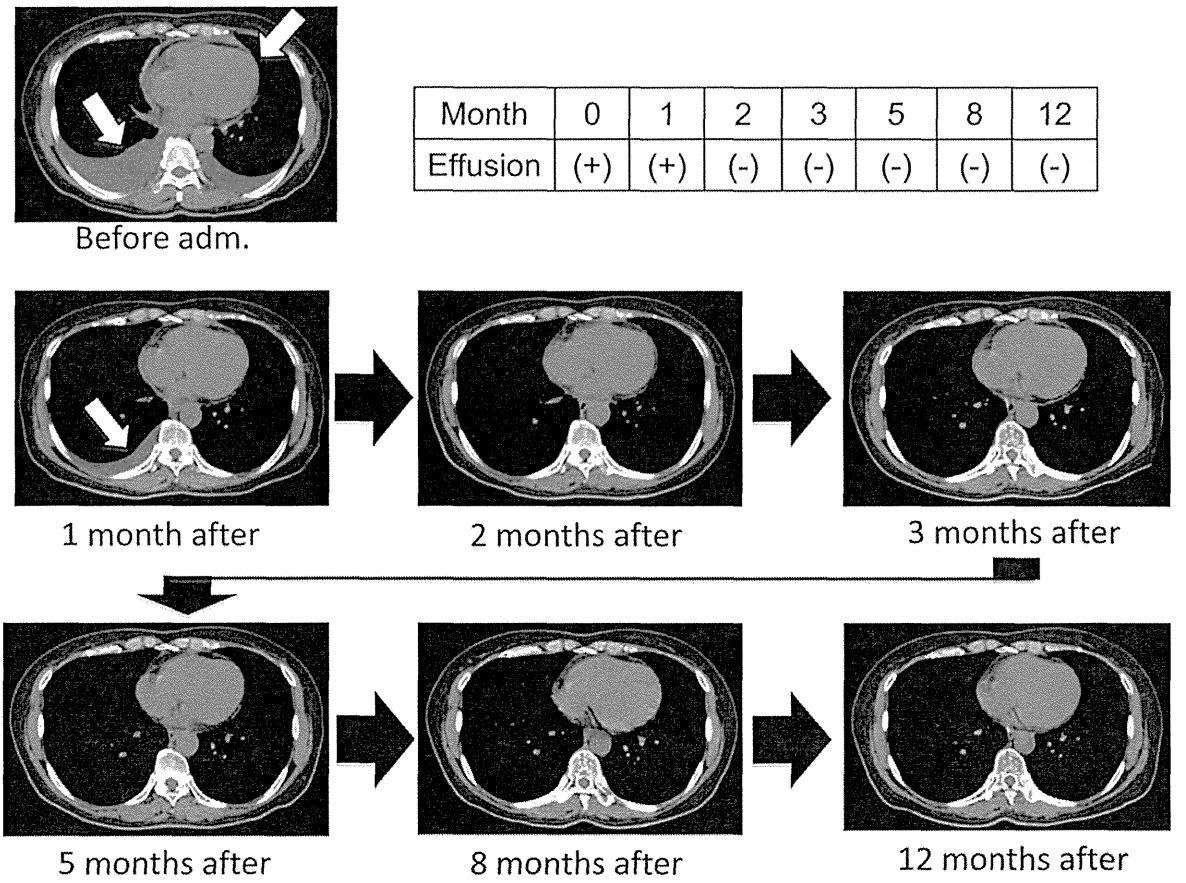
Patients received intradermal injections of 3.0 mg HLA-A*2402-restricted adjuvant (EPPIC S.A., Paris, France). Vaccinations were scheduled weekly for 12 consecutive weeks (13). Efficacy was based on computed tomography (CT) obtained at baseline and after 4, 8 and 12 weeks exposure to the vaccine.

Case Report

A 53-year-old woman was diagnosed as having serous ovarian adenocarcinoma in November 2007. After omentectomy of a pelvic mass with peritoneal dissemination, tri-weekly combination chemotherapy with paclitaxel and carboplatin produced SD and tumor shrinkage of 25%. This was followed by weekly administration of docetaxel, which also contributed to SD.

The patient participated in our WT1 vaccine trial beginning in October 2008. She received HLA-A*2402, and met the inclusion criteria for the phase II clinical study. She was administered WT1 at weekly intervals.

Decrease in tumor size and normalization of tumor marker (CA125). According to the internationally approved Response Evaluation Criteria in Solid Tumors (RECIST) guidelines, sum of the longest diameter (SLD) of the target (pelvic) lesion was reduced: the length was 96.5 mm before administration, 97.8 mm (+1.3%) after 1 month, 84.2 mm (–12.8%) after 2 months,



Month	0	1	2	3	5	8	12
Effusion	(+)	(+)	(-)	(-)	(-)	(-)	(-)

Figure 2. *Computed tomography of the patient before, during and after treatment. White arrows indicate pleural and cardiac effusion.*

and 82.7 mm after 3 months (–14.7%); after 3 months' administration, SLD continued to decrease (Figure 1). In addition, pleural and cardiac effusions disappeared within two months of beginning administration, and remained absent (Figure 2). Dosage of vaccine was increased from 41 units/ml to 160 units/ml after 1 month of administration, and the level of CA125 normalized after 3 months. According to the patient's wishes, we continued to administer the vaccine, with concomitant normalization/suppression of CA125 (Figure 3a).

No adverse effects of vaccination were observed other than a local inflammatory response with erythema at the injection sites.

Mononuclear phagocyte/lymphocyte ratio (Mo/Ly ratio) and CA125. To evaluate the immunological response to WT1 peptide vaccination, we analyzed the correlation between CA125 and the Mo/Ly ratio (Figure 3b). A weak positive correlation was observed (Spearman's ϱ=0.275, p=0.015).

Although WT1 peptide vaccination was continued for one year, CA125 began to gradually increase approximately 9 months following initial administration (Figure 3a). The patient subsequently dropped out of the clinical trial due to receiving another newly approved chemotherapy regimen for ovarian cancer.

Discussion

Ovarian cancer is a common malignant gynecological cancer of perimenoposal women. Patients with metastatic disease have a poor prognosis, with 5-year progression-free survival usually less than 30% in Japan.

Our patient had primary disease in the ovary, with metastases in the uterus, peritoneum, pelvic lymph nodes and omental. She also developed a pelvic mass with ascites and pleural effusion during chemotherapy, indicating poor response to chemotherapy. Because of her poor prognosis, she was selected for WT1 peptide immunotherapy. Immediately following inception of peptide immunotherapy, the size of the pelvic mass increased, but within two months, a decrease in tumor size and normalization of the level of tumor marker

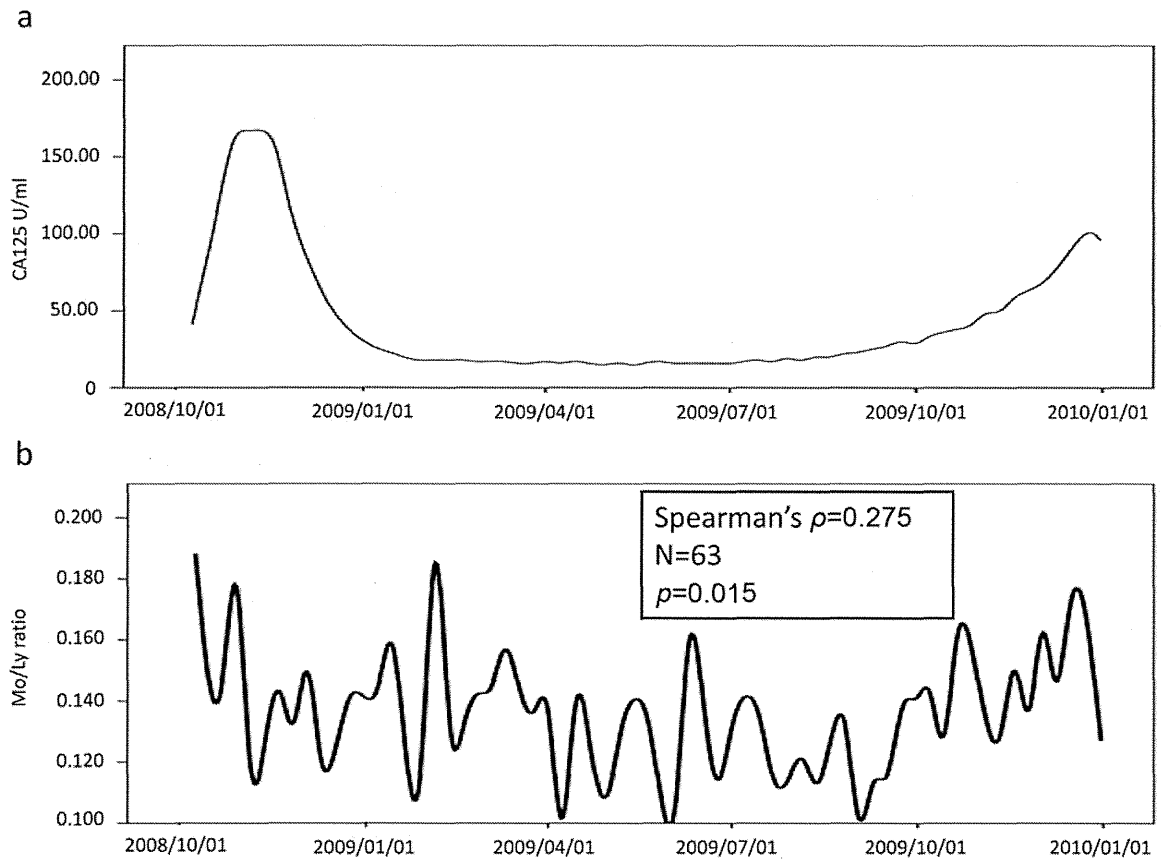


Figure 3. *a: Time series of CA125 by WT1 peptide (weekly subcutaneous 3 mg injection). Normal level <35 units/ml. b: Time series of mononuclear phagocyte/lymphocyte ratios (Mo/Ly ratio). There was a weak positive correlation between CA125 and Mo/Ly ratio.*

CA125 were observed. Despite the initial resistance to chemotherapy, stabilization of her disease for nearly a year suggests the efficacy of WT1 peptide vaccination.

Alterations of peripheral monocytes and lymphocytes might be good parameters for evaluating immunologic status and predicting recurrence in patients with gastric cancer (14). In our case, there was a weak positive correlation between CA125 and the Mo/Ly ratio (Spearman's ϱ=0.275, p=0.015). We believe in this case that the Mo/Ly ratio indicated immunologic status and predicted recurrence.

Belli *et al.* reported that in a study of 28 patients with resected metastatic melanoma, two showed complete response and three manifested long-term disease stabilization with HSPPC-96 (autologous tumor-derived heat-shock protein GP96-peptide complex) vaccine (15). Bolonaki *et al.* also reported that disease stabilization occurred in 8 out of 22 patients with advanced non-small cell lung cancer vaccinated with an optimized cryptic human telomerase reverse transcriptase peptide (16). Ohta *et al.* reported a case of WT1 peptide immunotherapy for metastatic childhood rhabdomyosarcoma, with patient remission continuing more than 22 months (17). This latter case is significant, as there are few reports of long-term WT1 peptide vaccination for cancer – apart from our own, where the vaccine stabilized intractable ovarian cancer over a year.

Unfortunately, the pathogenesis of ovarian cancer relapse is unknown. In addition, the immunotherapeutic mechanism of WT1 peptide and immunological escape mechanism for carcinoma cells remain to be elucidated.

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ORIGINAL ARTICLE

Gemcitabine enhances Wilms' tumor gene WT1 expression and sensitizes human pancreatic cancer cells with WT1-specific T-cell-mediated antitumor immune response

Akitaka Takahara · Shigeo Koido · Masaki Ito · Eijiro Nagasaki · Yukiko Sagawa · Takeo Iwamoto · Hideo Komita · Toshiki Ochi · Hiroshi Fujiwara · Masaki Yasukawa · Junichi Mineno · Hiroshi Shiku · Sumiyuki Nishida · Haruo Sugiyama · Hisao Tajiri · Sadamu Homma

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Abstract Wilms' tumor gene (WT1), which is expressed in human pancreatic cancer (PC), is a unique tumor antigen recognized by T-cell-mediated antitumor immune response. Gemcitabine (GEM), a standard therapeutic drug for PC, was examined for the regulation of WT1 expression and the sensitizing effect on PC cells with WT1-specific antitumor immune response. Expression of WT1 was examined by quantitative PCR, immunoblot analysis, and confocal microscopy. Antigenic peptide of WT1 presented on HLA class I molecules was detected by mass spectrometry. WT1-specific T-cell receptor gene–transduced human T cells were used as effecter T cells for the analysis of cytotoxic activity. GEM treatment of human MIAPaCa2 PC cells enhanced WT1 mRNA levels, and this increase is associated with nuclear factor kappa B activation. Tumor tissue from GEM-treated MIAPaCa2-bearing SCID mice also showed an increase in WT1 mRNA. Some human PC cell lines other than MIAPaCa2 showed up-regulation of WT1 mRNA levels following GEM treatment. GEM treatment shifted WT1 protein from the nucleus to the cytoplasm, which may promote proteasomal processing of WT1 protein and generation of antigenic peptide. In fact, presentation of HLA-A*2402-restricted antigenic peptide of WT1 (CMTWNQMNL) increased in GEM-treated MIAPaCa2 cells relative to untreated cells. WT1-specific cytotoxic T cells killed MIAPaCa2 cells treated with an optimal dose of GEM more efficiently than untreated MIAPaCa2 cells. GEM enhanced WT1 expression in human PC cells and sensitized PC cells with WT1-specific T-cell-mediated antitumor immune response.

Keywords Pancreatic cancer · WT1 · Gemcitabine · NF kappa B · T-cell response

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A. Takahara · S. Koido · H. Komita · H. Tajiri
Division of Gastroenterology and Hepatology,
Department of Internal Medicine,
Jikei University School of medicine, Tokyo, Japan

M. Ito · Y. Sagawa · S. Homma (✉)
Department of Oncology, Institute of DNA medicine,
Jikei University School of Medicine,
3-25-8 Nishi-shimbashi Minato-ku, Tokyo 105-8461, Japan
e-mail: sahya@jikei.ac.jp

E. Nagasaki
Division of Oncology and Hematology,
Department of Internal Medicine,
Jikei University School of medicine, Tokyo, Japan

T. Iwamoto
Division of Biochemistry, Core Research Facilities,
Jikei University School of medicine, Tokyo, Japan

T. Ochi · H. Fujiwara · M. Yasukawa
Department of Bioregulatory Medicine,
Ehime University Graduate School of Medicine, Toon, Japan

J. Mineno
Center for Cell and Gene Therapy, Takara Bio, Inc., Otsu, Japan

H. Shiku
Department of Cancer Vaccine,
Mie University Graduate School of Medicine, Tsu, Japan

S. Nishida
Department of Cancer Immunotherapy,
Osaka University Graduate School of Medicine, Suita, Japan

H. Sugiyama
Department of Functional Diagnostic Science,
Osaka University Graduate School of Medicine, Suita, Japan

Introduction

Pancreatic cancer (PC) is a devastating disease with a 5% overall 5-year survival rate [1, 2]. This high mortality rate is due to a combination of factors that include a high incidence of metastatic disease at initial diagnosis, an aggressive clinical course, and the failure of systemic therapies used for treatment. Despite the fact that advanced loco-regional disease is found in 40% of patients [3], only 5–25% of patients with pancreatic cancer are treated surgically [4]. Even in cases where pancreatic cancer is discovered at a resectable stage, only 10–20% of patients are expected to survive for more than 5 years after curative resection [5].

Gemcitabine (GEM) is currently the most commonly used therapeutic drug prescribed in cases of advanced PC [6, 7]. Numerous phase III trials testing gemcitabine in combination with other cytotoxic drugs have failed to reveal any additional benefit compared with gemcitabine alone [8]. Erlotinib, a small molecule inhibitor of the epidermal growth factor receptor tyrosine kinase, is a notable exception in that it is the only drug reported to confer a significant improvement in survival over gemcitabine alone [9]. Recently, Folfirinox was reported to be a more efficient, but more toxic, regimen for pancreatic cancer and might be promising for the patients with good performance status [10]. Ultimately, improved treatment of advanced PC will likely require additional selected and targeted agents that provide the benefit of prolonged survival with minimum risk.

The Wilms' tumor gene WT1 encodes a zinc finger transcription factor. Although the WT1 gene was originally defined as a tumor suppressor gene [11–13], additional reports demonstrate that it is highly expressed in leukemia and various types of malignant tumors [14] and can confer oncogenic functions [15]. WT1-specific cytotoxic T lymphocytes (CTLs) and WT1 antibodies have both been shown to be induced spontaneously in tumor-bearing leukemia patients [16]. These results indicate that WT1 protein is highly immunogenic and establish it as a promising tumor antigen for recognition by specific CTLs [17]. The safety and clinical efficacy of major histocompatibility complex (MHC) class I-restricted WT1 epitope peptides against various malignancies have been confirmed in clinical immunotherapy trials [14, 15].

Reports indicate that WT1 is frequently overexpressed in human pancreatic cancer cells [18]. Recent clinical reports on treatments combining GEM drug therapy with peptide vaccine immunotherapy have demonstrated safe and promising results in cases of advanced PC [19, 20]. In our recent phase I clinical trial that tested a combination of WT1 peptide vaccine and GEM in treatment of advanced PC, several cases showed marked tumor regression (manuscript in preparation). These results suggest that the actions of WT1-targeted antitumor immunity and GEM can function synergistically against PC cells. In the present study, we demonstrate that GEM treatment up-regulates WT1 expression in PC cell lines, and that antitumor immune activity against PC cells via a WT1-specific T-cell response is augmented by GEM treatment.

Materials and methods

Cell lines, antibodies, and mice

Human pancreatic cancer cell lines (MIAPaCa2, PANC-1, AsPC-1, BxPC-3, Capan-1 and Capan-2) were obtained from the American Type Culture Collection (Manassas, VA, USA) [21]. A rabbit polyclonal antibody against WT1 (C-19) and a goat polyclonal antibody against Lamin B (C-20) were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Eight- to ten-week-old SCID mice were supplied by Nihon SCL Co., Ltd. (Hamamatsu, Japan) and were maintained in our specific pathogen-free facilities. Mice received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institute of Health (NIH publication 86-23 revised 1985).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Tissue or cell samples were lysed directly in Buffer RLT Plus (Qiagen, Hilden, Germany) and homogenized. Reverse transcription (RT) was performed using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). TaqMan primers and non-fluorescent quencher probes complementary to WT1 (Assay ID:Hs00240913_m1) and 18S ribosomal RNA (rRNA, Assay ID:Hs99999901_s1) genes were purchased from Applied Biosystems. qRT-PCR was performed using the 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). WT1 expression levels were normalized relative to those of 18S rRNA.

Inhibition of nuclear factor kappa B (NF-kB)

Inhibition of NF-kB activity in human PC cells was achieved using an NF-kB p65 (Ser276) inhibitory peptide kit (IMGENEX, San Diego, CA, USA). Briefly, MIAPaCa2 cells (6×10^4/well) were seeded in 24-well culture plates and incubated for 24 h. Growth medium was then changed to medium containing GEM (0 or 30 ng/ml) with NF-kB blocking peptide (50 μM) or control peptide (50 μM). After 24-h incubation, cellular expression of NK-kB was determined using qRT-PCR.

Immunoblot analysis

The nuclear fraction of MIAPaCa2 cells used for the detection of WT1 protein was isolated using an Active Motif extraction kit (Carlsbad, CA, USA). Protein samples (30 μg/well) separated by electrophoresis in 15% sodium dodecyl sulfate-polyacrylamide gels were then transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). After blocking in 5% nonfat milk for 1 h, membranes were exposed to antibodies specific to WT1 (1:100) and beta-actin (1:10,000; Sigma–Aldrich, St. Louis, MO, USA) and then to horseradish peroxidase-conjugated secondary antibodies. The ECL-PLUS Detection System (GE Healthcare, Buckinghamshire, UK) was used for chemiluminescent detection of secondary antibodies.

Confocal microscopy

MIAPaCa2 cells cultured on glass coverslips were incubated with or without GEM (30 ng/ml) for 24 h. Cells were then washed and fixed in 4% paraformaldehyde. Immunofluorescent visualization of cells expressing WT-1 was achieved by incubating slides in rabbit anti-WT1 antibody (1/200), followed by Amaxa488-conjugated donkey anti-rabbit IgG antibody (Molecular probes, Eugene, OR, USA). Cell nuclei were stained with TO-PRO-3 iodide (Molecular Probes), and a laser scanning confocal microscope (LSM510, CarlZeiss, Thornwood, NY, USA) was used to obtain fluorescence images.

Positive ion ESI LC–MS/MS analysis of MHC class I binding peptides from MIAPaCa2 cells

MIAPaCa2-bearing mice were injected intraperitoneally with PBS or GEM (3.75 mg/mouse). After 48 h, tumors were resected and digested using collagenase to obtain single cells. MHC class I binding peptides were isolated from 10^8 cells using the method described by Storkus et al. [22]. Isolated peptides were dissolved in 50% methanol and analyzed via electrospray ionization (ESI) liquid chromatography (LC)-tandem mass spectrometry (MS/MS) using a triple quadrupole mass spectrometer (Q TRAP) (Applied Biosystems, Foster City, CA, USA). The mass spectrometer interfaced with an Agilent 1100 liquid chromatography (Agilent Technologies, Wilmington, DE, USA) was employed. The WT1 antigenic peptide (aa 235–243 CMTWNQMNL; MW = 1,139.5 Da) in 50% methanol was easily produced m/z 1171.5 as a methanol adduct ion $(M + MeOH)^+$. The multiple reaction monitoring (MRM) transition monitored for the detection of this peptide was m/z 1,171.5/1,154.5. This peptide was eluted at a flow rate 0.2 mL/min from an Intersil C8-3 column [50 × 2.1 mm, 3 μm particle size] (GL Science Inc., Tokyo Japan) using a linear gradient of 9.5% min^{-1} of 5–100% acetonitrile containing 1% formic acid. To estimate cellular peptide concentrations, a standard curve was prepared by increasing concentrations (0–1,000 pmol) with chemically synthesized WT-1 antigenic peptide. The response was considered to be linear if the correlation coefficient (r^2) was greater than 0.99, calculated by least-squares linear regression analysis.

Cytotoxicity assay

WT1-specific cytotoxic effector cells were generated as described below. Full-length WT1-specific T-cell receptor (TCR) a/b genes (Va20/J33/Ca for TCR-a and Vb5.1/J2.1/Cb2 for TCR-b, respectively) isolated from the HLA-A*2402-restricted $WT1_{235-243}$-specific $CD8^+$ CTL clone TAK-1 [23] were cloned into a pMEI-5 retroviral vector (Takara Bio, Shiga, Japan). WT1-specific TCR genes were then transduced into normal $CD8^+$ lymphocytes as described previously [24]. Cytotoxicity assays were performed using a standard 4-h culture ^{51}chromium (Cr) release assay described elsewhere [25].

Statistical analysis

The significance of differences between groups was analyzed using Student's *t* test for two independent groups and with Tukey's test for multiple-group comparisons. Values that did not fit a Gaussian distribution were analyzed with the Bonferroni method for multiple-group comparisons.

Results

Up-regulation of WT1 mRNA in human PC cells by in vitro treatment with GEM

Proliferation of MIAPaCa2 cells was inhibited for 48 h with stable numbers of viable cells following treatment with 30 and 100 ng/ml of GEM (Fig. 1a). Growth of MIAPaCa2 cells was also impaired by treatment with 10 ng/ml of GEM for 72 h. Levels of WT1 mRNA were enhanced significantly by treatment of MIAPaCa2 cells with 10, 30, and 100 ng/ml of GEM for 24, 48 and, 72 h, respectively (Fig. 1b). Enhancement of WT1 mRNA was also observed after 2-h treatment with GEM (100 ng/ml) in following 72 h (Fig. 1c). This GEM-mediated enhancement was suppressed by the addition of NF-kB blocking peptide in the culture (Fig. 1d).

GEM-mediated up-regulation of WT1 mRNA expression was examined in various human pancreatic cancer cell lines. GEM-treated Capan-2 cells showed a significant enhancement of WT1 mRNA expression (Fig. 2a). Low steady-state levels of WT1 mRNA expression in AsPC-1 and BxPC-3 cells were also enhanced by GEM treatment (Fig. 2b). In contrast, expression of WT1 mRNA in Capan-1 and PANC-1 cells was not up-regulated by GEM treatment (Fig. 2b, c).

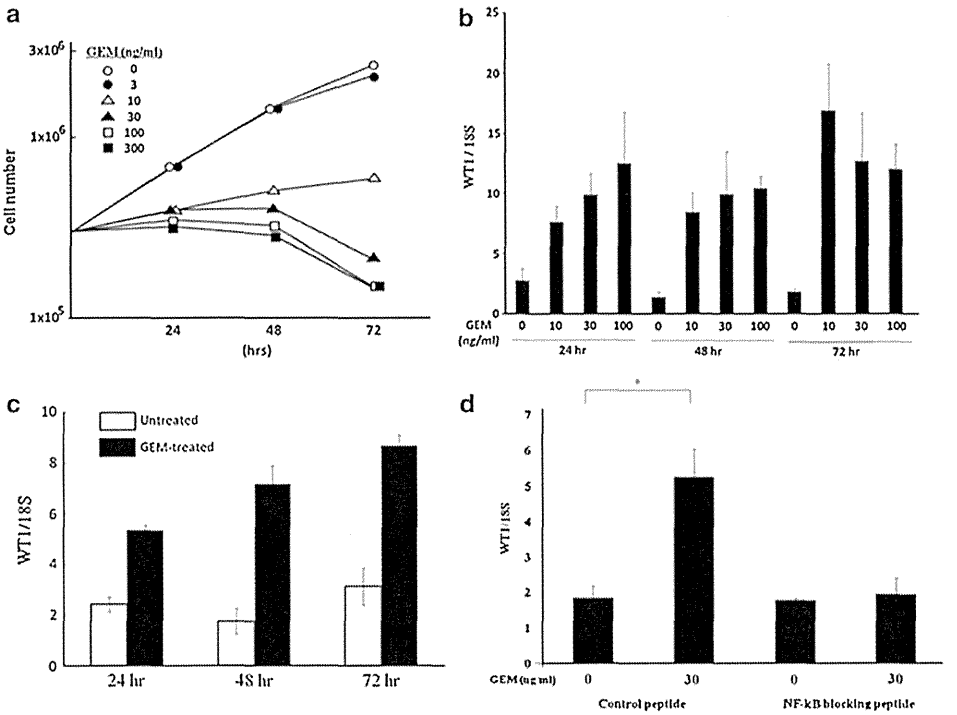

Fig. 1 **a** Proliferation of MIAPaCa2 cells in medium containing various concentrations of GEM. MIAPaCa2 cells (3×10^5/well) were seeded in 6-well culture plates in regular culture medium, which was then exchanged for GEM-containing medium after 24 h. At 24-h intervals, cells were detached using trypsin, and cell numbers were counted using a hemocytometer ($n = 3$). **b** Up-regulation of WT1 mRNA in MIAPaCa2 cells by GEM treatment. Twenty-four hours after plating, culture medium was exchanged to media containing GEM at indicated concentrations (0, 10, 30 and 100 ng/ml). MIAPaCa2 cells were harvested at 24-h intervals, and WT1 mRNA in cell homogenates was analyzed using qRT-PCR. WT1mRNA levels were normalized relative to those of 18S ribosomal RNA (18S). **c** Up-regulation of WT1 mRNA in MIAPaCa2 cells after short treatment with GEM. Twenty-four hours after plating, MIAPaCa2 cells were untreated or treated with 100 ng/ml of GEM for 2 h. MIAPaCa2 cells did not proliferate but kept alive for following 72 h by this treatment with GEM. After GEM treatment, cells were washed well, cultured in regular culture medium, and harvested at 24-h intervals. WT1 mRNA in cell homogenates was analyzed using qRT-PCR, and WT1mRNA levels were normalized relative to those of 18S ribosomal RNA (18S). **d** NF-kB suppresses GEM-induced up-regulation of WT1 mRNA. MIAPaCa2 cells (6×10^4/well) were seeded in 24-well culture plates. After 24 h, medium was exchanged for media containing GEM (0 or 30 ng/ml) and/or NF-kB blocking peptide (50 μM) or control peptide (50 μM). WT1 mRNA levels were quantified after 24-h incubation using qRT-PCR. $^*P < 0.01$

Changes in WT1 mRNA expression levels were also examined in MIAPaCa2 cells following in vitro treatment with various other chemotherapeutic agents. Oxaliplatin, Doxorubicin, and five-fluorouracil showed significant enhancement of WT1 mRNA expression, but cisplatin and irinotecan did not (Suppl. 1). Because GEM is the standard drug used to treat human PC, its effect on human PC cells was studied thereafter.

In vivo up-regulation of WT1 mRNA in tumor tissue by treatment of MIAPaCa2-bearing SCID mice with GEM

In order to clarify whether in vivo treatment of tumor cells with GEM induces an enhancement of WT1 mRNA expression, SCID mice implanted subcutaneously with MIAPaCa2 cells were treated with a clinical dosage of GEM. We observed a significant increase in the levels of WT1 mRNA 48 h after injection of GEM (Fig. 3).

GEM treatment shifts localization of WT1 from the nucleus to the cytoplasm

We used immunoblot analysis to examine the levels of WT1 protein in MIAPaCa2 cells cultured in the absence or presence of GEM. Relative to untreated cells, WT1 protein levels in GEM-treated MIAPaCa2 cells were augmented; however, after 36 h of cell culture, levels of WT1 protein diminished in both untreated and GEM-treated cells (Fig. 4a). This decline in WT1 protein levels was rescued by treatment with the proteasome inhibitor MG-132, indicating that WT1 protein is susceptible to proteasomal degradation (Fig. 4b).

Confocal microscopy images demonstrate that WT1 protein is primarily located in nuclei of untreated cells (Fig. 5a). However, in MIAPaCA2 cells treated with GEM, localization of WT1 protein shifted to the cytoplasm and the intensity of WT1 immunofluorescence in the nucleus decreased