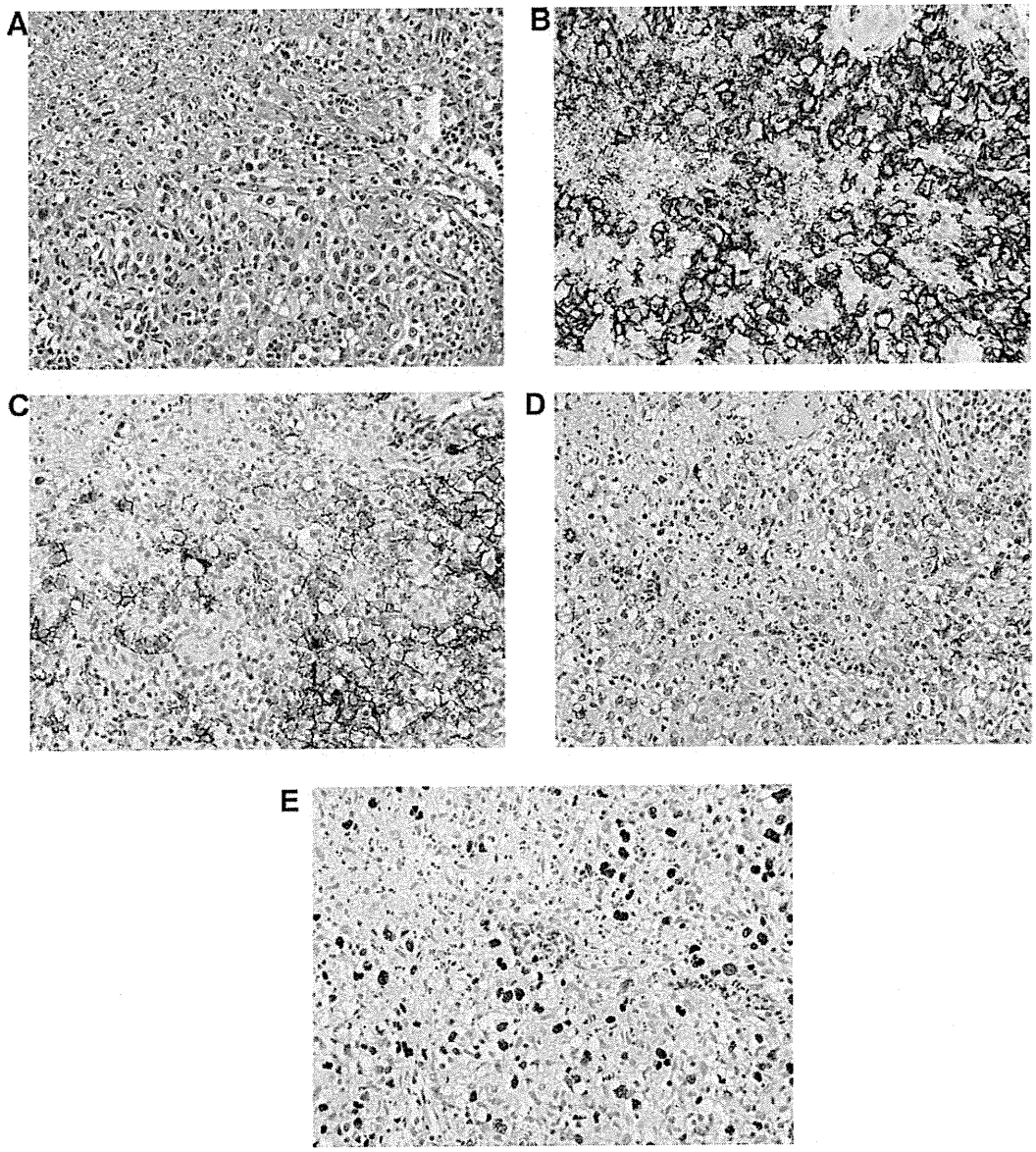

Fig. 1. **A**: Diffuse proliferation of large lymphoid cells with prominent nucleoli. H&E. Large lymphoid cells expressed CD20 (**B**) and less frequently CD30 (**C**), and LMP-1 (**D**). In situ hybridization with the EBER probe revealed positive signals in the nuclei of large lymphoid cells (**E**).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

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

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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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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).

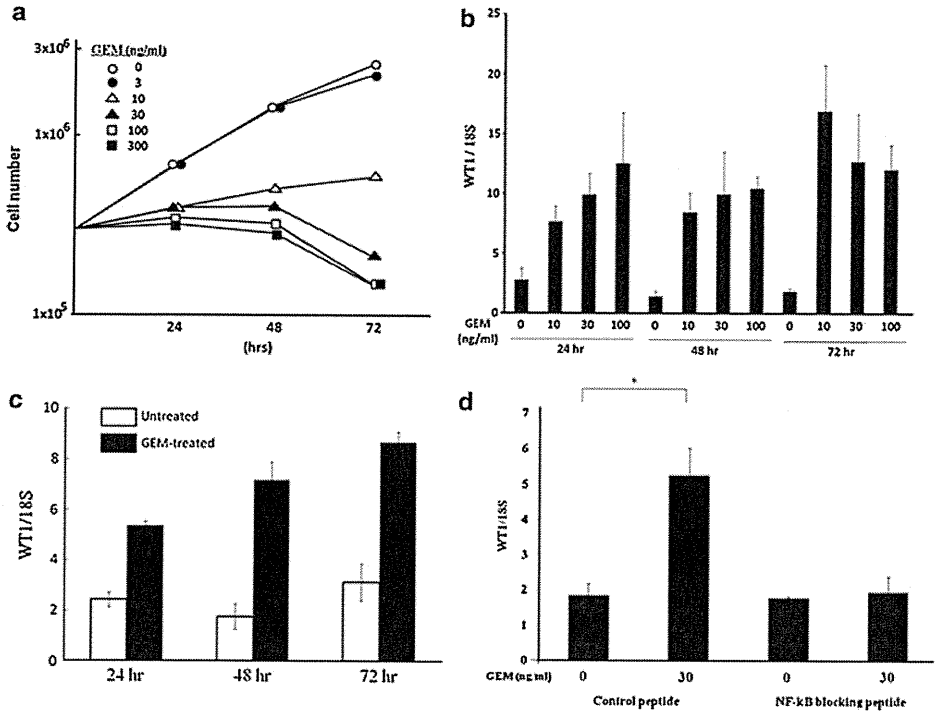

Fig. 1 **a** Proliferation of MIAPaCa2 cells in medium containing various concentrations of GEM. MIAPaCa2 cells (3×10^5/well) were seeded in 6-well culture plates in regular culture medium, which was then exchanged for GEM-containing medium after 24 h. At 24-h intervals, cells were detached using trypsin, 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

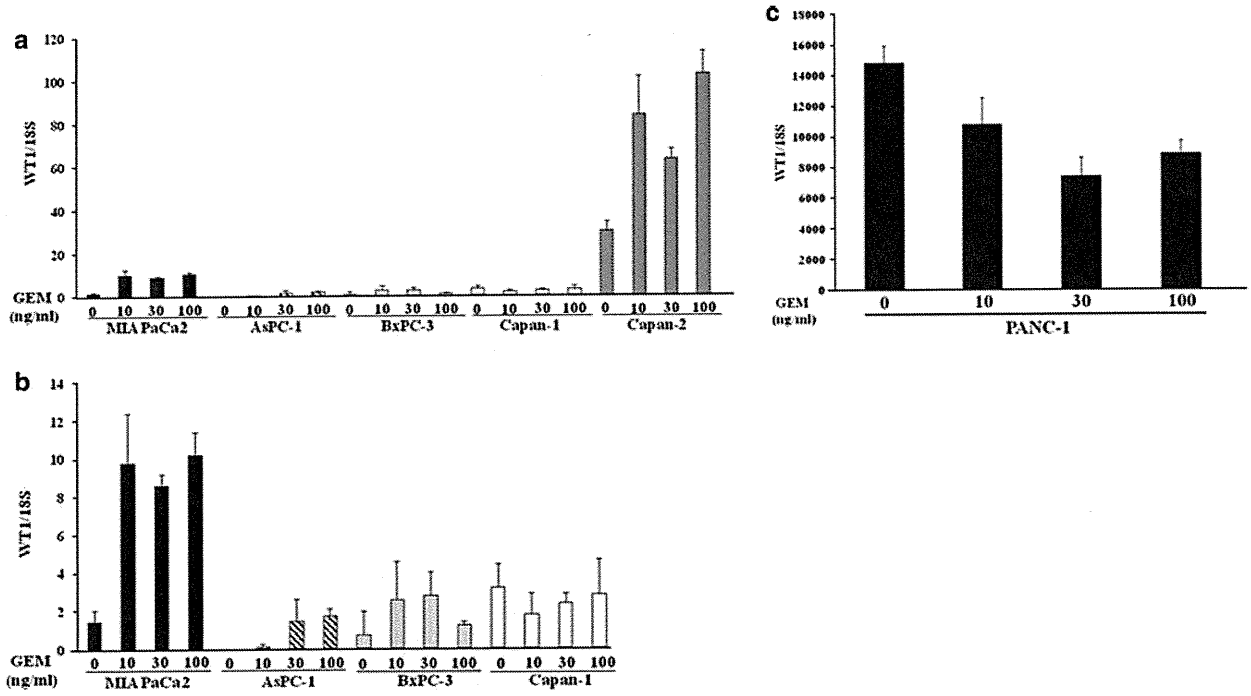

Fig. 2 **a** Up-regulation of WT1 mRNA levels in various human PC cell lines following GEM treatment. Human PC cells (1×10^6 MIAPaCa2, AsPC-1, BxPC-3, Capan-1 or Capan-2) were seeded in 10-cm culture plates. After 24-h incubation, medium was changed to media containing GEM (10, 30 or 100 ng/ml). After 48 h, we used qRT-PCR to quantify the relative ratio of WT1 to 18S mRNA levels in each cell line ($n = 3$). **b** GEM-induced up-regulation of WT1 mRNA in human PC cells with low basal levels of WT1 mRNA (MIAPaCa2, AsPC-1, BxPC-3 and Capan-1). To illustrate these results, we replotted data from (**a**) to represent a considerably narrower range of mRNA level ratios (0–14) on the *y*-axis. (**c**) Expression of WT1 mRNA in human PC cells with high basal levels of WT1 mRNA (PANC-1). To illustrate the results, we plotted data to represent a considerably wider range of mRNA level ratios (0–18,000) on the *y*-axis

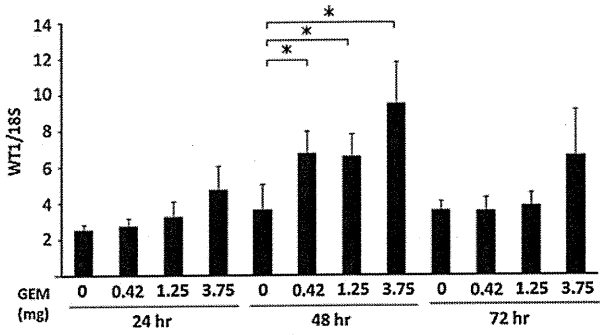

Fig. 3 Tumors in PC-bearing SCID mice treated with GEM show increased WT1 mRNA levels. Ten days after subcutaneous inoculation of SCID mice with 5×10^6 MIAPaCa2 cells (formation of approximately 1-cm diameter tumors), mice were injected intraperitoneally with GEM (0, 0.42, 1.25 and 3.75 mg/mouse). Tumors were resected every 24 h thereafter, and relative levels of WT1 mRNA were quantified using qRT-PCR ($n = 3$). Duplicate trials of the same protocol showed similar results. $^*P < 0.01$

(Fig. 5a). Decline in WT1 protein levels following GEM treatment was also observed in immunoblot analyses of the nuclear fraction of treated MIAPaCa2 cells (Fig. 5b).

Enhanced presentation of HLA-A*2402-restricted WT1 antigenic peptide following GEM treatment

Figure 6a shows typical standard curve obtained with increasing quantities of WT1 antigenic peptide. The data indicate a linear relation over a wide range (0–1,000 pmol) of analyte amount with correlation coefficients greater than 0.99. The data in the Fig. 6b demonstrate the sensitivity as well as the noise background of the LC–MS/MS. The noise background is less than 1 cps. The signal from injection of 10 pmol of this peptide spiked to MIAPaCa2 cells is approximately 16 cps, giving an S/N ratio of approximately 16. The low noise background and signal of 10 pmol of this peptide indicated the extrapolated limit of detection is less than 0.8 pmol on column under S/N = 2.

The level of the WT1 antigenic peptide was estimated among MHC class I binding peptides from MIAPaCa2 cells treated with either PBS or GEM to 6.49 pmol/10^8cell or 8.78 pmol/10^8cell, respectively. GEM treatment increased the presentation of HLA-A*2402-restricted WT1 antigenic peptide on MIAPaCa2 cells.

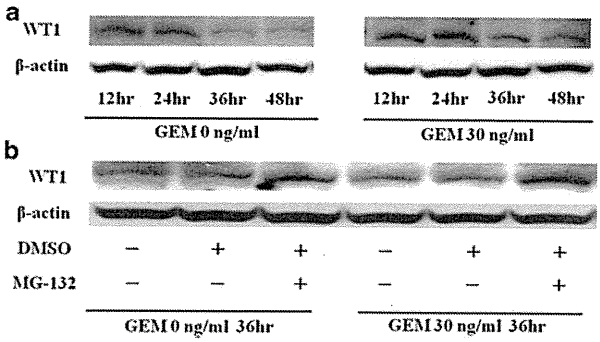

Fig. 4 **a** WT1 protein is degraded by proteasomal enzymes. Twenty-four hours after 3×10^5 MIAPaCa2 cells/well were seeded in 6-well culture plates, medium was exchanged from untreated to media containing GEM (0 or 30 ng/ml). Expression of WT1 protein in the cells was analyzed every 12 h thereafter from immunoblots described in Sect. "Materials and methods". **b** Protease inhibitors block WT1 degradation. Twenty-four hours after incubating MIAPaCa2 cells with GEM (0 or 30 ng/ml), MG-132 in DMSO or DMSO alone was added to each well at a concentration of 5 μM and 0.05%, respectively. Treated and control cells (in 0.05% DMSO alone) were incubated for 12 h before harvesting cells for immunoblot analysis of WT1 and beta-actin proteins

GEM-treated PC cells are killed efficiently by effector cells transduced with genes encoding a WT1-specific T-cell receptor

The susceptibilities of untreated and GEM-treated MIAPaCa2 cells to WT1-specific cytotoxic effector T cells were compared. The cytotoxic effect of WT1-specific effector cells on MIAPaCa2 cells was enhanced significantly when PC cells were treated with either 10 or 30 ng/ml of GEM for 48 h (Fig. 7). Notably, effector cell cytotoxicity was not enhanced by treatment of PC cells with 100 ng/ml of GEM, although this high dose of GEM was more toxic to PC cells than 10 or 30 ng/ml. Up-regulation of MHC class I in MIAPaCa2 cells by GEM treatment that possibly provides the similar results was not observed (data not shown).

Discussion

In the present study, we demonstrate that expression of WT1 mRNA in human PC cells is enhanced by treatment

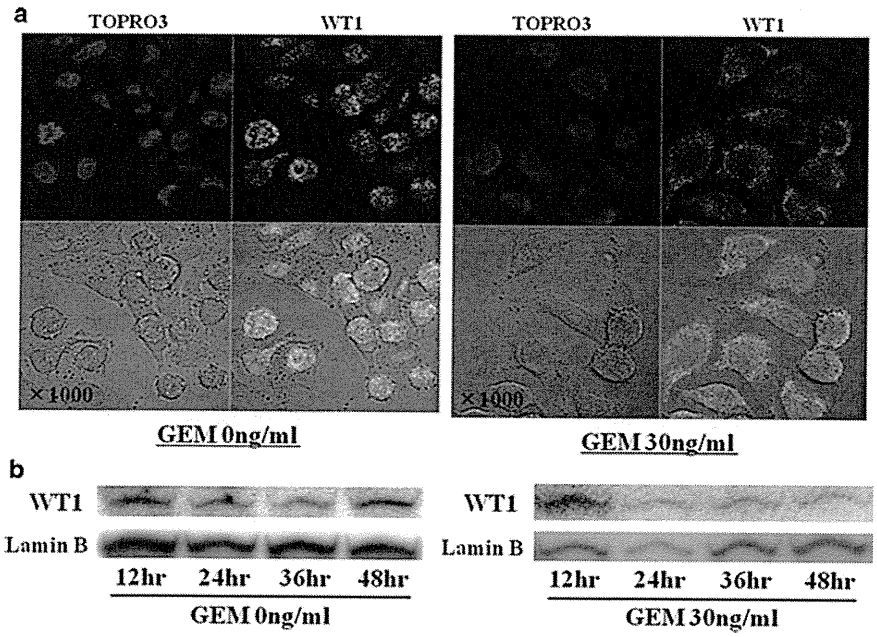

Fig. 5 **a** GEM treatment shifts WT1 protein localization from nucleus to cytoplasm. Twenty-four hours after seeding 3×10^5 MIAPaCa2 cells/well in 6-well culture plates, untreated medium was exchanged for fresh medium with or without GEM (0 or 30 ng/ml). After 24-h incubation, cells were fixed with paraformaldehyde, followed by nuclear staining with TO-PRO-3 iodide (*blue color*) and detection of WT1 with rabbit anti-WT1 polyclonal antibody and anti-rabbit IgG conjugated with fluorescein isothiocyanate (*green color*). Stained cells were observed using confocal microscopy (original magnification ×1,000). **b** GEM treatment diminishes nuclear localization of WT1 protein. Twenty-four hours after seeding 3×10^5 MIAPaCa2 cells/well in 6-well culture plates, medium was exchanged for fresh medium with or without GEM (0 or 30 ng/ml). At 12-hour intervals thereafter, nuclei were isolated and WT1 protein levels of nuclear extracts were analyzed on immunoblots as described in Sect. "Materials and methods"

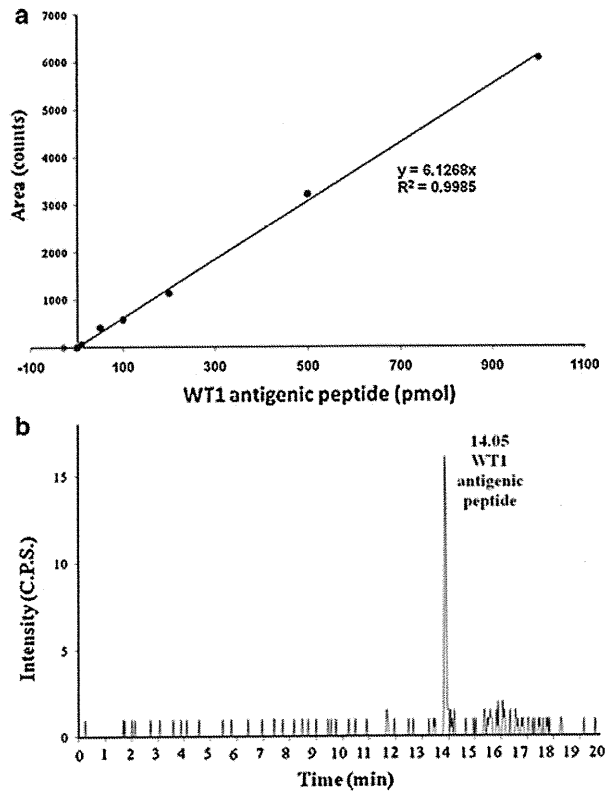

Fig. 6 **a** Standard curve for HLA-A*2402 restricted WT1 antigenic peptide. **b** Trace of MRM signal during LC–MS/MS analysis of spiked HLA-A*2402-restricted WT-1 antigenic standard peptide (10 pmol) in MIAPaCa2 cells

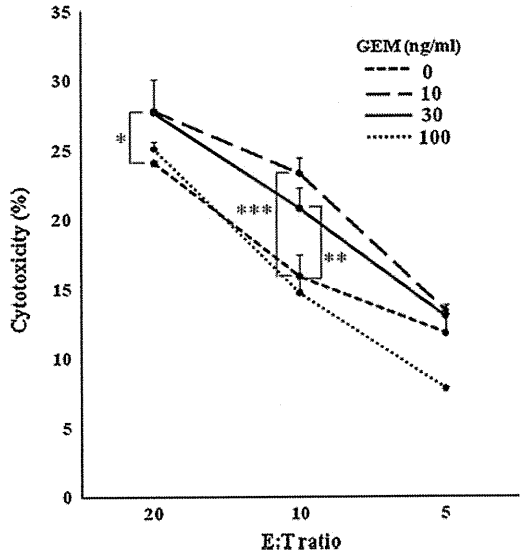

Fig. 7 WT1-specific CTLs kill GEM-treated MIAPaCa2 cells efficiently. MIAPaCa2 cells pretreated with 0, 10, 30, or 100 ng/ml GEM for 48 h were labeled with ^{51}Cr. ^{51}Cr release assays were used to measure the cytotoxic activity of WT1-specific effector cells against untreated or GEM-pretreated MIAPaCa2 cells. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$

with GEM. MIAPaCa2 cells demonstrating GEM-mediated enhancement of WT1 mRNA levels did not proliferate but maintained stable numbers of viable cells with impaired growth by continuous treatment with low-dose GEM as well as short treatment with high-dose GEM. WT1 is a transcription factor with oncogenic potential, in that it can induce malignant cellular phenotypes, suppress apoptosis, and promote cell proliferation [15]. We hypothesize that up-regulation of WT1 levels in PC cells aids cell survival by conferring chemoresistance against GEM's toxic effects.

Based on the fact that GEM-mediated augmentation of WT1 mRNA expression was attenuated by addition of an NF-kB blocking peptide in the culture, activation of NF-kB also appears to play a significant role in WT1 enhancement. NF-kB is known to be active in many malignant tumors and has been implicated in cellular resistance to cytotoxic agents and escape from apoptosis [26]. Previous reports demonstrate that GEM activates NF-kB [27] and that the ensuing regulatory cascade activates the WT1 gene downstream [28]. Human PC cell lines with high NF-kB activity are resistant to GEM [27], and that silencing or suppression of NF-kB increases the sensitivity of PC cells to GEM and induces apoptosis [29–31].

It is of note and interest that some chemotherapeutic agents other than GEM showed capability on up-regulation of WT1mRNA expression. Especially, treatment with oxaliplatin (L-OHP) induced marked enhancement of WT1mRNA expression. Folfirinox including L-OHP was recently reported to be a more efficient regimen for metastatic pancreatic cancer (10). However, combined treatment with Folfirinox and WT1 targeting immunotherapy might be unsuccessful because of severe leukopenia by Folfirinox. GEM has relatively low hematologic toxicity and thus seems to be preferable for combination therapy with WT1 targeting immunotherapy.

We also observed up-regulation of WT1 mRNA by GEM treatment in vivo. Within 48 h of treating MIAPaCa2-bearing SCID mice with a clinical dose of GEM, steady-state levels of WT1 mRNA in the tumor increased. Despite its rapid disappearance after intraperitoneal injection, the enhancement of WT1 mRNA expression in tumor tissue was significant. Enhancement of WT1 mRNA expression was also observed after in vitro short treatment with GEM. These results suggest strongly that GEM treatment of human PC in a clinical setting might induce up-regulation of WT1 in PC cells.

In the present study, we found that the localization of WT1 protein shifted from nucleus to cytoplasm following GEM treatment. WT1 protein has been shown to undergo nucleocytoplasmic shuttling [32], and the function of WT1 has been suggested to correlate with its cellular location: Siberstein et al. [33] described that WT1 was localized to

the cytoplasm and not to nuclei in some human breast cancers and suggested that such localization may be regulated by alternative splicing of WT1 mRNA. On the other hand, immunohistochemical studies of Nakatsuka et al. [34] demonstrate a majority of WT1-positive tumors with diffuse or granular staining in the cytoplasm. Ye et al. [35] report that phosphorylation of WT1 protein resulted in cytoplasmic retention of WT1, thereby inhibiting DNA binding and altering transcriptional activity. Through the activation of NF-kB, GEM treatment may mediate a similar phosphorylation and translocation of WT1 protein from nucleus to cytoplasm.

In order for MHC class I-restricted antigen to be presented and recognized by antigen-specific CTLs, tumor antigen must be degraded by proteasomal enzymes located in the cytoplasm [36]. Retention of an intra-nuclear tumor antigen such as WT1 in the cytoplasm should favor tumor antigen processing, and in fact, we observed enhanced presentation of HLA-A*2402-restricted WT1 antigenic peptide using ESI LC–MS/MS analyses. GEM-treated MIAPaCa2 cells showed greater susceptibility than untreated cells to the cytotoxic effects of WT1-specific CTLs generated by transduction of a gene encoding a WT1-specific T-cell receptor. Importantly, treatment with 10–30 ng/ml of GEM enhanced the susceptibility of MIAPaCa2 cells to CTL, but treatment with 100 ng/ml did not. This phenomenon indicates that the enhanced susceptibility of GEM-treated MIAPaCa2 cells to CTLs is not due to GEM toxicity, but to augmented expression of the WT1 target antigen.

GEM is a nucleoside analog with clinical relevance to the treatment of several solid tumors, including PC; nonetheless, its antitumor effect is limited. We observed significant clinical response in a phase I clinical study of combined treatment against advanced PC using a WT1 peptide vaccine and GEM (manuscript in preparation). The presumed actions of GEM up-regulating WT1 expression in vivo and WT1-specific CTLs killing GEM-treated tumor cells efficiently may prove valuable for the treatment of human PC. It has been reported that GEM may suppress the activity of myeloid-derived suppressor cells that inhibit antitumor immunity [37]. In addition, GEM has been shown to increase the number of dendritic cells in blood without affecting T-cell activity in patients with PC [38]. We propose that combining GEM's proven role as an immunopotentiator with its ability to up-regulate target WT1 expression of PC cells will enhance the susceptibility of PC cells to WT1-specific CTLs. Furthermore, PC cells already acquired GEM resistance by the activation of NF-kB might be injured by WT1-specific CTLs. Assessment of the clinical response to combined therapy with WT1 peptide vaccine and GEM is presently underway.

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Conflict of interest There are no financial disclosures of any of the authors to declare.

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

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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).

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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.

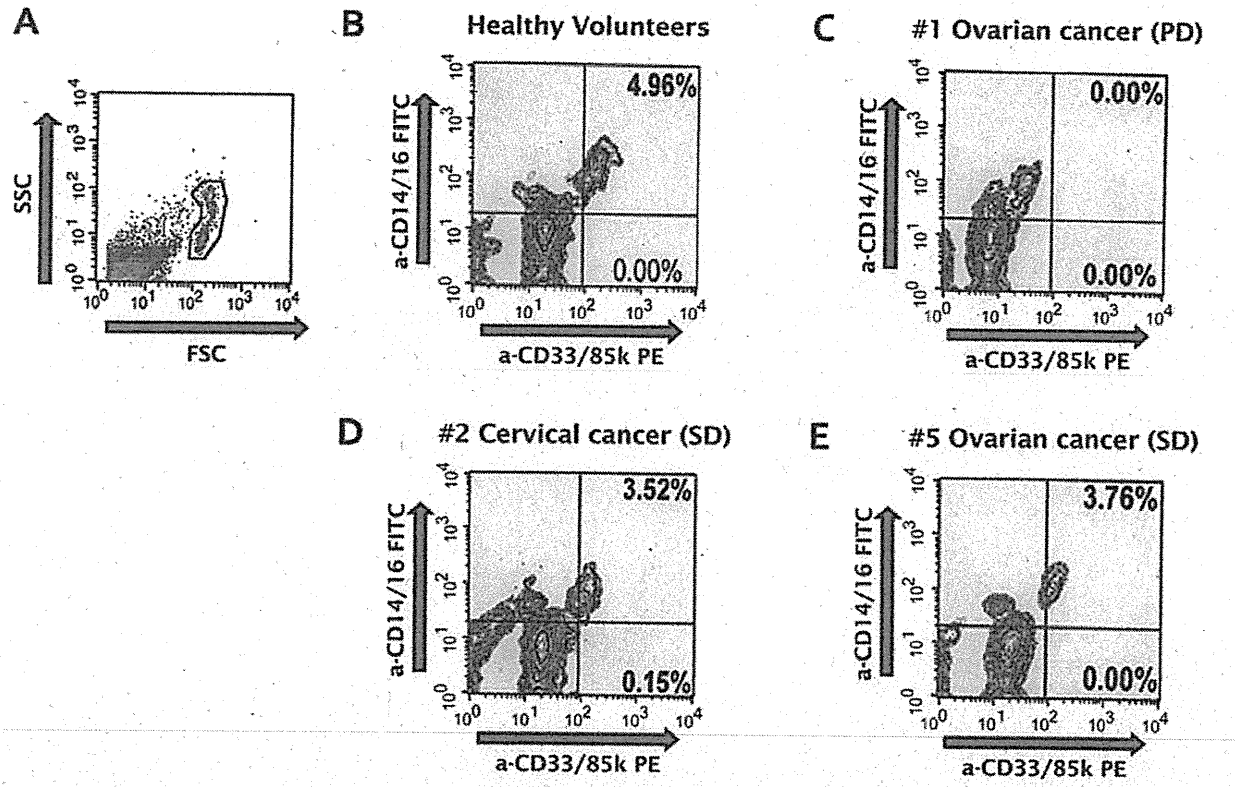

Figure 1. *Flow cytometric profiles of DCs in peripheral blood mononuclear cells (PBMCs). PBMCs collected from a healthy volunteer or cancer patients were stained with lineage-specific FITC- or PE-conjugated markers including anti-CD14-, anti-CD16-, anti-CD33- and anti-CD85 monoclonal antibodies. Gates 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

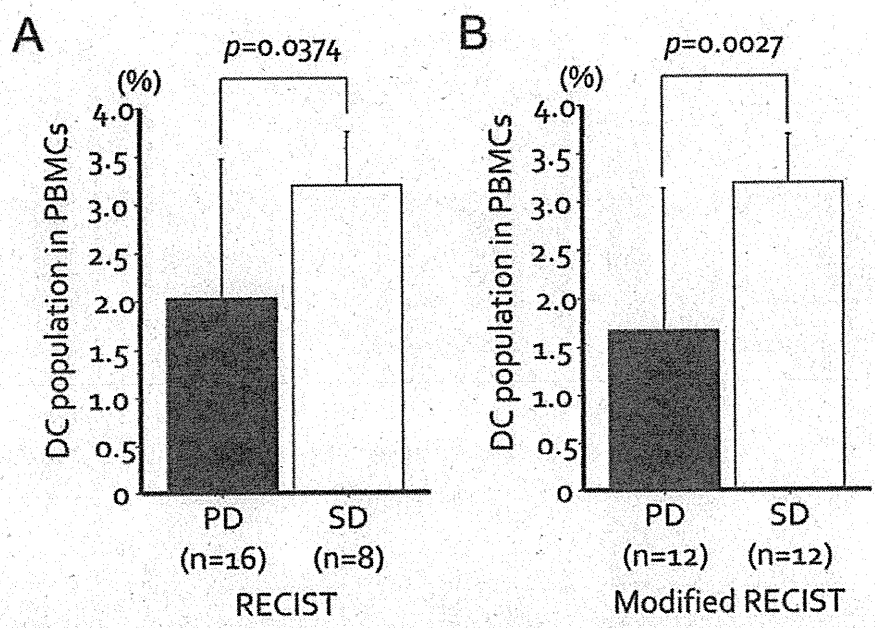

Figure 2. *Validation for circulating myeloid DCs in peripheral blood. Among the 6 gynaecological cancer patients with WT1 peptide vaccination, those in the progressive disease (PD) group (black histograms) showed a depletion of the myeloid DC subset, which was statistically significant compared with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

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WT1 peptide vaccination following allogeneic stem cell transplantation in pediatric leukemic patients with high risk for relapse: successful maintenance of durable remission

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Wilms tumor gene, *WT1*, is highly expressed in acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL), and has an essential role in leukemogenesis.[1] The gene product (WT1 protein) could be a good target antigen for immunotherapy against leukemia. Indeed, WT1 peptide vaccination trials in adult patients with AML or myelodysplastic syndromes with WT1 expression have provided good results,[2,3] indicating that WT1-specific cytotoxic T lymphocytes (CTLs) elicited by WT1 vaccination killed WT1-expressing leukemia cells. Although allogeneic stem cell transplantation (SCT) has been used as a curative treatment for pediatric high-risk hematological malignancies, prognosis of patients with relapse after SCT is very poor. Strategies to enhance graft versus leukemia (GVL) response are, therefore, needed to prevent recurrence after SCT. This is the first study of WT1 peptide vaccination against minimal residual disease (MRD) after SCT for pediatric patients with high-risk hematological malignancy and we report the clinical course for the first three cases.

The WT1 peptide-based phase II clinical study was approved by the Institutional Review Board of Osaka University Hospital. Inclusion criteria were as follows: patients with human leukocyte antigen (HLA)-A*2402 aged <20 years; donors with HLA-A*2402 and WT1 mRNA expression in leukemic cells determined by reverse transcriptase-PCR. The HLA-A*2402-restricted, 9mer-modified WT1 peptide (a.a.235–243 CYTWNQML) emulsified in Montanide ISA 51 adjuvant was injected intradermally at four different regions. The dose of WT1 peptide depended on patient weight. The vaccinations were scheduled to be given weekly for 12 consecutive weeks and if no recurrence was observed, vaccination was continued.

Case 1 was a 1-year-old boy who presented pancytopenia in June 2005. Bone marrow (BM) aspiration demonstrated B-precursor ALL. He was treated with the JACLS (Japan Association of Childhood Leukemia Study) ALL 02 protocol. Although he attained complete remission (CR), his disease recurred during maintenance treatment. He received chemotherapy and achieved re-remission in September 2007. However, he had 71% marrow blasts during consolidation chemotherapy. The patient received allogeneic SCT from an HLA-2 antigen mismatched father without CR after receiving a conditioning regimen consisting of total body irradiation (TBI), topotecan and melphalan in March 2008. Acute graft-versus-host disease (GVHD) of the skin (stage 2) was observed, but resolved with corticosteroids. Immunosuppressive treatment was stopped on day 37. WT1 mRNA level was higher than normal.[4] Case 2 was a 13-year-old girl who developed tumor of the upper eyelid and showed pancytopenia in February 2008. BM aspiration revealed AML with AML/MTG8 translocation on fluorescence *in situ* hybridization analysis. She received chemotherapy according to the JPLSG (Japan Pediatric Leukemia/Lymphoma Study Group) AML-05 protocol. She achieved CR after the second course of chemotherapy. Because a high WT1 mRNA level (3500 copies/μg RNA in BM) was observed, she received HLA-matched unrelated umbilical cord blood transplantation after a conditioning regimen consisting of TBI and cyclophosphamide in October 2008. Acute cutaneous GVHD (stage 3), observed on day 19 post-transplant, resolved after prednisolone administration. WT1 and AML/MTG mRNA levels remained abnormally high (Table 1). Case 3 was a 1-year-old boy without Down syndrome who presented high fever and thrombocytopenia in June 2008. A diagnosis of acute megakaryoblastic leukemia was made following BM aspiration. He was successfully treated with the JPLSG AML-05 protocol but relapsed 3 months after the end of treatment. He achieved morphological CR with topotecan-based combination chemotherapy, but WT1 mRNA level remained high (180 000 copies/μg RNA in BM). He received allogeneic bone marrow transplantation from an HLA-identical unrelated donor in July 2009. The conditioning regimen consisted of busulfan and melphalan. He developed no GVHD and immunosuppressive treatment was stopped on day 35 post-transplant. WT1 mRNA levels increased to as high as 2300 copies/μg RNA in BM on day 34 post-transplant.

WT1 vaccinations were started at 1-week interval in these three cases on day 41–173 post-SCT. WT1 mRNA levels in BM were as high as 1500–2600 copies/μg RNA before WT1 vaccination. After vaccination, WT1 mRNA levels decreased to 150–470 copies/μg RNA on day +180 in all cases, whereas WT1-specific CTL frequencies increased from 0–0.14% to

Table 1 Outcome after WT1 peptide vaccination

Case	*Vaccine doses administered*	*Outcome*	*Survival from SCT (months)*	*Adverse effect*	*WT1 transcripts (per μgRNA) in BM*			*AML/MTG8 transcripts (per μgRNA) in BM*		
					Before SCT	*Before vaccination (day)*[a]	*After vaccination (day)*[a]	*Before SCT*	*Before vaccination (day)*[a]	*After vaccination (day)*[a]
1	60	CR	40.1	Skin ulcer	3700	1500 (−10)	150 (+180)	ND	ND	ND
2	60	CR	33.5	Local erythema	2600	2600 (−9)	550 (+180)	5600	4800 (−9)	520 (+180)
3	23	Relapse	6.9[b]	Local erythema	180 000	2300 (−40)	1000 (+180)→ 120 000	ND	ND	ND

Abbreviations: BM, bone marrow; CR, complete response; ND, not detected; SCT, stem cell transplantation.
[a]Day a after the start of vaccination.
[b]Death.

Table 2 Frequency of WT1-specific CTLs and subpopulation analysis

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

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

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

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

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

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

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

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

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

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

Conflict of interest

The authors declare no conflict of interest.

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Functional role of BAALC in leukemogenesis

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

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

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