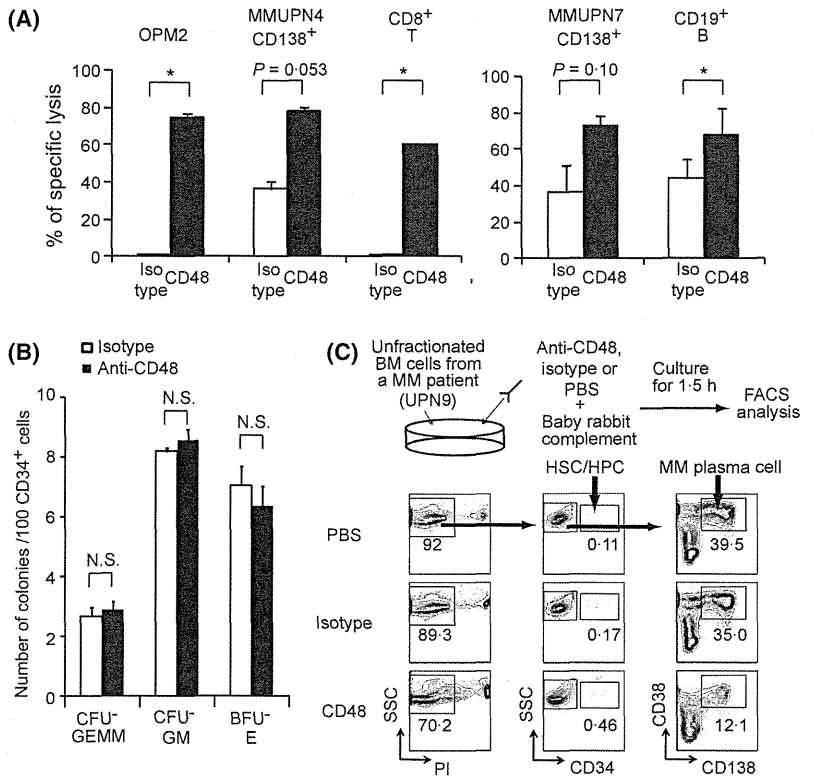

Fig 6. The anti-CD48 mAb does not damage $CD34^+$ HSCs/HPCs. (A) (Left) Analysis of CDC induced by the anti-CD48 mAb (1B4) using the OPM2 MM cell line, $CD138^+$ MM plasma cells form a MM patient (UPN4) and $CD8^+$T cells from a healthy donor as target cells. (Right) A CDC assay using MM plasma cells from a MM patient (UPN7) and $CD19^+$B cells from a healthy donor. Shown is mean plus SE of triplicate wells. *$P < 0{\cdot}05$. (B) $CD34^+$ cells from cord blood were incubated with baby rabbit complement and either the anti-CD48 mAb or isotype control, and then subjected to a colony-forming assay. Mean numbers of colonies produced from 100 $CD34^+$ cells are shown with error bars representing SEs of triplicate plates from one representative of two independent experiments. CFU-GEMM, mixed lineage colony-forming units (CFU); CFU-GM, granulocyte-macrophage CFU; BFU-E, erythroid burst-forming units; N.S.: no significant differences. (C) Unfractionated BM cells from an MM patient were incubated with phosphate-buffered saline (PBS), isotype control or the anti-CD48 mAb in medium supplemented with complement and then subjected to FACS analysis. Numbers represent percentages of gated cells among all cells analysed. Note that the percentages of $CD38^{++}$ MM plasma cells, but not of $CD34^+$ HSCs/HPCs, decreased significantly by anti-CD48mAb treatment. Representative results from two independent experiments are shown.

FACS-sorted $CD34^+$ HPCs from cord blood were incubated with the complement and either the anti-CD48 mAb or isotype control (10 μg/ml) for 1·5 h, and then subjected to haematopoietic colony-forming assays. The numbers of mixed lineage colony-forming units (CFU-GEMM), granulocyte-macrophage CFU (CFU-GM) and erythroid burst-forming units (BFU-E) colonies formed from $CD34^+$ cells treated with the anti-CD48 mAb were similar to those formed from $CD34^+$ cells treated with isotype control (Fig 6B), indicating that anti-CD48 mAb did not induce CDC against $CD34^+$ HPCs. Furthermore, CDC induced by the anti-CD48 mAb against un-fractionated BM cells from MM patients was examined (Fig 6C). After incubation with the anti-CD48 mAb (10 μg/ml) and complement for 1·5 h, $CD38^{++}CD138^+$ MM plasma cells, but not $CD34^+$ HSCs/HPCs, significantly decreased. This demonstrated that anti-CD48 mAb could selectively kill MM plasma cells, but not normal HSCs/HPCs by induction of CDC.

Discussion

The present study has provided evidence that CD48 is highly expressed on almost all plasma cells in the majority of MM patients, and that a new anti-CD48 mAb, 1B4, can induce significant cytotoxic effects against MM cells both *in vitro* and *in vivo*. CD48 expression levels on $CD34^+$ HSCs and HPCs were much lower than those on MM plasma cells and the anti-CD48mAb did not cause cytotoxicity. CD48 is not expressed on erythrocytes, platelets or any non-haematopoietic tissues (Vaughan *et al*, 1983), suggesting that anaemia, thrombocytopenia and tissue toxicity are not major concerns. $CD48^{++}$ cells in BM of MM patients overlapped with cells expressing high levels of CD38, which is a promising candidate antigen for therapeutic mAb against MM (Stevenson *et al*, 1991; Ellis *et al*, 1995; Stevenson, 2006; Tai *et al*, 2009; van der Veer *et al*, 2011; de Weers *et al*, 2011), while $CD34^+$ HPCs were $CD38^+CD48^{low/-}$. Targets of clinically effective mAbs against

haematological malignancies, such as CD20 and CD52, are constitutively expressed on entire target malignant cells at high levels, but not expressed on non-haematopoietic tissues. Similarly, CD48 is expressed on almost all MM plasma cells, but not on non-haematopoietic tissues. It has been reported that a soluble form of CD48 exists, but its concentration in serum was as low as that of soluble CD20 or CD52 (Smith *et al*, 1997; Giles *et al*, 2003). Taken together, these findings indicate that CD48 is a good candidate for a therapeutic target against MM.

Sintes *et al* (2008), in a study that used the anti-CD48 mAb clone 99A, which has thus far not been available to us, showed that CD48 was expressed on normal human $CD34^+$ HSCs and HPCs, while expression levels of CD48 on $CD34^+$ cells were unclear. We were able to show clearly in the present study, that CD48 expression levels on $CD34^+$ HSCs and HPCs were much lower than those on MM plasma cells, and confirmed these results by staining with three different clones of anti-CD48 mAb (HuLy-m3, MEM-102, ebio156-4H9). Importantly, we demonstrated that the anti-CD48 mAb did not induce CDC against $CD34^+$ HSCs or HPCs, suggesting that faint CD48 expression on $CD34^+$ HSCs and HPCs is not a major obstacle to the development of anti-CD48 mAb as a therapeutic mAb.

The *in vivo* anti-MM effects of the anti-CD48 mAb were remarkable in subcutaneous MM tumour models. Furthermore, we demonstrated that the anti-CD48 mAb treatment was effective against MM cells engrafted in a BM microenvironment. The inhibitory effect on MM was much more prominent in SCID mice than NOD/SCID mice, suggesting that CDC is likely to be a major mechanism of these more prominent anti-MM effects. In addition, the fact that 1B4 mAb was still effective against MM cells in NOD/SCID mice suggest that other mechanisms may be involved. While ADCC induced by the mouse anti-CD48 mAb *in vitro* was not very strong, the potential for inducing ADCC against MM plasma cells will need to be assessed after the humanized mAb is developed. In this regard, it was reported that a chimeric anti-CD48 mAb could induce significant ADCC against Raji B cell lymphoma cells (Sun *et al*, 2000).

A major concern regarding CD48 as a therapeutic target is its broad expression on normal lymphocytes and monocytes, which may cause severe cytopenia and immunosupression when anti-CD48 mAb is used as a therapeutic drug. In fact, normal T and B cells are also sensitive to *in vitro* CDC induced by the anti-CD48mAb. Normal mature lymphocytes and monocytes may be depleted together with MM cells as a result of anti-CD48 mAb treatment, whereas normal $CD34^+$ HSCs or HPCs are not damaged and the normal haematopoietic system is re-established after discontinuation of the mAb treatment. The fact that a mAb against CD52, which is also widely expressed on normal leucocytes, has been used in clinics suggests that an antigen that is widely expressed on normal leucocytes still has the potential to serve as a target of therapeutic mAb. However, it has been reported that alemtuzumab causes pancytopenia (Keating *et al*, 2002; Enblad *et al*, 2004) as well as severe virus infections (Keating *et al*, 2002; Ghobrial *et al*, 2003; Herbert *et al*, 2003; Kluin-Nelemans *et al*, 2008), while immunosuppression induced by alemtuzumab can be tolerated if accompanied by appropriate prophylaxis for virus infection (Hillmen *et al*, 2007; Gribben & Hallek, 2009; Stilgenbauer *et al*, 2009). The potential haematological toxicity of anti-CD48mAb should therefore be very carefully tested at the pre-clinical stage.

Anti-CD48mAb may not be suitable for long-term maintenance therapy because of haematological toxicities. For induction therapy, however, we may be able to take advantage of the broad and high CD48 expression on all MM plasma cells for the total eradication of MM plasma cells. In addition, consolidation therapy with anti-CD48 mAb may also benefit MM patients. Recent progress in MM therapy has resulted in complete response or good partial response in many patients (Palumbo & Anderson, 2011). However, these patients are rarely cured because a sub-fraction of MM cells remains resistant to the therapies currently in use. Anti-CD48 mAb may have the potential to eradicate such resistant MM cells. These indicate that anti-CD48 mAb may well turn out to be an effective tool for the survival improvement of MM patients.

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Author Contributions

NH designed the research study, performed research, analysed data and wrote the paper. HI, AM, YA, YF, SK, YM, HN, MK, TY, SF, HT, TN, SN, AT, SI, MH, YO and YO performed the research. HS wrote the paper.

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Enhanced tumor immunity of WT1 peptide vaccination by interferon-β administration☆

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ABSTRACT

To induce and activate tumor-associated antigen-specific cytotoxic T lymphocytes (CTLs) for cancer immunity, it is important not only to select potent CTL epitopes but also to combine them with appropriate immunopotentiating agents. Here we investigated whether tumor immunity induced by WT1 peptide vaccination could be enhanced by IFN-β. For the experimental group, C57BL/6 mice were twice pre-treated with WT1 peptide vaccine, implanted with *WT1*-expressing C1498 cells, and treated four times with WT1 peptide vaccine at one-week intervals. During the vaccination period, IFN-β was injected three times a week. Mice in control groups were treated with WT1 peptide alone, IFN-β alone, or PBS alone. The mice in the experimental group rejected tumor cells and survived significantly longer than mice in the control groups. The overall survival on day 75 was 40% for the mice treated with WT1 peptide + IFN-β, while it was 7, 7, and 0% for those treated with WT1 peptide alone, IFN-β alone or PBS alone, respectively. Induction of WT1-specific CTLs and enhancement of NK activity were detected in splenocytes from mice in the experimental group. Furthermore, administration of IFN-β enhanced expression of MHC class I molecules on the implanted tumor cells. In conclusion, our results showed that co-administration of WT1 peptide + IFN-β enhanced tumor immunity mainly through the induction of WT1-specific CTLs, enhancement of NK activity, and promotion of MHC class I expression on the tumor cells. WT1 peptide vaccination combined with IFN-β administration can thus be expected to enhance the clinical efficacy of WT1 immunotherapy.

1. Introduction

Induction and activation of tumor-associated antigen (TAA)-specific cytotoxic T lymphocytes (CTLs) is essential for cancer immunotherapy. For this purpose, it is important to co-administer appropriate immunopotentiating agents, including adjuvants or cytokines, together with a TAA-derived peptide that serves as a CTL epitope, because injection of a CTL epitope alone cannot sufficiently induce and activate the TAA-specific CTLs. Furthermore, if the co-administered agents not only help induction/activation of the CTLs but also activate other effector cells such as NK cells, this may further enhance anti-tumor responses.

The Wilms' tumor gene *WT1* was originally isolated as a gene responsible for Wilms' tumor, a pediatric renal cancer [1,2]. This gene encodes a zinc finger transcription factor involved in organ development, cell proliferation and differentiation, as well as apoptosis. The *WT1* gene product regulates the expression of various genes either positively or negatively, depending upon how it combines with other regulatory proteins in different types of cells. Although *WT1* was categorized at first as a tumor suppressor gene [3], we have proposed that the wild-type *WT1* gene plays an oncogenic rather than a tumor-suppressor gene function in leukemogenesis/tumorigenesis on the basis of the following

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findings: (i) the wild-type *WT1* gene was highly expressed in leukemias and solid cancers [4–17]; (ii) high expression levels of *WT1* mRNA correlated with poor prognosis in leukemia and several kinds of solid cancer [4]; (iii) growth of *WT1*-expressing leukemia and solid cancer cells was inhibited by treatment with *WT1* antisense oligomers *in vitro* [18]; and (iv) in wild-type *WT1* gene-transfected myeloid progenitor cells, differentiation was blocked but proliferation was induced in response to granulocyte colony-stimulating factor [19,20]. These findings indicate that *WT1* over-expression and leukemogenesis/tumorigenesis may be closely related, which suggests that the wild-type *WT1* gene product could be a promising tumor rejection antigen for cancer immunotherapy. In fact, we [14–17,21] and others [22,23] have generated human WT1-specific CTLs *in vitro*, and we were able to show that mice immunized with MHC class I-restricted WT1 peptide or with *WT1* plasmid DNA elicited WT1-specific CTLs and rejected the challenge of *WT1*-expressing cancer cells *in vivo* [14–17,24,25], while the induced CTLs did not damage normal tissue cells that physiologically expressed *WT1*, including kidney podocytes and bone marrow (BM) stem/progenitor cells. Furthermore, we demonstrated that WT1 peptide vaccination combined with *Mycobacterium bovis* bacillus Calmette-Guérin cell wall skeleton (BCG-CWS) [26], which was injected one day previously at the same site as the WT1 peptide was more effective for eradication of *WT1*-expressing tumors than treatment with WT1 peptide alone or BCG-CWS alone [27]. BCG-CWS strongly activated dendritic cells (DCs) of the injection sites, i.e. activated of innate immunity, and also induced/activated of TAA (WT1)-specific CTLs.

Interferon-β (IFN-β) is a Type I interferon, and is known for its various immunopotentiating properties: (i) enhancement of the expression of many surface molecules that are essential for binding and/or activation of CTLs, in particular the major histocompatibility complex (MHC) class I as well as the receptors B7-1 (CD80) and intercellular adhesion molecule-1 (ICAM-1) [28,29], on antigen-presenting cells (APCs) or cancer cells; (ii) activation of NK, B, and T cells [30,31]; (iii) a direct anti-proliferation effect on cancer cells by promoting cell cycle arrest at the G1 phase [32]; (iv) induction of apoptosis of cancer cells [33]; and (v) inhibition of angiogenesis [34]. In fact, it was reported in mouse models that type I interferon was essential in the induction of CTL and eradication of EG-7 tumors expressing ovalbumin in mice by vaccination with CpG-adjuvanted ovalbumin [35], and that type I interferon augmented induction of CTL through DNA-based vaccination [36]. Furthermore, IFN-β has already been in use for cancer immunotherapy in clinical settings [37–40], and the mechanism for the enhancement of immunity against cancer has been thoroughly investigated. The results show that IFN-β should be considered as one of the most promising immunopotentiating agents for use with TAA-directed cancer vaccines.

We examined whether WT1 peptide vaccination combined with IFN-β administration leads to greater enhancement of tumor cell rejection than WT1 peptide vaccination alone in a mouse model and we tried to elucidate the mechanisms of enhancement of WT1 immunity by the co-administration of IFN-β.

2. Materials and methods

2.1. Mice

Male C57BL/6 (H-2D^b) mice were purchased from Clea Japan, Inc. (Tokyo, Japan), maintained in a specific pathogen-free (SPF) containment facility in accordance with the guidelines of Osaka University, and used for experiments at 6–8 weeks of age.

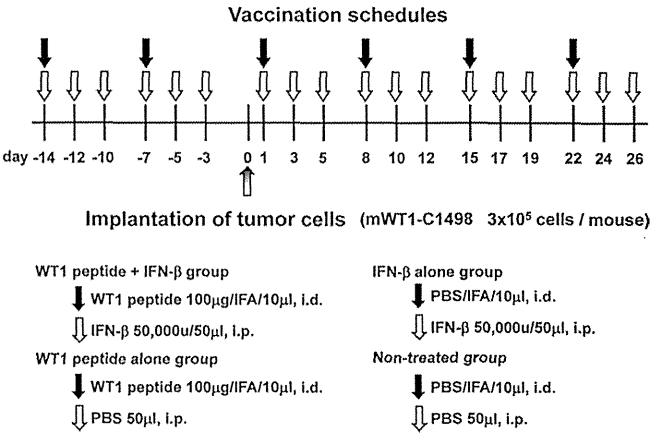

Fig. 1. *In vivo* tumor cell challenge and vaccination schedule. Mice were intradermally (i.d.) and abdominally pre-immunized with 100 μg WT1 peptide emulsified in incomplete Freund's adjuvant (IFA, Montanide ISA51) on day −14 and −7. Concomitantly, 50,000 units of murine IFN-β was intraperitoneally (i.p.) injected three times per week during the two weeks before tumor cell implantation. On day 0, mice were subcutaneously implanted with 3×10^5 mWT1-C1498 cells in 100 μl of PBS. This was followed by abdominal injection of 100 μg WT1 peptide emulsified in IFA on days 1, 8, 15 and 22. In addition 50,000 units of murine IFN-β was also i.p. injected three times per week until day 26 (WT1 peptide + IFN-β group). Mice in the control groups were injected with WT1 peptide emulsified in IFA and PBS (WT1 peptide alone group), PBS emulsified in IFA and IFN-β (IFN-β alone group), or PBS emulsified in IFA and PBS (non-treated group).

2.2. Reagents

An MHC class I (H-2D^b)-binding peptide, Db126 peptide (a.a.126-134 RMFPNAPYL), was synthesized by SIGMA Genosys (Ishikari, Japan) [24]. The peptide was dissolved in PBS and stored at −20 °C until use. Murine IFN-β was kindly donated by Toray Industries (Tokyo, Japan). Montanide ISA 51, an incomplete Freund's adjuvant (IFA), was purchased from Seppic S.A. (Orsay, France). Anti-CD8 and anti-NK1.1 mAbs for cell depletion were produced by 53-6.7.2 and PK136 hybridoma clones, respectively. Both hybridoma were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA).

2.3. Cells

C1498, a *WT1*-nonexpressing murine leukemia cell line of C57BL/6 origin, was obtained from ATCC (Rockville, MD, USA). *WT1*-expressing murine WT1-C1498 (mWT1-C1498) was generated by transduction of C1498 cells with CMV promoter driven murine WT1 17AA(+)KTS(+) isoform full length cDNA that was inserted into pcDNA3.1(+) mammalian expression vector (Invitrogen, Tokyo, Japan). YAC-1 cells that were used as target cells for NK activity were obtained from ATCC. RMAS, a TAP-deficient subline of RMA (Rauscher leukemia virus-induced lymphoma cell line of C57BL/6 origin), was kindly provided by Dr. K. Kärre (Karolinska Institute, Sweden) through Dr. H.-G. Rammensee (University of Tübingen, Germany) [24].

2.4. In vivo tumor cell challenge and vaccination schedule

The implanted dose of the tumor cells was optimized by preliminary experiments in which more than 90% of the non-treated mice transplanted with the tumor cells died within two months due to tumor development. We therefore adopted an observation period of 75 days after the tumor cell implantation (day 0). Tumor implantation and vaccination schedule are shown in Fig. 1. Mice were intradermally (i.d.) pre-immunized with an abdominal injection of 100 μg WT1 peptide emulsified with IFA on days −14 and

–7. During the same period, 50,000 units of murine IFN-β was intraperitoneally (i.p.) injected three times per week. On day 0, mice were subcutaneously (s.c.) implanted with 3×10^5 mWT1-C1498 cells in 100 μl of PBS, followed by abdominal i.d. injection of 100 μg WT1 peptide emulsified with IFA on days 1, 8, 15, and 22. In addition, 50,000 units of murine IFN-β was also injected i.p. three times per week until day 26. Mice in control groups were vaccinated with WT1 peptide + IFA + PBS (WT1 peptide alone group); PBS + IFA + IFN-β (IFN-β alone group); and PBS + IFA + PBS (non-treated group). Tumor growth was monitored by measuring the longest diameter of the palpable mass.

For the assessment of immunological effector cells, we performed *in vivo* experiments independently from those for the assessment of survival. Splenocytes and bone marrow cells from mice immunized as shown in Fig. 1 were recovered on day 30 (8 days after the last vaccination) and used for ^{51}Cr release cytotoxicity assay (CTL and NK activities) and colony assay, respectively. Furthermore, the resected tumors were used for analysis of MHC class I expression.

2.5. ^{51}Cr release cytotoxicity assay and mice treatment schedule for the assay

Splenocytes were stimulated with the 5 μg/ml WT1 peptide and cultured in complete medium containing 10% heat-inactivated FCS, 45% RPMI1640 medium, 45% AIM-V medium, 1 × non-essential amino acid (Gibco), 25 ng/ml 2-mercaptoethanol, 50 IU/ml penicillin and 50 μg/ml streptomycin. Two and four days later, recombinant interleukin-2 (rIL-2; kindly donated by Shionogi Biomedical Laboratories, Osaka, Japan) was added to the culture at a concentration of 20 IU/ml. After six days of culture, a ^{51}Cr release cytotoxicity assay was performed against WT1 peptide-pulsed or -unpulsed RMAS cells for WT1-specific CTL activity, and against YAC-1 cells for NK cell activity, as described previously [24]. Target cells (1×10^4 cells) labeled with ^{51}Cr were added to wells containing varying numbers of effector cells in 96-well plates. After 4 h of incubation at 37 °C, cell lysates were centrifuged and 100 μl of the supernatant was collected and measured for radioactivity. The percentage of specific lysis (% specific lysis) was calculated as follows: percentage of specific lysis = (cpm of experimental release − cpm of spontaneous release)/(cpm of maximal release − cpm of spontaneous release) × 100. Radioactivity of the supernatant, either of the target cell cultures without effector cells, or of the target cells that were completely lysed by the treatment with 1% Triton X-100 was used for spontaneous and maximal release, respectively.

2.6. Analysis of MHC class I expression

Tumors were resected from the tumor-bearing mice on day 30, and tumor cell suspensions were prepared with the tissues in the center of the tumor mass. The resected tissues contained only tumor mass with the naked eye. The cells were stained with FITC-conjugated anti-mouse H-2D^b monoclonal antibody (KH-95, BD Biosciences, Franklin Lakes, NJ, USA) and analyzed with the FACSort (BD). Live cells were determined by means of FSC and SSC gating.

2.7. Colony assay

For CFU-GM (colony-forming-unit granulocyte-macrophage) assay, bone marrow cells were recovered from mice on day 30, plated at 1×10^4 cells/plate in methylcellulose medium containing 10 ng/ml IL-3, 10 ng/ml IL-6, 50 ng/ml SCF, and 3 U/ml erythropoietin (EPO) (Methocult M3434; Stem Cell Technologies, Vancouver, BC, Canada), and cultured at 37 °C in a humidified incubator under 5% CO_2. Colonies with more than 50 cells were counted on days 8 and 12.

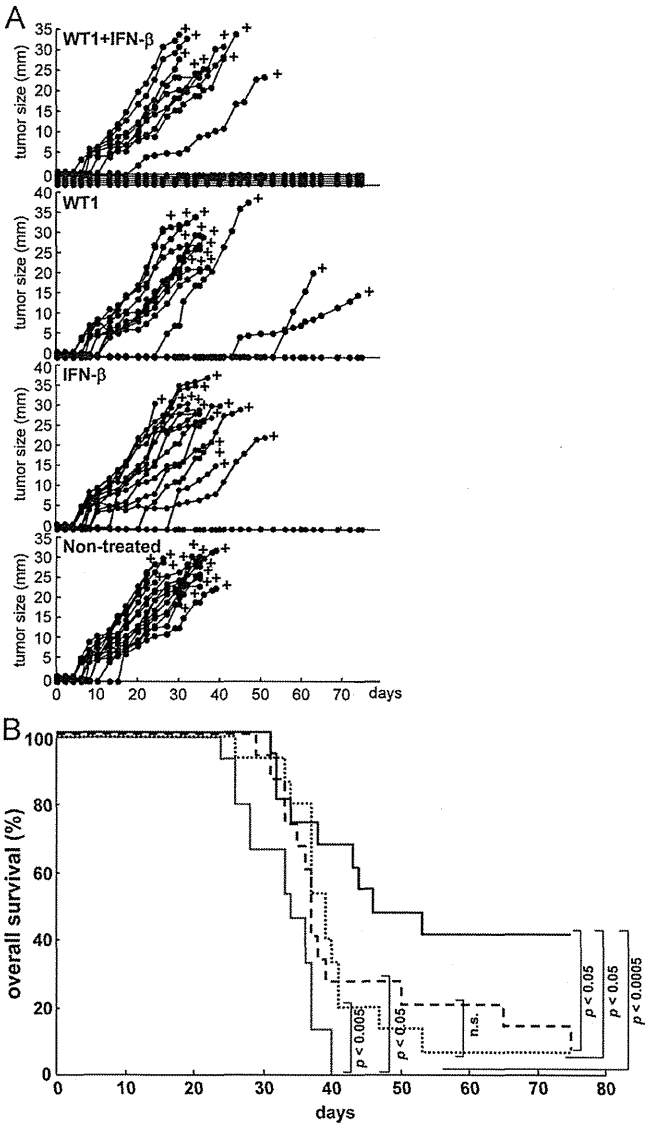

Fig. 2. Effect of WT1 peptide vaccination combined with IFN-β administration on rejection of implanted tumor cells. (A) Time course of size of tumors developed in individual mice of the four groups. Tumor sizes represent the longest diameters. (B) Overall survival curves of the four groups. Solid black, broken, dotted, and solid gray lines represent overall survival curves of mice treated with WT1 peptide vaccine + IFN-β, WT1 peptide vaccine alone, IFN-β alone, and non-treated mice, respectively.

2.8. In vivo CD8$^+$ T and NK cell depletion experiments

Mice were implanted with 3×10^5 mWT1-C1498 cells and treated with WT1 peptide vaccine + IFN-β as shown Fig. 1. The WT1- and IFN-β- treated mice were injected with PBS or 200 μg of anti-CD8 and/or 200 μg of anti-NK mAbs on days −15, −8, −1, 4, 7, 11, 14, 18, 21 and 25 [35,41].

2.9. Statistical analysis

Significant differences in overall survivals among experimental groups were evaluated with the Logrank test. The Student's *t*-test was used to calculate the differences in the expression levels of H-2D^b on tumor cells in mice among experimental groups.

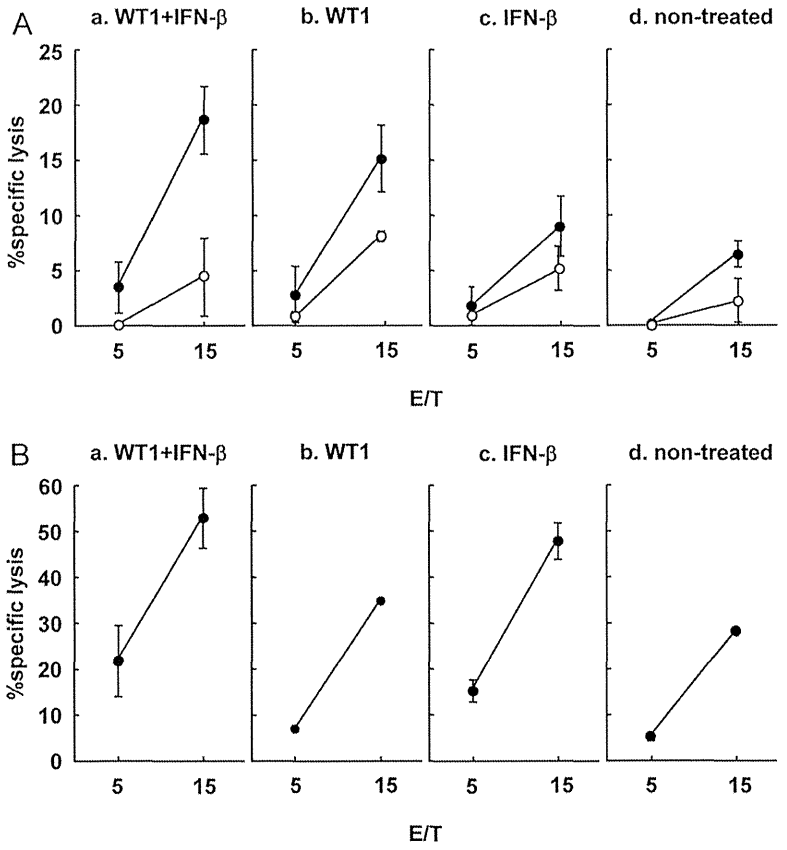

Fig. 3. Induction of WT1-specifc CTLs and enhancement of NK activity by treatment with WT1 peptide vaccine + IFN-β. Eight days after the last vaccination, splenocytes from the mice in each group were stimulated *in vitro* with WT1 peptide-pulsed synergistic splenocytes. WT1-specific CTL and NK cell activities were assayed in triplicate as cytotoxic activities against WT1 peptide-pulsed, -unpulsed RMAS or YAC-1 cells, respectively, at the indicated E/T ratio. (A) WT1-specific CTL activity. Closed and open circles represent cytotoxic activities against WT1 peptide-pulsed or -unpulsed RMAS, respectively. (B) NK activity. NK activity is shown as cytotoxic activities against YAC-1 cells. Bars indicate standard errors.

3. Results

3.1. IFN-β promotes efficacy of WT1 peptide vaccination

To investigate whether IFN-β promoted tumor cell rejection by WT1 peptide vaccination, mice were twice immunized with Montanide ISA51-emulsified WT1 peptide with or without IFN-β administration before transplantation of *WT1*-expressing tumor cells (mWT1-C1498) and then repeatedly WT1-immunized, followed by assessment of the tumor growth and their survival (Fig. 1). Optimization of cell number and determination of the observation period are described in Section 2.

Nine of the 15 mice treated with WT1 peptide vaccine + IFN-β developed tumors and died, while the remaining 6 mice were alive without tumors on day 75 (Fig. 2A). In contrast, 14 of the 15 mice treated with WT1 peptide vaccine alone, 14 of the 15 mice treated with IFN-β alone and all of the 15 non-treated mice had died of tumor growth by day 75. Overall survival rates on day 75 were 40% for mice treated with WT1 peptide vaccine + IFN-β, but 7, 7 and 0% for mice treated with WT1 peptide vaccine alone or IFN-β alone or for non-treated mice, respectively. The overall survival rates of mice treated with WT1 peptide vaccine + IFN-β were significantly higher than those of the other three groups (WT1 peptide vaccine + IFN-β versus WT1 peptide vaccine alone, IFN-β alone or non-treated: $p < 0.05$, $p < 0.05$, and $p < 0.0005$, respectively). The overall survival rates of mice treated with WT1 peptide vaccine alone or IFN-β alone were significantly higher than those of non-treated (WT1 peptide vaccine alone versus non-treated, IFN-β alone versus non-treated: $p < 0.05$ and $p < 0.005$, respectively). There was no significant difference in survival rate between WT1 peptide vaccine alone and IFN-β alone (Fig. 2B).

3.2. WT1 peptide vaccine + IFN-β enhances induction of WT1-specific CTLs and activates NK cell activity

In order to analyze immune responses, tumor-bearing mice treated with WT1 peptide vaccine + IFN-β as shown in Fig. 1 were sacrificed on day 30. The splenocytes of each mouse were stimulated *in vitro* with WT1 peptide and assayed for WT1 peptide-specific CTL activity against WT1 peptide-pulsed and -unpulsed RMAS cells and for NK activity against YAC-1 cells. Representative data are shown in Fig. 3. Splenocytes from mice treated with WT1 peptide vaccine + IFN-β showed the strongest WT1 peptide-specific cytotoxic activity while splenocytes from non-treated mice showed the weakest activity. WT1-specific cytotoxic activity was in the following order: WT1 peptide vaccine + IFN-β > WT1 peptide vaccine alone > IFN-β alone > non-treated. These findings convincingly showed that WT1-specific CTL activity was higher in the two groups with WT1 peptide vaccine than in the two groups without it. It appeared that the WT1-specific CTL activities in splenocytes from IFN-β-treated or non-treated mice were endogenously induced as a result of immunological stimulation by *WT1*-expressing tumor cells implanted.

Next, NK cell activity was examined (Fig. 3B). Mice of all four groups were sacrificed on day 30 and their splenocytes were analyzed for their NK cell activity. NK cell activity was higher in both

WT1 peptide vaccine + IFN-β and IFN-β alone groups. These results suggested that NK activity was endogenously induced in *WT1*-expressing tumor-bearing mice and that this activity was enhanced by administration of IFN-β, which is a potent enhancer of NK activity.

Taken together, these results indicated that the strongest rejection of implanted tumor cells in the mice treated with WT1 peptide vaccine + IFN-β resulted from the generation of the highest levels of both WT1-specific CTLs and NK cells.

3.3. *WT1 specific CTLs and NK cells play crucial roles in the treatment by WT1 peptide vaccine + IFN-β*

To confirm that WT1-specific CTLs and NK cells played crucial roles in the tumor rejection, *in vivo* depletion of $CD8^+$ T and/or NK cells was performed. Mice that were implanted with mWT1-C1498 cells and vaccinated with WT1 peptide vaccine + IFN-β as shown in Fig. 1 were treated with both or either of anti-CD8 and anti-NK mAbs.

Tow of five mAb-non-treated mice developed tumors and died, while the remaining three survived without development of tumors. In contrast, all of the mice that were treated with both or either of anti-CD8 and anti-NK mAbs and vaccination-non-treated mice died of tumor development. It should be noted that appearance of tumors in mice treated with both or either anti-CD8 and anti-NK mAbs was earlier than that in mAb-non-treated mice (Fig. 4).

These results strongly indicated that both WT1-specific $CD8^+$ CTLs and NK cells played crucial roles in the rejection of tumor cells.

3.4. *Enhancement of MHC class I (H-2D^b) expression on transplanted tumor cells by the administration of IFN-β*

Since WT1 (Db126) peptide is produced from WT1 protein through processing in tumor cells and presents on the cell surface in association with MHC class I (H-2D^b) [29,32], H-2D^b expression levels of target cells are thought to exert a major influence on the susceptibility of the cells to attack by vaccination-induced WT1 (Db126)-specific CTLs. For this reason, the H-2D^b expression levels on the transplanted tumor cells (*WT1*-expressing C1498 cells) were examined. Tumor-bearing mice were sacrificed 30 days after tumor cell implantation, the tumors were resected, and the tumor cells were stained with anti-H-2D^b antibody (Fig. 5). The expression levels of H-2D^b on tumor cells was significantly higher in mice treated with WT1 peptide vaccine + IFN-β or IFN-β alone than in those treated with WT1 peptide vaccine alone or non-treated mice ($p < 0.05$) (Fig. 5B). These results indicated that IFN-β administration enhanced the expression of H-2D^b on tumor cells, which should make tumor cells more susceptible to attack by WT1-specific CTLs.

3.5. *No inhibition of colony-forming ability of bone marrow cells from mice immunized with WT1 peptide vaccine + IFN-β*

WT1 is expressed in some tissues of normal adult mice, including hematopoietic stem/progenitor cells, podocytes of kidney glomeruli, gonads and mesothelial structures. To evaluate the risk of induction of autoimmunity by immunization with WT1 peptide vaccine + IFN-β, the colony-forming ability of bone marrow cells, as shown by the numbers of CFU-GM colonies, were examined. No differences in numbers of CFU-GM colonies were found among the five groups (WT1 peptide vaccine + IFN-β, WT1 peptide vaccine alone, IFN-β alone, tumor-bearing non-treated, and non-tumor-bearing non-treated) (Fig. 6). These results showed that induced

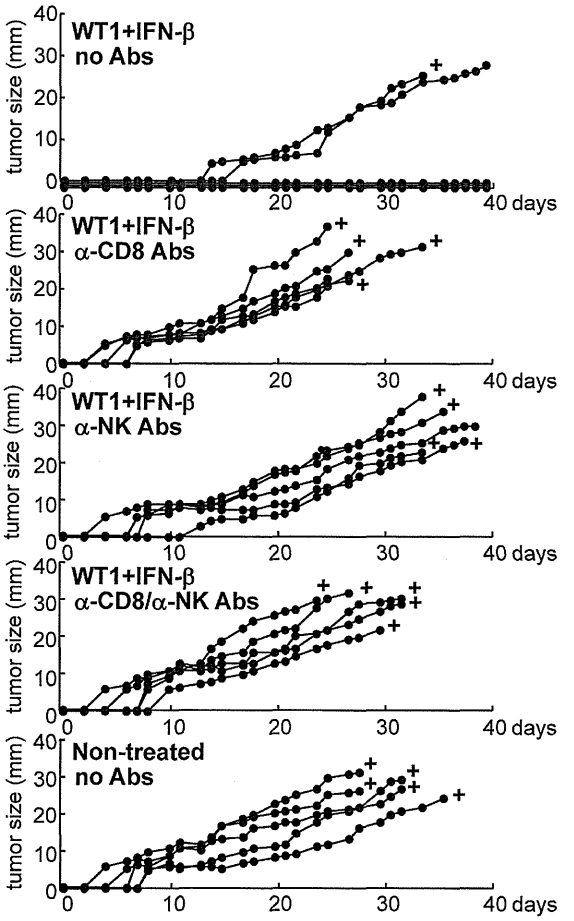

Fig. 4. Cancellation of tumor rejection by WT1 peptide vaccine + IFN-β by the administration of anti-CD8 and/or anti-NK mAbs. Mice were implanted with 3×10^5 mWT1-C1498 cells and treated with WT1 peptide vaccine + IFN-β as shown in Fig. 1. The WT1- and IFN-β- treated mice were injected with PBS or 200 μg of anti-CD8 and/or 200 μg of anti-NK mAbs on days −15, −8, −1, 4, 7, 11, 14, 18, 21 and 25. Time course of size of tumors developed in individual mice from the five groups. Tumor sizes represent the longest diameters.

WT1-specific CTLs did not recognize normal cells that physiologically expressed *WT1*.

4. Discussion

In the study presented here, we demonstrated that co-treatment with WT1 peptide vaccine (Db126; CTL epipope) + IFN-β enhanced rejection of *WT1*-expressing tumor cells in a mouse model. Enhanced induction of WT1-specific CTLs and NK cell activity was considered to be largely responsible for the successful rejection of the implanted tumor cells. The important roles of WT1-specific $CD8^+$ T cells and NK cells in the tumor rejection were confirmed by depletion experiments using anti-CD8 and/or anti-NK mAbs.

The most likely mechanism for the induction of the strongest WT1-specific cytotoxic activity in mice treated with WT1 peptide vaccine + IFN-β is the following: IFN-β activates NK cells [30,42,45], which generate IFN-γ, which in turn activates DCs and T cells [42–44]. Furthermore, IFN-β can also activate T cells directly [30]. These conditions lead to a more efficient induction of WT1-specific CTLs by the WT1 peptide vaccine. The WT1 peptide-specific cytotoxic activity observed in tumor-bearing non-treated mice may be due to the spontaneous induction of WT1-specific CTLs as a result of immune stimulation by implanted *WT1*-expressing tumors. Enhancement of NK cell function induced by *in vivo*

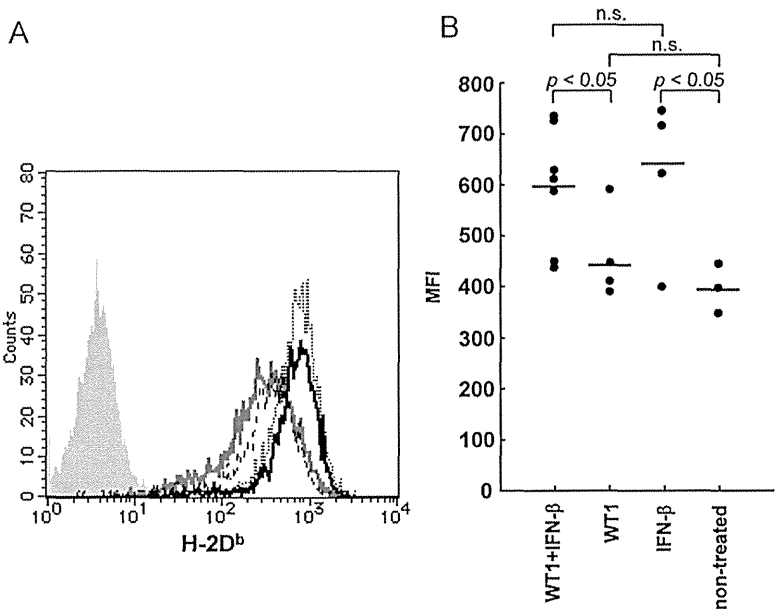

Fig. 5. IFN-β enhanced MHC class I (H-2D^b) expression of tumor cells *in vivo*. (A) H-2D^b expression levels of tumor cells recovered from mice. Solid black, broken, dotted, and solid gray lines represent the expression levels of tumor cells from mice treated with WT1 peptide vaccine + IFN-β, WT1 peptide vaccine alone, or IFN-β alone, and non-treated mice, respectively. (B) The mean fluorescence intensity (MFI) of H-2D^b expression of tumor cells from mice.

administration of IFN-β contributed to a high rejection rate of tumors in the present experiment system. However, the exact mechanism of the enhancement was not addressed in this study, while a series of investigations regarding the effect of IFN-β on NK cells were reported, including that IFN-β upregulated TRAIL on NK cells [45] and enhanced production of IFN-γ from NK cells. Besides NK cells, NKT cells might also have important roles in enhancement of tumor rejection in the present experiment system, considering that it was reported that IFN-β enhanced up-regulation of CD1d on DCs, which leads to NKT cell activation [46]. Further studies are needed to address the mechanism of enhancement of NK and NKT cell function by IFN-β in the context of tumor immunity.

At least two merits of IFN-β administration could be confirmed. One was that, as shown in Fig. 3B, greater enhancement of NK cell activity was observed in mice treated with WT1 peptide vaccine + IFN-β or with IFN-β alone than in the other two groups. This indicates that IFN-β activated NK cells *in vivo*, and that the enhanced NK activity contributed to eradication of MHC class I-negative tumor cells or those with low MHC class I expression. Another merit was that MHC class I expression on the WT1-C1498 leukemia cells was enhanced. WT1 peptides were generated through intracellular processing of the WT1 protein in tumor cells and presented on the surface of these cells in association with MHC class I molecules, followed by the recognition of the WT1 peptide/MHC class I complex by WT1-specific CTLs. Consistent with previously reported findings [28,29], MHC-class I expression on the WT1-C1498 leukemia cells was enhanced in mice treated with WT1 peptide vaccine + IFN-β or IFN-β alone. Higher expression of MHC class I molecules contributes the recognition and attack by CTLs [29]. It is possible that in mice treated with WT1 peptide vaccine + IFN-β MHC class I expression on the WT1-C1498 leukemia cells was enhanced, resulting in a heightened vulnerability to attack by WT1-specific CTLs. Taken together, it seems likely that target cells (mWT1-C1498 cells), of which the MHC class I expression was enhanced by IFN-β, were efficiently killed by WT1-specific CTLs, while the remaining target cells with negative or low MHC class I expression were efficiently killed by NK cells whose activity was enhanced by IFN-β. IFN-α is another type I IFN and has the similar structure and function to IFN-β [31–36,45,47]. Furthermore, both IFN-α and IFN-β were approved for human use [30,37–40,48]. Therefore, it would be interesting to examine, using this experiment system, whether IFN-α, as well as IFN-β, is effective in the context of a combined use with WT1 peptide vaccine for the treatment of malignancies.

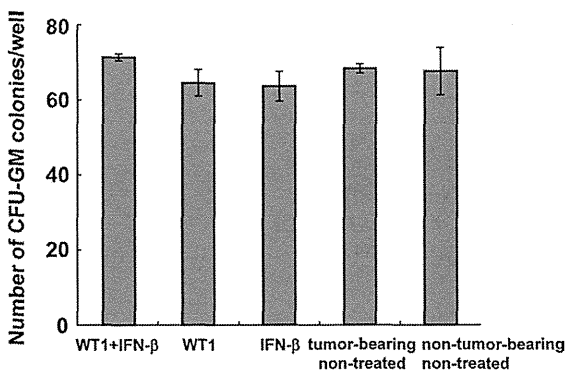

Fig. 6. No inhibition of colony-forming ability of bone marrow cells from mice immunized with WT1 peptide vaccine + IFN-β. Numbers of colonies generated by CFU-GM (colony-forming-unit granulocyte-macrophage) from mouse bone marrow cells on day 30. Values represent the means of the results from four mice in each group. Bars indicate standard errors.

Other functions of IFN-β in tumor rejection enhancement, that is, non-immunological mechanisms such as direct anti-tumor and anti-angiogenesis effect [32–34] may also have contributed to such rejection.

Although WT1 is physiologically expressed in some type of normal cells, including hematopoietic stem/progenitor cells and kidney glomeruli, WT1 vaccination combined with IFN-β treatment did not diminish the GM colony-forming ability of BM cells (Fig. 6), which is in agreement with previous reports [25,27]. These findings indicate that WT1-specific CTLs did not recognize normal cells that physiologically expressed *WT1*. The reason for this lack of recognition appears to be that WT1-specific CTLs can

discriminate only between *WT1*-expressing tumor cells and physiologically *WT1*-expressing normal cells, resulting in the selective killing of tumor cells with no damage to normal tissues. These results suggested that the mechanisms involved in processing of WT1 protein and/or presentation of WT1 peptide might be different between tumor and normal cells, resulting in no or weak presentation of the WT1 peptide on the cell surface of normal cells. Further studies to address this issue are clearly warranted.

Immunopotentiating agents play a key role in the success of cancer immunotherapy, because injection of CTL epitope peptide alone cannot sufficiently induce and activate the TAA-specific CTLs. Co-administration of CTL epitope peptides and immunopotentiating agents proved to be effective for induction and activation of the CTLs and/or activation of other effector cells such as NK cells. We previously reported that the WT1 peptide vaccine combined with *M. bovis* bacillus Calmette-Guérin cell wall skeleton (BCG-CWS), which activates DCs through TLRs 2 and 4, had a synergistic effect on tumor rejection in mice [27]. In the current study, we could demonstrate the immunopotentiating activities of IFN-β leading to the enhancement of WT1-specific CTLs, NK cells, and MHC class I expression. It is anticipated that WT1 peptide vaccination combined with both IFN-β and BCG-CWS will be more effective for tumor rejection. The combination of CTL epitope vaccine with some immunopotentiating agents with various mechanisms for enhancement of anti-tumor immunity can be expected to become part of effective strategies for the cancer immunotherapy. Clinical trials of WT1 peptide cancer vaccine have already been started, and WT1 peptide vaccination was shown to have good potential for the treatment of cancer [14–17,49–54]. So far, we have performed immunization using WT1 peptide with Montanide ISA 51 adjuvant, and another group used KLH and GM-CSF [55]. Since the safety and toxicity of IFN-β have been confirmed to a considerable extent [37–40], WT1 peptide vaccination combined with IFN-β should be ready for use in the clinical settings in the near future.

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IMAGING, DIAGNOSIS, PROGNOSIS

Low Wilms' Tumor Gene Expression in Tumor Tissues Predicts Poor Prognosis in Patients with Non-Small-Cell Lung Cancer

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We elucidated the relationship between prognosis of non-small-cell lung cancer (NSCLC) and Wilms' tumor gene (*WT1*) mRNA expression in tumor tissue. The WT1 mRNA expression levels of the fatal cases were lower as compared with those of the survival cases. Overall survival (OS) and disease-free survival (DFS) of the high WT1 expression group were longer than of the low expression group. As for squamous cell lung cancer (SQLC), low WT1 expression was significantly associated with lymph node metastasis. Cox analysis revealed that the gene level was a significant prognostic factor in OS and DFS. Low WT1 expression predicted poor prognosis in patients with NSCLC.

Keywords Lung cancer; Oncogenes; Tumor suppressors; Tumor immunology

INTRODUCTION

The Wilms' tumor gene (*WT1* gene), which was cloned from pediatric renal tumor (Wilms' tumor), is located at 11p13 (1, 2). The gene encodes zinc finger transcription factor (1) and is associated with normal development of the renal system as well as with Wilms tumor (2). Originally, the *WT1* gene was reported to be a tumor-suppressive gene (3). In sporadic unilateral Wilms' tumor, one allele of this gene contains a 25-bp deletion, while such deletion is not observed in the germline of affected individuals. These observations are consistent with somatic inactivation of a tumor-suppressive gene. The gene product suppressed transcription of some growth factors *in vitro*, such as insulin-like growth factor (IGF)-II, IGF-I receptor, platelet-derived growth factor-A, transforming growth factor-beta (4–8), and proto-oncogenes *bcl-2* and *c-myc* (9). Moreover, it has also been demonstrated that the *WT1* gene inhibits ras-mediated transformation (10). These data suggest that the *WT1* gene acts as a tumor suppressor.

On the contrary, several investigations have reported that the *WT1* gene acts as a proto-oncogene. Aberrant overexpression of the *WT1* gene was detected in leukemia cells (11–13), and the gene was associated with leukemogenesis (14). As described above, the biological function of the *WT1* gene is diverse, and according to types or situation of tumors, the gene may act either as a proto-oncogene or as a tumor-suppressive gene.

In non-small-cell lung cancer (NSCLC) cells, we have reported on the overexpression of the *WT1* gene by reverse transcriptase-polymerase chain reaction (RT-PCR) (15). However, the relation between gene expression level and prognosis of lung cancer patients has not been fully investigated. Most studies hitherto have focused on hematological tumors (16, 17) and sarcomas (18–20), and for carcinomas, very few reports exists (21). In this study, we planned to clarify the relationship between WT1 mRNA expression and survival rate of patients who underwent surgical resection of NSCLC.

MATERIALS AND METHODS

Patients

From May 2002 to November 2004, a total of 356 patients with lung tumor received surgical resection at the Kinki-Chuo Chest Medical Center, Osaka, Japan. Of the 319 patients who were diagnosed as having primary NSCLC in surgical specimens, a total of 98 patients met our eligibility criteria. Patient characteristics are shown in Table 1. NSCLC stages were classified according to the UICC TNM classification (22). The follow-up algorithm after surgery was as follows: The patients of stages I and II had physical examination, chest X-ray examination, and tumor marker tests every 3 or 4 months for the first 2 years postoperatively, and thereafter every 6 months. For the patients of stages III and IV,

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Table 1. Clinical Background of the Patients

Characteristics		
Age, year	Range	38–81
	Median	68
Sex, no. (%)	Male	55 (56.1)
	Female	43 (43.9)
Histology, no. (%)	Adenocarcinoma	63 (64.3)
	Squamous cell carcinoma	28 (28.6)
	Large-cell carcinoma	7 (7.1)
Tumor size, no. (%)	11~20 mm	15 (15.3)
	21~30 mm	38 (38.8)
	31~40 mm	23 (23.5)
	41~50 mm	11 (11.2)
	~51 mm	11 (11.2)
pathological stage, no. (%)	IA	30 (30.6)
	IB	34 (34.8)
	IIA	6 (6.1)
	IIB	6 (6.1)
	IIIA	15 (15.3)
	IIIB	6 (6.1)
	IV	1 (1.0)
Adjuvant therapy, no. (%)	None	60 (61.2)
	UFT	30 (30.6)
	Others	8 (8.2)

interval and modality of examinations were chosen according to clinical condition of the patients. Five-year postoperative mortality was observed. This study was approved by the Institutional Review Board of the National Hospital Organization Kinki-Chuo Chest Medical Center. All patients gave their written, informed consent before enrollment.

RNA purification and RT-PCR

Cancer tissues were obtained just after the surgical resection of lung, snap frozen in Isogen (Nippon Gene, Toyama, Japan) and stored at –20°C until use. The tissues were soaked in RNAlater (Qiagen, Valencia, CA) at 4°C overnight and then were stored at –80°C until use. Total RNA was isolated from frozen lung tissues using Isogen according to the manufacturer's instruction. RNA was dissolved in diethylpyricarbonate (DEPC)-treated water and quantified by a spectrophotometer. Total RNA was isolated from the sample tissues using Trizol (Invitrogen, Leek, the Netherlands) according to the manufacturer's instruction, dissolved in DEPC-treated water and quantified by a spectrophotometer according to the absorbance at 260 nm. RNA was converted into cDNA, as described previously, with a minor modification (17). In brief, 3 μg of total RNA in DEPC-treated water was incubated at 65°C for 5 min and then mixed with 25 μl of RT buffer (50 mM Tris-HCl, pH 8.3; 75 mM KCl; 3 mM $MgCl_2$; and 10 mM dithiothreitol) containing 600 U of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI), 500 μM of each dNTP, 200 ng of oligo dT primers, and 80 U of RNase inhibitor (Promega). The reaction mixture was then incubated at 37°C for 2 h, boiled for 5 min, and stored at –20°C until use. To determine relative WT1 expression levels, cDNA (3.0 μl for WT1 and 2.0 μl for β-actin) was added to the PCR buffer (100 mM Tris-HCl, pH8.3; 500 mM KCl; and 3 mM $MgCl_2$) containing 200 μM of each dNTP, 1.25 U of AmpliTaq Gold (PE Applied Biosystems, Foster city, CA), 0.5 μM forward and reverse primers, and 200 nM TaqMan probe in a total volume of 50 μl. The sequences of primers and probes used are as follows: WT1: forward primer (F1), 5'GATAACCACACAACGCCCATC3'; reverse primer (R1), 5'CACACGTCGCACATCCTGAAT3'; probe, 5'FAM-ACACCGTGC GTGTGTATTCTGTATTGG-TAMRA3'. β-actin: forward primer, 5'CCCAGCACAATGAAGATCAA GATCAT3'; reverse primer, 5'ATCTGCTGGAAGGTGGA CAGCGA3'; probe, 5'FAM-TGAGCGCAAGTACTCC GTGTGGATCGGCG-TAMRA3'. After activation of AmpliTaq Gold polymerase at 95°C for 10 min, PCR was performed for 40 cycles (95°C for 30 sec/63°C for 60 sec). Sequences of WT1 reverse and β-actin forward primers spanned two consecutive exons, from exon 6 to 7 and from exon 4 to 5, of respective gene in order to avoid amplification of the corresponding genome sequences. Standard curves for the quantification of WT1 and β-actin were constructed from the results of simultaneous amplification of serial dilutions of the cDNA from WT1-expressing K562 leukemic cells, whose WT1 expression level was defined as 1.0, as described previously (11). Real-time PCR and subsequent calculations were performed on an ABI Prism 7700 Sequence Detector System (PE Applied Biosystems). To normalize the difference in RNA degradation and in RNA loading for RT-PCR in individual samples, the values of levels of WT1 gene expression divided by those of β-actin gene expression were defined as relative WT1 expression levels in the samples. All experiments were performed in duplicate.

Statistical analysis

Survivals were calculated by the Kaplan–Meier method, and the log-rank test was used to evaluate the difference in survival.

Chi square test was used for comparison of the background of each subgroup. The Kendall's tau or Spearman's rho rank correlation coefficient was used to measure correlation of parameters. The Mann–Whitney test was used for comparison of the WT1 mRNA expression level of each subgroup. For multivariate analysis, the Cox proportional hazard regression analysis with a step-up procedure was employed, utilizing likelihood ratio as the criterion for adding significant variables. The SPSS version 15.0J software was used for statistical calculation. Statistical significance was assumed for $p < .05$.

RESULTS

Of the 319 patients who were diagnosed with primary NSCLC in surgical specimens, we excluded 36 patients whose tumor size was 10 mm or less with a longer axis from this study because we gave priority to clinical necessity of formalin fixation for pathological staging. Out of the 283 patients, 103 patients who was able to understand the purpose of this investigation and gave written informed consent to this study became candidates for this investigation, and RNAs were extracted from their tumor tissues. Among them, five

patients were excluded because their RNAs had degraded. Consequently, a total of 98 patients met our eligibility criteria.

No patients received chemo- or radiotherapy before surgery. For the patients with stage IA tumor, no adjuvant therapy was carried out. For the patients with stage IB and IIIA tumor, options of adjuvant therapy were presented. For the seven patients with stage IIIB and IV tumor, therapy was selected according to clinical condition of the patients. As a result, 30 patients received postoperative tegafur-uracil (UFT) therapy. Eight patients received postoperative therapy other than UFT: five patients radiotherapy, one chemoradiotherapy, and two combination chemotherapy.

During the postoperative follow-up of the 98 patients for 5 years, 20 patients died: 15 patients died of lung cancer, two of respiratory failure due to interstitial pneumonia, two of cerebrovascular disease, and one of respiratory failure of unknown cause. The WT1 mRNA expression did not show normal distribution, and median of the fatal cases and the survival cases was 0.0043 (range 0.0018–0.5220, interquartile range 0.0008–0.0250) and 0.0141 (range 0.0020–0.6100, interquartile range 0.0025–0.0677), respectively. Thus, for the fatal cases, the WT1 mRNA expression level was over a lower range as compared with that of the survival cases.

A cutoff value of WT1 mRNA expression to predict survival was estimated from the receiver operating characteristic (ROC) curve analysis (Figure 1). The patients were divided into two groups based on the optimal cutoff value of WT1 mRNA expression level 0.0057 (sensitivity was 0.679, and 1 – specificity was 0.350): the high WT1 expression group (60 patients) and the low WT1 expression group (38 patients). Overall survival (OS) of the high WT1 expression group was significantly longer ($p < .01$) than that of the low expression

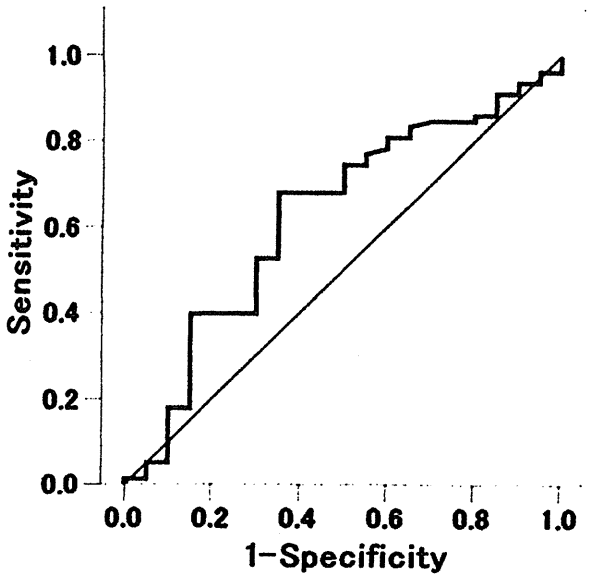

Figure 1. Receiver operating characteristic (ROC) curve analysis using WT1 mRNA expression rate and overall survival rate. The optimal cutoff value of WT1 mRNA expression was 0.00565 (sensitivity was 0.679, and 1 – specificity was 0.350).

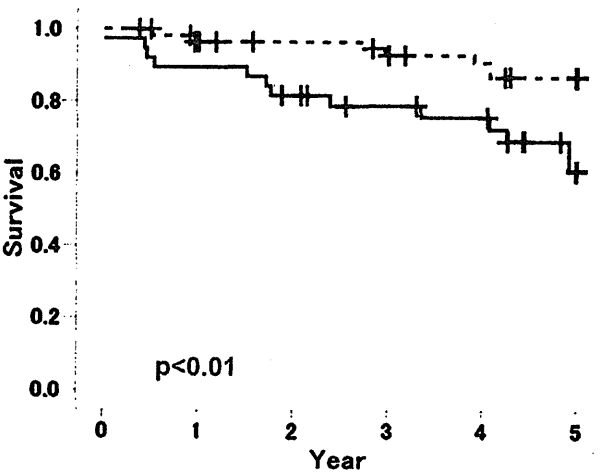

Figure 2. Overall survival (OS) rates of the high (broken line) and low (solid line) WT1 expression groups. OS rate of the low WT1 expression group was significantly lower than the high WT1 expression group ($p < .01$).

group (Figure 2). With regard to disease-free survival (DFS) for all the 98 patients, the low WT1 expression group showed a trend toward lower DFS compared with the high WT1 expression group ($p = 0.07$) but no significant differences were observed between the two groups (Figure 3A). In subset analysis for patients at stages I and II, no significant difference in DFS was observed between the high and the low WT1 expression group (Figure 3B). For patients at stages III and IV, the DFS of the low WT1 expression group was significantly lower than that of the high WT1 expression group ($p < .03$) (Figure 3C).

Then, we evaluated the relationship between WT1 mRNA expression and status of lymph node metastasis (Table 2). In subset analysis for histology, weak but significant negative correlation was observed in the 27 SQLC patients between WT1 mRNA expression level and lymph node metastasis (p-n factor) by the Kendall's tau ($p < .03$) and Spearman's rho ($p < .02$) rank correlation coefficient tests. The number of SQLC patients without lymph node metastasis was significantly larger (chi square test, $p < .01$) in the low WT1 expression group than in the high expression group. On the other hand, for the 63 ADLC patients, no significant correlation was observed between WT1 mRNA level and lymph node metastasis.

Table 2. Correlation of WT1 mRNA Expression Level and Lymph Node Metastasis

	All cases ($n = 96$)		Squamous cell carcinoma ($n = 27$)		Adenocarcinoma ($n = 63$)	
	r[a]	p	r	p	r	p
Kendall's tau	−0.018	.82	−0.355	.03	0.056	.60
Spearman's rho	−0.022	.83	−0.438	.02	0.068	.60

[a]correlation coefficient.

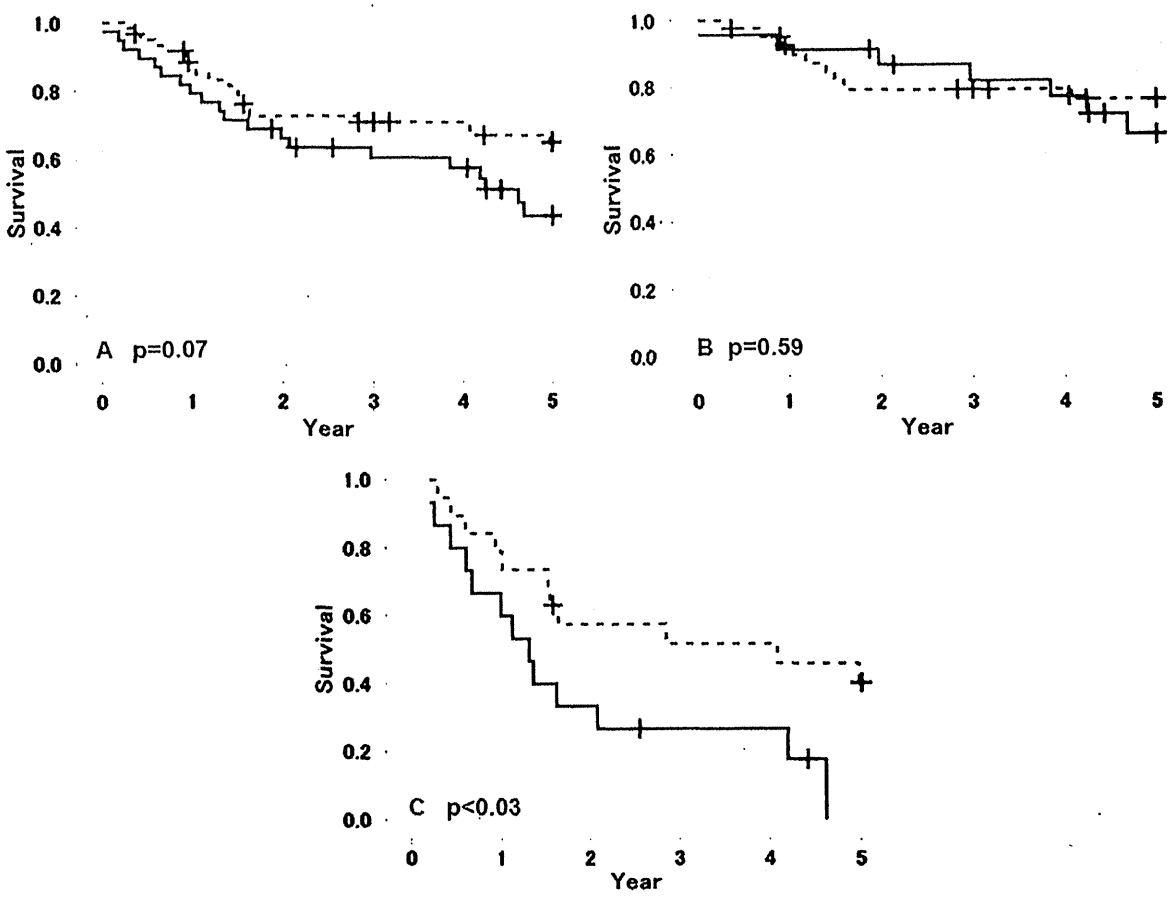

Figure 3. Disease-free survival (DFS) rates of the high (broken line) and low (solid line) WT1 expression groups. (A) DFS rates of the high (broken line) and low (solid line) WT1 expression groups for all stages. No significant difference was observed between the two groups. (B) DFS rates for patients at stages I and II of the high (broken line) and low (solid line) WT1 expression groups. No significant difference was observed between the two groups. (C) DFS rates for patients at stages III and IV of the high (broken line) and low (solid line) WT1 expression groups. In this subset, DFS rate of the low WT1 expression group was significantly lower than that of the high WT1 expression group ($p < .03$).

We evaluated association between various clinicopathological parameters and the WT1 mRNA expression. No significant association was observed between WT1 mRNA expression and age or tumor size (data not shown). In addition, no significant differences between the expression and parameters of sex, clinical stage or histological type of lung cancer were observed (data not shown).

In a multivariate analysis, significant and independent variables which influence OS were WT1 expression in the tumor tissue, pathological stages, and the absence or presence of subjective symptoms at the time of diagnosis (Table 3). As for DFS, significant and independent variables were WT1 expression, tumor size, and pathological stages (Table 4).

DISCUSSION

This is the first report that showed the relationship between WT1 gene expression and prognosis of NSCLC patients who underwent lung surgery. As for the OS and DFS of stages III and IV of NSCLC patients, WT1 expression level was a significant prognostic marker, independent of other established prognostic factors.

There have been a number of reports which show that low expression of WT1 mRNA is associated with malignant

Table 3. Multivariate Analysis of Prognostic Factors of Overall Survival

	Partial regression coefficient	p	Hazard ratio	95% CI
WT1 group	1.593	.003	4.921	1.75–13.85
Complaint at diagnosis	−1.312	.009	0.269	0.10–0.72
pathological stage	−1.203	.013	0.300	0.12–0.78

Table 4. Multivariate Analysis of Prognostic Factors of Disease-Free Survival

	Partial regression coefficient	p	Hazard ratio	95% CI
WT1 group	0.767	.025	2.152	1.10–4.22
Tumor size	0.041	.001	1.042	1.02–1.07
pathological stage	−1.261	.000	0.283	0.14–0.57

alteration. One of the growth factors whose gene expression is regulated by WT1 is vascular endothelial growth factor (VEGF). It has been reported that vegf promoter has several potential WT1 binding sites (23), and VEGF is associated with neovascularization and promotion of metastasis in lung cancer (24–26) and other solid tumors (27–29). Therefore, highly expressed WT1 might suppress expression of VEGF in lung cancers and inhibit their neovascularizaion and metastasis, resulting in favorable prognosis in patients with high expression of WT1. However, WT1 can also activate VEGF expression in a cellular context-dependent manner (23), and co-expression of WT1 and VEGF in the same area was observed in endometrial cancer tissue (30). Further study is needed to elucidate the role of WT1–VEGF pathway in lung cancers.

Moriya *et al.* reported that high level of WT1 expression was associated with suppression of lymph node metastasis in patients with SQLC, and that the invasive ability of an SQLC cell line was enhanced by suppression of WT1 gene expression (31). In all of the 27 SQLC cases in our investigation, lymph node metastasis and WT1 mRNA expression level showed significant negative correlation, which was consistent with the report by Moriya *et al.* This trend was not observed for the ADLC (antibody-dependent lymphocyte cytotoxicity) cases in our present study.

On the other hand, by *in-vitro* analysis of various types of cancers cells, there is accumulating evidence showing that the wild-type *WT1* gene is overexpressed and plays oncogenic functions, such as anti-apoptosis (32, 33) and promotion of cell migration (34). There are also a number of reports that show association between high expression of WT1 mRNA and poor prognosis. Sotobori *et al.* quantified the WT1 mRNA expression for soft tissue sarcoma in 52 patients using real-time PCR method (19). They reported that disease-specific survival rate and DFS for patients with high WT1 mRNA expression levels was significantly lower compared with that for patients with low WT1 mRNA expression levels. Srivastava *et al.* reported that high WT1 mRNA expression was associated with poor survival of patients with osteogenic sarcoma metastasis (20). As for an epithelial tumor, Miyoshi *et al.* quantified expression of WT1mRNA in breast carcinoma tissue using real-time PCR (21) and reported that poor prognosis was significantly associated with higher WT1 mRNA expression. Our data for NSCLC is apparently contradictory to the result for breast carcinoma, and the reason is unclear at present. Because cellular origin is different in NSCLC and breast carcinoma, their 5-year relative survival rates differ from one another (35). Hence, it may not necessarily be surprising that a discrepancy exists in the relationship between prognosis and WT1 gene expression. Another possibility is the difference in the induction of immune response depending on the types of tumors. Regulatory T cells as well as WT1-specific killer T cells are detected in patients with WT1-expressing tumors (36, 37). If regulatory T-cell activity differs between lung cancer and other tumors, the apparent contradictory result may be explained.

The present study showed a favorable association between WT1 expression and prognosis of NSCLC patients. This may be explained in the context of antigen-specific immune responses elicited in cancer patients. WT1 gene product is a potent pan-tumor-associated antigen, and WT1-targeting cancer immunotherapy is being demonstrated for its therapeutic potential (38). Recently, Chiba *et al.* analyzed the impact of WT1 protein expression on the prognosis of patients with recurrent or progressive glioblastoma multiforme in a phase II clinical trial of WT1 immunotherapy. The study showed that the high WT1 expression group had significantly longer OS and progression-free survival compared with the low WT1 expression group (36). These results may suggest that WT1 expression in glioblastoma cells have positive effects on their sensitivity to cytotoxic cellular immune responses targeting WT1 and correlates with favorable clinical outcome. In NSCLC, we have previously demonstrated that humoral immune responses against WT1 were elicited, as demonstrated by the enhanced production of WT1 IgG antibody (39). Interestingly, elevation in WT1 IgG antibody titers was significantly associated with longer DFS in patients with stages I–III NSCLC, suggesting that WT1-specific immune responses played an important role in anti-cancer immunity in NSCLC. In view of the above, high expression of WT1 in lung cancer cells, such as in glioblastoma cells, might have positive effects on their sensitivity to WT1-specific T cells, which correlates with favorable prognosis in advanced NSCLC.

Diversity in WT1 gene product functions may be attributable to the presence of five types of splice variants (3). One alternative splice alters the zinc finger region of WT1, resulting in modification of binding of WT1 to DNA (40). This observation suggests that each splice variant may have variable biological functions. Burwell *et al.* studied expression of different WT1 isoforms in mammary epithelial cell lines and observed that transformed phenotypes induced by transfection of the gene depended on the WT1 isoforms (41). Moriya *et al.* reported that only one isoform with a 3-amino acid deletion (–KTS) of the *WT1* gene enhanced a WT1 target gene *p21*(*Waf1*/Cip1), a gene associated with the regulation of lymph node metastasis of cancer (31). Detailed analysis of the relevancy of expression of each splice variant and prognosis of NSCLC is one of the important future issues.

In conclusion, we showed that low WT1 mRNA expression is associated with poor prognosis, and WT1 expression level will serve as a novel marker predicting prognosis of NSCLC. Moreover, our results add new information on the biological function of WT1 gene product, which may act on NSCLC as a tumor suppressor.

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DECLARATION OF INTEREST

The authors have no conflict of interest in connection with this paper. The authors alone are responsible for the content and writing of the paper.

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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.