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WT1 Peptide Therapy for a Patient with Chemotherapy-resistant Salivary Gland Cancer

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Abstract. *Wilms' tumor (WT1) protein is one of the most promising target antigens for cancer immunotherapy. In fact, clinical responses, such as growth stabilization or shrinkage of tumor with immunological responses, have been reported in patients vaccinated with WT1 peptide. Here, we performed WT1 peptide-based immunotherapy for a patient with chemotherapy-resistant salivary gland cancer, whose histologic type was carcinoma ex pleomorphic adenoma. The patient with its pulmonary metastasis, refractory to chemotherapy, was intradermally injected with 3 mg of WT1 peptide emulsified with Montanide ISA51 adjuvant at one-week intervals for 12 weeks. The considerably rapid growth of tumor was inhibited after WT1 vaccination, and stable disease, lasting three months, was achieved. Concomitantly, immunological responses, i.e. an increase in frequencies of WT1 tetramer$^+$ CD8$^+$T cells and delayed type hypersensitivity response, were detected after the vaccination. These results indicate the potential of WT1 peptide-based immunotherapy for the treatment of chemotherapy-resistant salivery gland cancer.*

The Wilms' tumor gene *WT1* was first isolated and categorized as a tumor suppressor gene that was inactivated in Wilms' tumor and mutated in the germline of children with genetic predisposition to Wilms' tumor, a kidney neoplasm of childhood (1). The *WT1* gene encodes a zinc finger transcription factor, controls the expression of many genes associated with cell growth, cell differentiation, and apoptosis, and plays a role in mRNA splicing (1).

Our group and others have demonstrated high expression of the *WT1* gene and/or WT1 protein in leukemia and various kinds of solid cancers (2). Based on a series of experimental evidence, we proposed that the *WT1* gene has an oncogenic rather than a tumor-suppressive function in most malignant diseases (1). These results indicated that the wild-type *WT1* gene product could be the most promising target antigen for cancer immunotherapy (2). WT1 peptide or *WT1* cDNA-vaccinated mice rejected the challenge by WT1-expressing tumor cells without damage to normal tissues that physiologically expressed WT1 (2). Human WT1 protein-derived peptides that were able to elicit human leukocyte antigen (HLA)-class I-restricted WT1-specific cytotoxic T lymphocyte (CTL) response were also identified by us and other groups (2-5).

Based on these pre-clinical findings, we performed a phaseI clinical study of WT1 peptide vaccination for patients with acute myeloid leukemia (AML), myelodysplastic syndromes (MDS), lung cancer, and breast cancer (2, 6). In this study, 0.3-3.0 mg of natural or modified 9-mer WT1 peptide emulsified with Montanide ISA51 adjuvant were intradermally injected at biweekly intervals. This study demonstrated that WT1 vaccination was able to induce WT1-specific CTLs and cancer regression without damage to normal tissues in the clinical setting (6). In the present study, we report a case of chemotherapy- and radiotherapy-resistant salivary gland cancer, histologically diagnosed as carcinoma ex pleomorphic adenoma, in which WT1 peptide vaccination

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Key Words: Wilms' tumor protein, WT1, salivary gland cancer, cancer immunotherapy, cancer vaccine.

considerably inhibited rapid growth of the tumor, leading to stable disease (SD) for three months.

Case Report

A 56-year-old man was diagnosed as having salivary gland cancer in February 2004. The histological diagnosis was carcinoma ex pleomorphic adenoma. The tumor was estimated as cT3N2bM1, and there was enlargement of the mediastinal lymph nodes. Left neck dissection, including submandibulectomy, was performed in February 2004, followed by postoperative radiotherapy, concurrent with S-1, targeted for the locoregional area and mediastinum. Since recurrence occurred with enlargement of mediastinal lymph nodes and elevation of tumor marker cytokeratin 19 fragment(CYFRA) in March 2005, chemotherapy with cisplatin and 5-fluorouracil was performed for three courses. However, since enlargement of mediastinal lymph nodes and lung tumor appeared after the three courses, chemotherapy with nedaplatin and docetaxel was performed for four courses. However, the disease eventually progressed with enlargement of mediastinal lymph nodes and lung tumor after the four courses. Metastasis in the right frontal lobe of brain was found in August 2006. The metastatic lesion in the brain was completely resected, followed by whole-brain radiotherapy. Histological examination of the brain tumor was compatible with metastasis of salivary gland cancer.

Since the patient was HLA-A*2402-positive, and WT1 expression of cancer tissue was proven by immunohistochemical examination (Figure 1), he was enrolled into this clinical study of WT1 peptide vaccination (7,8). The clinical course and the immunological responses, including frequencies of WT1-Tetramer $(Tet)^+$ $CD8^+$ T-cells in peripheral blood (PB) (Figure 2) and the WT1 peptide-specific delayed type hypersensitivity (DTH), were evaluated (8). During the month before the start of vaccination (weeks -4 to 0), the tumor was considerably rapidly growing and a small amount of pleural effusion at the right side appeared. Representative data of the computed tomographic examination from weeks -4 to 12 (the end of the clinical study period) are shown in Figure 3. The CT examinations revealed that the sum of the longest diameter (SLD) of target lesions increased from 67% at week -4 to 100% at the vaccination start (week 0) (SLD at week 0 was defined as 100%) (Figure 4, upper panel).

The first injection of WT1 vaccine was performed on December 20, 2006. The WT1 vaccine was composed of 3 mg of a modified WT1 peptide (amino acids 235-243: CYTWNQMNL) for HLA-A*2402 type and Montanide ISA51 adjuvant, and the vaccination was scheduled to be performed 12 times at weekly intervals (8). After WT1 vaccination was begun, rapid growth of the tumor declined (Figure 4, upper panel). SLD slightly decreased from 100% at week 0 to approximately 90% at week 5, and was then stable for a further 7 weeks until the end of this clinical study period (week 12) (Figure 4, upper panel). Furthermore, necrotic lesion in the tumor was found on CT examination at week 12 (Figure 3, upper panel). The amount of pleural effusion, which appeared before the vaccination, did not increase during the 12 weeks. The patient's quality of life was also maintained (performance status: 0) and he was able to carry out his daily life without any limitation during the three months of this clinical study period. At the end of this clinical study (week 12), the clinical response was assessed as stable disease (SD). As for adverse effects, only local skin erythema at the injection sites of the WT1 vaccine was observed.

Delayed-type hypersensitivity (DTH) skin test for WT1 peptide was performed for the monitoring of immunological response (9, 10). The DTH test was negative at the beginning of WT1 vaccination, but turned positive at weeks 5, 9 and 13 (Figure 4). Frequencies of WT1-Tet^+ $CD8^+$ T-cells among $CD8^+$ T-cells was 0.098% before the vaccination, but increased to 0.16% at week 4, and the increased percentage was maintained at week 8 (Figure 4, lower panel).

Discussion

This report demonstrates the potential of WT1 vaccination for the treatment of salivary gland cancer, histologically diagnosed as carcinoma ex pleomorphic adenoma. WT1 peptide vaccination for a patient with recurrent, chemotherapy-resistant, considerably rapidly growing submandibular gland cancer induced a cessation of tumor growth, followed by a slight decrease in tumor size (10% decrease of SLD). The decrease in tumor size was revealed with CT examination at week 4 and remained unchanged until the end of this clinical trial (week 12) (Figure 4). Furthermore, necrotic lesion in the tumor was found on CT examination at week 12 (Figure 3, upper panel). Since the tumor growth was rapid before the beginning of WT1 vaccination, it is reasonable to consider that WT1 vaccination induced a clinical response to suppress tumor growth.

The frequency of WT1-Tet^+ $CD8^+$ T-cells in PB of the patient before the vaccination was 0.098%, while the mean value of those from healthy donors was 0.094%, indicating that the frequency of WT1-Tet^+ $CD8^+$ T-cells in the PB of the patient was at the same level as the ones of healthy donors. However, the frequency of WT1-Tet^+ $CD8^+$ T-cells increased after the vaccination from 0.098% at week 0 to 0.16% at week 4 (1.63-fold increase). We previously reported a clear correlation between clinical response and 1.5-fold increase in the frequency of WT1-Tet^+ $CD8^+$ T-cells after WT1 vaccination (6). The change of DTH from negative to positive reaction after the vaccination also supports the elicitation of a WT1-specific immunological response by the vaccination. Taken together, these data strongly suggest that

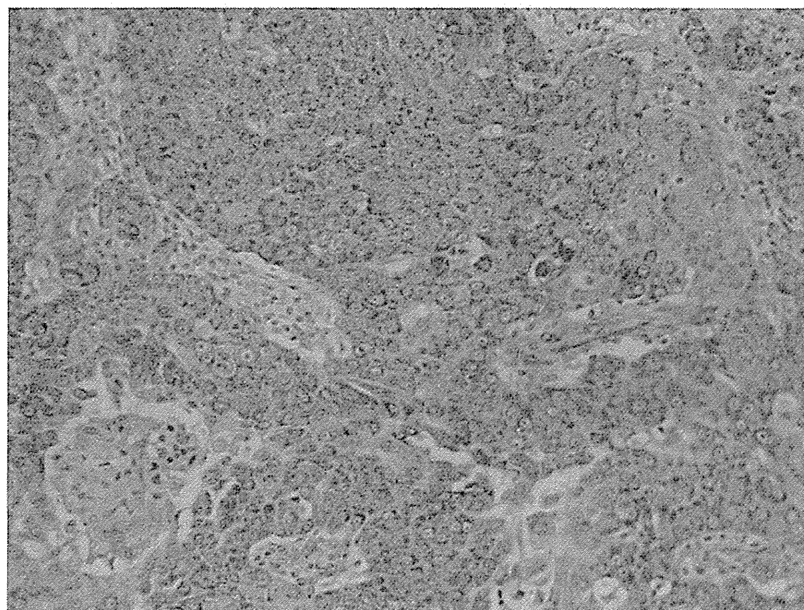

Figure 1. *Wilms' tumor (WT1) protein expression of tumor tissue. This tissue was stained with anti-WT1 protein antibody. The majority of cancer cells exhibit positive staining of WT1 protein, mainly in their cytoplasm.*

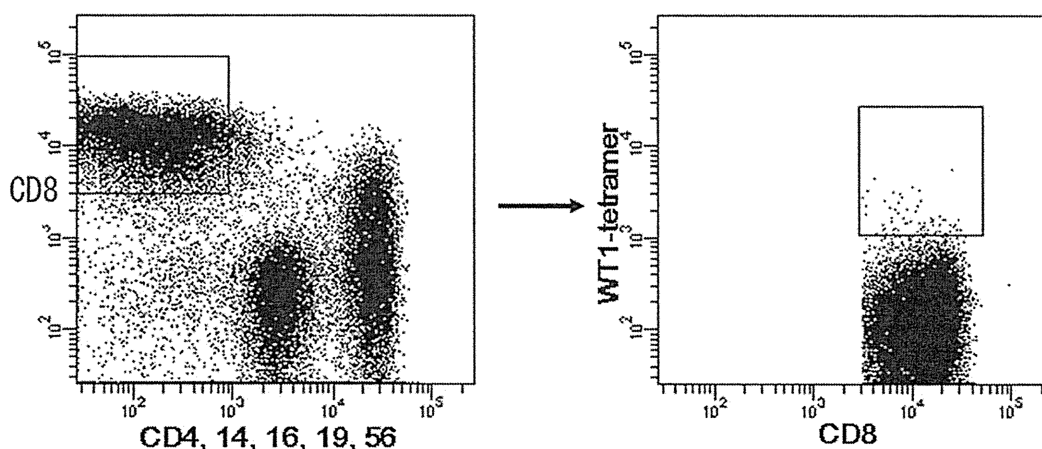

Figure 2. *Detection of WT1 tetramer+ CD8+ T-cells in the patient. CD4-, CD14-, CD16-, CD19- and CD56-positive cells were gated out from peripheral blood mononuclear cells, and these marker-negative, CD8- and WT1 tetramer-positive cells were defined as WT1-specific cytotoxic lymphocytes.*

a WT1 peptide vaccination-induced immunological response, detected by tetramer assay (*ex vivo* immune monitoring) and DTH reaction (*in vivo* immune monitoring), led to the clinical response, *i.e.* stabilization of the disease.

Surgical resection, followed by local radiotherapy, when needed, is a standard therapy for patients with stage I or II salivary gland cancer, and the prognosis is not so poor (11). However, the prognosis of patients with advanced stages of the disease, such as patients with its distant metastasis, is very poor (11). Carcinoma ex pleomorphic adenoma, presented in this report, accounts for about 12% of salivary malignancies and is a subtype of highly malignant tumor (12). Although novel therapies, including a combination therapy of trastuzumab and capecitabine, are being tested,

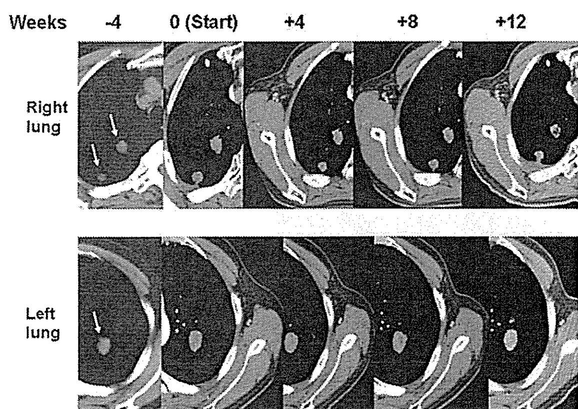

Figure 3. *Representative data of computed tomographic examination. Arrows indicate tumors in the chest. The growth of tumors was suppressed by WT1 vaccination.*

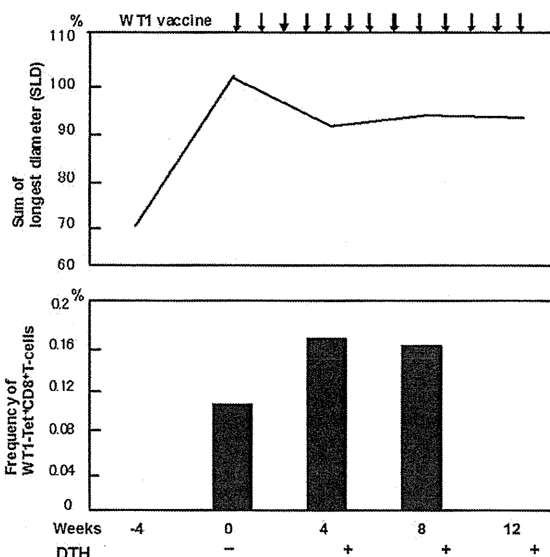

Figure 4. *The clinical course and immunological response of the patient to WT1 peptide vaccination. Upper panel: Sum of longest diameter (SLD); Lower panel: Frequencies of WT1-tetramer (Tet)$^+$ CD8$^+$ T-cells calculated as [(number of WT1-tetramer$^+$ CD8$^+$ T-cells/total number of CD8$^+$ T-cells)×100%] in peripheral blood. DTH: Delayed-type hypersensitivity.*

standard therapy for patients with advanced disease stages has not been established (12). In this context, immunotherapies, including WT1 peptide vaccine, may become alternatives for the treatment of this disease. In the course of the preparation of this manuscript, Sasabe *et al.* reported a case of pulmonary metastasis from adenoid cystic carcinoma of salivary gland that was successfully treated with WT1 peptide vaccination (13). Their report along with the current study strongly suggest that WT1 peptide vaccination has therapeutic potential for salivary gland cancer.

Besides salivary gland cancer, the favorable response of WT1 immunotherapy in various types of malignancies such as AML, MDS, multiple myeloma, glioblastoma multiforme, rhabdomyosarcoma, lung, breast, renal, ovarian cancers has been previously reported, which strongly suggests the superiority of WT1 protein as a target antigen for cancer immunotherapy (2-6, 8, 10, 14-16). In fact, WT1 protein was rated as the most promising target antigen in a recent review article (17). On this basis, WT1 peptide-based immunotherapy is expected to become a novel treatment for salivary gland cancer.

Conflict of Interest Statement

None declared.

Acknowledgements

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ORIGINAL ARTICLE

CD138-negative clonogenic cells are plasma cells but not B cells in some multiple myeloma patients

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Clonogenic multiple myeloma (MM) cells reportedly lacked expression of plasma cell marker CD138. It was also shown that CD19^{+} clonotypic B cells can serve as MM progenitor cells in some patients. However, it is unclear whether CD138-negative clonogenic MM plasma cells are identical to clonotypic CD19^{+} B cells. We found that *in vitro* MM colony-forming cells were enriched in CD138^{-}CD19^{-}CD38^{++} plasma cells, while CD19^{+} B cells never formed MM colonies in 16 samples examined in this study. We next used the SCID-rab model, which enables engraftment of human MM *in vivo*. CD138^{-}CD19^{-}CD38^{++} plasma cells engrafted in this model rapidly propagated MM in 3 out of 9 cases, while no engraftment of CD19^{+} B cells was detected. In 4 out of 9 cases, CD138^{+} plasma cells propagated MM, although more slowly than CD138^{-} cells. Finally, we transplanted CD19^{+} B cells from 13 MM patients into NOD/SCID IL2R$\gamma c^{-/-}$ mice, but MM did not develop. These results suggest that at least in some MM patients CD138-negative clonogenic cells are plasma cells rather than B cells, and that MM plasma cells including CD138^{-} and CD138^{+} cells have the potential to propagate MM clones *in vivo* in the absence of CD19^{+} B cells.

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Keywords: multiple myeloma; progenitor cells; CD138

INTRODUCTION

Multiple myeloma (MM) is characterized by the clonal expansion of malignant plasma cells.[1,2] The immunoglobulin gene sequences in MM plasma cells are somatically hyper-mutated and remain constant throughout the clinical course, suggesting that the disease arises from a post-germinal center B cell or a more differentiated cell.[3–5] Previous studies have found that MM patients harbor phenotypic B cells expressing the immunoglobulin gene sequence and the idiotype unique to the individual myeloma clone.[6–9] These findings imply that clonotypic B cells may be involved in the disease process but offer no definitive proof that B cells in fact correspond to the proliferating tumor compartment.

Clonogenic MM cells are thought to be responsible for disease progression[10,11] so that it is important to identify and target them. The first successful *in vitro* system capable of growing human MM colonies was described by Hamburger and Salmon.[10] They showed that the clonogenic frequency of clinical myeloma specimens ranged from 0.001 to 0.1% of BM cells from MM patients. In a later study utilizing methylcellulose media supplemented with lymphocyte conditioned media as growth factors, clonogenic MM progenitor cells were found in BM cells lacking expression of the plasma cell marker CD138.[11–15] It was further reported that rituximab inhibited MM colony formation[11] and that CD20^{+} B cells from some MM patients could produce MM plasma cells in a 3-D culture *in vitro*,[16] which suggests that CD138^{-} clonogenic MM cells might be B cells. However, it is still unclear whether clonogeic MM cells are B cells or plasma cells, because some CD38^{++} MM plasma cells lack CD138 expression.[17]

CD19^{+} B cells isolated from MM patients could reportedly generate MM disease upon transplantation into NOD/SCID mice,[11,12,18,19] indicating that clonotypic CD19^{+} B cells served as MM progenitor cells in these MM patients. However, B cell depletion by means of rituximab in MM patients was not clinically effective in most cases, at least for short periods, in which plasma cells did not express CD20.[20] It is therefore still unclear whether CD19^{+} or CD20^{+} clonogenic MM progenitor cells are responsible for disease progression and maintenance. Studies using the SCID-hu or rab models, which are SCID mice implanted with human fetal or rabbit bone fragments, respectively, to create a humanized or human-like microenvironment, suggested that malignant plasma cells have tumorigenic properties.[21–23]

For the development of effective treatment, it is important to know whether clonogenic cells in MM are B cells or plasma cells. In the study presented here, we aimed to clarify whether CD138^{-} clonogenic MM cells are B cells or plasma cells by using an *in vitro* colony assay and two types of xeno-transplant assays.

MATERIALS AND METHODS

Patient samples

BM cells from MM patients were collected from iliac bone after the patients' informed consent had been obtained. Mononuclear cells were purified using Ficoll Paque (GE Healthcare, Piscataway, NJ, USA) and analyzed. Cord blood cells were obtained from the Keihan Cord Blood Bank (Osaka, Japan).

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The research was approved by the institutional review boards of Osaka University School of Medicine and the Keihan Cord Blood Bank.

A total of 50 patients diagnosed with multiple myeloma were used in this study, 16 of whom were subjected to *in vitro* clonogenic assay. Twelve patients were used for the SCID-rab experiments with un-fractionated BM cells, and the samples of nine patients were subjected to SCID-rab experiments with fractionated BM cells. Finally, Samples from 13 patients were used for transplants into NOG mice.

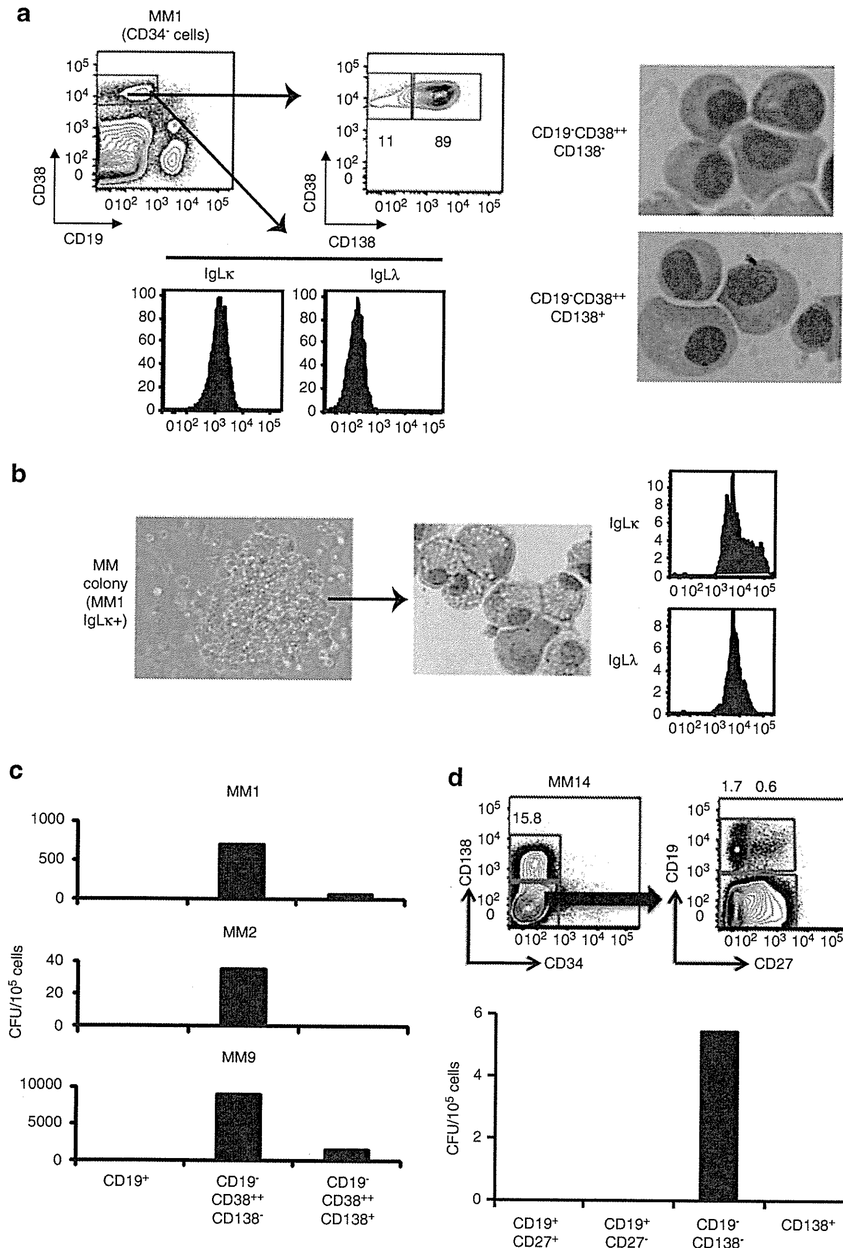

Figure 1. Clonogenic MM progenitor cells were enriched in the CD19$^-$CD38^{++}CD138$^-$ plasma cells. (**a**) FACS analysis of BM cells from an MM patient (MM1). CD19$^-$CD38^{++} MM plasma cells were divided into two populations according to CD138 expression. Results of May-Giemsa staining of FACS-sorted CD19$^-$CD38^{++}CD138$^-$ and CD19$^-$CD38^{++}CD138$^+$ cells are also shown. (**b**) An MM colony derived from CD19$^-$CD38^{++}CD138$^-$ BM cells. May-Giemsa staining of cytospin specimens of MM colonies and FACS analysis for IgLκ/λ expression in cells from MM colonies are also shown. (**c**) *In vitro* colony assay with FACS-sorted CD34$^-$CD19$^+$, CD34$^-$CD19$^-$CD38^{++}CD138$^-$ and CD34$^-$CD19$^-$CD38^{++}CD138$^+$BM cells from MM samples. (**d**) FACS analysis of the expression of CD34, CD138, CD19 and CD27 on BM cells from an MM patient. The results of an *in vitro* colony assay with FACS-sorted cells are also shown. All fractions were CD34$^-$.

Figure 2. Both CD138$^-$ and CD138$^+$ plasma cells, but not CD19$^+$ B cells, could engraft and propagate MM clones in the SCID-rab model (**a**) FACS analysis of rabbit BM transplanted with whole BM cells from an MM patient 12 weeks after transplant (Exp. 12, Table 1). MCD45, hCD19 and hCD38 denote mouse CD45, human CD19 and human CD38. Human CD38^{++} cells were further analyzed for the expression of cytoplasmic IgLκ/λ or CD138. May-Giemsa staining of FACS-sorted hCD38^{++} cells is also shown. (**b**) Transplantation of purified CD138$^-$CD34$^-$ or CD138$^+$ cells from MM BM cells into SCID-rab recipients (Exp.2, Table 2). Concentration of human IgLκ in serum at 12 weeks post transplant and the results of analysis of BM cells at 12 weeks (CD138$^-$) or 24 weeks (CD138$^+$) are shown.

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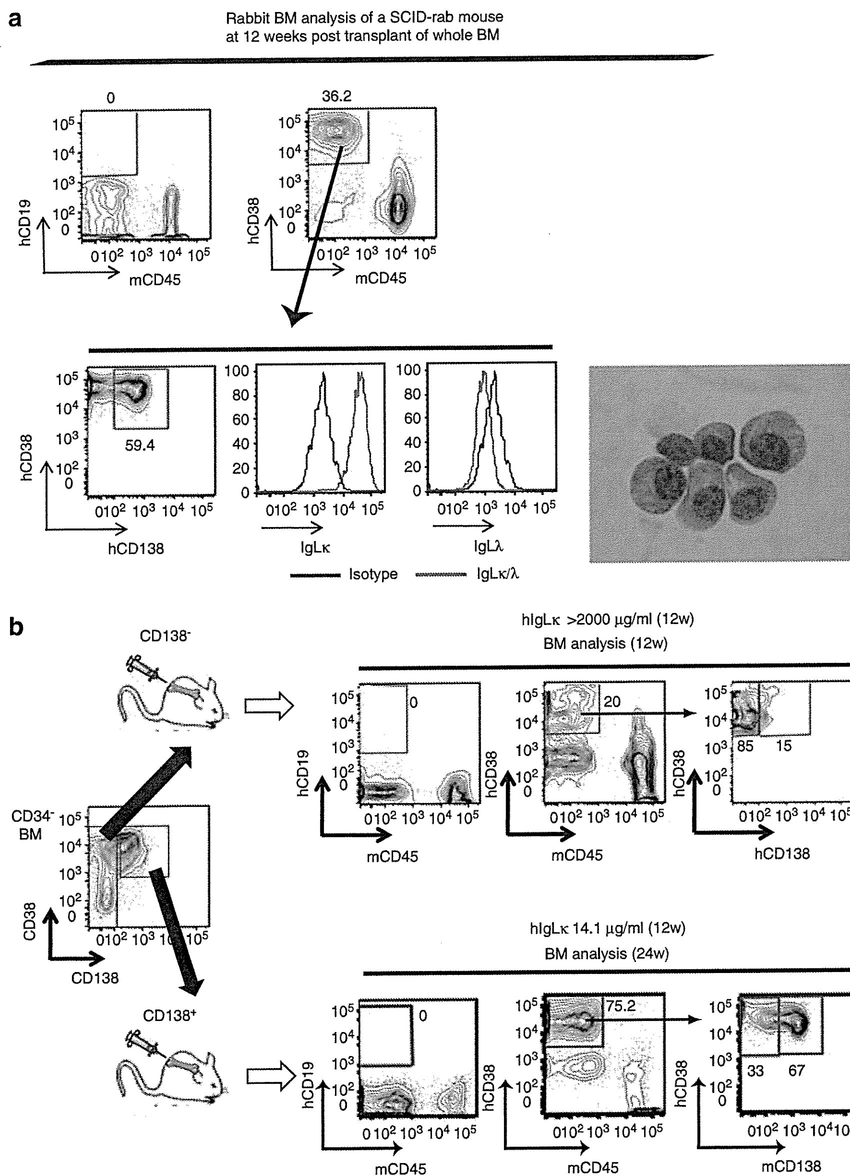
Table 1. SCID-rab experiments with whole BM cells from MM patients

Experiment	*Cell no.*	*% of CD38++ plasma cells*	*No. of plasma cells*	*Engraftment*[a]	*Serum human M protein in recipient mice (μg/ml)(10–16w)*
Exp 1	1×10^6	4.6	4.6×10^4	+	λ 324
Exp 2	3×10^6	16.6	5×10^5	−	
Exp 3	3.3×10^6	47.0	1.6×10^6	−	
Exp 4	5×10^6	4.8	2.4×10^5	+	κ 239
Exp 5	1×10^6	11.6	1.2×10^5	−	
Exp 6	3×10^6	6.3	1.9×10^5	−	
Exp 7	5×10^6	57.5	2.9×10^6	+	κ 104
Exp 8	5×10^6	75.0	3.8×10^6	−	
Exp 9	3×10^7	38.3	1.1×10^7	+	λ 25
Exp 10	4×10^6	4.0	1.6×10^5	−	
Exp 11	2.5×10^6	10.1	2.5×10^5	−	
Exp 12	1×10^7	52.8	5.3×10^6	+	N.A.[b]

[a]Engraftment was monitored by measuring IgLκ/λ and finally determined by FACS analysis of rabbit BM cells 12 or more weeks post transplant. [b]Engraftment of MM cells was examined only by FACS analysis



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Flow cytometry and cell sorting

Single cell suspensions from BM were treated with ACK solution (150 mM NH_4Cl, 10 mM $KHCO_3$) for 3 min on ice to lyse erythrocytes, then washed with PBS containing 2% FCS, incubated with 10% human AB serum for 20 min to prevent nonspecific antibody binding, and finally stained with fluorochrome-conjugated CD138 (MI15), CD38 (HB7), CD34 (8G12) and CD19 (H1B19) mAbs (BD Pharmingen, San Jose, CA, USA) on ice for 30 min. After washing, the cells were re-suspended in 1 μg/ml propidium iodide. Analysis and cell sorting were performed on FACS Aria (BD Biosciences, San Jose, CA, USA). The BD Cytofix/Cytoperm kit (BD Pharmingen) and phycoerythrin-conjugated anti-IgLκ(G20-193) or IgLλ(JDC-12) (BD Pharmingen) for staining cytoplasmic immunoglobulin were used according to the manufacturer's instructions.

Colony forming assay

Methylcellulose culture assays were performed in Methocult M3223 (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with 10% lymphocyte conditioned media, which was generated by culturing human peripheral blood mononuclear cells in RPMI1640 supplemented with 10%fetal bovine serum and 2.5 μg/ml PHA. Colonies were counted and scored on culture days 14–21.

SCID-rab model

SCID-rab mice were constructed as previously described.[23] The Institutional Animal Care and Use Committee of Osaka University Graduate School of Medicine approved the experimental procedures and protocols. Six- to eight-week-old CB.17/Icr-SCID mice were obtained from Crea Japan (Kanagawa, Japan) and 4-week-old New Zealand rabbits from Kitayama Rabesu (Nagano, Japan). The femurs and tibias from the rabbits were cut into two pieces with the proximal and distal ends kept closed. One piece of bone was inserted subcutaneously through a small (5–mm) incision, which was then closed with sterile surgical staples. Four to eight weeks after, BM cells from MM patients were injected directly into the implanted rabbit bone. The mice were periodically bled from the tail vein and changes in levels of circulating human immunoglobulin light chain (hIgL) of the M-protein isotype were used as an indicator of MM growth. Serum human immunoglobulin was measured by means of ELISA using the human kappa/lambda ELISA kit (Bethyl Laboratories, Montgomery, TX, USA). To enrich $CD138^-CD34^-$ or $CD138^+$ cells, CD138-microbeads and CD34-microbeads kits (Miltenyi Biotech, Auburn, CA, USA) were used according to the manufacture's instruction.

Transplantation into NOG mice

Intra-BM transplantation was performed as previously reported.[24] Seven-week-old female NOD/Shi-scid, IL-2 Rγnull (NOG) mice (Central Institute for Experimental Animals, Kawasaki, Japan) irradiated with 200 cGy 4-24 h before transplantation were injected into the tibia with FACS-sorted PB or BM cells from the MM patients. Transplantation into newborn NOG mice was performed within 72 h after birth. The mice were irradiated with 100 cGy 4-24 h before transplantation and injected with sorted cells via the anterior facial vein.[25] Development of MM was monitored by measuring human immunoglobulin light chain (IgL)κ or λ by means of ELISA. Twelve or more weeks after transplantation, the mice were sacrificed and BM cells were collected from the tibias and femurs and analyzed by means of FACS. Cells were stained with human CD45 (HI30)-APC, CD138-PE, CD38-FITC, CD19-Cy7APC (BD Pharmingen) and mouse CD45 (30-F11)-Cy7PE (eBioscience, San Diego, CA, USA), followed by analysis using FACS.

RESULTS

Clonogenic cells are enriched in $CD19^-CD38^{++}CD138^-$ plasma cells of some MM patients

MM plasma cells expressing a monotypic immunoglobulin light chain, could be identified as $CD34^-CD19^-CD38^{++}$ cells by FACS analysis in the BM samples of most MM patients (Figure 1a). $CD34^-CD19^-CD38^{++}$ MM plasma cells were separated into $CD138^-$ and $CD138^+$ cells (Figure 1a). $CD34^-CD19^+$ B cells, $CD34^-CD19^-CD38^{++}CD138^-$ plasma cells, or $CD34^-CD19^-CD38^{++}CD138^+$ plasma cells were FACS-sorted and subjected to colony assay. An *in vitro* colony assay was performed in methylcellulose medium supplemented with lymphocyte conditioned media, as previously reported.[11] Formation of MM colonies consisting of plasma cells (Figure 1b) was detected in 3 out of 13 MM samples. In those 3 MM samples, the frequencies of colony formation in the $CD34^-CD19^-CD38^{++}CD138^-$ plasma cells were 700, 35, and 9053 colonies per 10^5 cells, while those in $CD34^-CD19^-CD38^{++}CD138^+$ plasma cells were much lower (Figure 1c) and $CD34^-CD19^+$ B cells did not form any MM colonies. These results indicate that clonogenic cells were found in BM cells lacking the expression of a plasma cell marker CD138, but only in $CD138^-CD19^-CD38^{++}$ plasma cells, not in $CD19^+$ B cells.

We further investigated whether colony-forming cells could be detected in $CD19^+CD27^+$ B cells, which are reportedly enriched with clonogenic cells.[12] Samples from 3 patients were examined, and MM colony-forming cells were detected in one. In that sample, MM colonies were formed not from $CD19^+CD27^+$ B cells, but from $CD19^-CD138^-$ cells (Figure 1d).

In the SCID-rab model, only $CD38^{++}$ MM plasma cells engrafted and expanded *in vivo* without engraftment of $CD19^+$ B cells

SCID-rab mice were constructed as previously reported.[23] A rabbit bone fragment was inserted under the skin of a SCID mouse more than 4 weeks before transplantation of MM cells. First, whole BM cells from MM patients were transplanted and engraftment of MM cells was monitored by measuring human IgLκ and λ in serum of

Table 2. SCID-rab experiments with $CD138^-$ or $CD138^+$ BM cells from MM patients

	Population	Cell no.	Serum human M protein (μg/ml) in recipient mice (12–24 w posttransplant)
Exp1	$CD138^-$	1.2×10^6	—
	$CD138^+$	1.6×10^6	—
Exp2	$CD138^-$	1×10^7	>2000
	$CD138^+$	1×10^7	14
Exp2-2[a]	$CD138^-$	2×10^6	>2000
	$CD138^+$	2×10^6	95
Exp3	$CD138^-$	4×10^6	—
	$CD138^+$	2×10^5	313
Exp4	$CD138^-$	2.6×10^6	>2000
	$CD138^+$	4×10^5	—
Exp5	$CD138^-$	1.6×10^6	—
	$CD138^+$	2×10^5	11
Exp6	$CD138^-$	2×10^6	>2000
	$CD138^+$	2×10^6	81
Exp7	$CD138^-$	4×10^6	—
	$CD138^+$	2×10^5	—
Exp8	$CD138^-$	2×10^6	—
Exp9	$CD138^-$	2×10^6	—
	$CD138^+$	2×10^4	—

[a]Secondary transplantation.

Figure 3. Neither B cells nor plasma cells from MM samples engrafted in NOG mice. (**a**) Analysis of cytoplasmic immunoglobulin light chain (cIgL) κ or λ expression in $CD38^{++}$ plasma cells or in $CD19^+$ cells from the MM BM sample used for exp. 6 in Table 3. Findings of FACS analysis of BM cells of NOG mice transplanted with $CD19^+$ B cells from the patient's BM (exp. 6-2 in Table 3). Corresponding data for an NOG mouse transplanted with cord blood-derived $CD34^+$ cells is shown for reference (exp. CB, Table3). Analyses were performed 12 weeks post-transplant. (**b**) Analysis of cytoplasmic immunoglobulin light chain (cIgL) κ or λ expression in $CD38^{++}$ plasma cells, or $CD19^+$ B cells from MM BM sample used for exp. 13 shown in Table 3. FACS analysis findings of NOG mice transplanted with $CD3^-$ BM cells from the patient sample 12 weeks after transplant. Expression of cytoplasmic IgLκ and λ in $CD38^{++}CD138^+$ cells was also analyzed to determine whether they were clonal MM cells.

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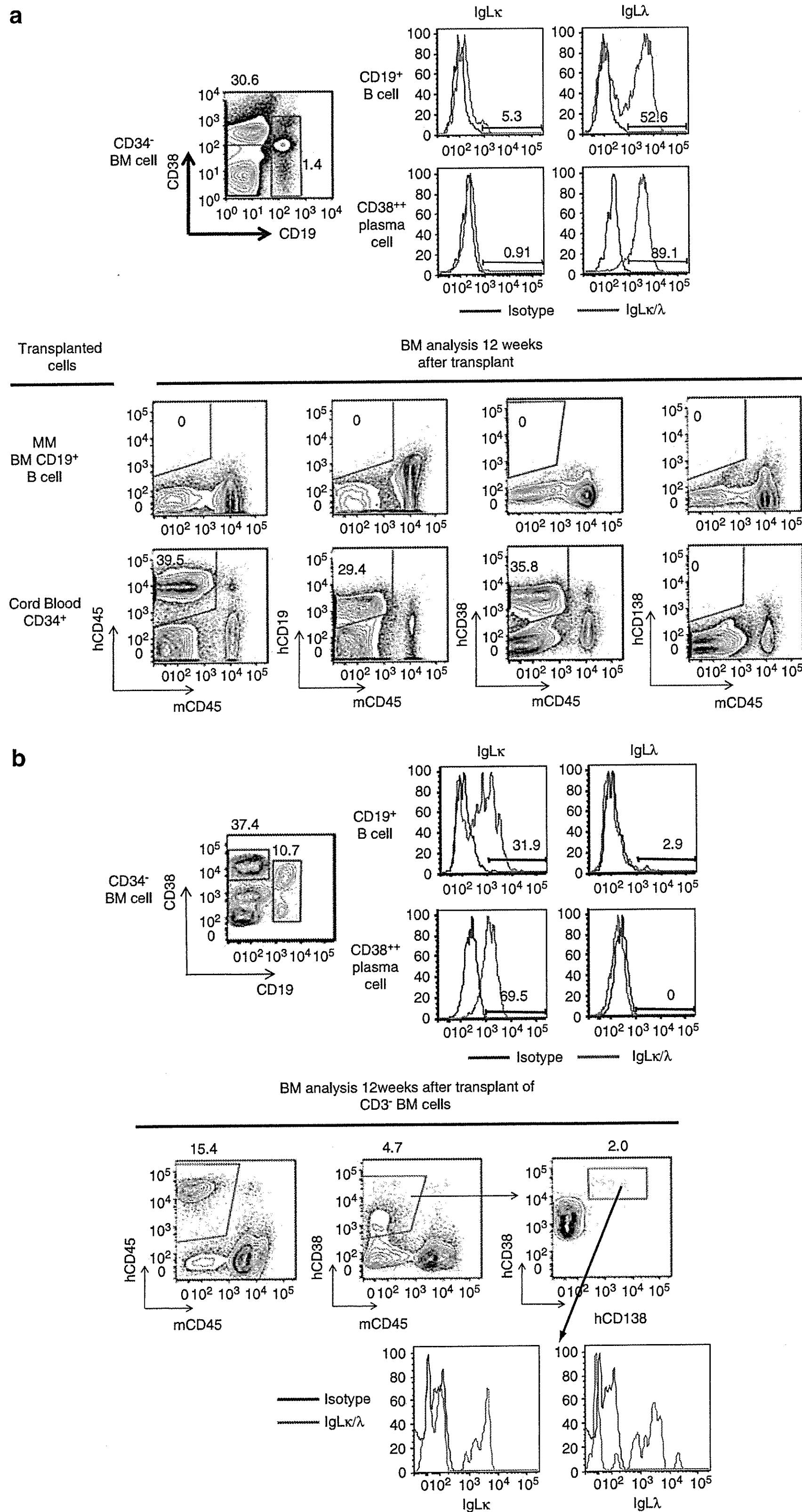
the recipient mice. Engraftment and expansion of MM cells were observed in 5 out of 12 cases (Table 1). Rabbit BM was analyzed 12 weeks or more after transplant to determine whether engraftment of not only MM plasma cells but also CD19$^+$ B cells had taken place. Robust engraftment of human CD38^{++} MM plasma cells expressing the monotypic immunoglobulin light chain and



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containing both CD138$^-$ and CD138$^+$ cells was detected in the rabbit BM, but no human CD19$^+$ B cells were detected (Figure 2a). These results indicate that CD19$^-$CD38^{++} plasma cells could engraft and expand at least for several months without engraftment of CD19$^+$ B cells.

Both CD138$^-$ and CD138$^+$ plasma cells could engraft and propagate MM clones in SCID-rab model

Next, we transplanted purified CD138$^-$ or CD138$^+$ BM cells into SCID-rab mice to test whether proliferating cell compartments were present in the CD138$^-$ population. Transplants with 9 MM samples were performed and in 3 of the samples, rapid increase of either human IgLκ or λ was observed in serum of the mice transplanted with CD138$^-$ cells (Table 2). In the rabbit BM, CD38^{++} MM plasma cells including CD138$^-$ and CD138$^+$ cells, but not CD19$^+$ cells, were detected (Figure 2b). In 4 of 8 cases CD138$^+$ plasma cells also engrafted and expanded, although more slowly than CD138$^-$ BM cells (Table 2, Figure 2b). In addition, CD138$^-$ cells from the SCID-rab mice that had initially been engrafted with CD138$^-$ BM cells could be secondarily transplanted to another SCID-rab recipient and propagate MM disease more rapidly than CD138$^+$ cells (Table 2, exp. 2-2). These results indicate that CD138$^-$ plasma cells of some MM patients have the potential to propagate MM disease rapidly in the SCID-rab model, while CD138$^+$ plasma cells can also engraft and propagate MM slowly.

CD19$^+$ B cells from MM patients did not engraft to NOD/Shi-scid,IL-2Rγnull (NOG) mice

To examine whether CD19$^+$ B cell fractions contain MM-initiating cells upon transplantation to immune-deficient mice, CD19$^+$ B cells were FACS-sorted from PB of 5 MM patients and BM cells from 4 MM patients including one patient whose CD19$^+$ B cells exclusively expressed IgL λ (Figure 3a), and transplanted directly into BM of NOD/Shi-scid,IL-2Rγnull (NOG) mice or intravenously to new born pups of NOG mice (Table 3, exp. 1-9). Engraftment of human MM cells was monitored by measuring human immunoglobulin light chain (IgL) κ and λ in serum of the recipient mice, but no human IgL was detected at any time. We also analyzed BM of the recipient mice 12–20 weeks after transplant, but no human CD19$^+$ or CD38^{++} cells were detected (Table 3, Figure 3a). CD19$^-$CD38^{++} plasma cells from the MM BM samples were also transplanted into NOG mice, but did not engraft (Table 3, exp. 6–9). In contrast, robust engraftment of human cells was observed upon transplantation of cord blood-derived CD34$^+$ cells (Table 3, exp. CB, Figure 3a). In three experiments (Table 3, exp. 10–12), CD3$^-$CD34$^-$CD138$^-$ cells were transplanted into BM of NOG mice, but did not engraft. Finally, CD3-depleted BM cells from an MM patient whose CD19$^+$ B cells exclusively expressed IgLκ (Figure 3b) were transplanted intravenously into a newborn NOG mouse (Table 3, exp. 13). When the BM cells were analyzed 12 weeks after transplant, significant engraftment of human CD45$^+$ cells was observed because CD3$^-$ BM cells contained many CD34$^+$ hematopoietic stem and progenitor cells. Small numbers of human CD38^{++}CD138$^+$ plasma cells were also detected, but analysis of their IgL κ and λ expression showed that they were not clonal MM plasma cells (Figure 3b). This result suggests that normal human hematopoietic cells, but not MM cells, engrafted in the recipient mice.

Table 3. Transplantation of cells from MM patients into NOG mice

Experiment	BM or PB	Population	Transplant method[a]	Cell no.	Engraftment
Exp 1	PB	CD19$^+$	iBMT	3.5×10^4	–
Exp 2	PB	CD19$^+$	iBMT	3.0×10^5	–
Exp 3	PB	CD19$^+$	iBMT	1.5×10^5	–
Exp 4	PB	CD19$^+$	iBMT	1.6×10^5	–
Exp 5	PB	CD19$^+$	iBMT	7.5×10^5	–
Exp 6-1	BM	CD19$^+$	iBMT	2.5×10^4	–
	BM	CD19$^-$CD38^{++}	iBMT	5×10^5	–
Exp 6-2	BM	CD19$^+$	Newborn	1×10^4	–
	BM	CD19$^-$CD38^{++}	Newborn	1.3×10^5	–
Exp 7	BM	CD19$^+$	Newborn	4×10^3	–
	BM	CD19$^-$CD38^{++}	Newborn	5×10^4	–
Exp 8	BM	CD19$^+$	iBMT	1×10^5	–
	BM	CD19$^-$CD38^{++}	iBMT	1×10^5	–
Exp 9	BM	CD19$^+$	iBMT	4.5×10^4	–
	BM	CD19$^-$CD38^{++}	iBMT	5×10^4	–
Exp 10	BM	CD3$^-$CD34$^-$ CD138$^-$	iBMT	4×10^5	–
Exp 11	BM	CD3-CD34$^-$ CD138$^-$-	iBMT	5×10^5	–
Exp 12	BM	CD3-CD34$^-$ CD138$^-$	iBMT	3×10^5	–
Exp 13	BM	CD3$^-$	Newborn	5×10^6	–[b]
Exp-CB	CB	CD3$^-$CD34$^+$	Newborn	1×10^5	+

[a]iBMT denotes intra-BM transplantation, and new born denotes transplantation to new born pups. [b]Engraftment of MM plasma cells was not detected, but normal hematopoietic cells engrafted robustly.

DISCUSSION

In this study, we showed that *in vitro* clonogenic cells that were detected in some MM patients lacked expression of the plasma cell marker CD138,[11,13,14] and that they were CD19$^-$CD38^{++}CD138$^-$ plasma cells, not CD19$^+$ B cells. Consistent with our results, it has been recently reported that CD138$^-$CD38^{++}plasma cells contain more cycling cells compared to CD138$^+$ plasma cells.[17] An *in vitro* colony assay of CD138$^-$CD19$^-$CD34$^-$ cells showed colony formation only in 4/16 (25%) patients which is a lower ratio than the one previously reported (88%) by Matsui *et al.*[11] even though our method for *in vitro* colony assay was the same. Only cells that proliferate extensively in response to stimulation by lymphocyte-conditioned medium (LCM) can be detected with the clonogenic assay used in our study. When the survival of clonogenic MM cells depends on factors other than the soluble factors contained in LCM, for example attachment to stromal cells, clonogenic MM cells cannot be detected with the assay used in our study. On the other hand, cells from aggressive or advanced MM cases may be more independent of several cell extrinsic factors and efficiently produce colonies. Heterogeneity of MM patients should thus be the reason for the differences in the frequencies of MM colony formation between our study and the one by Matsui *et al.*[11]

The SCID-rab experiments showed that highly proliferative myeloma cells were present in the CD138-negative fraction in some patients, but those cells were MM plasma cells, not B cells. Consistent with the findings of previous studies,[22,23] we also found that MM developed in the SCID-hu/rab mice transplanted with CD138$^+$ plasma cells. MM plasma cells thus have the potential to propagate and maintain MM clones, at least for several months, in the absence of clonotypic B cells. This may explain why B cell depletion by rituximab was not clinically effective, at least in the short run, for most MM patients.

Interestingly, CD138$^-$CD38^{++} plasma cells were detected in the SCID-rab mice transplanted with CD138$^+$ plasma cells. This suggests that CD138 expression on MM plasma cells may be reversible, although we cannot exclude the possibility of minor contamination of CD138$^-$ cells in the purified CD138$^+$ cells. The significance of CD138 expression on clonogenic MM cells thus needs to be carefully interpreted. It was reported that interaction with bone marrow stromal cells induced expression of CD138 in MM plasma cells,[26] suggesting that changes in CD138 expression depend on the microenvironment. In addition,

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Jakubikova *et al*. recently reported that clonogenic side populations in MM cell lines were not enriched in the CD138$^{low/+}$ but not in the CD138^{-} population, although it is not clear whether clonogeic side populations in primary MM cells are also enriched in CD138$^{low/+}$ cells.[27]

CD19^{+} B cells in some MM patients generated MM disease upon transplantation into NOD/SCID mice,[11,12,18,19] indicating that CD19^{+} B cells of some MM patients definitely contain MM progenitor cells. In our experiments, however, we could not find any MM patients whose CD19^{+} B cells induced MM disease upon transplantation into NOG mice. However, this does not necessarily mean that CD19^{+} clonotypic B cells cannot be MM progenitor cells in MM patients. It should be noted that there are many difficulties involved in the engraftment of human cells in xeno-graft models. For example, mouse IL6, which is one of the major growth factors for plasma cells, does not transduce its signals through human IL6 receptors, and probably other factors lack inter-species cross reactivity between human and mice. This means that MM progenitor cells can be detected in xeno-graft assays only when they can survive independently of human IL6 or other human factors. Thus, CD19^{+} B cells from advanced MM patients may be independent of several survival factors and effectively engraft and propagate MM disease upon transplant into immuno-deficient mice. In addition, signals from B cell receptors (BCRs) on clonotypic CD19^{+} B cells need to be taken into consideration. When BCRs of clonotypic CD19^{+} B cells show very high affinity to xeno-antigen in mice, they may be depleted and cannot survive in xeno-graft models. Thus, assuming that CD19^{+} MM progenitor cells are present, they can be detected in xeno-graft assays only when their BCRs are suitable for survival in mice. We can therefore not deny the possibility that CD19^{+} B cells have potential as MM progenitor cells, even if CD19^{+} B cells do not engraft in immunodeficient mice.

Taken together, our findings show that CD138^{-} clonogeic cells are plasma cells and not B cells in some MM patients. Furthermore, we suggest that MM plasma cells, which include CD138^{-} and CD138^{+} cells, have potential to propagate and maintain MM clones for at least several months without the need for CD19^{+} clonotypic B cells. Our findings also indicate that clonogenic MM plasma cells should be considered important therapeutic targets. The Hedgehog signaling pathway was reported to be a promising candidate as a therapeutic target against clonogenic MM cells.[15] In addition, since clonogenic MM plasma cells mainly reside in the BM microenvironment, it is also important to understand the mechanisms involved in how the BM microenvironment supports clonogenic MM plasma cells and targets them. The Notch signaling pathway may be a good candidate for such a target.[28]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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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)

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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

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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

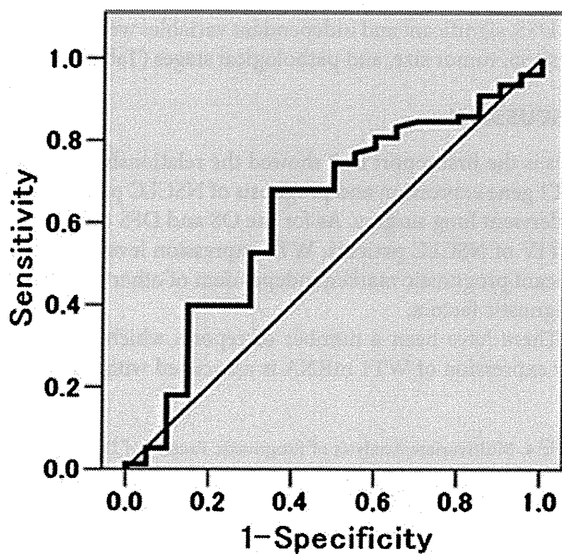

Figure 1. Receiver operating characteristic (ROC) curve analysis using WT1 mRNA expression rate and overall survival rate. The optimal cutoff value of WT1 mRNA expression was 0.00565 (sensitivity was 0.679, and 1 – specificity was 0.350).

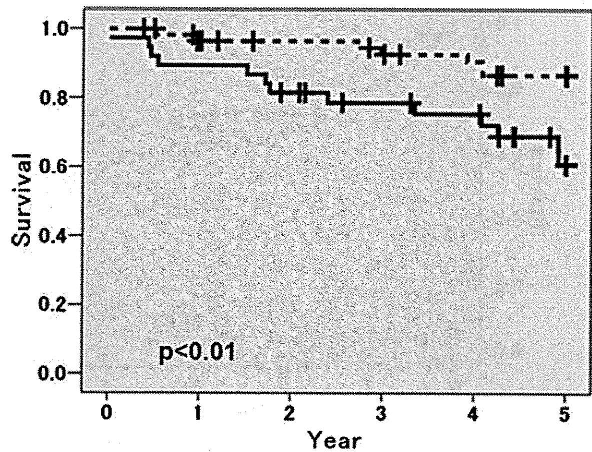

Figure 2. Overall survival (OS) rates of the high (broken line) and low (solid line) WT1 expression groups. OS rate of the low WT1 expression group was significantly lower than the high WT1 expression group ($p < .01$).

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.

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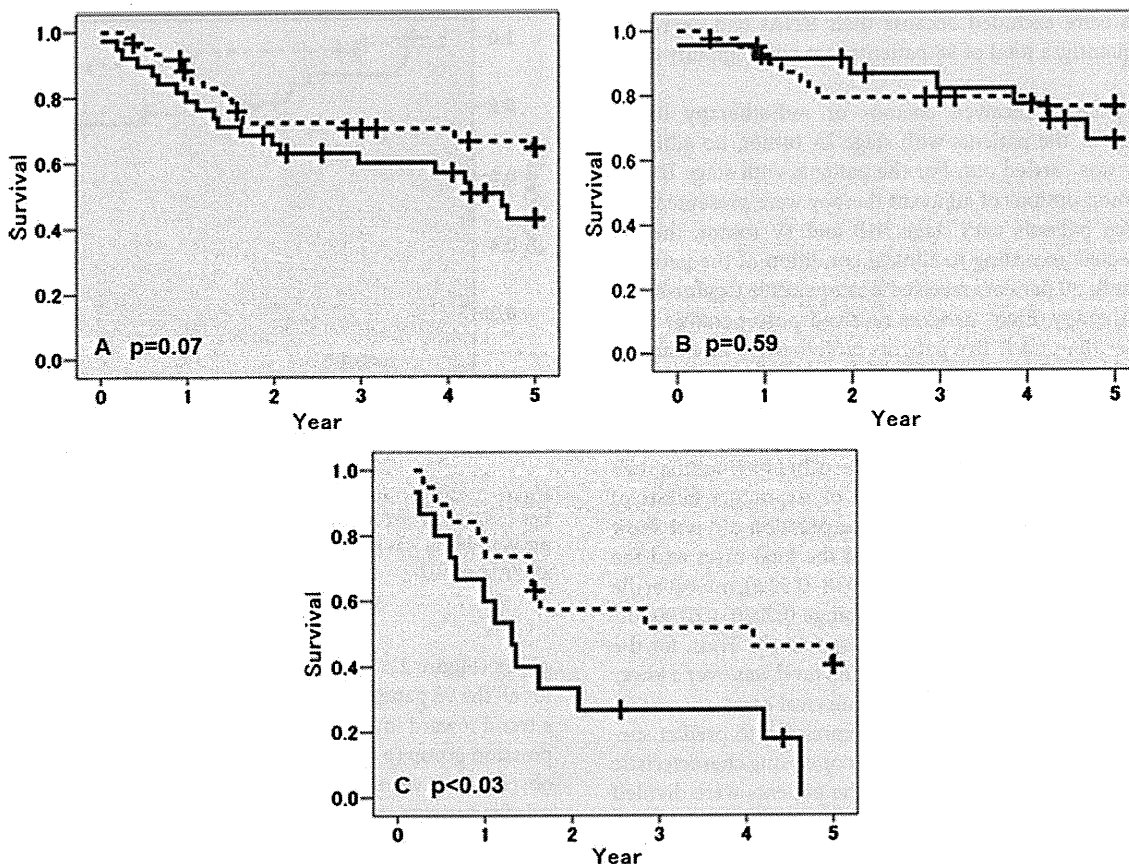

Figure 3. Disease-free survival (DFS) rates of the high (broken line) and low (solid line) WT1 expression groups. (A) DFS rates of the high (broken line) and low (solid line) WT1 expression groups for all stages. No significant difference was observed between the two groups. (B) DFS rates for patients 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 ($p < .03$).

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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

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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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