Th1-biased humoral immune responses against Wilms tumor gene *WT1* product in the patients with hematopoietic malignancies

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The Wilms' tumor gene *WT1* is highly expressed in leukemias and myelodysplastic syndrome (MDS), and *WT1* expression levels increase along with the disease progression in chronic myeloid leukemia and MDS. We previously reported that IgM and IgG WT1 antibodies were detected with significantly higher detection rate and antibody titers in leukemias and MDS compared to those in healthy volunteers. In this study, whether IgG humoral immune responses against WT1 protein were Th1- or Th2-type were determined by measurement of four subclasses of IgG WT1 antibody, IgG1, IgG2, IgG3, and IgG4. In leukemias and MDS, Th1-type WT1 antibodies such as IgG1, IgG2, and IgG3 were significantly increased in both detection rate and antibody titers compared to those in healthy volunteers, whereas Th2-type WT1 antibody such as IgG4 did not increase. These results showed that Th1-biased humoral immune responses against WT1 protein were generated in leukemias and MDS. These results should allow us to consider that Th1-biased cellular immune responses against WT1 protein, which was essentially needed for cancer immunotherapy targeting WT1, should be elicited in patients with hematopoietic malignancies.

Introduction

The Wilms' tumor gene *WT1*, first isolated as a gene responsible for a pediatric renal neoplasm, Wilms' tumor,[1,2] encodes a zinc-finger transcription factor involved in cell proliferation and differentiation, in apoptosis, and in organ development. Although the *WT1* gene was originally categorized as tumor-suppressor gene,[3,4] we proposed that the wild-type *WT1* gene performed an oncogenic rather than a tumor-suppressor function[5] on the basis of the accumulated evidence such as (i) high expression levels of the wild-type *WT1* gene in hematopoietic malignancies including leukemia and myelodysplastic syndrome (MDS) and in various kinds of solid tumors,[6–9] (ii) growth inhibition of human leukemic and solid tumor cells by *WT1* antisense oligodeoxynucleotides,[8,10] and (iii) growth promotion and differentiation inhibition of murine myeloid progenitor cells by the constitutive expression of the *WT1* gene.[11,12]

The above findings indicated that WT1 product could become a target for cancer immunotherapy. In fact, we and others generated human WT1-specific cytotoxic T lymphocytes (CTLs) *in vitro*.[5,13–16] Furthermore, it was shown that mice immunized with WT1 peptides or *WT1* cDNA elicited WT1-specific CTLs and rejected challenges of *WT1*-expressing tumors,[17,18] indicating that the WT1 protein could *in vivo* serve as a tumor rejection antigen. It was also shown that WT1-specific CTL precursors (CTLp) were detected more frequently in the patients with hematopoietic malignancies than in healthy volunteers.[19]

Recently, we and others detected IgM and IgG WT1 antibodies in the patients with hematopoietic malignancies.[20,21] Disease progression of MDS from refractory anemia to overt leukemia was associated with isotype class-switching of WT1 antibody from IgM to IgG.[21] These findings indicated that WT1-specific cellular immune responses that induced isotype class-switching of WT1 antibody had been elicited, suggesting that WT1 protein was immunogenic. Thus these results should provide us with the rationale for elicitation of CTL responses against WT1 product in cancer immunotherapy targeting it.[21]

Determination of IgG subclasses of the antibodies against WT1 protein should lead to further understanding of the immune responses against WT1 protein in the *WT1*-expressing tumor-bearing patients, because IgG subclasses in humoral immune responses were associated with different helper T cells, Th1 or Th2, which stimulated B cells in different ways, respectively.[22] In this study, IgG subclasses of WT1 antibody were analyzed to determine whether humoral immune responses against WT1 protein in patients with hematopoietic malignancies are Th1- or Th2-type.

Materials and methods

Patients

Sera and peripheral blood mononuclear cells (PBMCs) were obtained from 96 patients with hematopoietic malignancies (28 acute myeloid leukemia (AML), 12 acute lymphoid leukemia (ALL), 16 chronic myeloid leukemia (CML) (eight in chronic phase (CP), eight in blast crisis (BC)) and 40 MDS) (Supplementary Table 1). The MDS patients included 15 with refractory anemia (RA), 15 with RA with excess of blasts (RAEB), and 10 with RAEB in transformation (RAEB-t). The patients had not received chemotherapy at the time point when blood samples were obtained, except the patients with CML in BC. The control group was comprised of 53 healthy volunteers who gave their informed consent for this study.

Reverse transcription-polymerase chain reaction for quantitation of WT1 expression levels

RNA was prepared from PBMCs at diagnosis and converted into cDNA. Polymerase chain reaction (PCR) was performed for optimized cycles with a DNA thermal cycle as described previously.[7,23] *WT1* expression levels in the samples were shown relatively to that (defined as 1.0) in K562 leukemic cells.[6,7,23]

Dot-blot assay for quantitation of IgG subclasses of WT1 antibody

Recombinant human WT1 protein hWT3 (1–294 amino acids) were produced and purified as described previously.[21] Serum #223 obtained from an AML patient was used as a reference serum because it contained high titers of all the four subclasses of IgG WT1 antibody in preliminary experiments.

Standardization of titers of IgG subclasses of WT1 antibody in the reference serum #223 was performed by calibration with heterologous interpolation as described previously.[21,24–26] Briefly, the mixture of mouse anti-human kappa and lambda antibodies (BDBiosciences Pharmingen, San Diego, CA, USA) was bound on nitrocellulose membrane Optitran (Schleicher & Schuell, Dassel, Germany), and WT1 protein (hWT3) was bound to another membrane. After blocking with 4% skim milk, the two membranes were loaded onto dot-blot apparatus (Schleicher & Schuell, Dassel, Germany). Two-fold serially diluted purified human myeloma proteins (IgG1, IgG2, IgG3, and IgG4) (Athens Research and Techenology, Inc., Athens, GA, USA) were applied to the membrane coated with the mixture of mouse anti-human kappa and lambda antibodies. Two-fold serial dilutions of the reference serum #223 were applied to the other membrane coated with hWT3. After washing, the two membranes were reacted with horseradish-peroxidase-conjugated sheep anti-human IgG1, IgG2, IgG3, and IgG4 (The Binding Site Limited, Birmingham, UK), followed by incubation with the substrate solution, Renaissance (NEN life Science Products, Boston, MA, USA), and exposure to Hyper film (Amersham Pharmacia Biotech, Buckinghamshire, England) with the same exposure time. Densities of dot blots were measured in densitometric units with a computerized scanning analyzer system (Molecular Dynamics, Sunnyvale, CA, USA). Concentrations of IgG1, IgG2, IgG3, and IgG4 WT1 antibodies in the reference serum #223 were determined as 28.7, 87.2, 3.7, and 2.5 μg/ml, respectively, from the respective calibration curves of purified human IgG1, IgG2, IgG3, and IgG4 myeloma proteins using Abelbeck software.

Concentrations of IgG subclasses of WT1 antibody of each serum sample were determined according to the calibration curves for IgG subclasses of WT1 antibody of reference serum #223, as described previously,[25,27] using Abelbeck software.

Statistics

Cutoff values of individual IgG subclasses of WT1 antibody were determined to be 10, 40, 1.5, and 1.5 μg/ml for IgG1, IgG2, IgG3, and IgG4, respectively, on the basis of two receiver-operating characteristic plots.[28] Mann–Whitney test was used for the measurement of statistical difference in WT1 antibody titers between two groups. Fisher's exact test was used for the measurement of statistical difference in detection rate of WT1 antibody between two groups and for evaluation of the correlation between the presence of IgG subclasses of WT1 antibody and either sex or clinical performance of the patients. Linear regression coefficient (r) was used for evaluation of the correlation between WT1 antibody concentrations and either *WT1* expression levels or patient ages. Statistical analysis was performed by JMP (SAS Institute, Cary, NC, USA) and StatView software.

Results

WT1 expression levels in PBMC of patients with hematopoietic malignancies

To demonstrate that *WT1* expression levels, which reflected the amount of leukemic tumor burden, increased in company with the disease progression of CML and MDS, indicating an increase in an antigenic stimulation of immune system by *WT1*-expressing cells along with the disease progression, *WT1* expression levels were measured by quantitative reverse transcription (RT)-PCR in 72 of 96 patients (Supplementary Figure 1). In 70 of 72 patients with hematopoietic malignancies (25 AML, 10 ALL, 16 CML, and 19 MDS) except for two RA patients, *WT1* expression levels were $\geqslant 1 \times 10^{-5}$, while they were $<1 \times 10^{-5}$ in all of 53 healthy volunteers. *WT1* expression levels increased in parallel with disease progression from CP to BC in CML, and from RA to RAEB and further to RAEB-t in MDS. These results confirmed previous reports of ours.[6,7,23]

Detection of IgG subclasses of WT1 antibody in sera from patients with hematopoietic malignancies

IgG subclasses of WT1 antibodies were measured by dot-blot assay using reference serum #223 in 96 patients with hematopoietic malignancies (28 AML, 12 ALL, 16 CML, and 40 MDS) and 53 healthy volunteers (Table 1, Figure 1).

IgG1 WT1 antibody was detected in 35 (36.5%) of the 96 patients with hematopoietic malignancies, whereas they were detected in only four (7.5%) of the 53 healthy volunteers (Table 1, Figure 1a). Both detection rate ($P<0.0001$) and titers ($P<0.0001$) of IgG1 WT1 antibody were significantly higher in patients with hematopoietic malignancies than in healthy volunteers. When detection rates of IgG1 antibody in individual disease of hematopoietic malignancies were compared with those in healthy volunteers, they were significantly higher in patients with AML ($P<0.001$), CML ($P<0.01$), or MDS ($P<0.001$) than in healthy volunteers (Figure 1a). The antibody titers of IgG1 WT1 antibody were also significantly higher in the patients with hematopoietic malignancies except ALL than in healthy volunteers (Figure 1a).

Both detection rate ($P<0.05$) and titers ($P<0.05$) of IgG2 WT1 antibody were significantly higher in the patients than in healthy volunteers (Figure 1b). When detection rate and antibody titers of IgG2 WT1 antibody in individual disease were compared with those in healthy volunteers, both ($P<0.05$ for detection rate, and $P<0.001$ for titers) and detection rate ($P<0.05$) alone were significantly higher in AML and MDS, respectively, than in healthy volunteers (Figure 1b).

As for IgG3 WT1 antibody, only patients with CML had significantly higher detection rate ($P<0.05$) and antibody titers ($P<0.05$) than healthy volunteers (Figure 1c).

Neither detection rate nor antibody titers of IgG4 WT1 antibody were significantly different between the patients and

Table 1 Summary of detection rate of IgG subclasses of WT1 antibody in healthy volunteers and patients

	IgG1	IgG2	IgG3	IgG4
Healthy volunteers	4/53 (7.5)	5/53 (9.4)	5/53 (9.4)	8/53 (15.1)
Patients	**35/96 (36.5)******	**23/96 (24.0)***	19/96 (19.8)	24/96 (25.0)
AML	**12/28 (42.9)*****	**9/28 (32.1)***	5/28 (18.9)	9/28 (32.1)
ALL	2/12 (16.7)	1/12 (8.3)	1/12 (8.3)	3/12 (25)
CML	**5/16 (31.2)****	3/16 (18.8)	**5/16 (31.3)***	4/16 (25.0)
CP	1/8 (12.5)	1/8 (12.5)	1/8 (12.5)	1/8 (12.5)
BC	**4/8 (50.0)*****	2/8 (25.0)	**4/8 (50.0)****	2/8 (25.0)
MDS[a]				
IPSS	**14/36 (38.9)*****	**9/36 (25.0)***	8/36 (22.2)	8/36 (22.2)
Early stage	4/18 (22.2)	4/18 (22.2)	3/18 (16.7)	4/18 (22.2)
Advanced stage	**10/18 (55.6)******	5/18 (27.8)	5/18 (27.8)	4/18 (22.2)
FAB	**16/40 (40.0)*****	**10/40 (25.0)***	8/40 (20.0)	8/40 (20.0)
RA	1/15 (6.7)	4/15 (26.7)	2/15 (13.3)	5/15 (33.3)
RAEB	**8/15 (53.3)******	3/15 (20.0)	3/15 (20.0)	1/15 (6.7)
RAEB-t	**7/10 (70.0)******	3/10 (30.0)	3/10 (30.0)	2/10 (20)

Numbers in the parenthesis are percentages of detection rate. *P*-values shown in the table are those for difference in detection rates of WT1 antibody between the individual disease and healthy volunteers. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.
The detection rates significantly higher than those in healthy volunteers are shown in bold. [a]The MDS patients were fractionated into early (Low risk and Int-1) and advanced (Int-2 and High risk) stages according to IPSS (International Prognostic Scoring System), or RA, RAEB, and RAEB-t according to FAB (French–American–British) classification. Four of 40 patients could not be classified by IPSS because of the lack of some information needed for it.
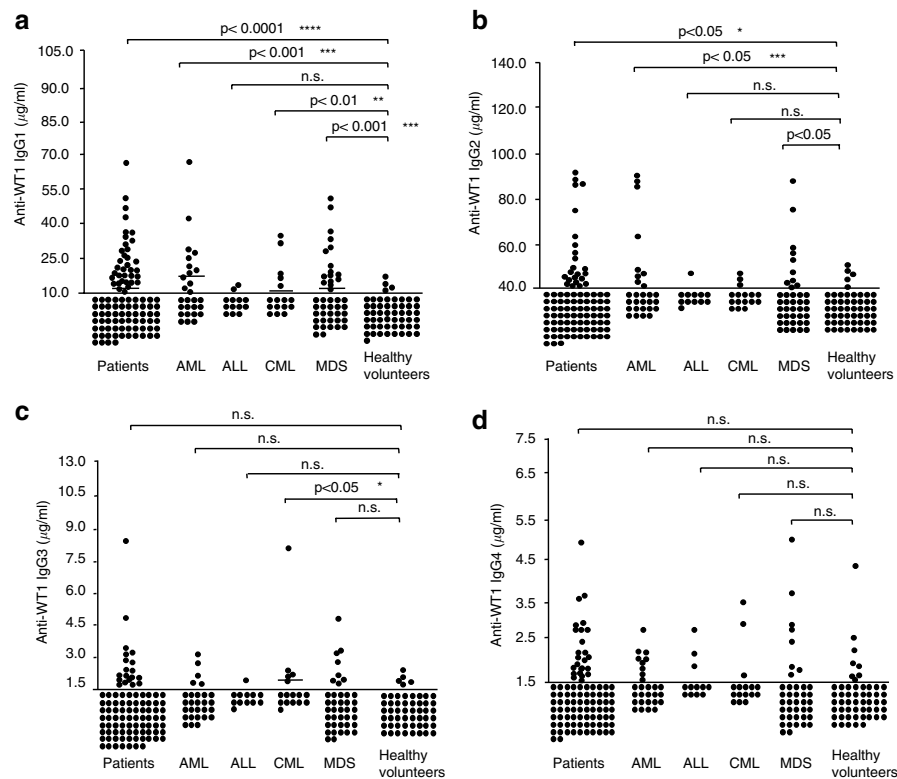

Figure 1 Titers of IgG1 (a), IgG2 (b), IgG3 (c), and IgG4 (d) WT1 antibodies in patients with hematopoietic malignancies and in healthy volunteers are shown. *P*-values shown in the figures are those for difference in detection rate between two groups. *P*-values for difference in antibody titers between two groups are shown with asterisks: *$P < 0.05$; **$P < 0.005$; ***$P < 0.001$; ****$P < 0.0001$.

healthy volunteers and between AML, ALL, CML, or MDS and healthy volunteers (Figure 1d).

No correlation was found between the detection of IgG1, IgG2, IgG3, or IgG4 WT1 antibodies and tumor burden such as *WT1* expression levels or percentages of blasts, age, sex, performance status, or outcome of the patients (data not shown).

An increase in IgG1 and IgG3 WT1 antibodies along with disease progression of CML

A relationship between the disease stages of CML and detection of IgG WT1 antibody was examined (Figure 2, Table 1).
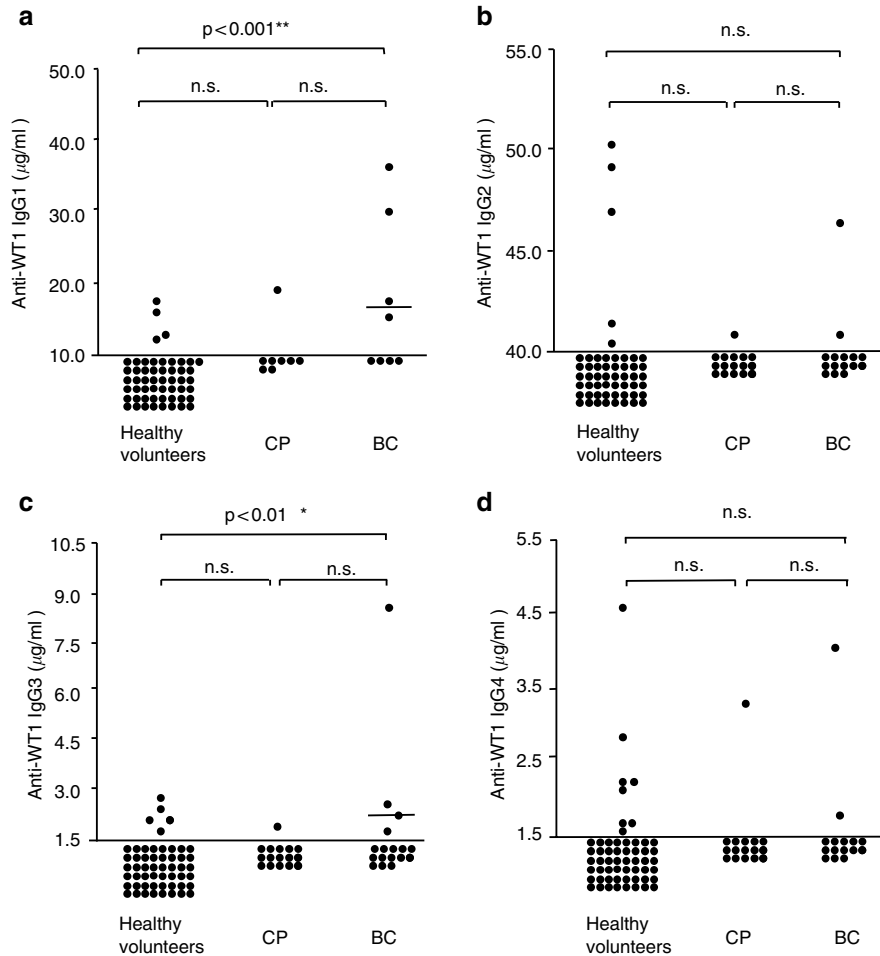
Figure 2 Titers of IgG1 (a), IgG2 (b), IgG3 (c), and IgG4 (d) WT1 antibodies in healthy volunteers and in patients with CML CP or BC are shown. *P*-values shown in the figures are those for difference in detection rate between two groups. *P*-values for difference in antibody titers between two groups are shown with asterisks: *$P<0.05$; **$P<0.005$; ***$P<0.001$.

IgG1 WT1 antibody was detected in one (12.5%) of eight patients with CP and in four (50%) of eight patients with BC. The detection rate ($P<0.001$) and titers ($P<0.005$) of IgG1 WT1 antibody were significantly higher in the patients with BC, but not CP than in healthy volunteers (Figure 2a).

As for IgG2 WT1 antibody, no significant difference in detection rate was observed between healthy volunteers, CP, and BC (Figure 2b).

IgG3 WT1 antibody was detected in one (12.5%) of eight patients with CP and in four (50.0%) of eight patients with BC. The detection rate ($P<0.01$) and titers ($P<0.05$) of IgG3 WT1 antibody were significantly higher in the patients with BC, but not CP, than in healthy volunteers (Figure 2c).

As for IgG4 WT1 antibody, no significant difference in both antibody titers and detection rate was observed between healthy volunteers, CP, and BC.

Taken together, disease progression of CML from CP to BC was accompanied by a significant increase in IgG1 and IgG3 WT1 antibody, because only the advanced stage (BC) of CML had significantly higher detection rate and titers of IgG1 and IgG3 WT1 antibody than healthy volunteers (Figure 2 and Table 1).

An increase in IgG1 WT1 antibodies along with disease progression of MDS

A relationship between the disease stages of MDS (15 RA, 15 RAEB, and 10 RAEB-t) and detection of IgG WT1 antibody was examined (Figure 3, Table 1, and Supplementary Figure 2). The patients were divided into two groups according to IPSS (International Prognostic Scoring System).[29] A total of 18 patients were included in early MDS (Low risk and Int-1), and 18 patients in advanced MDS (Int-2 and High risk), but the remaining four patients could not be classified because of the lack of some information needed for it (Figure 3).

As for IgG1 WT1 antibody, both detection rate ($P<0.0001$) and antibody titers ($P<0.0001$) were significantly higher in advanced MDS but not in early MDS than in healthy volunteers. Furthermore, both detection rate and antibody titers were significantly higher in advanced MDS than in early MDS (Figure 3a).

As for IgG2 WT1 antibody, titers alone were significantly higher ($P<0.05$) in advanced MDS than in healthy volunteers (Figure 3b).

As for IgG3 and IgG4 *WT1* antibody, no significant difference in both detection rate and titers was observed between healthy volunteers, early MDS, and advanced MDS (Figure 3c and d).
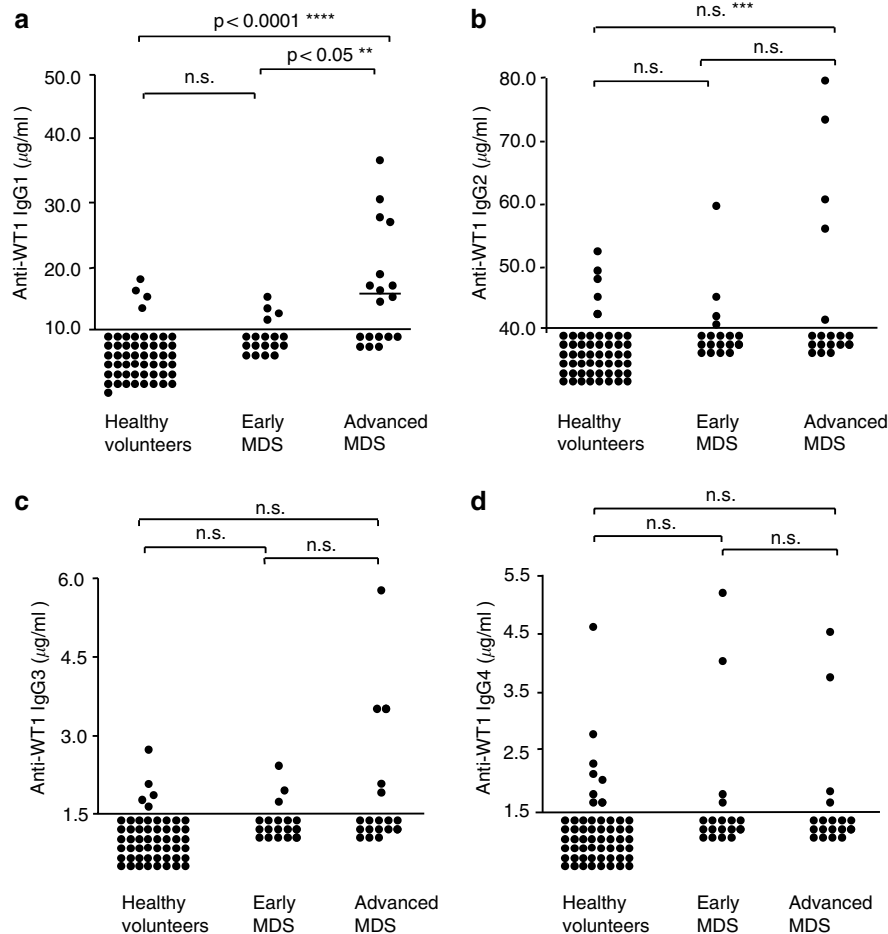
Figure 3   Titers of IgG1 (a), IgG2 (b), IgG3 (c), and IgG4 (d) WT1 antibodies in healthy volunteers and in patients with early or advanced MDS are shown. The MDS patients were divided into early (Low risk and Int-1) or advanced (Int-2 and High risk) stages according to IPSS. *P*-values shown in the figures are those for difference in detection rate between two groups. *P*-values for differences in antibody titers between two groups are shown with asterisks: *$P<0.05$; **$P<0.005$; ***$P<0.001$; ****$P<0.0001$.

We also analyzed IgG WT1 antibody in the MDS patients who were fractionated according to FAB classification (Table 1, Supplementary Figure 2). Detection rate in IgG1 WT1 antibody was significantly higher in RAEB and RAEB-t but not in RA than in healthy volunteers (Table 1). As for IgG2, IgG3, and IgG4 WT1 antibody, no significant difference in detection rate was observed between healthy volunteers, RA, RAEB, and RAEB-t.

Taken together, disease progression of MDS from early to advanced stages was accompanied by a significant increase in IgG1 WT1 antibodies (Figure 3, Supplementary Figure 2, and Table 1).

IgG WT1 antibody in one MDS patient could be measured at two different stages (RA and RAEB) at 15-month intervals. IgG1 WT1 antibody titer increased from 12.6 μg/ml (RA) to 24.7 μg/ml (RAEB), while the WT1 antibody titers of the other three subclasses (IgG2, IgG3, and IgG4) were under the cutoff levels at both stages.

Discussion

Considerable investigations reported that IgG antibodies against tumor-associated antigen (TAA) were detected in the patients with malignancies, implying that the TAA had immunogenicity

in the patients, and that the antigen-specific helper T cells, which induced immunoglobulin class-switch from IgM to IgG, should be activated in the patients.[20,21,30] However, intensive analysis of IgG subclasses of the antibodies against TAA was not performed so far. Determination of IgG subclasses of the antibodies against TAA should lead to further understanding of the immune responses against the TAA in the patients, because the association of the IgG subclasses of humoral immune responses with Th1- or Th2-type immune responses had been elucidated. It was reported that Th1-type immune responses in mice were associated with production of antibodies of IgG2a and IgG3 subclasses, and that the equivalent subclasses in humans were IgG1 and IgG3, respectively.[22,31–33] It was reported that production of IgG2 antibody in human was also associated with Th1-type responses,[34,35] while production of IgG4 antibody was associated with Th2-type responses.[22] The present study demonstrated an increase of detection rate in IgG1 WT1 antibody in AML, CML (BC), and MDS (advanced stages), in IgG2 WT1 antibody in AML and MDS, and in IgG3 WT1 antibody in CML(BC) when IgG WT1 antibody responses in hematopoietic malignancies were compared to those in healthy volunteers (Table 1). No IgG4 WT1 antibody responses were observed in all the patients with hematopoietic malignancies. These findings strongly indicated that Th1-biased immune

responses against the WT1 protein were generated in these patients.

We previously reported that class switching of WT1 antibody from IgM to IgG occurred along with disease progression of MDS from RA to RAEB and further to RAEB-t.[21] In the present study, detection rate and titers of IgG1, and titers of IgG2 WT1 antibodies increased along with the disease progression from RA to RAEB and RAEB-t (Table 1, Supplementary Figure 2). Thus, class switching of WT1 antibody from IgM to IgG along with the disease progression will be ascribed to an increase in IgG1 (and possibly IgG2) production in association with the disease progression. Furthermore, examination of IgG WT1 antibody in MDS patients who were fractionated according to IPSS revealed that both detection rate and antibody titers in IgG1, and titers in IgG2 WT1 antibodies in advanced MDS (Int-2 and High risk) but not in early MDS (Low risk and Int-1) were higher than in healthy volunteers (Table 1, Figure 3). These results might allow us to imagine that immune responses against WT1 protein were biased toward Th1-type by increased WT1 antigenic stimulation as a result of an increase in *WT1*-expressing tumor burden along with the disease progression of MDS.

We also reported previously that IgG WT1 antibody titers are higher in patients with CML than in healthy volunteers.[21] The present study demonstrated that IgG1 and IgG3 WT1 antibodies, Th1-biased IgG subclasses, were higher in both detection rate and titers in patients with BC, but not CP, than in healthy volunteers, indicating that Th1-type humoral immune responses against WT1 protein were elicited along with the disease progression of CML. Continuous and stronger stimulation of immune system by WT1 protein due to longer disease period and higher tumor burden in CML BC patients may lead to production of Th1- type IgG of WT1 antibody. Therefore, in both MDS and CML, an increase in Th1-type IgG subclasses of WT1 antibody such as IgG1 and IgG3 was associated with the disease progression (Table 1).

IgG1 and IgG2-, IgG1 and IgG3-, and IgG1-biased WT1 antibody responses were detected at higher rates in patients with AML, advanced stages of CML, and advanced stages of MDS, respectively, than in healthy volunteers (Table 1). The reason for the difference in dominant IgG subclasses of WT1 antibodies between disease types is unknown at the moment. Difference in the disease types and in the periods for which the patients are suffering from the diseases (patients with advanced stages of CML or MDS should have longer disease periods than the patients with AML) may lead to a difference in antigenic stimulation by WT1 protein. The different stimulation may activate B cells in different ways, leading to the production of different subclasses of WT1 antibody, which have the subclass-specific unique function. Further studies are needed to address this issue.

Based on the recent investigations,[22,31–35] the patients with hematopoietic malignancies with increased production of IgG1, IgG2, and IgG3 WT1 antibodies are considered to have generated Th1-biased immune responses against WT1. Since Th1-type immune responses promoted cellular immune responses such as CTL induction, the results in the present study should provide us with the rationale for an idea that injection of WT1 gene products as cancer immunotherapy should be able to elicit WT1-specific CTLs. The WT1 peptide-based cancer immunotherapy may be more effective in patients with Th1-biased humoral immune responses against WT1 than in those without them. It would be interesting to see whether Th1-biased antibody production against the WT1 protein was associated with induction of WT1-specific CD8^{+} CTL responses in the

patients with hematopoietic malignancies. If the correlation between Th1-biased antibody responses against WT1 and CTL responses towards WT1 is found, prediction of efficacy of WT1-peptide-based cancer immunotherapy may be possible by measuring IgG subclasses of WT1 antibody in the patients.

Supplementary Information

Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu).

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