cytopenia certainly exist. For example, MDS associated with isolated del(5q) (5q– syndrome) shows remarkable dysplastic features of megakaryocytic lineage in bone marrow (BM). However, the platelet counts of patients with 5q– syndrome do not usually decrease.[3] Previously, we reported a detailed cytomorphologic analysis of refractory anemia according to the FAB classification (FAB-RA).[5] Using this data set, we analyzed the relationship between dysplastic lineage and cell count in the present study.

Table 1
Patient Characteristics

Characteristic	Value
Age, median (range), y	57 (15-88)
Male sex, No. (%)	53 (53)
Hb, median (range), g/dL	8.3 (2.9-14.3)
ANC, median (range), $\times 10^9$/L	1.397 (0.260-6.201)
PLT, median (range), $\times 10^9$/L	35 (4-760)
Chromosome (IPSS), No. (%)	
Good	76 (76)
Intermediate	15 (15)
Poor	9 (9)

ANC, absolute neutrophil count; Hb, hemoglobin; IPSS, International Prognostic Scoring System; PLT, platelet count.

Materials and Methods

Patients

The data set of Japanese patients from our previous study[5] was used for this study. Patients included those with primary MDS excluding refractory anemia with ringed sideroblasts, refractory cytopenia with multilineage dysplasia and ringed sideroblasts, refractory anemia with excess of blasts (RAEB), or 5q– syndrome according to WHO classification (version 3).[6] Therefore, all patients in this study had FAB-RA except those with 5q– syndrome. Patients were diagnosed at the Saitama Medical University Hospital, Nagasaki University Hospital, or affiliated hospitals between April 1976 and January 2002. BM cellularity and fibrosis were evaluated by BM trephine biopsy and/or clot section. Patients with BM fibrosis were excluded, because the accuracy of morphologic evaluations might be not reliable owing to the few BM cells of the films. Disorders other than MDS (eg, aplastic anemia, paroxysmal nocturnal hemoglobinuria, megaloblastic anemia, autoimmune hemolytic anemia, anemia of chronic disorders, large granular lymphocytic leukemia, hairy cell leukemia, chronic liver disorders, and hypersplenism) were excluded. Patients who had previous therapy (antineoplastic drugs and/or ionizing radiation) or other prior hematologic disease also were excluded from the study. This study was approved by the Institutional Review Board of Saitama International Medical Center, Saitama Medical University. Retrospective analysis was performed in 100 Japanese patients. Age, sex, and cytogenetic findings of patients at diagnosis are summarized in **Table 1**.

Cytomorphologic Study

Microscopic examinations were performed using standard methods (BM Wright-Giemsa [WG] or May-Giemsa [MG], Prussian blue and periodic acid–Schiff [PAS] stained films, and peripheral blood [PB] WG- or MG-stained films).

In the present study, we analyzed the correlation between dysplasia and cell count using the data set of our previous morphologic study, in which we performed a detailed cytomorphologic analysis. We limited dysplasias to only dysplasias described in the WHO classification (version 3)[6] as follows. Dysplasias of the nucleus in erythroid lineage cells were defined as having budding, bridging, internuclear, karyorrhexis, multinuclearity, or megaloblastoid changes. Dysplasias of the cytoplasm in erythroid lineage cells were defined as having ring sideroblasts, vacuolization, or PAS positivity (diffuse or granular). With regard to granulocytes, dysplasias were defined as having the following characteristics: small size, nuclear hyposegmented mature neutrophils (Pelger), hypersegmentation, hypogranularity, or pseudo–Chédiak-Higashi granules. Dysplasias of megakaryocytes were defined as having micromegakaryocytes (mMgk), nonlobulated nuclei, or multiple widely separated nuclei. A minimum of 25 megakaryocytes, 200 erythroblasts, and 200 neutrophils in BM were examined in each patient. The cutoff levels for dyserythropoiesis (dys E) and dysgranulopoiesis (dys G) were defined as 10% according to the WHO classification.[6] Dysmegakaryopoiesis (dys Mgk) was evaluated with 2 cutoff levels: 10% according to the WHO classification or 40% according to data previously reported from the German group.[7,8] Since the accuracy of the quantitative evaluation of dysmegakaryopoiesis might be not reliable when there are few megakaryocytes to examine, we excluded patients who did not have at least 25 examined megakaryocytes from the morphologic evaluation of the megakaryocytic lineage. In our previous study, we reported that 2 distinct dysplastic changes, Pelger and mMgk **Image 1**, had negative prognostic impacts. Therefore, Pelger and mMgk were also evaluated. We defined hyposegmented mature neutrophils with strikingly clumpy chromatin as "Pelger" and mononucleated or binucleated megakaryocytes with a size equal to or smaller than promyelocytes as "mMgk." Positivity for Pelger (Pelger+) was defined as the presence of 10% or more Pelger among 200 mature neutrophils. Positivity for mMgk (mMgk+) was defined as the presence of 10% or more mMgk among 25 or more megakaryocytes. Patients with decreased megakaryocytes were assessed as being negative for mMgk (mMgk–).

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Definition of Cytopenias

To analyze the relationship between dysplasia and cytopenia, we compared hemoglobin (Hb) concentrations, absolute neutrophil counts (ANCs), platelet counts, and types of cytopenia. The definitions of cytopenias were as follows: Hb concentration less than 10 g/dL, ANC less than 1.8×10^9/L, and platelet count less than 100×10^9/L.[3]

Statistical Analysis

Continuous data were compared using the nonparametric Mann-Whitney test, and proportions were compared using the χ^2 test. A 2-sided *P* value of less than .05 was considered statistically significant.

Results

Cytomorphologic Study

In our previous data set, we evaluated suitable BM preparations for the detailed assessments of myelodysplasia.[5] Results of morphologic analysis are shown in Table 2 and Table 3. Some BM preparations could not be examined in detail. In particular, observing the granules of neutrophils was difficult due to the poor staining condition of the films. Four cases could not be evaluated for the frequency of dys G, and 1 case could not be evaluated for the frequency of Pelger. Nineteen patients showed decreased megakaryocytes. Therefore,

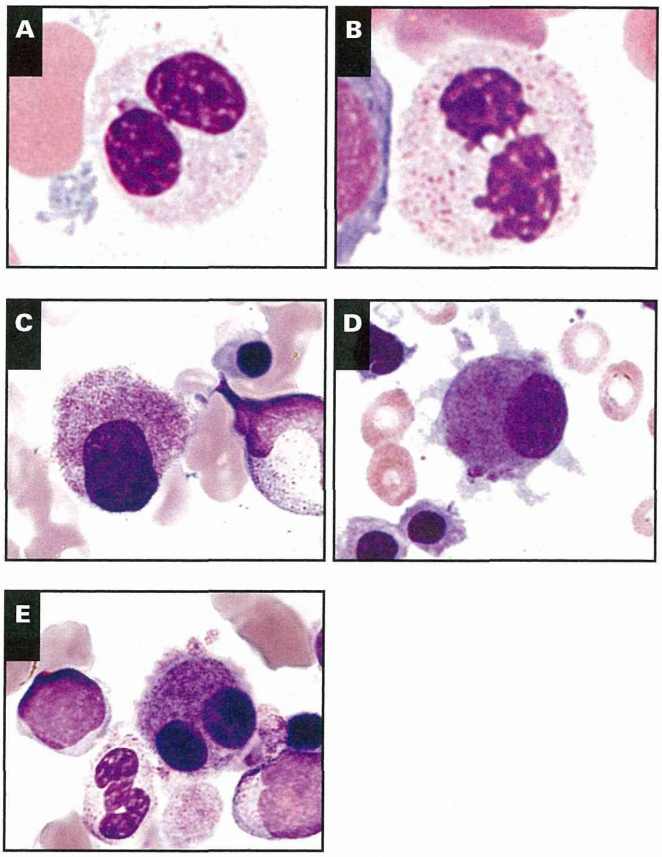

Image 1 **A** and **B**, Nuclear hyposegmented mature neutrophils (Pelger) (May-Giemsa). **C-E**, Micromegakaryocytes (May-Giemsa).

Table 2
Relationship Between Dysgranulopoiesis and Absolute Neutrophil Count

Characteristic	No. of Patients	ANC, Median (Range), $\times 10^9$/L	*P* Value	No. (%) of Patients With Neutropenia[a]	*P* Value
dys G ≥10%	17	1.394 (0.492-6.201)	.83	13 (76.5)	.22
dys G <10%	79	1.397 (0.260-4.708)		48 (60.8)	
Pelger ≥10%	12	1.364 (0.492-6.201)	.86	9 (75.0)	.42
Pelger <10%	87	1.394 (0.260-4.708)		55 (63.2)	

ANC, absolute neutrophil count; dys G, dysgranulopoiesis; Pelger, nuclear hyposegmented mature neutrophils.
[a] Definition of neutropenia is ANC $<1.8 \times 10^9$/L.

Table 3
Relationship Between Dysmegakaryopoiesis and Platelet Count

Characteristic	No. of Patients	Platelets, Median (Range), $\times 10^9$/L	*P* Value	No. (%) of Patients With Thrombocytopenia[a]	*P* Value
dys Mgk ≥10%	69	48 (8-760)	.08	51 (73.9)	.18
dys Mgk <10%	12	26 (5-313)		11 (91.7)	
dys Mgk ≥40%	38	70 (15-760)	.02	26 (68.4)	.10
dys Mgk <40%	43	36 (5-343)		36 (83.7)	
mMgk ≥10%	12	99 (29-760)	.004	7 (58.3)	.03
mMgk <10%	88	33 (4-390)		7 (84.1)	

dys Mgk, dysmegakaryopoiesis; mMgk, micromegakaryocytes.
[a] Definition of thrombocytopenia is platelet count $<100 \times 10^9$/L.

these cases could not be evaluated for the frequency of dys Mgk. All patients showed dys E of 10% or more. No patients with unilineage dysplasia had dys G or dys Mgk of 10% or more. Patients with unilineage dysplasia had an erythroid lineage. Seventeen (18%) and 12 (12%) patients had dys G of 10% or more and were Pelger+, respectively. Sixty-nine (85%) and 38 (47%) patients had dys Mgk of 10% or more and 40% or more, respectively. Twelve (12%) patients were mMgk+. All patients with mMgk+ showed dys Mgk of 40% or more.

Table 4
Dysplastic Lineage(s) in Patients With Unilineage Cytopenia[a]

Case No.	dys E ≥10%	dys G ≥10%	dys Mgk ≥10%
Patients with only anemia			
103	+	−	+
131	+	+	−
136	+	−	+
257	+	−	+
Patients with only neutropenia			
135	+	+	+
230	+	+	+
245	+	−	+
286	+	−	+
Patients with only thrombocytopenia			
129	+	−	−
154	+	−	+
233	+	−	+
248	+	−	Unknown[b]
252	+	−	−
269	+	−	+
270	+	+	+
271	+	−	+
273	+	−	+
278	+	−	+
290	+	−	+

dys E, dyserythropoiesis; dys G, dysgranulopoiesis; dys Mgk, dysmegakaryopoiesis.
[a] Definitions of cytopenias are hemoglobin concentration <10 g/dL, absolute neutrophil count <1.8 × 10^9/L, and platelet count <100 × 10^9/L.
[b] Could not be evaluated for the frequency of dys Mgk due to decreases in megakaryocytes.

Table 5
Relationship Between Dysplastic Lineage and the Type of Cytopenia in Patients With Unilineage Dysplasia[a]

Case No.	Dysplastic Lineage	Anemia	Neutropenia	Thrombocytopenia
109	Erythroid	+	+	+
120	Erythroid	−	+	+
125	Erythroid	+	+	+
127	Erythroid	−	+	+
129	Erythroid	−	−	+
134	Erythroid	+	−	+
140	Erythroid	+	−	+
202	Erythroid	+	+	+
204	Erythroid	+	+	+
236	Erythroid	+	+	+
252	Erythroid	−	−	+

[a] Definitions of cytopenias are hemoglobin concentration <10g/dL, absolute neutrophil count <1.8 × 10^9/L, and platelet count <100 × 10^9/L.

Relationship Between Dysplasia and Cell Count

Results of the relationship between dysplasia and cell count are shown in Tables 2 and 3. There was no relationship between the presence of dys G of 10% or more and ANC. Similarly, Pelger+ was not related to ANC. Interestingly, platelet counts of patients with dys Mgk of 10% or more tended to be higher than those of patients without dys Mgk of 10% or more (P = .08). Moreover, in patients with dys Mgk of 40% or more, this difference in platelet counts was significant (dys Mgk ≥40% vs <40%, P = .02). In particular, in patients with mMgk+, the sign became even clearer (P = .004). In patients with dys Mgk of 40% or more, the platelet count of those with mMgk+ was not different from those without mMgk+ (P = .47). Of the 19 patients who showed unilineage cytopenia, only 2 had unilineage dysplasia. However, dysplastic lineages were different from the lineages of cytopenia **Table 4**. Of the 11 patients who showed unilineage dysplasia, only 2 had unilineage cytopenia. However, lineages of cytopenia were different from dysplastic lineages **Table 5**.

Discussion

Generally, it seems that cytopenias correspond to dysplastic lineage, but this has not been studied completely. Increases in blasts in BM may reduce blood cell counts due to hematopoietic injury. Patients with 5q– syndrome show anemia due to erythroid hypoplasia.[9,10] Increases in blasts or the existence of del(5q) may influence the decrease in cell count. To examine the correct relationship between dysplastic lineage and cell count, we excluded patients with RAEB or 5q– syndrome. Our previous data set used for the present study also does not include these cases. Therefore, it seems that this data set is suitable for the purpose of the present study.

Recently, it was reported that there was no clear correlation between the presence of any distinct dysplastic sign and cell counts in patients with MDS.[11] In the present study, we could not find any correlation between the presence of dys G of 10% or more and ANC. Low ANC cannot be directly explained by the presence of dys G. This result suggests that dysplastic features in granulocytic lineage may be unrelated to ineffective hematopoiesis explained by apoptosis. Interestingly, the platelet count of patients with dys Mgk was higher than that of patients without dys Mgk (dys Mgk ≥10% vs <10%, P = .08; dys Mgk ≥40% vs <40%, P = .02; mMgk+ vs mMgk–, P = .004). Although patients with 5q– syndrome show remarkable dysplastic features of megakaryocytic lineage in BM, their platelet counts are usually normal or increased. Thrombocytopenia is uncommon.[3,9,10] In the present study, a correlation between dys Mgk and platelet counts was similar to the characteristics of 5q– syndrome. These findings suggest that dys Mgk is not related to a direct sign of apoptosis, at least in the megakaryocytic lineage. In addition, we analyzed

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the correlation between the frequency of dys Mgk and the number of megakaryocytes. Evaluation of the megakaryocyte count was performed using specimens of the BM trephine biopsy and/or clot section. Of the 38 patients with dys Mgk of 40% or more, 21 (55%), 10 (26%), and 7 (17%) had increased, normal, and decreased megakaryocyte counts, respectively. In patients with dys Mgk of 40% or more, megakaryocyte counts tended to increase. In contrast, of the 43 patients without dys Mgk of 40% or more, 5 (12%), 17 (40%), and 21 (48%) had increased, normal, and decreased megakaryocyte counts, respectively. In patients without dys Mgk of 40% or more, megakaryocyte counts did not tend to increase. This finding was similar to a correlation between megakaryocyte count and mMgk. In 12 patients with mMgk+, 9 (75%), 3 (25%), and 0 had increased, normal, and decreased megakaryocyte counts, respectively. In patients with mMgk+, megakaryocyte counts tended to increase. In contrast, of the 88 patients without dys mMgk+, 17 (19%), 24 (27%), and 47 (53%) had increased, normal, and decreased megakaryocyte counts, respectively. In patients without mMgk+, megakaryocyte counts did not tend to increase. Increases in megakaryocyte counts may be a reason for the increase in platelet counts in patients with dys Mgk of 40% or more or mMgk+.

Only 6 patients had hypoplastic BM. There was no significant difference in Hb concentrations between hypoplastic BM and nonhypoplastic BM patients ($P = .51$). Similarly, there was no significant difference in ANC between the 2 groups ($P = .44$). Nineteen patients had decreased megakaryocytes. Interestingly, the platelet count of patients with decreased megakaryocytes (median, 18×10^9/L) was significantly lower than that of patients without decreased megakaryocytes (median, 48×10^9/L) ($P < .001$). This finding is similar to the mechanism of thrombocytopenia in patients with aplastic anemia. It seems that the presence of decreased megakaryocytes may be associated with the presence of thrombocytopenia even in patients with MDS.

We could not find a correlation between dysplastic lineage and low cell counts. Low ANC cannot be explained by the presence of dys G. The platelet count of patients with dys Mgk was higher than that of patients without dys Mgk. Therefore, we suggest that dysplastic features do not directly relate to apoptosis in MDS patients with FAB-RA except 5q– syndrome. To clarify the conclusion of our study, quantitative analysis of apoptosis by immunohistochemistry should be performed in the future studies.

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ORIGINAL ARTICLE: CLINICAL

Clinical evaluation of WT1 mRNA expression levels in peripheral blood and bone marrow in patients with myelodysplastic syndromes

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Abstract

A study to evaluate WT1 mRNA expression levels in peripheral blood (PB) and bone marrow aspirate (BM) was conducted in 172 patients, including 115 with myelodysplastic syndromes (MDS), in Japan. The level of WT1 mRNA expression was evaluated according to the French–American–British (FAB) and World Health Organization (WHO) classifications (2001, 2008) and using the International Prognostic Scoring System and the WHO Prognostic Scoring System scales. WT1 mRNA expression levels in PB and BM were well correlated ($r = 0.85$), and they tended to increase with disease stage progression and in those at higher risk of leukemic transformation. WT1 mRNA expression can be a useful marker for the diagnosis and risk evaluation of MDS.

Keywords: Myelodysplastic syndromes, WT1 mRNA expression, classification system, peripheral blood, bone marrow

Introduction

Myelodysplastic syndrome (MDS), a clonal disorder of pluripotent hematopoietic stem cells, is a blood disease characterized by dysplasia and ineffective hemopoiesis. Approximately 20–30% of cases of MDS undergo transformation to acute myeloid leukemia (AML) [1].

The expression of Wilms' tumor gene (WT1) has been found to be a new prognostic factor and marker for the detection of minimal residual disease (MRD) in acute leukemia, including AML and acute lymphocytic leukemia (ALL) [2]. A recent study has revealed the clinical relevance of measuring WT1 mRNA for monitoring MRD in AML, primarily due to its high rate of expression (93.9%) in the peripheral blood (PB) of incipient untreated patients with AML, secondarily due to its ability to predict relapse after complete remission (CR), and finally because its levels after consolidation therapy

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show a significant correlation between disease-free survival, overall survival and early relapse [3]. WT1 mRNA expression occurs not only in AML but also in the PB and bone marrow (BM) of patients with MDS [4–9].

Tamaki *et al.* [4] examined the level of WT1 mRNA expression in PB and BM from 57 patients with MDS grouped by the French–American–British (FAB) classification, and 12 patients experienced AML-MDS progression. The results revealed that WT1 mRNA expression in both PB and BM progressively increased with disease stage progression, from refractory anemia (RA), refractory anemia with excess of blasts (RAEB), refractory anemia with excess of blasts in transformation (RAEB-t), and to AML, suggesting the possibility that the WT1 mRNA expression level reflects the disease stage progression of MDS. Particularly, the patient group who developed leukemia from RAEB or RAEB-t within 6 months showed significantly higher WT1 mRNA expression in PB compared with the group who did not [4].

In accordance with that study, Cilloni *et al.* [6] measured WT1 mRNA expression levels in PB and BM from 131 patients with MDS, and found that: (1) WT1 mRNA expression in PB and BM was confirmed in 78% and 65% of patients with RA, respectively; (2) WT1 mRNA expression in PB and BM was confirmed in all patients with RAEB and secondary AML; (3) the level of WT1 mRNA expression increased with disease stage progression; and (4) the WT1 mRNA expression level was well correlated with the International Prognostic Scoring System (IPSS) scores established by Greenberg *et al.* [10].

In addition to the IPSS, the World Health Organization (WHO) Classification-Based Prognostic Scoring System (WPSS) has been proposed as a prognostic scoring system for MDS [11]. The WPSS consists of three characteristics: WHO subtype classification, considered to be important as a prognostic factor; IPSS-based karyotype abnormalities; and transfusion dependency.

Both the IPSS and WPSS require a chromosomal test as a primary parameter. However, because there are cases in which chromosomal abnormalities cannot be determined [12–14], it is necessary to establish molecular- and genetic-based methods to diagnose and determine the prognosis of MDS. The relatively rapid quantitation of WT1 mRNA is considered to be a useful test to determine the prognosis of MDS and has potential for clinical application, to become a novel marker to complement the current IPSS and WPSS criteria. We performed a clinical study in patients with MDS to demonstrate the usefulness of measuring the WT1 mRNA expression level in PB and BM in the diagnosis and treatment of MDS.

Patients and methods

This study was conducted in accordance with the Declaration of Helsinki, and preliminary approval was obtained from the Institutional Review Board or equivalent organization of each participating institution. Explanations of the study protocol were provided to all patients, and written informed consent was obtained from them before study enrollment.

Patients

From December 2008 to September 2009, 175 patients with MDS, suspected MDS and AML-MDS examined at 17 Japanese medical institutions were enrolled in the study. The subjects were 20 years of age or older and entered in the study regardless of gender, inpatient/outpatient status, or presence or absence of treatment. The 175 patients comprised 106 men (age range 27–88 years, average 65.5 years) and 69 women (age range 22–85 years, average 64.5 years). PB and BM samples from each patient were collected on the same day and used for WT1 mRNA measurement. Three of the 175 enrolled patients were excluded because BM could not be collected due to a dry tap or because the subtype could not be diagnosed. A total of 172 patients were therefore included in the final analysis set.

Diagnosis

Diagnosis of MDS was carried out using a central review format based on the FAB classification [15], the 2001 WHO classification [16] and the 2008 WHO classification [17]. Central review of the bone marrow smear-stained specimens, blood smear-stained specimens, iron-stained specimens, and clot hematoxylin and eosin-stained specimens was carried out by two individuals, one each in the Department of Hemato-Oncology, Saitama International Medical Center, Saitama Medical University, and the Department of Laboratory Medicine, Kawasaki Medical School.

WT1 mRNA measurement method

mRNA was extracted from PB leukocytes and BM nucleated cells at SRL, Inc., Tokyo, Japan using the RNeasy Mini-Kit (Qiagen, Valencia, CA), and the amount containing WT1 mRNA was measured at the Research Laboratory, Diagnostic Division, Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan using a WT1 mRNA Assay Kit (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan). cDNA was synthesized from 1 μg of extracted RNA in a reverse-transcription reaction using random hexamer primers. The amounts of WT1 and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) mRNA were quantitated using real-time polymerase chain reaction (PCR) with a COBAS TaqMan48 analyzer (Roche Diagnostics, Pleasanton, CA), and the respective amounts of WT1 and GAPDH RNA in the sample were calculated by simultaneous reaction with standards of known concentrations.

Method for calculating WT1 mRNA expression

mRNA of the universally expressed housekeeping gene GAPDH was used for correction of variations in the efficiencies of RNA extraction and reverse transcription. As shown in the following formula, the level of WT1 mRNA expression was calculated by dividing the measured amount of WT1 mRNA by the measured amount of GAPDH mRNA and multiplying that value by the average number of copies of GAPDH mRNA found in 1 μg of RNA from PB leukocytes of healthy adults (GAPDH mRNA expression). The average GAPDH mRNA expression in PB leukocytes of healthy adults was reported to be 2.7×10^7 copies/μg RNA based on independent tests in healthy adults [3].

WT1 mRNA expression (copies/μg RNA) = (measured WT1 mRNA [copies/mL]/measured GAPDH mRNA [copies/mL]) × 2.7 × 10^7 (copies/μg RNA)

PB cut-off value

The lower limit of the WT1 mRNA measurement range in the WT1 assay kit is 2500 copies/mL, or 50 copies/μg RNA when converted to copies per microgram of RNA. In this study, a value of 50 copies/μg RNA was set as the cut-off value for WT1 mRNA expression, and a value of 50 or more copies/μg RNA was judged as positive according to the instruction manual of the WT1 mRNA assay kit.

Statistical analysis

The mean ± SD for the log-transformed values of WT1 mRNA expression (copies/μg RNA) was calculated, and then converted back to base 10 and used as the geometric mean. All data below the detection limit were shown as 49 copies/μg RNA. For intergroup comparison of WT1 mRNA expression, a Tukey-Kramer honestly significant difference (HSD) test was performed at the level of significance of $p < 0.05$ using log-transformed values of WT1 mRNA expression (copies/μg RNA). For comparison of WT1 mRNA expression between the aplastic anemia (AA) and RA groups, a Wilcoxon rank-sum test and Steel test were performed at the level of significance of $p < 0.05$ using log-transformed values of WT1 mRNA expression (copies/μg RNA). The Pearson correlation coefficient was used for analysis of each correlation.

Results

As a result of the central review conducted on all 172 patients, 115 were classified as patients with MDS in the FAB classification, excluding chronic myelomonocytic leukemia (CMML). Similarly, 98 patients in the 2001 WHO classification and 97 in the 2008 WHO classification were classified as patients with MDS (Figure 1).

Analytical results based on FAB classification

WT1 mRNA expression in PB and BM

The 172 patients eligible for analysis were categorized by disease type, and their WT1 mRNA expression levels in PB and BM are shown in Table I. The mean WT1 mRNA expression level in the 115 patients with MDS (excluding CMML) was 360 copies/μg RNA in PB and 2240 copies/μg RNA in BM, and these values were the second highest after the values obtained in patients with AML-MDS (PB: 12 600 copies/μg RNA; BM: 33 100 copies/μg RNA). On the other hand, the WT1 mRNA expression level was less than 50 copies/μg RNA in PB and 90–630 copies/μg RNA in BM in patients with AA, idiopathic cytopenia of unknown significance (ICUS), idiopathic thrombocytopenic purpura (ITP), paroxysmal nocturnal hemoglobinuria (PNH), pure red-cell aplasia (PRCA) and erythroid hypoplasia, which were all lower compared with the level in MDS.

The relationship between WT1 mRNA expression in PB and BM was evaluated in all patients. The regression line formula $y = 0.7329x + 1.4407$ was obtained, indicating a strong correlation ($r = 0.85$) (Figure 2).

WT1 mRNA expression in PB and BM for each MDS disease stage

When the WT1 mRNA expression levels in PB and BM were compared for each MDS subtype based on the FAB classification [Figure 3(a)], the level in both increased proportionally with each MDS classification as the disease

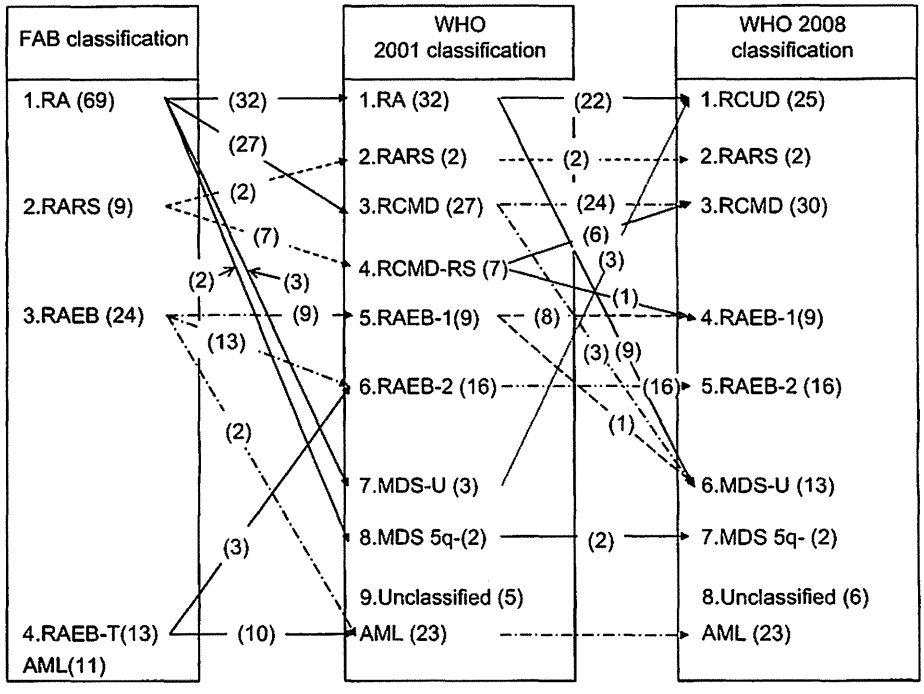

Figure 1. FAB and WHO classification of myelodysplastic syndromes in this study. FAB classification-based MDS subtypes (four subtypes: RA, RARS, RAEB and RAEB-t), 2001 WHO-based MDS subtypes (eight subtypes: RA, RARS, RCMD, RCMD-RS, RAEB-1, RAEB-2, MDS-U and MDS 5q−), 2008 WHO-based MDS subtypes (seven subtypes: RCUD, RARS, RCMD, RAEB-1, RAEB-2, MDS-U and MDS 5q−). Numbers in parentheses represent numbers of patients.

Table I. WT1 mRNA expression levels in PB and BM from patients with different MDS subtypes and AML-MDS according to FAB classification.

		WT1 mRNA expression level			
		Peripheral blood		Bone marrow	
Disease	No. of patients	Log (mean ± SD)	Geometric mean (copies/μg RNA)	Log (mean ± SD)	Geometric mean (copies/μg RNA)
MDS	115	2.56 ± 1.05	360	3.35 ± 0.87	2240
AML-MDS	11	4.10 ± 0.96	12 600	4.52 ± 0.77	33 100
AML-MDS (CR)	2	1.89 ± 0.20	80	2.98 ± 0.39	1000
CMML	3	2.17 ± 0.54	150	3.04 ± 0.54	1100
CLL	1	1.92	80	3.33	2140
Atypical CML	1	—	< 50	1.95	90
AA	8	—	< 50	2.64 ± 0.37	440
ICUS	3	—	< 50	2.16 ± 0.36	140
ITP	1	—	< 50	2.13	130
PNH	1	—	< 50	2.8	630
PRCA	2	—	< 50	2.17 ± 0.12	150
Erythroid hypoplasia	1	—	< 50	1.94	90
Unclassified	23	2.14 ± 0.56	140	2.96 ± 0.61	910
Total	172	2.50 ± 1.05	320	3.27 ± 0.90	1860

PB, peripheral blood; BM, bone marrow; MDS, myelodysplastic syndromes; AML-MDS, acute myeloid leukemia-evolved MDS; FAB, French-American-British; CR, complete remission; CMML, chronic myelomonocytic leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; AA, aplastic anemia; ICUS, idiopathic cytopenia of unknown significance; ITP, idiopathic thrombocytopenic purpura; PNH, paroxysmal nocturnal hemoglobinuria; PRCA, pure red-cell aplasia.

stage progressed. Significant differences in both PB and BM expression were seen between RA and RAEB, RA and RAEB-t, refractory anemia with ringed sideroblasts (RARS) and RAEB, and RARS and RAEB-t ($p < 0.05$).

WT1 mRNA expression in PB and BM for each IPSS risk group

WT1 mRNA expression levels in PB and BM for each IPSS risk group were compared in the 115 patients with MDS. A tendency for WT1 mRNA expression to increase in both PB and BM was observed in each IPSS risk group as the risk of transformation to AML increased from low to high. Significant differences ($p < 0.05$) in WT1 mRNA expression were observed in risk groups between low and intermediate-2, low and high, intermediate-1 and intermediate-2, and intermediate-1 and high in PB samples; and between low and intermediate-1, low and intermediate-2, low and high, intermediate-1 and intermediate-2, and intermediate-1 and higher in BM samples [Figure 3(b)]. The correlation between IPSS score and WT1 mRNA expression was evaluated, and a correlation of $r = 0.57$ was found for both PB and BM samples.

Next, the WT1 mRNA expression levels in PB and BM between IPSS risk groups were compared in the 69 patients with RA [Figure 3(c)]. As the risk increased from low to intermediate-2, the level of WT1 mRNA expression in both PB and BM increased. Moreover, when the distribution of WT1 mRNA expression between each risk group was evaluated, a significant difference ($p < 0.05$) was found in PB between low and intermediate-2; in BM, significant differences were found between low and intermediate-1, and low and intermediate-2.

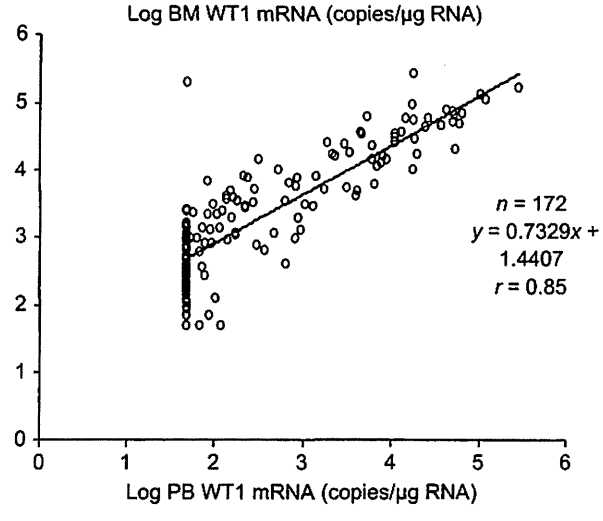

Figure 2. Correlation of WT1 mRNA expression in PB and WT1 mRNA expression in BM.

Correlation between IPSS karyotype and WT1 mRNA expression

A total of 114 patients with MDS were categorized into the three prognostic groups of good, intermediate and poor in accordance with their IPSS karyotype, and the levels of WT1 mRNA expression in their PB and BM samples were compared. One patient with MDS was excluded from this analysis because chromosome testing was not performed. The WT1 mRNA expression level increased in both PB and BM samples as the karyotype indicated a poorer prognosis. Among karyotypes, significant differences ($p < 0.05$) in WT1 mRNA expression were found between the good and intermediate and between the good and poor groups [Figure 3(d)].

Correlation between WT1 mRNA expression and percentage of blasts in BM

The correlation between blast ratio and WT1 mRNA expression in PB and BM was investigated in 114 patients with MDS (excluding one patient in whom the blast ratio could not be measured). The correlation between blast ratio and PB WT1 mRNA expression was $r = 0.51$, and the correlation between blast ratio and BM WT1 mRNA expression was $r = 0.48$.

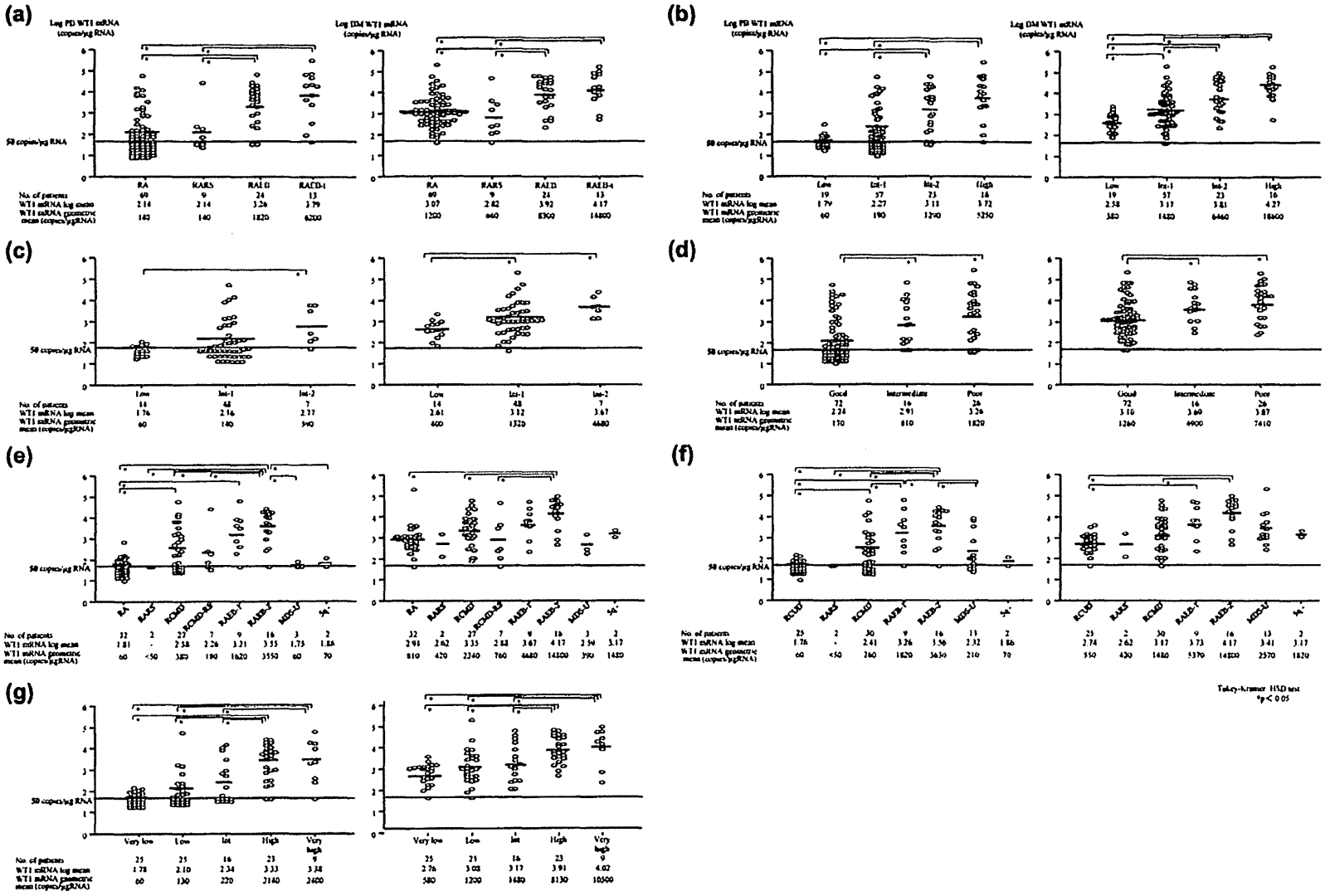

Figure 3. WT1 mRNA expression levels in PB and BM from patients with MDS (a) according to FAB classification, (b) according to IPSS category, (c) patients with RA according to IPSS category, (d) according to chromosomal karyotype, (e) according to WHO 2001 classification, (f) according to WHO 2008 classification, (g) according to WPSS category. In intergroup comparison of WT1 mRNA expression, the Tukey-Kramer HSD test was performed using log-transformed values of WT1 mRNA expression with a level of significance of $p < 0.05$. Bold lines represent mean WT1 mRNA expression after log transformation. Fine lines represent lower limit of detection of WT1 mRNA (50 copies/μg RNA).

Analytical results based on 2001 WHO classification

WT1 mRNA expression in PB and BM for each MDS disease stage based on 2001 WHO classification

Figure 3(e) shows the assay results for WT1 mRNA expression in PB and BM in 98 patients in various MDS disease stages categorized on the basis of the 2001 WHO classification. The WT1 mRNA expression levels in both PB and BM tended to increase with the progression to each MDS subtype. When the levels of WT1 mRNA expression in each disease stage were investigated, significant differences ($p < 0.05$) were found in PB between RA and refractory cytopenia with multilineage dysplasia (RCMD), RA and RAEB-1, RA and RAEB-2, RARS and RAEB-2, RCMD and RAEB-2, RCMD with ringed sideroblasts (RCMD-RS) and RAEB-2, RAEB-2 and unclassified MDS (MDS-U), and RAEB-2 and 5q− syndrome; in BM, significant differences were found between RA and RAEB-2, RCMD and RAEB-2, and RCMD-RS and RAEB-2.

Correlation between WT1 mRNA expression and percentage of blasts in BM based on 2001 WHO Classification

The correlation between the blast ratio and WT1 mRNA expression in PB and BM was investigated in 97 patients with MDS (excluding one patient in whom the blast ratio could not be measured). The correlations between the blast ratio and WT1 mRNA expression were $r = 0.50$ in PB and $r = 0.46$ in BM.

Analytical results based on 2008 WHO classification

WT1 mRNA expression in PB and BM for each MDS disease stage based on 2008 WHO classification

Figure 3(f) shows the assay results for WT1 mRNA expression in PB and BM in a total of 97 patients in various MDS disease stages categorized on the basis of the 2008 WHO classification. WT1 mRNA expression in both PB and BM tended to increase with the progression to each MDS subtype.

When the distribution of WT1 mRNA expression for each disease stage was examined, significant differences ($p < 0.05$) were found in PB between refractory cytopenia with unilineage dysplasia (RCUD) and RCMD, RCUD and RAEB-1, RCUD and RAEB-2, RARS and RAEB-2, RCMD and RAEB-1, RCMD and RAEB-2, RAEB-1 and MDS-U, and RAEB-2 and MDS-U; in BM, significant differences were found between RCUD and RAEB-1, RCUD and RAEB-2, and RCMD and RAEB-2.

Correlation between WT1 mRNA expression and percentage of blasts in BM based on 2008 WHO classification

The correlations between blast ratio and WT1 mRNA expression in 96 patients (excluding one patient with MDS whose blast ratio could not be measured) were $r = 0.50$ in PB and $r = 0.46$ in BM.

WT1 mRNA expression in PB and BM for each WPSS risk group

WT1 mRNA expression in PB and BM was compared in 98 patients with MDS classified according to WPSS risk group [Figure 3(g)]. As the risk increased from very low to very high, WT1 mRNA expression in both PB and BM also tended to rise. When the distribution of WT1 mRNA for each risk group was evaluated, significant differences ($p < 0.05$) were found in both PB and BM between very low and high, very low and very high, low and high, low and very high, intermediate and high, and intermediate and very high. Moreover, when the correlation between the WPSS score and WT1 mRNA expression was investigated, the values were $r = 0.61$ in PB and $r = 0.55$ in BM.

Differential diagnosis between RA and AA

Differential diagnosis based on WT1 mRNA expression in PB samples

The WT1 mRNA expression level in PB was less than 50 copies/μg RNA in all eight patients with AA, whereas it was less than 50 copies/μg RNA in 34 patients with RA and 50–52 100 copies/μg RNA in 35 of 69 patients with RA. The statistical analysis by Wilcoxon rank-sum test revealed a statistical difference between eight patients with AA and 65 patients with RA ($p = 0.01$). Sixty-nine patients with RA were further categorized into three groups by bone marrow findings: hypoplastic RA ($n = 20$), hyperplastic RA ($n = 15$) and normoplastic RA ($n = 30$), excluding the non-categorized RA ($n = 4$). Significant differences were observed between AA and each of hypoplastic ($p = 0.04$) or normoplastic RA ($p = 0.02$), whereas no difference was shown between the AA and hyperplastic RA group ($p = 0.10$) by Steel test (Figure 4). From these findings, a differential diagnostic cut-off value between RA and AA of 50 copies/μg RNA for WT1 mRNA expression in PB is considered appropriate, for which the sensitivity was 50.7% (35/69) and the specificity was 100% (8/8).

Differential diagnosis based on WT1 mRNA expression in BM samples

The WT1 mRNA expression level in BM was 251–2600 copies/μg RNA in eight patients with AA, whereas it was less than 50 copies/μg RNA in one of 69 patients with RA and 69–196 000 copies/μg RNA in the others. The statistical analysis by Wilcoxon rank-sum test revealed no statistical difference between eight patients with AA and 65 patients with RA. Sixty-nine patients with RA were similarly categorized into three groups: hypoplastic, hyperplastic and normoplastic RA, excluding the non-categorized RA. Statistical analysis by Steel test revealed a significant difference between AA and normoplastic RA groups ($p = 0.04$), whereas there were no significant differences between the AA and each of hypoplastic RA and hyperplastic RA groups (Figure 4).

When receiver operating characteristic (ROC) analysis was performed to evaluate the performance of BM WT1 mRNA expression as an indicator to differentiate between RA and AA, the area under the curve was 0.713, and the Youden index [18] showed 432 copies/μg RNA. Moreover, the sensitivity was 69.6% (48/69), and the specificity was 75.0% (6/8) (Supplementary Figure to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2012.745074).

When the PB cut-off value of 50 copies/μg RNA was inserted into the regression line formula obtained