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

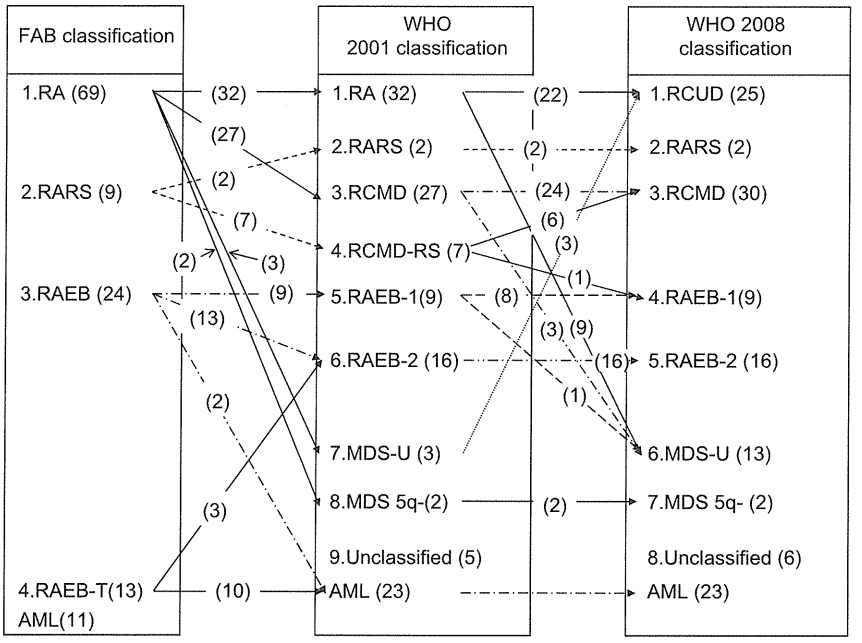

Figure 1. FAB and WHO classification of myelodysplastic syndromes in this study. FAB classification-based MDS subtypes (four subtypes: RA, RARS, RAEB and RAEB-t), 2001 WHO-based MDS subtypes (eight subtypes: RA, RARS, RCMD, RCMD-RS, RAEB-1, RAEB-2, MDS-U and MDS 5q−), 2008 WHO-based MDS subtypes (seven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

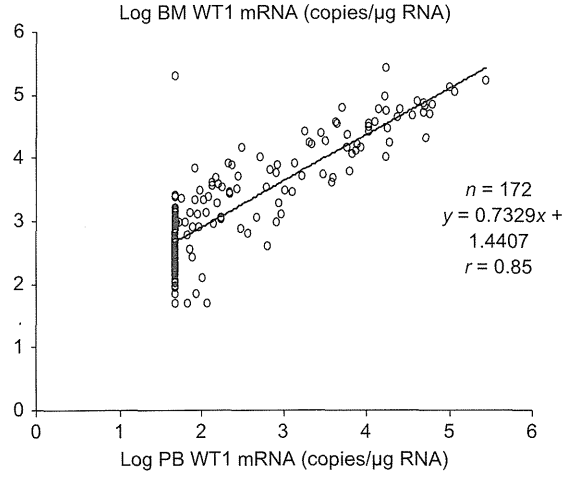

Figure 2. Correlation of WT1 mRNA expression in PB and WT1 mRNA expression in BM.

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 $r = 0.48$.

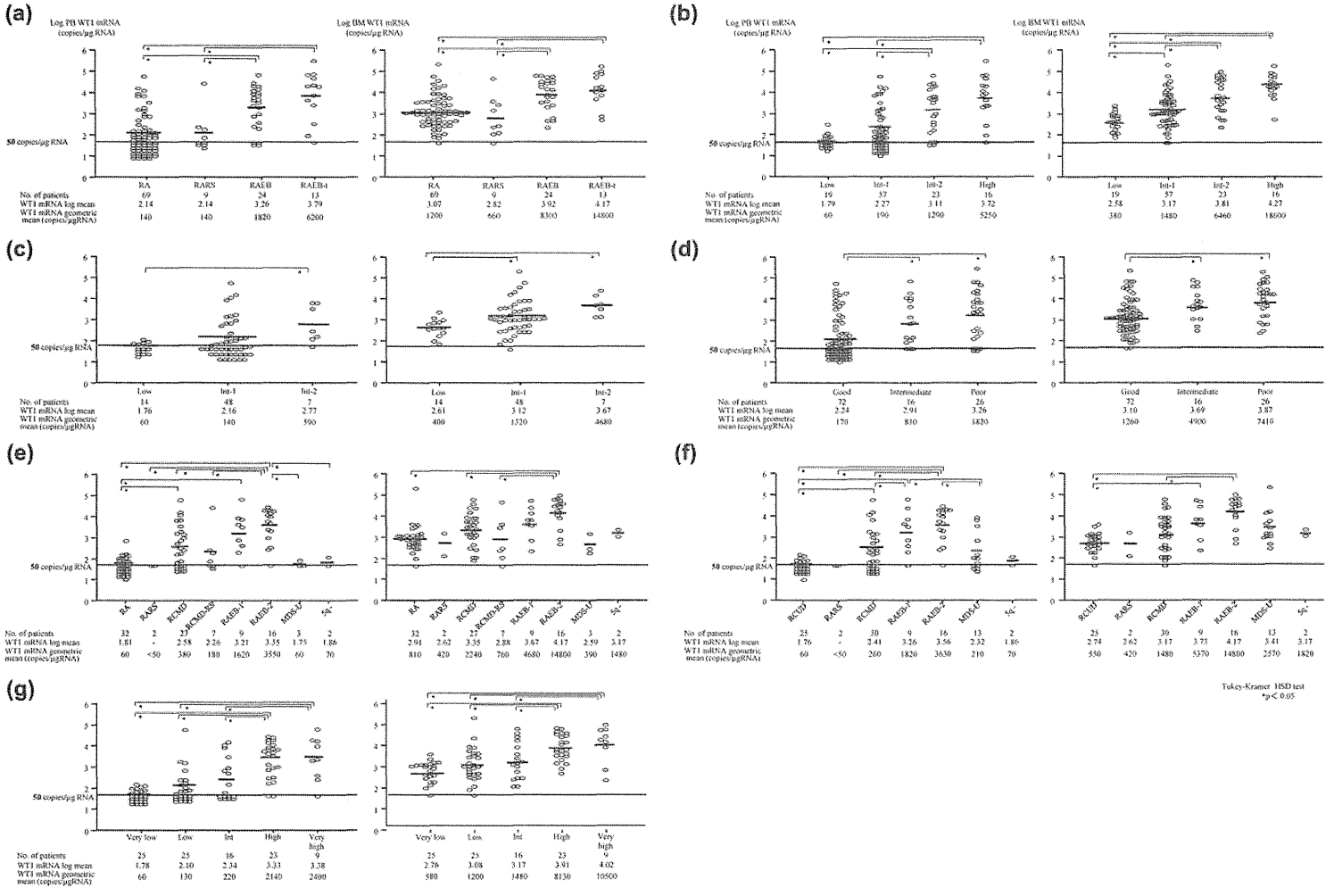

Figure 3. WT1 mRNA expression levels in PB and BM from patients with MDS (a) according to FAB classification, (b) according to IPSS category, (c) patients with RA according to IPSS category, (d) according to chromosomal karyotype, (e) according to WHO 2001 classification, (f) according to WHO 2008 classification, (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

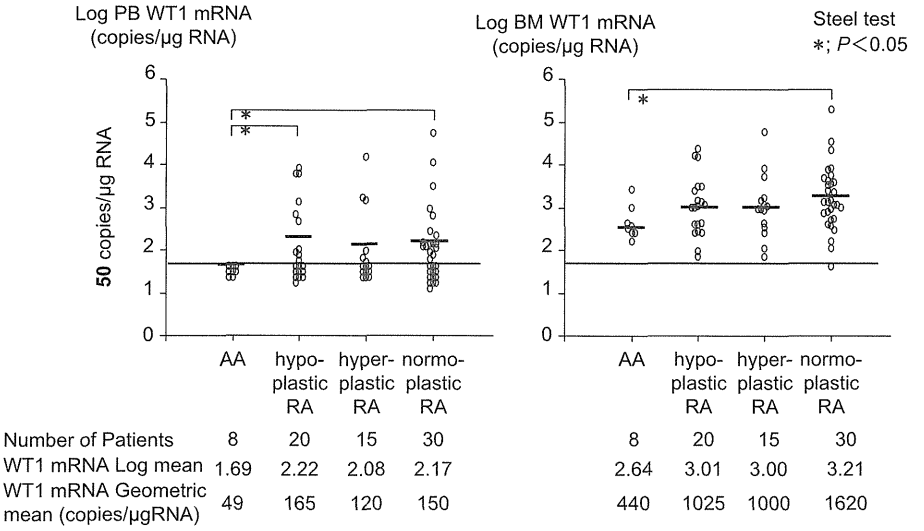

Figure 4. Comparison of WT1 mRNA expression between AA and RA groups (hypoplastic, hyperplastic and normoplastic RA). In intergroup comparison of WT1 mRNA expression, Steel test was performed using log-transformed values of WT1 mRNA expression with a level of significance of $p < 0.05$. Bold lines represent mean WT1 mRNA expression after log transformation. Fine lines represent lower limit of detection of WT1 mRNA (50 copies/μg RNA).

from the correlation between WT1 mRNA expression in PB and BM (Figure 2), BM WT1 mRNA expression became 480 copies/μg RNA. When 500 copies/μg was evaluated as the cut-off value for BM WT1 mRNA expression, the sensitivity was 68.1% (47/69) and the specificity was 75.0% (6/8). Based on these results, 500 copies/μg RNA was considered to be an appropriate cut-off value for the differential diagnosis between RA and AA using WT1 mRNA expression in BM.

Comprehensive analysis using cut-off values

The PB and BM samples in each disease and MDS subtype were further evaluated for their WT1-positive rates, using the WT1 mRNA expression cut-off values determined above (PB: 50 copies/μg RNA; BM: 500 copies/μg RNA) (Table II). For AML-MDS (11 patients), the WT1 mRNA-positive rates were a high 100% (11/11) for PB and 90.9% (10/11) for BM, and in MDS (115 patients), the WT1 mRNA-positive rates were 61.7% (71/115) for PB and 73.0% (84/115) for BM, which were the second highest after AML-MDS. In contrast, all patients with AA, ICUS, ITP, PNH, PRCA and erythroid hypoplasia had low positive rates of 0% for PB and 18.8% (3/16) for BM. The WT1 mRNA-positive rates for PB and BM increased with MDS disease stage progression (Table II).

Table II. WT1 mRNA-positive rate in PB and BM from patients with different MDS subtypes and AML-MDS according to FAB classification.

Subtype	No. of patients	WT1 mRNA-positive rate (%)	
		Peripheral blood	Bone marrow
RA	69	50.7 (35/69)	68.1 (47/69)
RARS	9	44.4 (4/9)	44.4 (4/9)
RAEB	24	83.3 (20/24)	87.5 (21/24)
RAEB-t	13	92.3 (12/13)	92.3 (12/13)
AML-MDS	11	100.0 (11/11)	90.9 (10/11)
Total	126	65.1 (82/126)	74.6 (94/126)

PB, peripheral blood; BM, bone marrow; MDS, myelodysplastic syndromes; AML-MDS, acute myeloid leukemia-evolved MDS; FAB, French-American-British; RA, refractory anemia; RARS, refractory anemia with ringed sideroblasts; RAEB, refractory anemia with excess of blasts; RAEB-t, refractory anemia with excess of blasts in transformation.

Discussion

In this study, the clinical usefulness of the measurement of WT1 mRNA expression in risk assessment of MDS was evaluated using a WT1 assay kit. Recently, a steady stream of reports has indicated the usefulness of WT1 mRNA measurement. The group of Cilloni [6] confirmed that WT1 mRNA expression potentially fulfills all the requirements for an additional marker for risk assessment in MDS, compared with the conventional methods. The measurement of WT1 can be effective, particularly in cases in which BM aspiration and/or cytogenetic analysis fail or are not informative [6].

Furthermore, in their findings in a long-term prospective study, Tamura *et al.* [19] reported that a significant correlation ($p = 0.0186$) was seen between WT1 mRNA expression and survival time when WT1 mRNA expression in PB was categorized into three groups of less than 10^2, 10^2–10^4, and greater than 10^4 copies/μg RNA, that the median survival time for each group was 62.7 months, 29.9 months and 11.6 months, respectively; and that the time until transformation to leukemia was the shortest in the group with the highest WT1 mRNA expression. In addition, they reported that in univariate analysis, WT1 mRNA expression was a predictive parameter for transformation to leukemia, and in multivariate analysis, it was a significant predictive parameter along with the IPSS score [19]. As described above, Tamaki *et al.* reported similar findings [4].

This study was conducted using not only the FAB classification system but also the 2001 and 2008 WHO classification systems. It was confirmed that in all three classification systems, WT1 mRNA expression in both PB and BM increases significantly in MDS subtypes with disease stage

progression. In addition, both PB and BM WT1 mRNA expression increased significantly as the risk of transformation to AML rose in the IPSS and WPSS risk groups. Furthermore, a correlation of $r = 0.57$ between the IPSS score and WT1 mRNA expression was seen in both PB and BM. The correlations between the WPSS score and WT1 mRNA expression were $r = 0.61$ in PB and $r = 0.55$ in BM. In comparison with the IPSS, the WPSS allows the assessment of survival time and progression of leukemic transformation at all time periods during the clinical course, leading to continued prognostic evaluation while reviewing the risk. WT1 mRNA expression correlates with the WPSS prognosis, and despite the single-point quantitation, the results in this study indicate that WT1 mRNA is useful as a time-course prognostic marker in the same manner as the WPSS.

At present, allogeneic hematopoietic stem cell transplant is the only curative treatment for MDS. However, determination of the timing of allogeneic transplant is very difficult because many patients are older, treatment-related deaths frequently occur, and there are large individual differences in the rate of disease progression. Allogeneic transplant is selected as the therapeutic regimen for MDS when no increase in blast cells is confirmed, taking into consideration the development of transfusion dependency and frequency of infections [20]. In addition, allogeneic transplant is selected when a future increase in blast cells is predicted by karyotypic analysis even though no increase is currently observed. It is recommended that transplant be performed before the progression to cytopenia caused by an increase in blast cell clones and before the progression to acute leukemia, although induction chemotherapy may be required when an increase in blast cells is observed [21]. On the other hand, another study suggested that delaying transplant until the advanced stage of disease results in a longer survival time for low and intermediate-1 IPSS risk groups, while early transplant was recommended for the intermediate-2 and high groups [22]. The period after CR is achieved is considered to be the standard timing to perform transplant for acute leukemia, but determining CR is extremely challenging. Our results revealed that periodic monitoring of WT1 mRNA expression in patients with MDS provided useful information for predicting the timing of transplant.

RA, a subtype in the early MDS disease stage, is often difficult to differentiate from AA [23]. In a previous study by Iwasaki *et al.*, no difference in WT1 mRNA expression was observed between RA and AA [9]. However, our data revealed the possibility of WT1 expression level to differentiate AA and RA groups using both peripheral blood and bone marrow samples (Figure 4). In the present statistical analysis, significant differences were observed between AA and hypoplastic RA ($p = 0.04$) in PB. The number of subjects was limited, and further trial is required for more detailed analysis. Moreover, tentative cut-off values for WT1 mRNA expression were set at 50 copies/μg RNA in PB and 500 copies/μg RNA in BM. Although the number of patients was small, the results showed that the level of WT1 mRNA expression could differentiate between RA and AA, with specificity in PB and BM of 100% (8/8) and 75.0% (6/8), respectively. This provides evidence that the measurement of WT1 mRNA expression can play a role in the differential diagnosis of RA and AA.

The WT1 assay kit is used clinically in Japan as a marker to monitor MRD in patients wth AML. In MDS, a clonal disorder of pluripotent hematopoietic stem cells, WT1 mRNA expression increases depending on the MDS subtype and disease stage. In contrast, the mechanism by which WT1 mRNA expression increases in MDS is not considered to correlate simply with the fluctuation in leukemic clones, as seen in AML. In normal hematopoiesis, WT1 mRNA is expressed mainly in CD34-positive cells. In contrast, in patients with MDS, WT1 mRNA is also expressed in CD34-negative cells, particularly in lineages exhibiting abnormalities [24]. In our study, the level of WT1 mRNA expression within the RA group was shown to increase with the increase in IPSS risk [Figure 3(c)]. Moreover, a similar trend of increasing WT1 expression was found in the RCUD and RCMD groups according to the 2008 WHO classification, although no significant increase in blast cells in BM was observed in these groups. Taken together, these findings indicate that the increase in WT1 mRNA expression in patients with MDS may reflect the divergence of MDS clones from normal clones and preleukemic changes.

In patients with MDS, evaluating the changes in WT1 mRNA levels simultaneously in PB and BM samples provides useful information on disease stage progression or risk assessment in individual patients. In addition, the WT1 mRNA-positive rate in each subtype of MDS was high (50–90%) in both PB and BM in this study, suggesting that a single measurement of WT1 mRNA is sufficient for MDS diagnosis, particularly for differentiating RA from AA.

Overall, this study provides evidence that the measurement of the level of WT1 mRNA expression in PB and BM serves as a supplemental marker for MDS diagnosis and prognostic assessment. This assay has great potential to contribute to more appropriate diagnoses and therapeutic decisions in patients with MDS and to evaluate the timing of allogeneic transplant.

Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article at www.informahealthcare.com/lal.

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Supplementary material available online

Supplementary figure showing ROC analysis of WT1 mRNA expression in BM in RA and AA groups

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巻頭言

ドナーアフェレシスにおけるインフラストラクチャーの整備を望む

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今回の特集では，自己，同種を問わず，血球成分を治療に用いるために採取することや，それに関連した治療をとりあげた．

まず最初に，面川論文は最も日常的に広範に行われている，赤十字血液センターにおける，ドナーアフェレシスについて述べている．実に152万人ものボランティアドナーが，アフェレシスによる献血を行っているが，すべて遠心式血液成分分離装置を用いた体外循環によるもので，その手技の安定性は，特筆すべきものであり，赤十字血液センターが積み上げてきた経験は大きな財産である．吉原論文は，現在の日本では承認されておらず，一部の施設でのみ行われている，ドナーからの顆粒球輸血（採取）について述べている．G-CSFの併用で，輸注して効果を発揮可能な数の顆粒球を採取することが技術的に可能となっており，無顆粒球が長期に続く場合の治療戦略のオプションとして，保険診療の中へ組み込まれるよう取り組んでゆく必要がある．佐川論文は，自己血輸血，特に貯血式自己血輸血について，最も盛んに行われている我が国の現状について，その背景から実践に至るまでを詳述している．一昨年より骨髄バンクにおいて，非血縁の同種末梢血幹細胞移植が開始された．従来の全身麻酔下の骨髄採取ではなく，ボランティアドナーへのG-CSF投与下の，アフェレシスを用いた末梢血の造血幹細胞採取による非血縁者間造血幹細胞移植である．日野論文は，日本での非血縁者間末梢血幹細胞移植の立ち上げの経緯とその問題点について述べており，長藤論文は，同種骨髄移植と同種末梢血幹細胞移植が，異なる意味を持つ治療である可能性について，欧米と我が国の研究成果をもとに検討している．アフェレシスによって得られた白血球を修飾して治療に用いる試みも始まっている．西田論文は，レシピエント傷害性と同時に，抗腫瘍効果，抗ウィルス効果を持つドナー単核球を用いて，ドナーのウィルス特異的細胞傷害性T細胞（CTL）を誘導増殖させ，難治性のウィルス感染症を治療する養子免疫療法について述べている．最後に北脇論文は，アフェレシスを用いて得られた患者単核球から，樹状細胞（dendritic cell，以下DC）を作成し，腫瘍免疫反応を誘導するワクチンとして投与するという，DCワクチン療法の臨床応用について述べている．これらの内容はすべて，ドナーもしくは自己血球を，直接もしくは修飾を加えた上で治療に用いるものであり，全血を用いた自己血貯血を除いて，すべて遠心式血液成分分離装置を用いた治療である．

Graphic presentationでも述べたが，現在我が国では，治療的ヘムアフェレシスの大半は，膜分離法や吸着法等の遠心分離以外の方法で行われている．しかしながら，今回の論文で取り上げられた様な，採取細胞を再び治療に用いるには，現在の技術では遠心法に頼らざるをえない．現在我が国で行われている，成分献血以外の遠心分離法によるアフェレシスの大半は，患者自身や血縁ドナーからの末梢血幹細胞採取であり，2010年の日本造血細胞移植学会の調査[1]では，自家末梢血幹細胞移植1,578件，血縁者間同種末梢血幹細胞移植634件が合計224施設で行われている．仮に1移植に

2アフェレシスが必要であったとして，1施設平均1年間に19.75アフェレシスが行われることになる．この状況では，遠心アフェレシス専任の看護師の配置や，アフェレシスの質の確保はきわめて困難である．日野論文でも述べられた，非血縁者間同種末梢血幹細胞移植開始にあたって，ボランティアドナーの末梢血幹細胞採取の施設要件を満たし参加する施設数が速やかに増加しない主な理由がここにあると思われる．一方，面川論文でも述べられているように，赤十字血液センターでは，年間152万回の遠心分離法によるアフェレシスが，アフェレシスナースを中心に行われている．様々な要件の調整は必要であろうが，我が国の現状で，安定した非血縁ドナー採取を確立させるには，遠心分離法とボランティアドナーへの対応に習熟した赤十字血液センターが大きな役割を担わざるをえないであろう．

我が国では，本年4月から，赤十字血液センターが集約化され，機能が分化され，ブロックセンター以外の各県のセンターの機能が，採血と供給に限定されることとなった．また，日本赤十字社は，献血以外のアフェレシスへの協力に消極的ともいわれる．我が国の現状を考えるとき，センター内でのアフェレシスの協力，もしくは出張しての技術協力が必要と思われる．先日，米国のアフェレシスを盛んに行っているいくつかの施設を訪れたが，院内もしくは血液センター内のアフェレシス部門では，多数の遠心分離器が置かれ，アフェレシスナースが主体となって，日々数多くの治療的アフェレシスが行われるとともに，自家もしくはドナーからの末梢血幹細胞採取，photopheresis，北脇論文で触れられている，DC療法のための単核球採取が，日常診療の中で行われていた．造血幹細胞移植については，まったく欧米と遜色ない水準まで到達している中で，薬剤で従来より指摘され，現在少しずつ改善の兆しが認められるdrug lagの状態が，細胞療法でも存在しているが，その一因は，遠心分離法による治療関連のアフェレシスを容易に行える環境が，整備されていないことにあると思われる．

細胞療法は，今後先進的な治療として，前臨床や日常診療の中に組み込み得る形を様々に提示してくるであろう．そして細胞療法への取り組みは，医療の先進性を示す指標のひとつとなるものと思われる．現在示されている細胞療法に関するインフラストラクチャーの脆弱さを，早急に改善する必要がある．各臨床施設で可能なことは，血液疾患，輸血，細胞治療等を専門にしながら，アフェレシスに習熟した医師の養成，膜による体外循環だけでなく，遠心分離法によるアフェレシスに習熟した臨床工学技士の育成，日本輸血・細胞治療学会が認定している，学会認定アフェレシスナース，学会認定臨床輸血看護師，学会認定自己血輸血看護師の資格をできればすべてとり，これらの業務だけで雇用が可能な看護師の養成と輸血部門の独立であろう．日々莫大な数の遠心分離法でのボランティアドナーに対するアフェレシスを経験している赤十字血液センターが，現状では十分な遠心分離法によるアフェレシスの経験を積むことが困難な我が国の臨床施設に対して，その経験を，場所の提供や，技術協力といった形で還元していくことは極めて重要である．行政に関しては，Graphic presentationで述べたように，ACD-A液が，体外循環の抗凝固薬として薬事承認されていないこと，遠心分離装置のディスポーザブル回路が保険償還されないこと等，基本的な無理解があるが，今後，腫瘍関連，免疫関連の細胞治療の進歩に我が国が取り残されないよう，早急なインフラストラクチャーの整備を希望したい．

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IX 治療的プラズマフェレーシスとサイタフェレーシス

IX-1 基本概念

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キーワード：plasmapheresis, cytapheresis, plasma exchange, plasma filtration, adsorption

血液の成分の一部（中分子量以上）や血球成分を，体外循環を用いて採取したり除去したり置換したりすることをヘムアフェレーシスと呼ぶ．この項では，治療的ヘムアフェレーシス（Therapeutic hemapheresis：TxHAP）について述べる．

TxHAPの治療概念としては，①有害物質の除去，②有用物質の注入，③生体の緩衝能，恒常性の回復，④標的細胞の採取，除去，処理，注入，⑤BRM（biological response modifier）作用が挙げられる．

TxHAPは，血球成分のアフェレーシス（サイタフェレーシス；cytapheresis：CAP）と血漿成分のアフェレーシス（プラズマフェレーシス；plasmapheresis：PAP）とにわかれる．

1 体外循環の方法

HAPに用いる体外循環の方法として，PAPの場合には，遠心分離式[1]と膜分離式[2]がある．CAPの場合には，遠心分離式のほかに，不織布，吸着剤を用いた吸着法がある．

1）遠心分離法

遠心分離は，血液を遠心することにより，比重によって血漿と各血球を分離し，必要な層より採取する．後述する膜分離法に比して血漿を効率よく分離でき，目標とする分画を比較的修飾を受けない形で扱うことができる反面，層自体が重層しているので，隣接した層に含まれる成分も混入する欠点がある．

2）膜分離法

膜を用いた血漿分離は，通常中空糸の側面に開いた細孔を通じて血漿が分離されていく．そのため中空糸の出口側に行くにつれて，ヘマトクリットが上昇し粘度が高くなる．このため，TMP（transmembrane pressure）の上昇による溶血が生じやすい．溶血を回避するため，血漿分離比を高くできないので，全血流量が多くなるが，血漿中への血球の混入はない特徴がある．

3）白血球除去器

詳細は各論で述べる．

2 抗凝固薬

体外循環の抗凝固薬として，ACD-A（acid citrate dextrose-A）液，ヘパリン，低分子ヘパリン，ナファモスタットメシル酸塩（nafamostat mesilate：NM），アルガトロバンが挙げられる．CAPにおいては，遠心式の場合には，ACD-A液を用いる．ヘパリンは，血小板凝集を生じるため使用されないが，ACD-A液にヘパリンを適当な濃度混和することで，ACD-A液の使用量を減らすことが可能である[3]．不織布や吸着剤によるCAPの場合には，ヘパリン，低分子ヘパリンや，半減期の短いNMが用いられる．PAPにおいては，遠心分離では各抗凝固薬が使用可能であるが，膜分離の場合には全血の流量が多くクエン酸中毒を生じやすいため，ACD-A液は通常使用されない．

3 血液の処理

1）処理量

交換効率から通常1～1.5循環血漿量の血漿処理を行うことが基本になる．

2）単純血漿交換［遠心分離器，血漿分離膜］（plasma exchange）

・置換液

新鮮凍結血漿（fresh frozen plasma：FFP）は，肝疾患等で凝固異常のある場合，止血系の異常を伴う場合，ADAMTS13（a-disintegrin-like and metalloproteinase with thrombospondin type 1 motifs 13）欠乏による血栓性血小板減少性紫斑病（thrombotic thrombocytopenic purpura：TTP）などで用いる．止血系諸因子の合成能に問題がない場合に5%アルブミンを用いる．循環動態，アルブミン合成能に問題がない場合には，低分子デキストランとアルブミンを併用することが可能である．患者血中アルブミン濃度が低い時には，肺水腫が生じないよう置換液のアルブミン濃度を下げる必要がある．

3）血漿分画

血漿全体を廃棄せず，血液（血漿）中の有害物質をできる限り選択的に除去しようという試みがなされている．現在使用可能なものとして，血漿分画膜や吸着器がある．

a）血漿分画膜

血漿を血漿分離膜より細孔の小さいカラムを用いて，免疫グロブリン，LDL（low density lipoprotein）コレステロール等のmacromoleculeを，アルブミン等は残したまま除去する（plasma filtration）．特にIgMやLDLコレステロールについては効率よい除去が可能である．

b）Cryofiltration[4]

ヘパリン化した血漿を冷却し，生じる免疫グロブリンやフィブロネクチンを含んだcryogelを膜分離法で除去する方法で，関節リウマチや血液型不適合腎移植で用いられる．

c）吸着器

吸着（adsorption）の方法を以下に挙げる．

①非特異的吸着（薬物，エンドトキシン，β2-ミクログロブリン）

②物理化学的吸着（LDLコレステロール，免疫グロブリン，ビリルビン等）

③免疫学的吸着（抗アセチルコリン受容体抗体等）を用いた吸着カラムが開発されている．非特異的吸着では全血を用い，物理化学的吸着，免疫学的吸着では血漿を吸着することが多い．

4）施行頻度

TTPでは血小板数が回復するまで，ほぼ連日施行する．家族性高コレステロール血症（ヘテロ）では，薬剤と併用で2週に1回行う．

その他の免疫関連疾患，神経疾患では2～3回/週を2～3週繰り返すことが多い（保険適応と異なる場合があるので留意が必要である）．

4 瀉血（赤血球除去；erythrocytapheresis）

多血症における赤血球除去，ヘモジデローシス，ヘモクロマトーシス，慢性C型肝炎における鉄過剰に対する治療として行う．通常，成人では400ml程度の血液を採取し廃棄する．多血症等で大量の赤血球除去が必要な場合には，遠心分離器で赤血球層を除去し，等量の補液を行う．

5 白血球除去

炎症性腸疾患(潰瘍性大腸炎，クローン病)，関節リウマチの治療として行う．通常白血球除去（leukapheresis）フィルターや白血球吸着カラムを用いる（遠心分離器でも可能）．非常に白血球数の多い白血病の治療開始時に行うことがある（遠心分離器）．

6 ECP（extracorporeal photopheresis）

体外循環で取り出した白血球浮遊液に，MOP（8-methoxypsoralen）を添加後UVA（ultraviolet A）を照射し活性化した後，体内に返す治療であるが[5]，現在日本では行われていない（IX章-2　適応参照）．

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Phase II study of dose-modified busulfan by real-time targeting in allogeneic hematopoietic stem cell transplantation for myeloid malignancy

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We aimed to evaluate the efficacy and safety of allogeneic hematopoietic stem cell transplantation with targeted oral busulfan (BU) and cyclophosphamide (CY) in a phase II study. Busulfan (1.0 mg/kg) was given initially in six doses. Based on the estimated concentration at steady state after the first dose of BU, subsequent (7th–16th) doses were adjusted to obtain a targeted overall concentration at steady state of 700–900 ng/mL. The primary endpoint was 1-year overall survival (OS). Fifty patients were registered and 46 (median age, 53 years; range, 18–62 years) received planned transplant, including 24 with AML, 16 with myelodysplastic syndrome, and six with CML. Fourteen patients were categorized as standard risk. Nineteen patients received transplant from human leukocyte antigen-identical siblings, 27 from unrelated donors. The BU dose required reduction in 32 patients and escalation in six patients. One-year OS was 65% (95% confidence interval, 50–77%). Cumulative incidence of hepatic sinusoidal obstruction syndrome was 11%. One-year transplant-related mortality was 18%. Both OS and transplant-related mortality were favorable in this study, including patients of older age and with high risk diseases. Individual dose adjustment based on BU pharmacokinetics was feasible and effective in the current phase II study. This trial is registered in the University Hospital Medical Information Network Clinical Trial Registry System (UMIN-CTR, ID:C000000156). (*Cancer Sci* 2012; 103: 1688–1694)

Busulfan is an alkylating agent widely used in high-dose chemotherapy regimens for HSCT.[1,2] The BU level in serum has been shown to be an important factor for graft rejection and regimen-related toxicity such as SOS.[3–5] Unfavorable profiles of oral BU include delayed and variable absorptive characteristics and high variability in drug metabolism.[6] Individualized dose adjustment of BU using the LSM, and its transplantation results, have been investigated widely in Caucasian patients and pediatric populations, but few prospective studies have investigated results in Asian patients.[7] Prior to the current study, we carried out a prospective PK study to analyze BU concentration using gas chromatography–mass spectrometry.[8] Nine patients were enrolled in the study, and received preparative regimen containing oral BU 1 mg/kg every 6 h for eight or 16 doses. Out of nine patients, only three met the average steady-state plasma concentration levels in the safety range of 650–1000 ng/mL[4,9] after the first and 13th dose. From the results, we developed LSM to estimate the AUC using two different formulas in order to fit even delayed clearance. Subsequently, we carried out a pilot study that used the same targeting method as the current study, and six patients with myeloid malignancy received tBU+CY conditioning with a targeting AUC of Css 700–900 ng/mL. Four patients received dose reduction after the seventh dose of BU, and overall Css of three patients met the safety range of 786–905 ng/mL (Akio Kohno, Mariko Fukumoto, Hiroto Narimatsu, Kazutaka Ozeki, Masashi Sawa, Shuichi Mizuta, Hitoshi Suzuki, Isamu Sugiura, Seitaro Terakura, Kazuko Kudo, and Yoshihisa Morishita, unpublished data, 2003).

From these results, we carried out a prospective phase II trial in Japanese patients with myeloid malignancies to evaluate the clinical results of allogeneic HSCT undergoing individualized high-dose oral BU+CY conditioning.

Materials and Methods

Eligibility criteria. Patients from 16 to 65 years old were eligible if they had a diagnosis of AML, CML, or MDS, with an Eastern Cooperative Oncology Group performance status of 0–2, and no previous history of HSCT. Standard risk was defined as AML in first complete remission, MDS in refractory anemia or refractory anemia with ringed sideroblasts, and CML in chronic phase. High risk was defined as the remaining disease type. Patients receiving T cell depletion, or those with clinically significant infection or severe abnormalities of cardiac, pulmonary, and hepatic functions were excluded. Included patient/donor pairs were either related HLA matched by serological typing of A, B, and DR locus, unrelated HLA matched, or HLA DRB1 one locus mismatched by genotypical typing of A, B, and DRB1 locus. Unrelated donors were chosen by coordination with the Japan Marrow Donor Program. Written informed consent was obtained from each patient according to the Declaration of Helsinki. The study

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protocol was approved by the Institutional Review Board of each center.

Conditioning regimen, GVHD prophylaxis, and supportive care. Patients received a conditioning regimen consisting of BU 1.0 mg/kg given orally four times a day for six doses on two consecutive days (dose 1–6). Six hours after dose 6, patients received an adjusted dose of BU four times a day for 10 doses (dose 7–16) on three consecutive days (Fig. S1). Cyclophosphamide 60 mg/kg was given i.v. on two successive days. Both BU and CY were dosed based on actual body weight if it was <120% of ideal body weight, and adjusted body weight for those exceeding 120%. Sodium valproate was given as seizure prophylaxis before and during BU treatment. Fluconazole was used as fungal prophylaxis.

Either cyclosporine or tacrolimus in combination with methotrexate was used for GVHD prophylaxis. Cyclosporine was given i.v. at a dose of 3 mg/kg per day in two divided doses starting on day −1. Tacrolimus was given i.v. at a dose of 0.025 mg/kg continuously starting on day −1. Methotrexate was given at a dose of 10 mg/m^2 on day 1 and 7 mg/m^2 on days 3 and 6. Oral cyclosporine or tacrolimus was substituted for i.v. administration when tolerated. In the absence of GVHD, the cyclosporine and tacrolimus doses were tapered after day 50. Acute GVHD of grade 2 or more was treated with methylprednisolone 1–2 mg/kg. Chronic GVHD was treated by the protocols of each institute.

Supportive care measures were used according to institutional guidelines. Daily granulocyte colony stimulating factor was started on day 6 and continued until absolute neutrophil count exceeded 500/μL for two consecutive days.

Pharmacokinetic studies of BU. For PK studies of BU, blood samples were obtained 0, 30, 60, 120, 300, and 360 min after the first oral dose. Frozen plasma samples were sent to the laboratory at Kitasato University, and plasma BU concentrations were assayed by gas chromatography–mass spectrometry.[8] The AUC was calculated by LSM using the formulas shown in Table 1.

Average Css levels of BU were determined by the ratio of the BU AUC_{LSM} over the dosing interval to the time between doses. The BU dose after the sixth dose was adjusted when Css after the first dose was not within 700–900 ng/mL. Dose adjustment was not carried out for patients whose Css after the first dose was 700–900 ng/mL. A targeted dose was calculated to achieve an average Css after all doses of 800 ng/mL. The optimal dose of BU was calculated as follows: optimal single dose of BU (mg/kg) = 800 (ng/mL) × first dose (mg/kg)/Css of first dose (ng/mL).

The dose of the 7th to 16th BU was calculated as follows: revised dose (mg/kg) = [optimal single dose (mg/kg) × 16 (times) − first dose (mg/kg) × 6 (times)]/10 (times).

Definitions of outcomes. The study was designed as a phase II prospective trial. The primary endpoint of the study was 1-year OS after transplantation. The secondary endpoint was DFS, PK of BU, aGVHD, and cGVHD, and the frequency and severity of SOS, regimen-related toxicity up to day 28, mortality at day 100, hematological recovery, and DFS and OS of each disease category.

All patients were prospectively monitored for engraftment,[10] post-transplant toxicities, GVHD, hepatic SOS, and infection. Failure to reach an absolute neutrophil count of 0.5 × 10^9 cells/L by day 28 after transplantation was defined as graft failure, and the patient was withdrawn from the study. The aGVHD was evaluated daily until day 28 and weekly from day 29 to 100 and graded by established criteria.[11] The cGVHD was evaluated up to day 365. Treatment and the outcome of aGVHD and cGVHD were also evaluated. Sinusoidal obstruction syndrome was clinically evaluated before day 28, and diagnosed,[12–14] then graded clinically[12] according to the published criteria. Liver toxicity that occurred after day 21 and fulfilled the above criteria of SOS was defined as late-onset SOS. Clinical data after day 29 until day 100 was additionally surveyed to evaluate late-onset SOS retrospectively.

Disease monitoring was carried out by bone marrow aspiration within 1 week before or after days 30, 60, and 90 after transplantation. Relapse was defined by hematological recurrence for AML,[15,16] and by hematological or cytogenetic relapse for CML. Deaths in the absence of persistent relapse were categorized as non-relapse mortality. Additional surveillance was carried out and the onset of SOS and regimen-related toxicities from days 29 to 100 were collected retrospectively. Long-term survival data and data of relapse after day 365 were also collected retrospectively.

Statistical analysis. The primary endpoint of the study was 1-year OS after transplantation. The expected 1-year OS was estimated to be 60%, and its threshold was estimated to be 40%. With a statistical power of 90% and a one-sided, type I error of 5%, the number of eligible patients required for this study was calculated to be 46 using a binominal analysis method. The projected sample size was 50 patients, with the expectation that 10% of patients would be deemed ineligible.

Disease-free survival was calculated from the date of transplantation until the date of relapse or the date of death in complete remission. This trial has been registered in the University Hospital Medical Information Network Clinical Trial Registry System (UMIN-CTR, ID:C000000156). Data were analyzed with Stata 9.2 statistical software (Stata, College Station, TX, USA).

Table 1. Formulas for limited sample model (LSM) in patients receiving allogeneic hematopoietic stem cell transplantation treated with targeted oral busulfan and cyclophosphamide

i	In cases C_6/C_2 = or <0.5
	$AUC_{LSM} = 0.5C_{0.5} + 0.75C_1 + 2.5C_2 + 2.0C_6 + 4C_6/(LnC_2 - LnC_6)$
ii	In cases $C_6/C_2 > 0.5$
	$AUC_{LSM} = 0.5C_{0.5} + 0.75C_1 + 2.5C_2 + 2.0C_6 + 2C_6/(LnC_2 - LnC_6)$

In the previous pilot study, formula (i) bore a strong approximation to actual area under the blood concentration time curve (AUC), but not in patients with an elongated absorption or a delayed elimination of busulfan. The formula of the LSM was modified in the case of $C_6/C_2 > 0.5$ and formula (ii) was used for those patients. C_x, serum busulfan level obtained at x hours after the first dose.

Results

Patient characteristics. Patients were registered from October 2003 through March 2007. Fifty patients were registered. One patient who developed severe hemorrhagic ulcer of the ileum after registration was considered to be ineligible. One patient developed metastatic breast cancer before receiving the conditioning regimen and was withdrawn. Forty-eight patients received tBU+CY conditioning. One patient developed systemic convulsion on day −6 before transplantation, and the study was discontinued. Another patient received cord blood transplantation due to unexpected emergent unavailability of the unrelated bone marrow and was included only in the PK analysis. The remaining 46 patients who completed tBU+CY conditioning and received the planned transplantation were analyzed in the subsequent outcome study. Characteristics and a transplantation summary of these 46 patients at the time of registration are shown in Tables 2 and 3, respectively.

Treatment-related toxicity and hepatic veno-occlusive disease. Forty-five of 46 patients undergoing tBU+CY conditioning (98%) experienced grade II or higher regimen-related toxicity, and 38 of 48 patients (79%) experienced grade III or more toxicity within 28 days post-transplantation (Table S1). Infection (70%), oral mucositis (52%), nausea and vomiting (30%), and

Table 2. Characteristics of patients receiving allogeneic hematopoietic stem cell transplantation (n = 46)

Characteristics	
Median age of patients (range), years	53 (18–62)
Sex of recipient (%)	
Male	29 (63)
Female	17 (37)
Sex, donor versus recipient (%)	
Match	25 (54)
Male to female	11 (24)
Female to male	10 (22)
Disease type (%)	
AML	24 (52)
1st CR	5
2nd CR	10
1st relapse	5
No treatment†	4
MDS	16 (35)
RA	4
RAEB	9
CMML	1
RAEB-t	2
CML	6 (13)
CP	5
AP	1
Disease risk‡ (%)	
Standard	14 (30)
High	32 (70)
Performance status§ (%)	
0	40 (86)
1	6 (13)
2	0 (0)
Donor (%)	
Related	19 (41)
Unrelated	27 (59)
HLA (%)	
HLA identical sibling	19 (41)
HLA 6/6 matched, unrelated	23 (50)
HLA mismatched, unrelated	4 (9)

†Two patients with overt leukemia from myelodysplastic syndrome (MDS) and another two patients with hypoplastic AML did not receive induction chemotherapy before transplantation. ‡Standard risk was defined as AML in 1st complete remission (CR), MDS in refractory anemia (RA) or RA with ringed sideroblasts, and CML in chronic phase (CP). §According to Eastern Cooperative Oncology Group criteria. AP, accelerated phase; CMML, chronic myelomonocytic leukemia; HLA, human leukocyte antigen; RAEB, refractory anemia with excess of blasts; RAEB-t, RAEB in transformation.

Table 3. Summary of transplantation in patients with AML (n = 24), myelodysplastic syndrome (n = 16), or CML (n = 6)

Stem cell source	
G-PBMC	7
Bone marrow	39
GVHD prophylaxis†	
sMTX+CyA	22
sMTX+FK	21
aGVHD, grade (%)	
None	26 (56)
I	4 (9)
II	11 (24)
III	4 (9)
IV	1 (2)
cGVHD, type (%)	
None	16 (43)
Lmt	9 (24)
Ext	12 (32)

†One patient received short-term methotrexate + tacrolimus prophylaxis and subsequently received short-term methotrexate + cyclosporine. aGVHD, acute graft versus host disease; chronic GVHD, chronic graft versus host disease; GVHD, graft versus host disease.

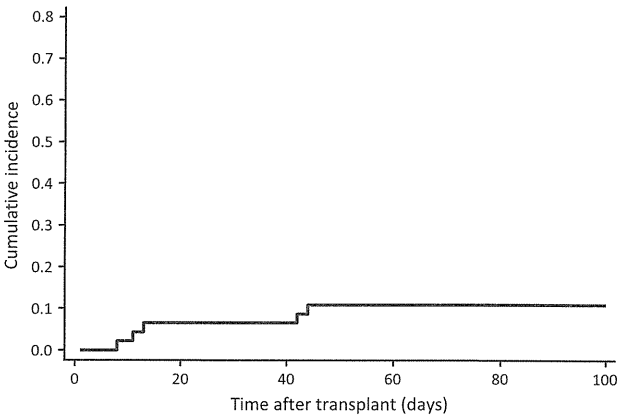

Fig. 1. Cumulative incidence of sinusoidal obstruction syndrome in patients receiving allogeneic hematopoietic stem cell transplantation treated with targeted oral busulfan and cyclophosphamide. The cumulative incidence of overall sinusoidal obstruction syndrome was 11% (95% confidence interval, 4–22%) at day 100 after transplantation.

diarrhea (30%) were frequent grade III or more adverse reactions. Severe neurological toxicity of grade III or more was observed in five patients (11%). One patient developed subarachnoid hemorrhage and died on day 1 after transplantation. Another patient developed tacrolimus encephalopathy on day 23 after transplantation. This patient died of acute bleeding from gastric ulcer on day 57. Another patient developed neurological toxicity during the course of septic shock and died on day 15. One patient who received dose reduction had delayed engraftment, but subsequently engrafted on day 31.

Among 46 patients undergoing planned transplantation, four patients experienced grade III or IV liver toxicity before day 28 (Table S1). Grade III or more long-term liver toxicity between days 29 and 100 was observed in nine patients (Table S2). Three patients were reported to have SOS before day 20, and two were reported to have late-onset SOS from days 21 to 100. Cumulative incidence of overall SOS was 11% (95% CI, 4–22%) at day 100 after transplantation (Fig. 1). Two patients had mild SOS on day 8 and 11 after transplantation, and both improved. One of these patients died of an unrelated cause (acute renal failure and infection). The third patient was reported to have moderate SOS on day 13. This patient died of an unrelated cause (septic shock) on day 15 after transplantation. Two patients developed severe SOS on days 42 and 44. These patients died of hepatic failure on day 64 and 81, respectively.

Graft versus host disease. The cumulative incidence of grade II–IV and III/IV aGVHD at day 100 were 35% and 11%, respectively. The cumulative incidence of grades II–IV aGVHD in the recipients who underwent transplant from an HLA-identical related donor or unrelated donor was 26% and 41%, respectively, and those of grades III/IV aGVHD was 11% and 11%, respectively. The cumulative incidence of cGVHD at 1 year after transplantation was 52%. Of the 21 patients who developed cGVHD, 12 had extensive disease and nine had limited disease.

Survival outcome. Twenty-six patients were alive with a median follow-up of 43 months (range, 11.9–65 months) after

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transplant. Overall survival was 65% (95% CI, 50–77%) at 1 year after transplantation, 66% (95% CI, 47–79%) for high risk and 64% (95% CI, 34–83%) for standard risk patients (Fig. 2a). Overall survival of AML was 71% (95% CI, 48–85%; $n = 24$) 1 year after transplantation, 50% (95% CI, 25–71%; $n = 16$) for MDS, and 83% (95% CI, 27–97%; $n = 6$) for CML patients. Two patients died before day 28 as described above. From days 28 to 100, seven patients died due to treatment-related mortality (four patients), infection (two patients), and relapse (one patient). Of the four patients who died of TRM, two died from hepatic toxicity, one from gastrointestinal bleeding, and one from thrombotic microangiopathy.

Disease-free survival was 57% (95% CI, 41–69%) 1 year after transplantation, 56% (95% CI, 38–71%) for high risk and 57% (95% CI, 28–78%) for standard risk patients (Fig. 2b). Disease-free survival of AML was 58% (95% CI, 36–75%) at 1 year, 44% (95% CI, 20–66%) for MDS, and 83% (95% CI, 27–97%) for CML patients.

Relapse and TRM. Thirteen patients (28%) experienced disease recurrence. Cumulative incidence of relapse was 24% at 1 year after transplantation. Cumulative incidence of relapse was 22% among patients with high risk disease, and 14% among patients with standard risk disease (Fig. 3a). Cumulative incidence of TRM was 18% at 1 year after transplantation (Fig. 3b).

Pharmacokinetic studies and dose modification. Among the 47 patients who completed the 16 BU doses, Css of the first dose was 1090 ± 318 ng/mL (range, 593–1673). The mean AUC_{inf} estimated after the first dose of BU was 6760 μg·h/L (range, 3656–13058 μg·h/L). The mean values of oral clearance, distribution volume, and elimination half-life were 0.159 L/h/kg (0.079–0.263 L/h/kg), 0.55 L/kg (0.178–0.989 L/kg), and 2.54 h (0.98–5.49 h), respectively. Six patients received dose escalation of BU, and 32 received dose reduction (Fig. 4a). Median decreasing dose of BU was 4.5 mg/kg (28% of 16 mg/kg). Mean actual dose of BU was 12.7 ± 3.7 mg/kg (range, 7.6–21.3 mg/kg).

One patient was excluded from the analysis due to systemic convulsions on day −6, as described above. The Css of the first dose was 683.1 ng/mL in this patient. Although dose escalation was carried out to receive 18.7 mg/kg, the conditioning regimen was not completed.

Busulfan targeting and transplant outcome. Overall survival was not different between patients who received dose reduction, no modification, or escalation of BU (68%, 67%, and 50% at 1 year, respectively). Significantly more grade III–IV toxicities from days 29 to 100 were observed in patients who received dose escalation (Fisher's exact test, $P = 0.023$) (Table S2). No difference in TRM was observed among these three groups.

All three patients who developed early-onset SOS within 20 days after transplant had received dose reduction of BU. Two developed grade II liver toxicity, and another developed grade IV liver toxicity before day 28 (Fig. 4b). Two patients who had late-onset SOS and died had received dose escalation (Fig. 4c).

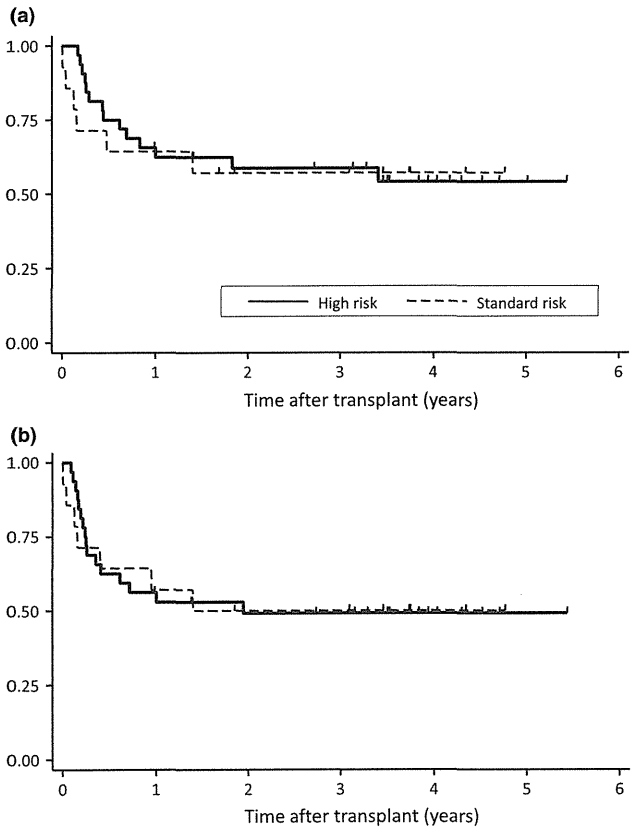

Fig. 2. *Overall survival and disease-free survival curves according to disease risk in patients receiving allogeneic hematopoietic stem cell transplantation treated with targeted oral busulfan and cyclophosphamide.* Overall survival (a) and disease-free survival (b), each stratified according to disease risk. Data were analyzed with the Kaplan–Meier method.

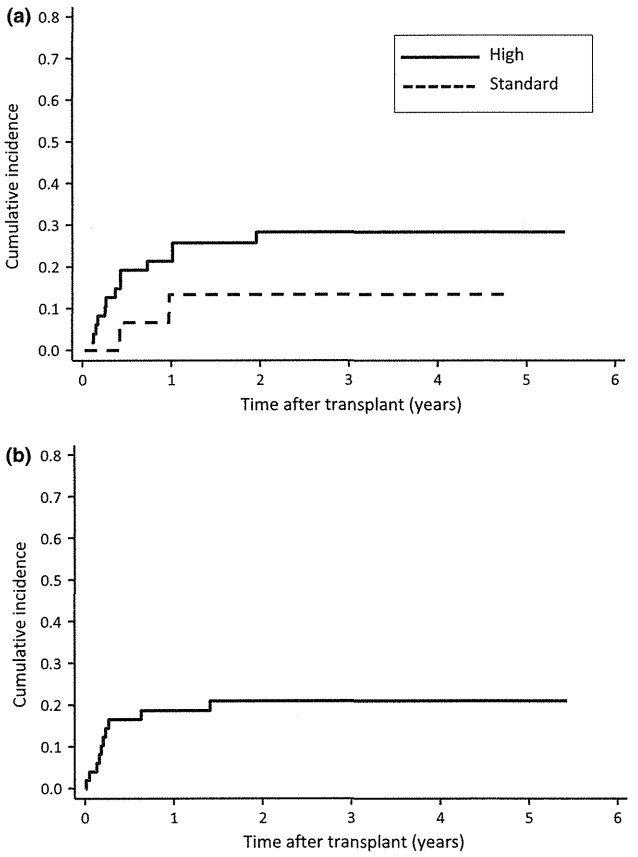

Fig. 3. *Cumulative incidence of relapse and transplant-related mortality in patients receiving allogeneic hematopoietic stem cell transplantation treated with targeted oral busulfan and cyclophosphamide.* Cumulative incidence of relapse with (a) high risk disease (22% at 1 year), standard risk disease (14%), and (b) cumulative incidence of treatment-related mortality (18%).

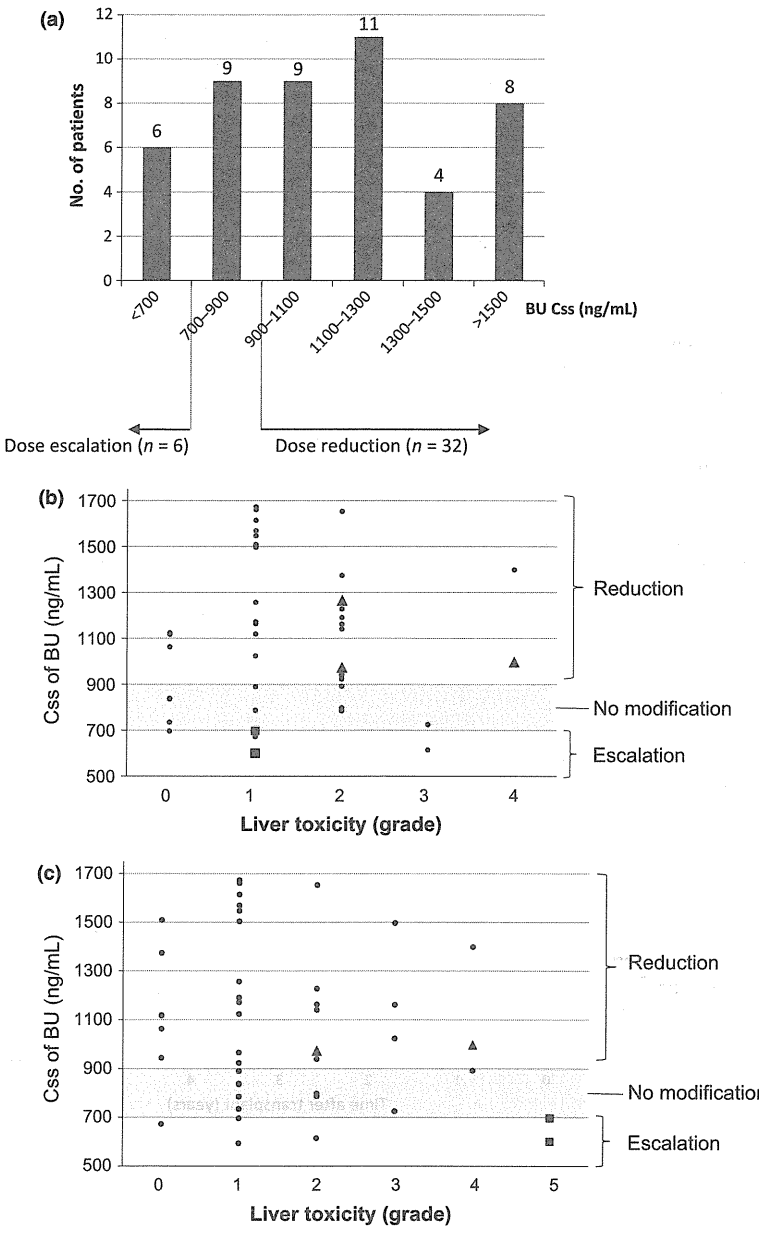

Fig. 4. Pharmacokinetic studies of busulfin (BU) in patients receiving allogeneic hematopoietic stem cell transplantation. (a) Number of the patients reaching concentration at steady state (Css) with the first BU dose. Six patients received dose escalation, nine patients received no modification, and 32 patients received dose reduction. (b) Liver toxicity in the first 28 days, sinusoidal obstruction syndrome (SOS), and BU dose modification. Triangles (▲) indicate patients diagnosed with SOS before day 20. Two of these patients developed grade II liver toxicity, and another developed grade IV liver toxicity. Two of these patients with early onset SOS died of an unrelated cause. Squares (■) indicate two patients who developed grade I liver toxicity in the first 28 days and were later diagnosed with severe late-onset SOS. (c) Liver toxicity from days 29 to 100, SOS and BU dose modification. Triangles (▲) and squares (■) indicate the same patients as (b). Two patients who had late-onset SOS and died had received dose escalation.

Discussion

We carried out a phase II study of individualizing the oral BU and CY conditioning regimen for adult allogeneic HSCT for myeloid malignancies. In the current study, 1-year OS (65%; 95% CI, 50–77%) clearly exceeded the threshold level of 40%.

Oral administration of BU had been associated with erratic gastrointestinal absorption and resulted in unpredictable systemic drug exposure.[3–5,17] Pharmacokinetic studies of BU and subsequent dose adjustment strategies for the BU and CY conditioning regimen have been reported, mainly among pediatric patients.[18–22] Although no essential difference in PK analysis has been reported between data from Japan and North America,[23] survival data and information on the benefit of the tBU+CY regimen for Asian adult populations are limited.[7] In this phase II study to target the BU Css range of 700–900 ng/mL, 32 patients received BU dose reduction and the median dose of total BU was reduced. Nevertheless, no increase in relapse was observed and the incidence of TRM was comparable to the BU+CY regimen using the i.v. form.[23] Notably, the incidence of SOS (11% at day 100) was relatively lower than in the previous report of an adult population receiving the CY+total body irradiation regimen[24] or oral non-targeted BU+CY.[6] Severe SOS was not observed within 20 days after transplantation, and this targeting strategy may contribute to reduce the severity of early-onset SOS. Our positive results could be a consequence of adjusting the BU dose, considering that 38 of 47 patients (81%) actually had not achieved optimal Css after the first dose. That is, the fixed dose of BU was not optimal in 81% of these Japanese patients.

In our previous study, SOS was not observed among patients whose Css range was within the target dose or when the BU dose

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was reduced (Akio Kohno, Mariko Fukumoto, Hiroto Narimatsu, Kazutaka Ozeki, Masashi Sawa, Shuichi Mizuta, Hitoshi Suzuki, Isamu Sugiura, Seitaro Terakura, Kazuko Kudo, and Yoshihisa Morishita, unpublished data, 2003). In the current study, three patients in the BU reduction group developed early-onset SOS, although the estimated cumulative Css remained within the targeted range. Liver toxicity in these patients might also be related to increased exposure to toxic metabolites of CY.[24] A dose-escalation study using test dose PK also showed that patients who showed a high level of AUC in the first dose developed severe toxicity, including hepatic SOS.[25]

Two of the six patients who received dose escalation experienced late-onset severe SOS. We may need to be cautious of possible late-onset severe SOS after dose escalation of BU. However, the causal relationship between dose escalation of BU based on low initial Css and SOS needs to be further evaluated, because individual oral BU PK are influenced by many factors. Glutathione S-transferase-mediated conjugation with GSH is the main mechanism to detoxify BU. Accumulation of the active metabolite of CY through depletion of the cellular GSH pool may contribute hepatic toxicity.[26] Hepatic GST activity and GST gene polymorphisms have been shown to be associated with BU clearance as well as transplant outcome. Polymorphism of GSTM1 is reported as a risk factor of SOS.[27] The heterozygous variant of GSTA1 (GSTA1*A/*B), which is observed in 26% of the Japanese population, resulted in slower elimination of BU than the wild-type.[28] Analysis using the Japan Marrow Donor Program showed a higher risk of TRM among recipients with the GSTM1-positive genotype, which was different from the Caucasian population.[29] We are currently investigating gene polymorphisms reported to be related with the risk factors of transplantation, such as GST genes and the UDP glucosyltransferase gene family[30] in a prospective trial.

Dose targeting possibly improves the OS by alleviating the variable absorptive characteristics among individuals. However, our results also suggest that dose modification might increase the chance of toxicity after day 28, especially in the case of dose escalation, although we should be careful of this interpretation. Dose reduction could generally lead to rejection of the graft. However, in this study, only one patient had 3 days' delay of engraftment in spite of the large number of patients in the study who received a dose reduction of BU. Busulfan in i.v. form has enabled us to accomplish narrow-ranged dose adjustment.[31] Careful validation of the clinical efficacy of PK-based targeting using i.v. BU is warranted.

In conclusion, individual dose adjustment based on BU PK was feasible and effective in the current phase II study.

Acknowledgments

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Disclosure Statement

The authors have no conflicts of interest.

Abbreviations

aGVHD	acute graft versus host disease
AUC	area under the blood concentration time curve
BU	busulfan
cGVHD	chronic graft versus host disease
CI	cumulative incidence
Css	concentration at steady state
CY	cyclophosphamide
DFS	disease-free survival
GSH	glutathione
GVHD	graft versus host disease
HLA	human leukocyte antigen
HSCT	hematopoietic stem cell transplantation
LSM	limited sample model
MDS	myelodysplastic syndrome
OS	overall survival
PK	pharmacokinetic
SOS	sinusoidal obstruction syndrome
tBU+CY	targeting BU+CY
TRM	transplant-related mortality

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Study scheme.

Table S1. Regimen-related toxicity before day 28 in patients receiving allogeneic hematopoietic stem cell transplantation treated with targeted oral busulfan and cyclophosphamide.

Table S2. Regimen-related toxicity (day 29–100) in patients receiving allogeneic hematopoietic stem cell transplantation treated with targeted oral busulfan and cyclophosphamide.

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