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transcript in ST1, SO4, and KK1 but increased it in KOB (B), which results were confirmed at protein level (C). (D, E) Effects of PS (LBH589) on *EZH2* transcript (D) or EZH2 protein expression (E) were examined. PS (LBH589) was added at final concentrations of 50 nM and 100 nM for (D) and 20 nM and 100 nM for (E). One hundred nM of PS (LBH589) decreased the expression of EZH2 at both transcript (D) and protein levels (E) after 24 hours of culture. (F) Effects of combined treatment with DZNep and PS (LBH589) on LM-Y1 and KOB cells were analyzed. Cells were treated with DZNep (0.3-5.0 μM) and PS (LBH589) (3-50 nM) for 48 hours. After evaluation of cell proliferation status by a MTS assay (upper panel), the combination index (CI) for each drug combination was obtained using commercially available software Calcusyn (lower panel). CI < 1 indicates synergism.

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Figure 1

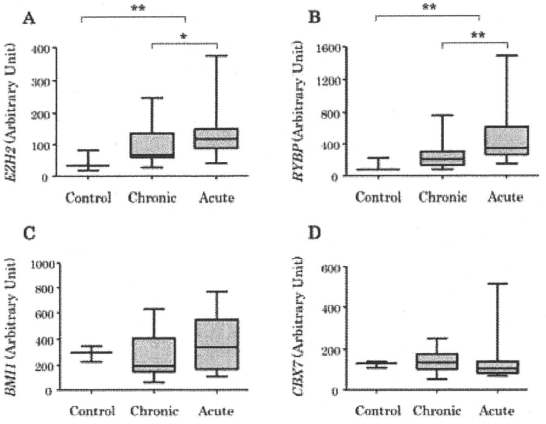

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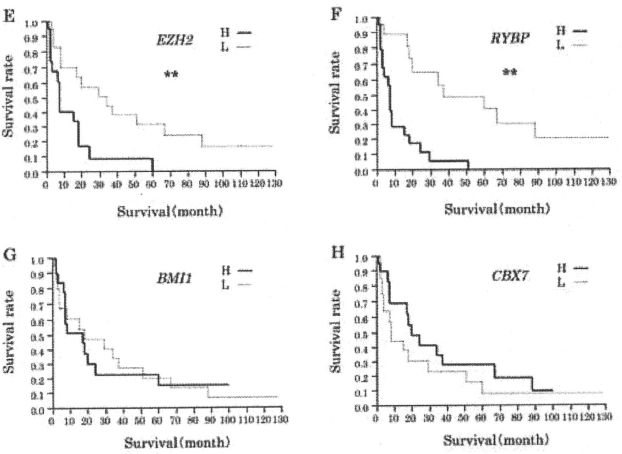
Figure 1



DOI: 10.3324/haematol.2010.028605

Figure 2

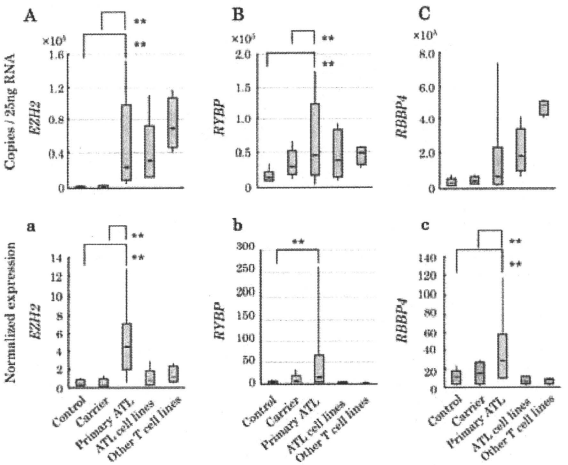


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Figure 2

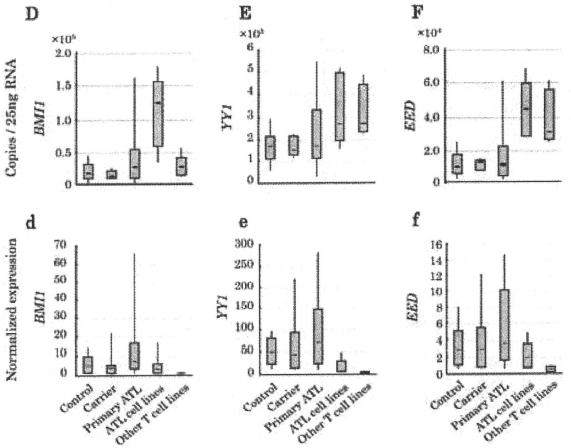

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Figure 3

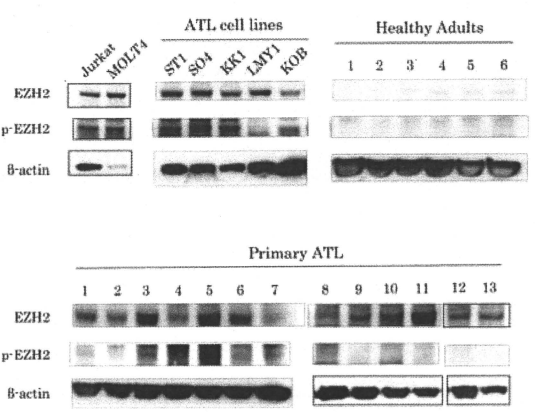

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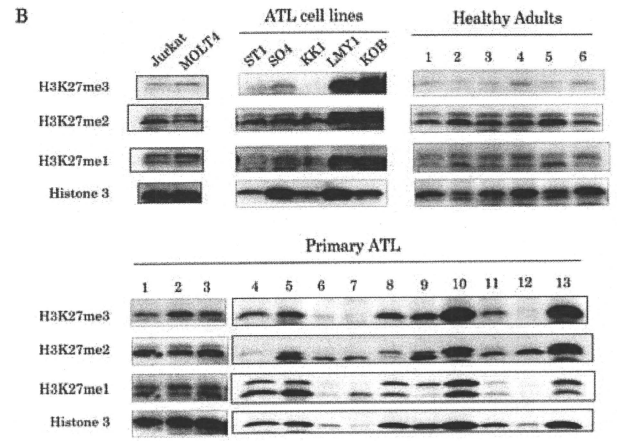
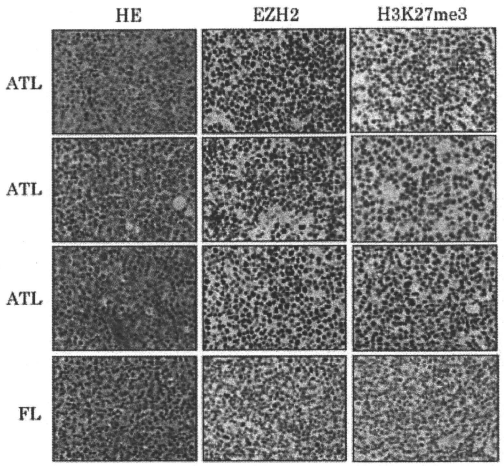
Figure 3



DOI: 10.3324/haematol.2010.028605

Figure 4



DOI: 10.3324/haematol.2010.028605

Figure 5

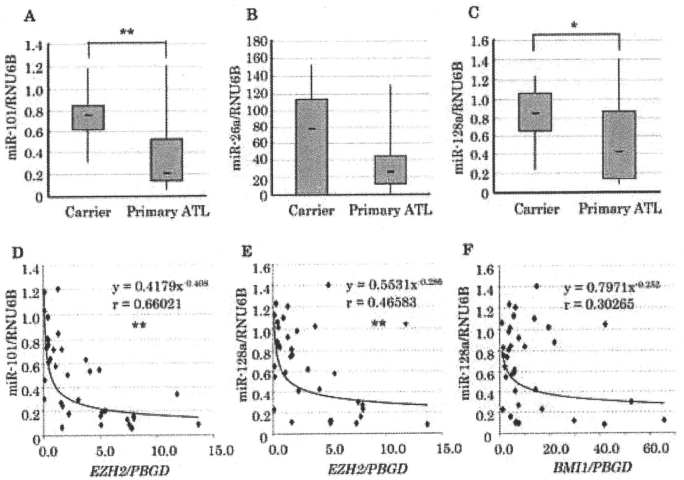


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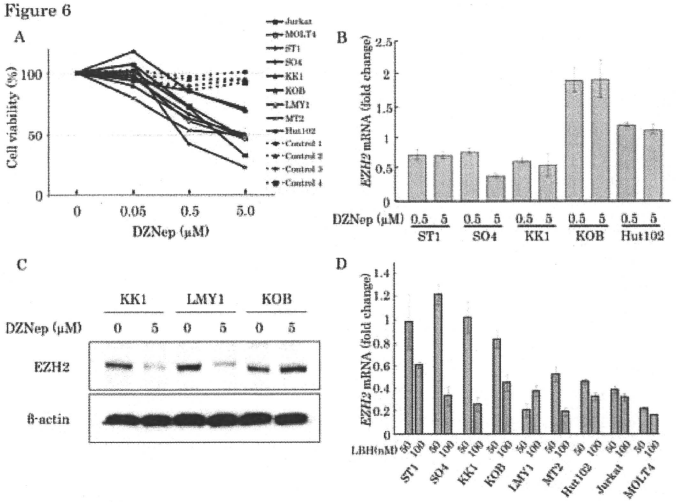
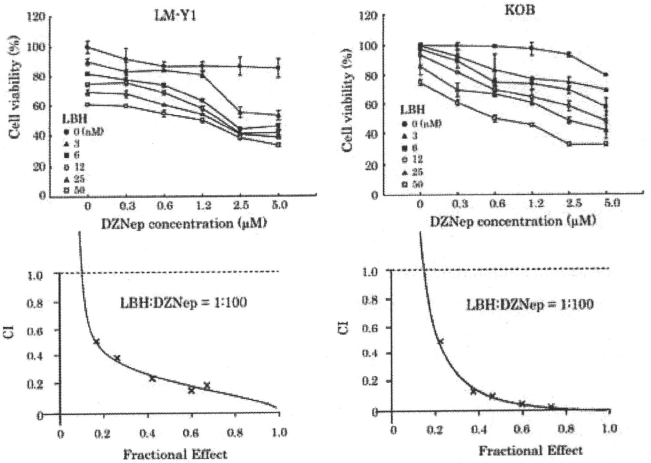
Figure 6



DOI: 10.3324/haematol.2010.028605

Figure 6 F



DOI: 10.3324/haematol.2010.028605

Supplementary Appendix

Figure legends for Supplementary Figures

Supplementary Figure 1. RYBP protein expression. Western blot analysis for RYBP protein was performed on primary ATL cells and cells from healthy adults. Most primary ATL samples showed a clear band for RYBP. In contrast, cells from healthy adults lacked the band.

Supplementary Figure 2. Quantitative genomic PCR for miR-101. PCR was performed in two loci, miR-101-1 (chromosome 1p31) and miR-101-2 (chromosome 9p24), in 10 primary ATL samples and cells from 10 HTLV-1 carriers as a control. Both loci were preserved in ATL cells, refuting the possibility that downregulation of miR-101 is caused by genomic loss of the gene.

Supplementary Figure 3. Analysis of 3'-UTR sequence of EZH2 to predict potential target sites for miRNA. In addition to the target sites for miR-101 and miR-26a, there is also a potential target site for miR-128a in the 3'-UTR of EZH2 near one of the miR-101 target sites.

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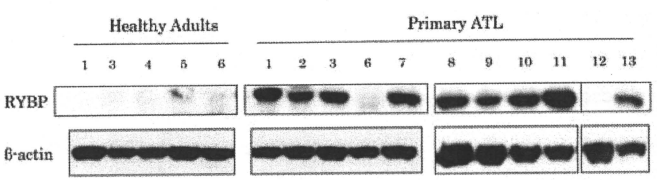
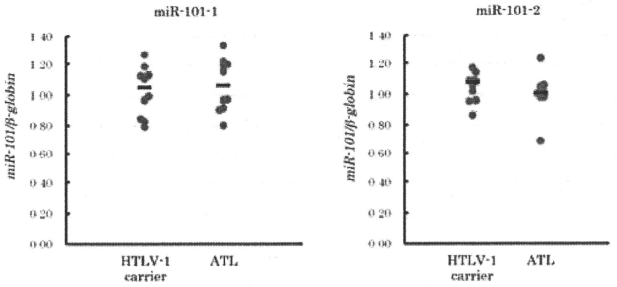
Supplementary Figure 4. Sequence analysis of EZH2. Pyrosequence analysis of EZH2 Try641 was performed in 10 ATL patients and 10 HTLV-1 carriers. Pyrograms of 6 ATL patients are shown. There were no mutations in the examined samples.

Supplementary Figure 1.



DOI: 10.3324/haematol.2010.028605

Supplementary Figure 2.



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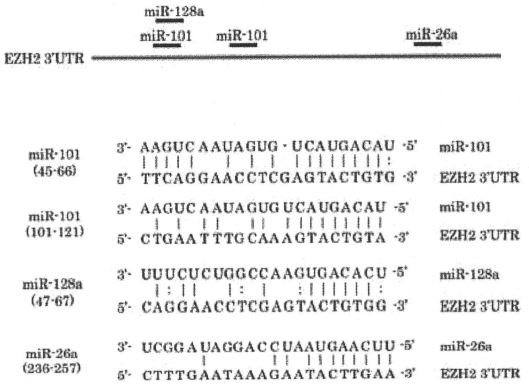
Supplementary Figure 3

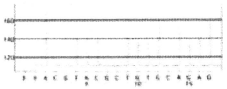


DOI: 10.3324/haematol.2010.028605

Supplementary Figure 4

Wild-type sequence: AATACTGTGGAGAGG

ATL patient 1: wild type

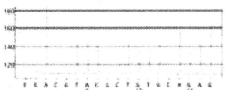


Sequence : AATAC TGTGGAGAGG

ATL patient 2: wild type

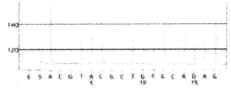
Sequence : AATAC TGTGGAGAGG

ATL patient 3: wild type



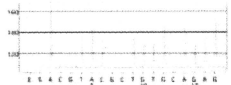
Sequence : AATAC TGTGGAGAGG

ATL patient 4: wild type



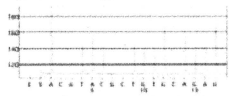
Sequence : AATAC TGTGGAGAGG

ATL patient 5: wild type



Sequence : AATAC TGTGGAGAGG

ATL patient 6: wild type



Sequence : AATAC TGTGGAGAGG

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Differences in the distribution of subtypes according to the WHO classification 2008 between Japanese and German patients with refractory anemia according to the FAB classification in myelodysplastic syndromes

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ABSTRACT

We reported the different clinical features between Japanese and German refractory anemia (RA) patients in FAB classification. We re-analyzed the clinical features by WHO classification revised in 2008. The frequencies of refractory cytopenia with unilineage dysplasia (RCUD) and myelodysplastic syndrome-unclassified (MDS-U) with pancytopenia in Japanese patients were higher than in German patients ($p < 0.001$). Refractory cytopenia with multilineage dysplasia patients showed the most unfavorable prognosis in both countries. The higher frequencies of MDS-U with pancytopenia and RCUD in Japanese patients may influence the different clinical characteristics between Japanese and German FAB-RA patients.

1. Introduction

Myelodysplastic syndromes (MDS) are acquired clonal stem cell disorders characterized by ineffective hematopoiesis with myelodysplasia [1] and are associated with a high risk of progression to acute leukemias [2]. MDS are very heterogeneous in terms of their morphology, clinical features, and survival [3]. There are several reports indicating possible differences in clinical features between Western MDS types and Eastern MDS types [4–9]. The median age of MDS patients in Korea and Thailand were reported to be 57 [8] and 56 [7], respectively. On the other hand, large MDS studies from Western countries showed a median or mean age of 68–73 years [10–13]. We have reported that the clinical features of refractory anemia with excess of blasts (RAEB) or RAEB in transformation (RAEB-t) according to the French–American–British (FAB) classification [14] seemed to be similar between Japanese and Western patients [15]. However, previous reports [5,15] indicated that Japanese MDS patients have a lower frequency of refractory anemia with ringed sideroblasts (RARS) according to the FAB classification and a higher frequency of refractory anemia according to the FAB classification (FAB-RA) than the Western International Prognostic Scoring System (IPSS) study [10], and we reported that the clinical and laboratory features of Japanese FAB-RA patients apparently differ from those of German patients after a precise morphologic consensus (FAB classification: concordance rate, 98.4%; κ, 0.94; $p < 0.001$; prior World Health Organization (WHO) classification (WHO classification 2001) [16]: concordance rate, 83.8%; κ, 0.73; $p < 0.001$) [17]. That was the first comparison report between Western and Eastern FAB-RA patients after confirming morphological consensus. Japanese FAB-RA patients were younger, showed more severe cytopenia(s), a lower frequency of abnormal karyotypes, a lower frequency of MDS with isolated del(5q) (5q- syndrome), and a more favorable prognosis in terms of the overall survival (OS) and leukemia free survival (LFS) in our previous study.

MDS subtypes in the WHO classification 2001 [16] was revised in 2008 (WHO classification 2008) [18]. Refractory anemia (RA), refractory neutropenia (RN), and refractory thrombocytopenia (RT) were combined into refractory cytopenia with unilineage dysplasia

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(RCUD) in the WHO classification 2008. The diagnosis of MDS-unclassified (MDS-U) according to the WHO classification 2008 can be made in the following instances:

1. Patients with the findings of RCUD or refractory cytopenia with multilineage dysplasia (RCMD) but with 1% blasts in the peripheral blood (PB) (PB blasts type).
2. Cases of RCUD which are associated with pancytopenia (RCUD/pancytopenia type).
3. Patients with cytopenia(s) with 1% or fewer blasts in the PB and fewer than 5% in the bone marrow (BM), unequivocal dysplasia in <10% of the cells in one or more myeloid lineages, and who have cytogenetic abnormalities (cytogenetic abnormalities type).

MDS-U (PB blasts type) is classified as RAEB according to the FAB classification because of 1% blasts in the PB. MDS-U (cytogenetic abnormalities type) is not diagnosed as MDS according to the FAB classification because of unequivocal dysplasia. Thus, FAB-RA patients are classified as RCUD, RCMD, MDS with isolated del(5q) (5q- syndrome) or MDS-U (RCUD/pancytopenia type) according to the WHO classification 2008. In the present study, we re-analyzed in detail the clinical features of Japanese and German FAB-RA patients by using revised MDS subtypes in the WHO classification 2008.

2. Patients and methods

The dataset of consecutive patients with primary FAB-RA of our previous study [17] (total 728 consecutive patients: Japan, 131 cases; Germany, 597 cases) were used for the present retrospective analysis. Japanese patients of this dataset were diagnosed at the Saitama Medical University Hospital, Nagasaki University Hospital or affiliated hospitals between April 1976 and January 1997. German patients were diagnosed at the Department of Hematology, Oncology and Clinical Immunology of the Heinrich-Heine University between January 1973 and December 2002. Patients who had previously been treated with anti-neoplastic drugs or ionizing radiation were excluded from the study. Patients without the available necessary data for the WHO classification 2008 were excluded from the present study. Cytogenetic analyses were performed with a trypsin-Giemsa banding technique on BM cells from aspirates. Ordinarily 20–30 metaphases were examined. Cytogenetic aberrations were grouped according to the IPSS publication [10]. Thresholds for cytopenia(s) were defined as those of the IPSS (hemoglobin (Hb) <10.0 g/dL, absolute neutrophil count (ANC) $<1.8 \times 10^9$/L, and platelet $<100 \times 10^9$/L). Criteria for dysplasia were defined as those of a previous German report [19]. Hypoplastic BM was defined as <30% cellular in patients <60 years old, or <20% cellular in patients ≥60 years old [20]. If hypoplastic BM and certain dysplasia more than 10% in one or more of major myeloid cell lines were present, a diagnosis of hypoplastic MDS was made. Patients were reclassified according to the definition of WHO classification 2008 for MDS subtyping by using PB and BM findings, morphologic findings, and cytogenetic findings of the previous dataset [17]. Comparisons of the clinical features at the time of diagnosis and OS and LFS were analyzed by using the dataset of our previous study [17]. OS was measured from the date of diagnosis until death due to any cause, the date of stem cell transplantation, or until the last patient contact. LFS was measured from the date of diagnosis until the date of diagnosis of acute leukemia. This study was approved by the Institutional Review Board of Saitama International Medical Center, Saitama Medical University, Saitama, Japan.

2.1. Statistical methods

The chi-square test and the nonparametric Mann–Whitney test were used to compare the proportions of patients and continuous data, respectively. The Kaplan–Meier method was used to generate the estimate of cumulative probabilities of OS and LFS. The difference in the cumulative probabilities within subcategories of patients was compared using a two-sided log-rank test. A two-sided p value of <0.05 was considered to be statistically significant. All statistical analyses were performed with the use of StatView (version 5.0, SAS Institute, Cary, NC).

3. Results

3.1. Comparison of frequencies of subtypes according to the WHO classification 2008 between Japanese and German FAB-RA patients

A total of 295 patients (Japan, 102 cases; Germany, 193 cases) could be classified according to the WHO classification 2008. A total of 433 patients (Japan, 29 cases; Germany, 404 cases) could not be classified according to the WHO classification 2008 due to a deficit of either cytogenetic data or adequate peripheral blood data, and 427 patients presented without available cytogenetic findings (Japan, 29 cases; Germany, 398 cases). There were 6 patients (Germany, 6 cases) without any data of peripheral blood.

MDS-U (PB blasts type) is classified as RAEB according to the FAB classification. MDS-U (cytogenetic abnormalities type) is not diagnosed as MDS according to the FAB classification due to unequivocal dysplasia. Therefore, patients with MDS-U (PB blasts type) or with MDS-U (cytogenetic abnormalities type) were not included in the previous dataset. Because the previous dataset used in the present study was that of FAB-RA patients, dysplasia existed in at least one lineage and the frequency of blasts in PB was <1% in all patients. Therefore, all MDS-U patients in the present study were diagnosed as RCUD/pancytopenia type. Most Japanese FAB-RA patients were classified as RCUD, RCMD, or MDS-U (RCUD/pancytopenia type) according to the WHO classification 2008 (Table 1A). Most German FAB-RA patients were classified as RCUD, RCMD, or 5q- syndrome (Table 1B). The frequency of RCUD in Japanese FAB-RA patients (45%) was significantly higher than that in German FAB-RA patients (19%) ($p < 0.001$). The frequency of patients with bicytopenia in Japanese RCUD patients was 59%, but that in the German RCUD patients was only 19%. Among 46 Japanese RCUD patients, number of patients with single cytopenia was 17 cases (37%) including 2 RA, 4 RN and 11 RT cases. Among 37 German RCUD patients, number of patients with single cytopenia was 22 cases (59%) including 7 RA, 11 RN and 4 RT cases. Frequency of RT was 2% of German FAB-RA patients. The frequency of RT of Japanese FAB-RA patients (11%) was higher than that of German FAB-RA patients. The frequency of MDS-U in Japanese FAB-RA patients (29%) was significantly higher than that in German FAB-RA patients (3%) ($p < 0.001$). The frequency of RCMD in Japanese FAB-RA patients (25%) was significantly lower than in German FAB-RA patients (58%) ($p < 0.001$). The frequency of 5q- syndrome in Japanese FAB-RA patients (3%) was significantly lower than in German FAB-RA patients (20%) ($p < 0.001$) (Table 1C).

3.2. Comparison of clinical and laboratory features at the time of diagnosis between Japanese and German patients could be classified according to the WHO classification 2008

The age of patients in RCUD, MDS-U and RCMD subtypes did not differ between the two countries. The MDS-U (RCUD/pancytopenia type) subtype was younger than other subgroups in Japanese patients. The gender ratios in the RCUD

Table 1
Laboratory features at the time of diagnosis in FAB-RA patients who could be classified according to the WHO classification 2008.

	RCUD	MDS-U	RCMD	5q- synd
(A) Japanese patients, $n = 102$				
Patients = n (%)	46(45)	28(29)	25(25)	3(3)
Gender (male/female)	28/18	12/16	11/14	2/1
Age (years)	57(16–86)	51(15–82)	63(16–88)	60(59–74)
Neutrophils ($\times 10^9$/L)	1.89 (0.44–4.69)	1.10 (0.26–1.77)	1.28 (0.05–10.24)	0.73 (0.50–2.54)
Hemoglobin (g/dL)	10.2 (3.0–14.3)	6.9 (4.2–9.1)	8.2 (2.9–14.0)	6.3 (4.6–10.8)
Platelets ($\times 10^9$/L)	41 (4–246)	29(7–98)	50(13–390)	207(134–212)
Abnormal karyotype = n (%)	12(26)	6(21)	9(36)	3(100)
Hypoplastic bone marrow = n (%)	3(7)	3(11)	0(0)	0(0)
(B) German patients, $n = 193$				
Patients = n (%)	37(19)	6(3)	111(58)	39(20)
Gender (male/female)	20/17	1/5	80/31	14/25
Age (years)	62(20–80)	56(19–59)	63(15–86)	62(32–78)
Neutrophils ($\times 10^9$/L)	1.92 (0.36–8.72)	1.41 (0.48–1.50)	1.60 (0.21–19.40)	1.95 (0.61–6.78)
Hemoglobin (g/dL)	11.0 (5.2–15.4)	9.4 (5.5–9.8)	9.2 (5.1–16.9)	8.7 (3.0–12.2)
Platelets ($\times 10^9$/L)	128(2–840)	33(10–90)	102(9–999)	250(28–1540)
Abnormal karyotype = n (%)	12(32)	3(50)	47(42)	39(100)
Hypoplastic bone marrow = n (%)	3(8)	2(33)	13(12)	5(13)
	Japan vs Germany			
(C) Comparison between Japanese and German patients				
(1) RCUD patients				
Frequency	$p < 0.001$			
Gender (male/female)	$p = 0.532$			
Age (years)	$p = 0.150$			
Neutrophils ($\times 10^9$/L)	$p = 0.466$			
Hemoglobin (g/dL)	$p = 0.087$			
Platelets ($\times 10^9$/L)	$p < 0.001$			
Abnormal karyotype (%)	$p = 0.526$			
Hypoplastic bone marrow (%)	$p = 0.782$			
(2) MDS-U patients				
Frequency	$p < 0.001$			
Gender (male/female)	$p = 0.239$			
Age (years)	$p = 0.557$			
Neutrophils ($\times 10^9$/L)	$p = 0.821$			
Hemoglobin (g/dL)	$p = 0.036$			
Platelets ($\times 10^9$/L)	$p = 0.752$			
Abnormal karyotype (%)	$p = 0.150$			
Hypoplastic bone marrow (%)	$p = 0.156$			
(3) RCMD patients				
Frequency	$p < 0.001$			
Gender (male/female)	$p = 0.007$			
Age (years)	$p = 0.401$			
Neutrophils ($\times 10^9$/L)	$p = 0.494$			
Hemoglobin (g/dL)	$p = 0.016$			
Platelets ($\times 10^9$/L)	$p = 0.030$			
Abnormal karyotype (%)	$p = 0.561$			
Hypoplastic bone marrow (%)	$p = 0.072$			
(4) 5q- synd patients				
Frequency	$p < 0.001$			
Gender (male/female)	$p = 0.290$			
Age (years)	$p = 0.920$			
Neutrophils ($\times 10^9$/L)	$p = 0.144$			
Hemoglobin (g/dL)	$p = 0.370$			
Platelets ($\times 10^9$/L)	$p = 0.188$			
Abnormal karyotype (%)	N/A			
Hypoplastic bone marrow (%)	$p = 0.509$			

Values for presentation characteristics are given as median and range where applicable. N/A, not applicable; RCUD, refractory cytopenia with unilineage dysplasia; MDS-U, MDS-unclassified; RCMD, refractory cytopenia with multilineage dysplasia; 5q- synd, MDS with isolated del(5q).

and MDS-U subtypes were not significantly different between the two countries. The frequency of male patients in Japanese RCMD subgroup was significantly lower than that in German RCMD subtype. Japanese patients had significantly lower platelet counts than German patients in both the RCUD and RCMD subtypes. Japanese MDS-U (RCUD/pancytopenia type) and RCMD patients showed significantly lower Hb concentrations than German MDS-U (RCUD/pancytopenia type) and RCMD patients. Japanese RCUD patients showed a tendency towards lower Hb concentrations than German RCUD patients. The ANC did not differ significantly between the two countries in RCUD, MDS-U (RCUD/pancytopenia type), and RCMD patients (Table 1). The frequency of cytogenetic abnormalities in the Japanese FAB-RA patients was significantly lower than in German patients ($p < 0.001$) (Tables 1 and 2). The frequencies of cytogenetic abnormalities in the RCUD, MDS-U (RCUD/pancytopenia type), and RCMD subtypes were not significantly different between the two countries (RCUD, $p = 0.526$; RCMD, $p = 0.561$; MDS-U (RCUD/pancytopenia type), $p = 0.150$). The frequency of isolated del(5q) in Japanese FAB-RA patients was significantly lower than in German patients

Table 2
Cytogenetic findings at the time of diagnosis in FAB-RA patients who could be classified according to the WHO classification 2008.

	RCUD	MDS-U	RCMD	5q- synd	Total
(A) Japanese patients, $n = 102$					
Patients = n	46	28	25	3	102
Good	37(80.4%)	23(82.1%)	16(64.0%)	3(100%)	79(77.5%)
Normal	34(73.9%)	22(78.6%)	16(64.0%)	0(0%)	70(68.6%)
−Y	0	1	0	0	1
del(5q)	0	0	0	3	3
del(20q)	3	0	0	0	3
Intermediate	8(17.4%)	3(10.7%)	4(16.0%)	0	15(14.7%)
Poor	1(0.2%)	2(7.2%)	5(20.0%)	0	8(7.8%)
Complex (≥3 abnormalities)	0	1	4	0	5
Chromosome 7 anomalies	1	1	1	0	3
(B) German patients, $n = 193$					
Patients = n	37	6	111	39	193
Good	27(73.0%)	3(50.0%)	72(64.9%)	39(100%)	141(73.1%)
Normal	25(67.6%)	3(50.0%)	64(57.7%)	0(0%)	92(47.7%)
−Y	2	0	2	0	4
del(5q)	0	0	0	39	39
del(20q)	0	0	6	0	6
Intermediate	4(10.8%)	2(33.3%)	23(20.7%)	0	29(15.0%)
Poor	6(16.2%)	1(16.7%)	16(14.4%)	0	23(11.9%)
Complex (≥3 abnormalities)	5	0	9	0	14
Chromosome 7 anomalies	1	1	7	0	9

Good indicates normal, −Y, del(5q), del(20q); poor, complex (≥3 abnormalities) or chromosome 7 anomalies; intermediate, other abnormalities not listed in good and poor subgroups.

($p < 0.001$) (Table 2). The most frequent cytogenetic aberration in the intermediate cytogenetic risk according to the IPSS publication was trisomy 8 (4 German RCMD cases, 3 Japanese RCUD cases, 1 Japanese MDS-U case). The frequencies of hypoplastic BM were not significantly different between the two countries in the RCUD and MDS-U (RCUD/pancytopenia type) subtypes. In the RCMD subtype, there were no Japanese patients presenting with findings concordant with hypoplastic BM. However, the frequency of German RCMD patients with hypoplastic BM was 12% (Table 1).

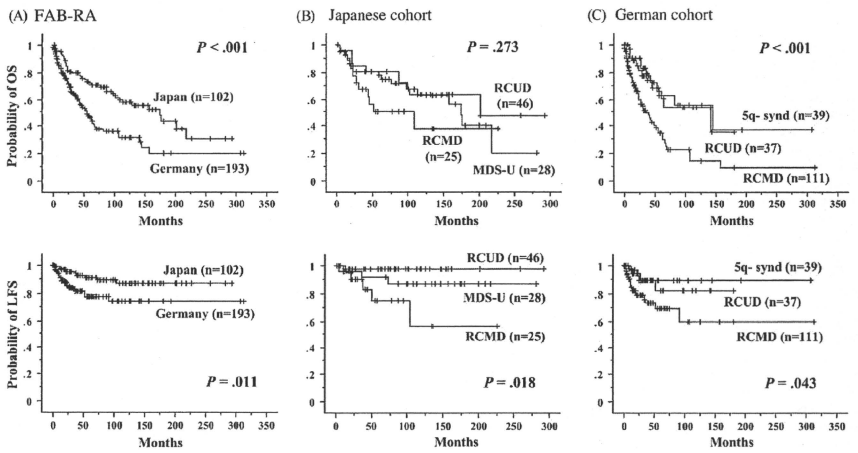

Fig. 1. Cumulative overall survival and leukemia free survival of FAB-RA patients who could be classified according to the WHO classification 2008. (Top) Overall survival (OS). (Bottom) Leukemia free survival (LFS). (A) In FAB-RA patients who could be classified according to the WHO classification 2008, Japanese patients had a more favorable OS than German patients ($p < 0.001$). Japanese patients had a more favorable LFS than German patients ($p = 0.011$). (B) In Japanese FAB-RA patients who could be classified according to the WHO classification 2008, RCMD patients showed the least favorable OS and LFS compared with the other subtypes excluding a rare subtype (5q- syndrome subtype). RCUD patients showed more favorable OS and LFS than RCMD patients (OS, $p = 0.128$; LFS, $p = 0.004$). MDS-U (RCUD/pancytopenia type) patients tended to show more favorable OS and LFS than RCMD patients (OS, $p = 0.218$; LFS, $p = 0.137$). (C) In German FAB-RA patients who could be classified according to the WHO classification 2008, RCMD patients showed the least favorable OS and LFS compared with the other subtypes excluding a rare subtype (MDS-U (RCUD/pancytopenia type) subtype). RCUD patients showed more favorable OS and LFS than RCMD patients (OS, $p = 0.003$; LFS, $p = 0.075$). 5q- syndrome patients showed more favorable OS and LFS than RCMD patients (OS, $p = 0.002$; LFS, $p = 0.043$).