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ORIGINAL ARTICLE

Myelodysplastic syndrome with chromosome 5 abnormalities: a nationwide survey in Japan

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Chromosome 5 abnormalities, deletion of the long arm of chromosome 5 (del(5q)) or monosomy 5 (−5), arise in about 10% of myelodysplastic syndromes (MDS), either as the sole cytogenetic abnormality or as part of complicated karyotype, and has distinct clinical implications for MDS. However, the prognostic factors of MDS patients with chromosome 5 abnormalities are not determined yet. In this study, 183 Japanese MDS patients with chromosome 5 abnormalities were analyzed. Estimated incidence of del(5q) and 5q- syndrome among MDS patients was 8.4 and 1.3%, respectively. Significant shorter overall survival (OS) and leukemia-free survival (LFS) were observed in −5 patients than del(5q) patients. Among del(5q) patients, addition of monosomy 7 or complex karyotype with more than three abnormalities were significantly related to shorter OS.

LFS of del(5q) patients was divided into two risk groups by international prognostic scoring system (IPSS): low/intermediate (Int)-1 and Int-2/high groups. LFS sorted by World Health Organization classification-based prognostic scoring system (WPSS) was also divided into two groups: very low/low/Int and high/very high, and WPSS was able to predict the outcome of del(5q) patients more clearly than IPSS.

Together with additional cytogenetic data, WPSS might be useful for clinical decision making in MDS patients with del(5q).
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Introduction

Loss of part of the long arm of chromosome 5 (del(5q)) is a frequent clonal chromosomal abnormality in patients with myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML),[1] and is thought to contribute to the pathogenesis of these diseases by deleting one or more tumor-suppressor genes.[2] MDS with del(5q) is a heterogeneous disease,[3,4] apart from 5q- syndrome,[5,6] and often accompanies additional cytogenetic abnormalities leading to the poor-risk karyotypes, or an increase of bone marrow and/or peripheral blasts irrespective of chromosomal complexity. These distinct disease subgroups have dramatically different prognostic features.[3,5,7,8]

The international prognostic scoring system (IPSS), defined by bone marrow blast percentage, number of peripheral cytopenias and cytogenetic pattern, has become a benchmark for clinical decision making.[9] Recently, on the other hand, the World Health Organization (WHO) classification-based prognostic scoring system (WPSS) has been proposed based on WHO classification,[10] cytogenetic pattern and transfusion dependency as independent indicators of disease severity.[11]

To elucidate the prognostic features of Japanese del(5q) MDS patients, we adapted IPSS, WHO criteria and WPSS to 131 MDS patients with del(5q), 52 patients with monosomy 5 (−5) and 375 MDS patients who did not carry chromosome 5 abnormality, to estimate the mortality rates and life expectancy of these groups as the base for adapting treatments.

Materials and methods

Patient data

A total of 50 MDS patients with chromosome 5 abnormalities were collected in the first series of the survey within 425 MDS patients recorded by the Japanese Cooperative Study Group for Intractable Bone Marrow Diseases. Of 375 patients who did not carry chromosome 5 abnormality in this series were used as controls. In addition to these 50 patients, we conducted a retrospective survey on MDS patients with chromosome 5 abnormalities across 285 hospitals in Japan. A total of 133 cases were newly collected.

A total of 558 MDS patients were collected and 183 patients with chromosome 5 abnormalities and 375 patients with a morphologically normal chromosome 5 were analyzed for (1) additional chromosomal abnormalities, (2) French American and British (FAB) and WHO criteria, (3) IPSS and WPSS,[9–12] (4) clinical outcome and (5) degree of cytopenia. MDS patients with chromosome 5 abnormalities were classified according to FAB and WHO criteria in Table 1.

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Table 1 Classification of MDS with chromosome 5 abnormalities

Case number	*Total*		*del(5q)*[a]		*Monosomy 5*		*Isolated del(5q)*[b]	
Classification	*183*		*131*		*52*		*35*	
	FAB	*WHO*	*FAB*	*WHO*	*FAB*	*WHO*	*FAB*	*WHO*
RA	61	25	51	18	10	7	24	
RCMD		14		11		3		2
RARS	5	3	4	2	1	1	1	
RCMD-RS		1		1				
RAEB-1	81	46	56	30	25	16	8	4
RAEB-2		45		31		14		5
RAEB-t	20		10		10			
CMML	2	2	2	2				
AML	14	24	8	14	6	10	2	2
5q- syndrome		21		21				21
Others		2		1[c]		1[d]		1[c]

Abbreviations: AML, acute myeloid leukemia; CMML, chronic myelomonocytic leukemia; FAB, French American and British; RA, refractory anemia; RAEB-t, refractory anemia with excess blast in transformation; RARS, RA with ringed sideroblast; RCMD-RS, refractory cytopenia with multilineage dysplasia with ringed sideroblast; WHO, World Health Organization.
[a]Includes all del(5q) cases with or without other chromosomal abnormalities.
[b]Includes the cases having only del(5q) and some cases besides 5q- syndrome.
[c]MDS/MPD, unclassifiable case.
[d]MDS, unclassifiable case.

Categorization of del(5q) MDS by additional chromosomal abnormalities

Fifty-two patients with −5 were initially separated, and then del(5q) patients were assigned to one of four cytogenetic categories according to their karyotypes: del(5q) alone as 5q- that included 5q- syndrome, del(5q) with additional chromosome 7 abnormality as (7+), del(5q) with more than three abnormalities as 'complex' and del(5q) patients with cytogenetic aberrations other than 7+ and complex defined as 'other'. Of 131 del(5q) patients, 35 patients were categorized as 5q-, 47 were 7+, 35 were complex and 14 were other.

Statistical analysis

First, statistical test of homogeneity between the two patient data collections was carried out. Actuarial probability of overall survival (OS) and leukemia-free survival (LFS) were estimated using the Kaplan–Meier product limit method. OS was defined as the time between diagnosis and death of any cause or end of follow-up. LFS was calculated from diagnosis to leukemic progression or end of follow-up. Patients who died before leukemic progression were considered as censored at the time of death and those who received stem cell transplantation were censored at the time of transplantation. Comparisons between Kaplan–Meier curves were carried out by Gehan's Wilcoxon's test. To assess the relation of cytogenetic abnormalities with hematological values, Mann–Whitney's *U*-test was carried out using the hemoglobin concentration, neutrophil and platelet count.

Univariate analyses were performed by χ^2-test and multivariate analyses were performed by Cox proportional hazard regression model. The data were considered statistically significant if *P*-values were less than 0.05. These analyses were carried out using SPSS for Windows version14.0. The whole study was in accordance with the modified Declaration of Helsinki.

Results

Patient characteristics

As the data of patients with chromosome 5 abnormalities were collected in two cohorts, we performed the statistical test of homogeneity between the two data collections on such factors as patient ages, gender, WHO classification, hemoglobin concentration, neutrophil and platelet counts, degree of red cell transfusion dependency and confirmed that the two groups were not statistically different each other.

Total patient characteristics are listed in Table 1. The median age of patients with chromosome 5 abnormalities was 69 years and the male-to-female ratio was 113/70, the median age of patients with del(5q) was 69 years and the male/female ratio was 80/51, consistent with the well-known male predominance of MDS. Of the 183 patients, 131 (71.5%) were del(5q) patients with or without other chromosomal abnormalities, 21 (11%) were 5q- syndrome and 52 (28.4%) were −5. Here we defined the cases with macrocytic red cells, isolated del(5q), bone marrow blasts less than 5% as 5q- syndrome. Two refractory cytopenia with multilineage dysplasia (RCMD) cases with isolated del(5q) were excluded because of microcytic anemia without iron deficiency or detection of t(5;17) by further analysis.

Of 425 Japanese MDS patients recorded in the Japanese Cooperative Study Group for Intractable Bone Marrow Diseases, 50 (11.8%) had chromosome 5 abnormalities; therefore, the estimated rate of del(5q) patients and 5q- syndrome among MDS patients was 8.4 and 1.3%, respectively. The incidence of 5q- syndrome proved to be quite rare in Japan.

Impact of chromosome 5 abnormalities on cytopenia

As compared with MDS patients who did not have chromosome 5 abnormality, patients with chromosome 5 abnormalities had significantly severe anemia, whereas no significant difference in the degree of anemia was observed between patients with del(5q) without 5q- syndrome, −5 and 5q- syndrome (Figure 1a and inset table of Figure 1c). Except for 5q- syndrome patients, neutropenia was significantly severe in patients with chromosome 5 abnormalities (Figure 1b and inset table of Figure 1c). Significant thrombocytopenia was observed in −5 patients as compared with del(5q) patients and patients without chromosome 5 abnormality (Figure 1c and inset table). As expected, the platelet count of 5q- syndrome patients remained within the normal range, which was significantly higher than that of del(5q) excluding 5q- syndrome.

Impact of chromosome 5 abnormalities on survival

Although the median OS of MDS patients without chromosome 5 abnormality was 2358 days, that of patients with chromosome 5 abnormalities was 454 days and significantly short (Figure 2a). To analyze more precisely, patients with chromosome 5 abnormalities were divided into 5q- syndrome, del(5q) excluding 5q- syndrome and (−5) patients. In total, 52 patients were classified as −5, 110 patients were classified as del(5q) excluding 5q- syndrome and 21 were categorized as 5q- syndrome. The median OS of 5q- syndrome was over 6000 days but that of del(5q) excluding 5q- syndrome and −5 was 501 days and 210 days, respectively.

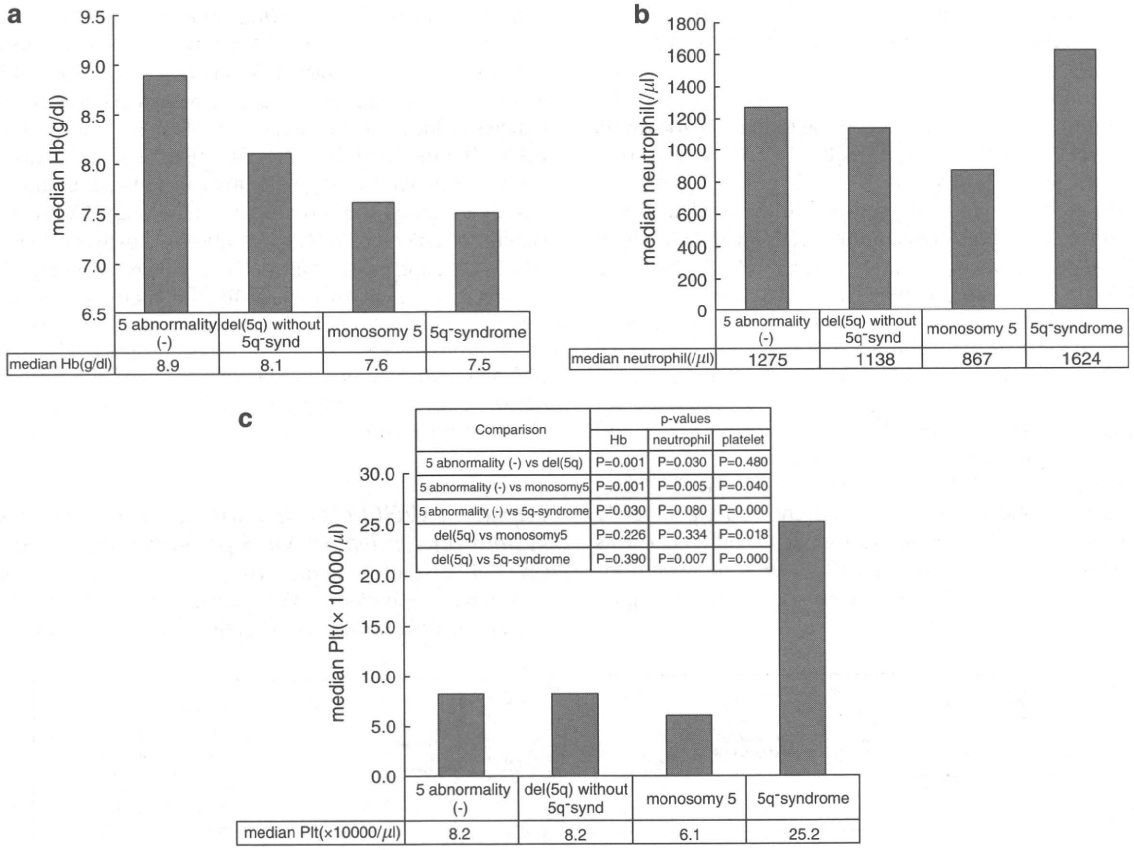

Figure 1 Cytopenias in four categories of myelodysplastic syndromes (MDS) with chromosome 5 abnormalities. (**a**) Hemoglobin concentration, (**b**) neutrophil count and (**c**) platelet count. Median values are indicated by a column and numerals. MDS patients were categorized into chromosome 5 abnormality (−; 375 cases), del(5q) without 5q- syndrome (110 cases), monosomy 5 (52 cases) and 5q- syndrome (21 cases). Mann–Whitney's *U*-test was performed between the groups and the *P*-values are indicated collectively in the inset table of Figure 1c.

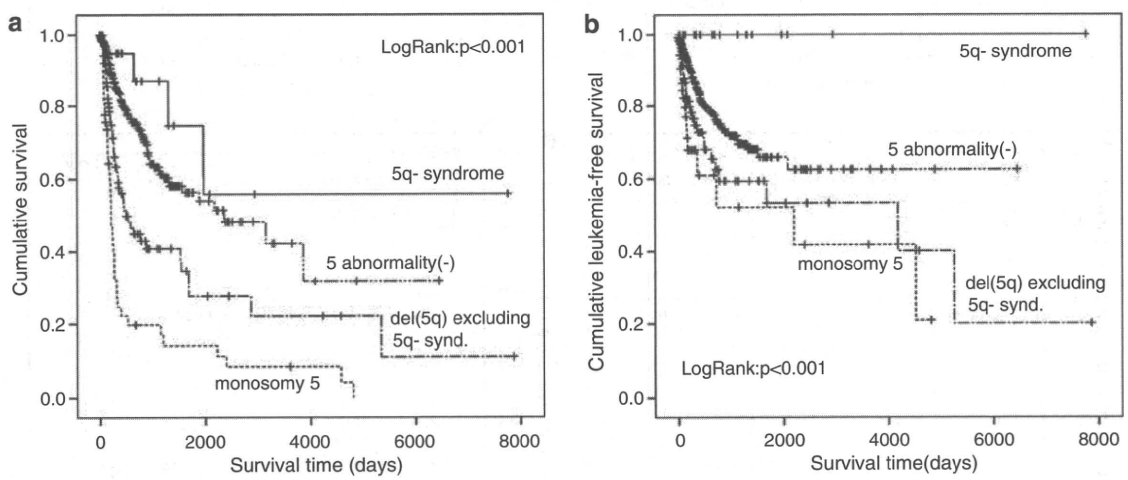

Figure 2 Impact of chromosome 5 abnormalities by three categories on the survival of myelodysplastic syndromes (MDS) patients. MDS patients were categorized into no chromosome 5 abnormality (375 cases), del(5q) (110 cases), monosomy 5 (52 cases) and 5q- syndrome (21 cases). (**a**) Overall survival curves and (**b**) leukemia-free survival curves were drawn by Kaplan–Meier method, and Wilcoxon's Log-rank test was performed in a lump.

It is noteworthy that none of the 5q- syndrome patients died of leukemic progression. The median LFS of patients without chromosome 5 abnormality was over 6000 days but that of patients with del(5q) excluding 5q- syndrome was 4176 days and significantly short (Figure 2b). The median LFS of −5 was 2199 days.

Of 52 patients with −5, 37 carried monosomy 7 together, whereas 47 of 131 del(5q) patients did, which indicated that MDS patients with −5 carry monosomy 7 together in a significantly higher incidence than del(5q) patients (χ^2-test, $P<0.001$).

Survival analysis of MDS patients with del(5q) according to categories of additional chromosomal abnormalities

We paid attention to the outcome of 131 MDS patients with del(5q) including 5q- syndrome. According to the categorization as mentioned in 'Materials and methods', 35 cases were categorized as 5q-, 47 cases as 7+, 35 cases as complex and 14 cases as other. Figure 3a shows that the median OS of 5q- and other was both over 6000 days with no significant difference between the two groups ($P=0.329$). The median OS of 7+ and complex was 240 and 458 days, respectively, and there were significant differences between 5q- and 7+ ($P<0.001$), between 5q- and complex ($P<0.001$). 7+ patients had a significant shorter OS than complex ($P=0.018$).

The median LFS of 5q- and other was over 6000 days and 4176 days, respectively, without significant difference ($P=0.699$). The median LFS of 7+ and complex was 770 days and 5247 days, respectively. 5q- group showed significantly longer LFS than 7+ ($P=0.006$). No significant difference was observed between 5q- and complex ($P=0.069$), between 5q- and other ($P=0.699$) and between 7+ and complex ($P=0.236$), respectively.

Survival analysis according to IPSS

According to IPSS, of 131 del(5q) patients, 11 were categorized as low-risk and their median OS was over 1800 days, 37 patients were as Intermediate (Int)-1 risk with a median OS of 2863 days, 50 patients as Int-2 risk with a median OS of 501 days and 33 as high risk with a median OS of 248 days (Figure 3b). Significant shorter OS was observed in high-risk than in low-risk group, Int-1 and Int-2. Int-1 group showed a significantly longer OS than Int-2. No significant difference in OS was observed between low and Int-1 ($P=0.423$), and low and Int-2 ($P=0.058$), respectively (Figure 3b).

Next, as shown in Figure 3c, the median LFS of low-risk group, Int-1, Int-2 and high risk was over 1800 days, over 6000 days, 1682 days and 770 days, respectively. Significant difference in LFS was observed between low-risk and Int-2, between low risk and high risk, between Int-1 and Int-2 and between Int-1 and high risk.

Impact of WHO classification-based prognosis scoring system on survival of MDS patients with del(5q)

Of 131 del(5q) patients, 106 had information concerning transfusion dependency. We categorized these patients according to the WPSS. Of these 6 patients were categorized as very

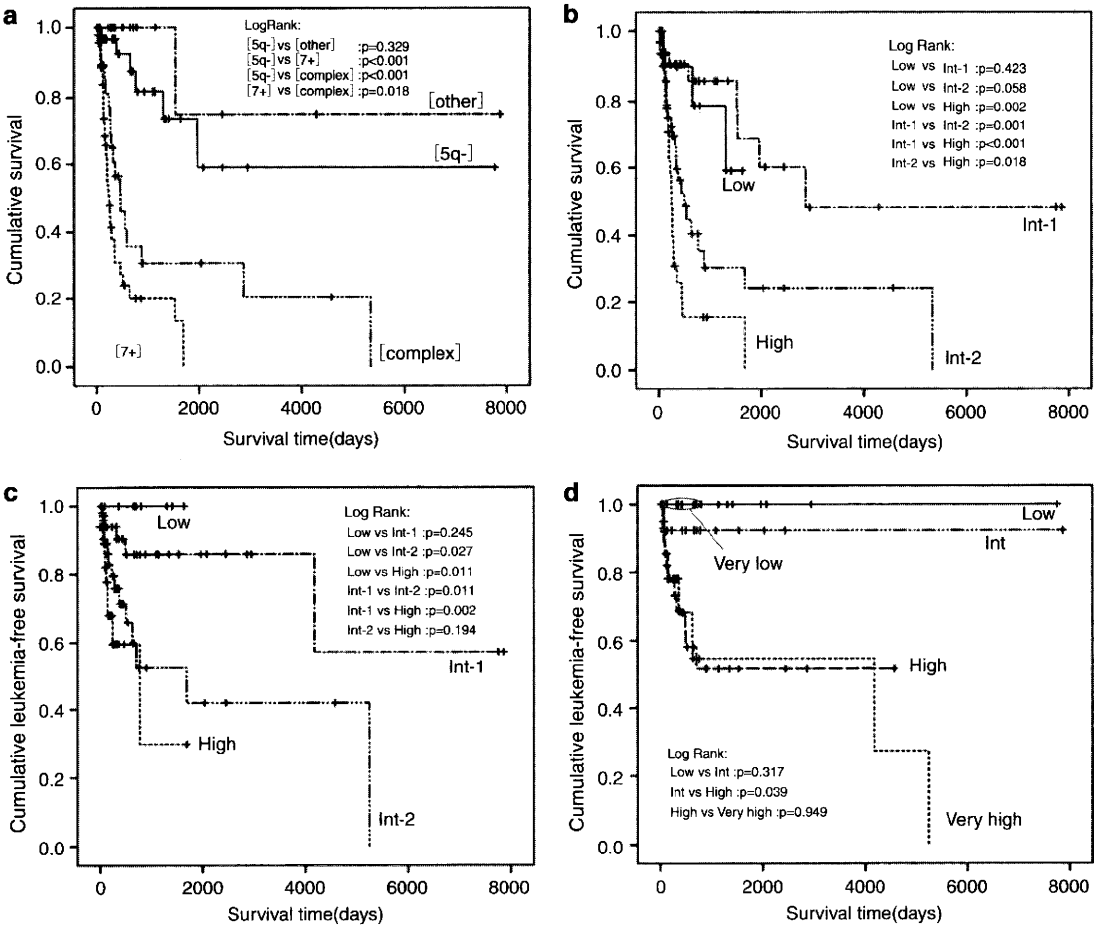

Figure 3 Impact of several factors on the survival of myelodysplastic syndromes (MDS) patients with del(5q). (**a**) Overall survival of MDS patients with del(5q) categorized by additional chromosomal abnormalities. A total of 131 MDS patients with del(5q) were categorized into 5q- (35 cases), 7+ (47 cases), complex (35 cases) and other (14 cases). (**b**) Overall survival of MDS patients with del(5q) categorized by IPSS. A total of 131 MDS patients with del(5q) were categorized into low cases, intermediate (Int-1, 37 cases), Int-2 (50 cases) and high (33 cases). (**c**) Leukemia-free survival of MDS patients with del(5q) categorized by IPSS. A total of 131 MDS patients with del(5q) were categorized into low (11 cases), Int-1 (37 cases), Int-2 (50 cases) and high (33 cases). (**d**) Leukemia-free survival of MDS patients with del(5q) categorized by WPSS. A total of 106 MDS patients with del(5q) were categorized into very low (6 cases), low (13 cases), Int (16 cases), high (29 cases) and very high (42 cases). The survival curves were drawn by Kaplan–Meier method, and Wilcoxon's Log-rank test was performed in the indicated pairs.

low risk, 13 as low risk, 16 as Int, 29 as high and 42 as very high. As for patients categorized by WPSS, the patterns of LFS were divided into two groups such as very low/low/Int and high/very high Figure 3d). Although the duration of observation was short, none of the very low-risk group and only one case of the low-risk group progressed to leukemia.

Hence, we divided IPSS and WPSS classifications into lower-risk and higher-risk groups; that is, low/Int-1 and Int-2/high in IPSS and very low/low/Int and high/very high in WPSS described above, respectively. We then applied our patient data to each scoring system and calculated the statistic sensitivity to divide the outcome of each patient into: (1) 'alive or dead' and (2) 'leukemia-free or leukemic transformation'. The sensitivity for dividing the events 'alive or dead' was 75.6% in IPSS and 85.4% in WPSS. Likewise, the sensitivity to divide into 'leukemia-free or leukemic transformation' was 78.3% in IPSS and 95.7% in WPSS. These data demonstrated that WPSS was able to predict the outcome of MDS patients with del(5q) more clearly than IPSS.

Determination of prognostic factors in MDS patients with del(5q)

As the univariate analysis, age, gender, hemoglobin concentration, platelet count, neutrophil count, percentage of bone marrow blasts, cytogenetic pattern, red cell transfusion dependency and platelet transfusion dependency were examined. Table 2 indicates the relevant factors for death and leukemic progression rates in the del(5q) patients. This analysis showed

Table 2 Prognostic risk factors of MDS patients with del(5q) by univariate analysis

Variables	*Number*	*Death rate*				*Leukemic progression rate*			
		Odds ratio	*95% CI*	χ^2-*value*	P-*value*	*Odds ratio*	*95% CI*	χ^2-*value*	P-*value*
Gender									
Male	65								
vs		1.630	0.717–3.704	1.370	0.242	0.976	0.379–2.516	0.003	0.96
Female	41								
Age									
>60	83								
vs		1.155	0.436–3.058	0.084	0.772	1.336	0.403–4.428	0.225	0.635
≤60	22								
Hb									
>9 g per 100 ml	77								
vs		2.485	0.949–6.504	3.559	0.059[a]	0.635	0.236–1.710	0.815	0.367
⩾9 g per 100 ml	29								
Plt									
<100 000 per μl	53								
vs		1.491	0.679–3.273	0.994	0.319	0.895	0.355–2.255	0.056	0.814
⩾100 000 per μl	53								
Neutrophil									
<1800 per μl	69								
vs		1.261	0.551–2.887	0.301	0.583	3.135	0.978–10.044	3.966	0.046[b]
⩾1800 per μl	37								
BM blasts									
⩾5%	50								
vs		2.500	1.121–5.576	5.114	0.024[b]	2.031	0.791–5.216	2.212	0.137
<5%	56								
Additional chromosome abnormalities									
7 Abnormality/complex	61								
vs		6.836	2.640–17.700	17.629	<0.001[b]	2.511	0.900–7.004	3.22	0.073
Isolated (5q) or others	45								
Red cell transfusion dependency									
Dependent	80								
vs		2.006	0.758–5.310	2.007	0.157	0.679	0.243–1.892	0.554	0.457
Independent	26								
Platelet transfusion dependency									
Dependent	31								
vs		3.425	1.406–8.344	7.662	0.006[b]	2.045	0.745–5.617	1.971	0.16
Independent	66								

Abbreviations: BM, bone marrow; CI, confidence interval; Plt, platelet
[a]Shows nearly significant difference.
[b]Shows statistically significant differences.

that the major risk factors for death rate were the percentage of bone marrow blasts, cytogenetic pattern and platelet transfusion dependency. It was not significant whether the degree of anemia influences the outcome. The only major factor for leukemic progression was neutrophil count.

We further investigated the prognostic factors by multivariate analyses using Cox proportional hazard regression model with fixed covariates and found that the most significant independent risk factors for determining outcome were the percentage of bone marrow blasts, cytogenetic pattern and platelet transfusion dependency (Table 3). The major factors predictive of leukemia progression were the cytogenetic pattern, the presence of neutropenia and thrombocytopenia. Multivariate analyses excluding the influence of red cell and platelet transfusion dependency revealed that the risk factors for OS were bone marrow blasts ($P=0.044$) and cytogenetic pattern ($P<0.001$), and the risk factors for LFS were neutrophil count ($P=0.026$), platelet count ($P=0.023$) and cytogenetic pattern ($P=0.008$). In contrast, Multivariate analyses excluding the influence of hemoglobin concentration and platelet count revealed that cytogenetic pattern was a risk factors for OS ($P=0.003$) but neither red cell nor platelet transfusion dependency was a statistically significant risk factor for OS.

Discussion

The biological and clinical significances of −5 and deletion of the long arm of chromosome 5 (del(5q)) are accepted as equivalent, or at least quite similar, in patients with AML and

Table 3 Prognostic risk factors of MDS patients with del(5q) by multivariate Cox hazards regression analysis

Variables	*Number*	*Overall survival*			*Leukemia-free survival*		
		P-value	*Hazard ratio*	*95% CI*	*P-value*	*Hazard ratio*	*95% CI*
Gender							
Male	65						
vs		0.162	1.796	0.790–4.085	0.688	1.248	0.424–3.678
Female	41						
Age							
>60	83						
vs		0.351	0.626	0.235–1.672	0.682	1.344	0.327–5.533
⩽60	22						
Hb							
<9 g per 100 ml	77						
vs		0.106	2.212	0.844–5.795	0.642	0.760	0.239–2.418
⩾9 g per 100 ml	29						
Platelets							
<100 000 per μl	53						
vs		0.145	0.501	0.197–1.271	0.038[a]	0.277	0.082–0.934
⩾100 000 per μl	53						
Neutrophil							
<1800 per μl	69						
vs		0.787	1.116	0.504–2.469	0.045[a]	3.377	1.030–11.072
⩾1800 per μl	37						
BM blasts							
⩾5%	50						
vs		0.047[a]	2.288	1.011–5.175	0.416	1.589	0.521–4.852
<5%	56						
Additional chromosome abnormalities							
7 Abnormality/ complex	61						
vs		0.002[a]	4.421	1.692–11.552	0.028[a]	4.333	1.169–16.056
Isolated 5q- or others	45						
Red cell transfusion dependency							
Dependent	80						
vs		0.398	0.637	0.224–1.812	0.422	0.578	0.152–2.202
Independent	26						
Platelet transfusion dependency							
Dependent	31						
vs		0.047[a]	2.403	1.013–5.703	0.316	1.841	0.558–6.068
Independent	66						

Abbreviations: BM, bone marrow; CI, confidence interval.
[a]Shows statistically significant differences.

MDS.[13–15] In fact, the prognostic value of these two chromosome aberration groups was not significantly different among AML patients,[15] but sufficient data for MDS were lacking. The present study demonstrates that the prognosis of patients who carry −5 and del(5q) are significantly different, as OS and LFS of −5 group were shorter than del(5q) patients even if 5q- syndrome patients were excluded from del(5q) patients (Figure 2 and data not shown). AML patients with monosomy 7 rather than with deleted 7q chromosome were reported to lead to poor prognosis.[16] Significantly poor prognosis of −5 group in our series might be explained by the observation that −5 was significantly correlated with presence of monosomy 7 as compared to del(5q) group ($P<0.001$). The co-presence of chromosomes 5 and 7 abnormalities has been associated with poor outcomes in MDS and AML.[17–22]

Although the cause of leukemic progression is unknown, susceptibility to leukemia clearly leads to higher mortality of −5 patients compared to del(5q) patients (Figure 2b). The −5 group also had significantly more severe neutropenia and thrombocytopenia and might exacerbate the survival of this group (Figures 1b and c). Neutropenia and thrombocytopenia, but not severe anemia, are reported to be common findings of patients with monosomy 7.[23] Taken together, laboratory findings shown in −5 group and high incidence of the co-presence of −5 and monosomy 7 might result in poor prognosis of the corresponding patients.

In this survey, the incidence of 5q- syndrome was quite rare in Japan. Recent studies suggest different genetic or environmental backgrounds between Asian and Western MDS populations.[24,25] According to the recent report by Haase *et al.*,[28] isolated del(5q) was seen in 14%, del(5q) with one additional abnormality in 5%, and complex abnormalities including del(5q) were seen in 11% of patients with clonal abnormalities. In the Korean study, isolated del(5q) was seen in 1.7% of the patients.[24] The incidence of del(5q), isolated del(5q) and 5q- syndrome patients was 8.4, 2.2 and 1.3%, respectively, in Japanese MDS study. These data are lower than those of Western patients but more similar to those of Asian patients.[24–26]

In the present study, we paid particular attention to MDS patients with del(5q) and classified them into four groups according to the cytogenetic complexity: 5q-, 7+, complex and other. 5q- patients have previously been well defined as having relatively good prognosis, whereas poor prognosis was indicated when it was combined with other anomalies.[19,20,26,27] OS and LFS of 7+ group were significantly shorter than 5q- group and OS of complex was significantly shorter than 5q-(Figure 3a and data not shown). It is suggested that the clinical outcome of MDS patients with del(5q) depends on the prognostic value of combined chromosomal abnormalities.

The most significant independent prognostic variables in MDS are the percentage of bone marrow blasts, the number of cytopenias and cytogenetic pattern. By weighting these variables according to their statistic power, IPSS separates MDS patients into four distinct risk groups regarding survival and the potential for leukemic progression: low risk, Int-1, Int-2 and high risk.[10] Even in the del(5q) patient group, which is considered to have a better prognosis, univariate analysis and multivariate analysis of del(5q) patients in our series showed that the cytogenetic pattern, percentage of bone marrow blasts and platelet transfusion dependency were the most relevant risk factors (Tables 2 and 3). Figures 3b and c show that IPSS critically determines OS and LFS of del(5q) patients. All 21 patients classified as 5q- syndrome were low risk of IPSS, and the patients classified as high risk were AML and refractory anemia with excess blasts (RAEB)-2 cases with adverse chromosomal abnormalities.

As for the del(5q) patients who are far from the risk of leukemic progression, red cell transfusion dependency often has an adverse impact on survival.[28] Although red cell transfusion dependency was not a significant prognostic factor by the present analyses, the degree of anemia had a tendency to affect survival of these patients from the result of univariate analysis ($P=0.059$, Table 2).

Although IPSS is based on FAB classification and does not take into account other prognostic factors such as dysplasia and transfusion requirement, WPSS has a relevant prognostic value.[29] Therefore, we applied WPSS to our patient data (Figure 3d) and confirmed that WPSS can predict the prognosis of del(5q) MDS patients more clearly than IPSS.

Our study demonstrated that −5 and del(5q) belong to different clinical entities and their biological behaviors are different from each other, and that del(5q) patients can be stratified according to their additional chromosomal abnormalities and IPSS or WPSS status. Severe anemia requires frequent transfusions, reduces quality of life and becomes often the major clinical problem for MDS patients with del(5q). The prognosis of del(5q) patients is related to their status of chromosomal abnormalities and transfusion dependency, but new agents such as lenalidomide improve the disorder and might provide new insights into more precise understanding of the disease.[30]

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Diagnosis and classification of myelodysplastic syndrome: International Working Group on Morphology of Myelodysplastic Syndrome (IWGM-MDS) consensus proposals for the definition and enumeration of myeloblasts and ring sideroblasts

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ABSTRACT

The classification of myelodysplastic syndromes is based on the morphological criteria proposed by the French-American-British (FAB) and World Health Organization (WHO) groups. Accurate enumeration of blast cells, although essential for diagnosis of myelodysplastic syndrome and for assignment to prognostic groups, is often difficult, due to imprecise criteria for the morphological definition of blasts and promyelocytes. An International Working Group on Morphology of Myelodysplastic Syndrome (IWGM-MDS) of hematopathologists and hematologists expert in the field of myelodysplastic syndrome reviewed the morphological features of bone marrows from all subtypes of myelodysplastic syndrome and agreed on a set of recommendations, including recommendations for the definition and enumeration of blast cells and ring sideroblasts. It is recommended that (1) agranular or granular blast cells be defined (replacing the previous type I, II and III blasts), (2) dysplastic promyelocytes be distinguished from cytologically normal promyelocytes and from granular blast cells, (3) sufficient cells be counted to give a precise blast percentage, particularly at thresholds that are important for diagnosis or prognosis and (4) ring sideroblasts be defined as erythroblasts in which there are a minimum of 5 siderotic granules covering at least a third of the nuclear circumference. Clear definitions and a differential count of a sufficient number of cells is likely to improve precision in the diagnosis and classification of myelodysplastic syndrome. Recommendations should be applied in the context of the WHO classification.

Key words: myelodysplastic syndrome, myelodysplastic syndrome, myeloblast, ring sideroblast.

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Introduction

The term myelodysplastic syndrome (MDS) is used to describe a heterogeneous group of disorders that are characterized by clonal and ineffective hematopoiesis, morphological dysplasia, peripheral blood cytopenias and progressive bone marrow failure. MDS transforms to acute myeloid leukemia (AML) in approximately 30% of cases. Survival following a diagnosis of MDS varies from a few months to more than ten years (comparable to age/sex matched normal popu-

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lations).[1] This highly variable prognosis underscores the importance of a classification system, supplemented by a prognostic index, to predict the survival of patients with MDS and the likelihood of transformation to AML. With the recent development and introduction of several effective treatment options for MDS,[2,3] the need for a classification system to predict responsiveness to treatment and clinical outcomes for individual patients has become even more important.

During the past 20 years, several MDS classification and prognostic scoring systems have been proposed.[4-7] Several of these systems have gained acceptance with the French-American-British (FAB) classification as modified by the World Health Organization (WHO), the International Prognostic Scoring System (IPSS) being the most widely used. In addition, the recent identification of transfusion burden and a modification of the IPSS by Malcovati and co-workers,[8] the so-called WPSS (World Prognostic Scoring System), have surfaced as an important component of our understanding of the natural history of MDS. Refinements in classification are needed as research continues to advance our knowledge of the etiology and the pathogenesis of MDS.

To address these issues, a panel of experts in the classification of MDS, the International Working Group on Morphology of MDS (IWGM-MDS) convened on three occasions in 2005/06 to review and refine the morphological criteria for the classification of MDS. This group consisted of both clinical hematologists and hematopathologists. The latter attended all three meetings and participated actively in the review and characterization of many individual cases (slide review). The former provided clinical input as to the relevance of the precise determination of morphological cell types in the assessment of patients with MDS. This model has been utilized with success in the development of the 2008 WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues.

The goals of this Working Group were to (i) define minimal diagnostic criteria for MDS; (ii) develop standardized definitions for myeloblasts and promyelocytes; and (iii) propose a classification for sideroblasts. This article details the proposals of the IWGM-MDS for the definition of myeloblasts, promyelocytes and ring sideroblasts. Proposals for minimal diagnostic criteria and for dealing with cases of possible MDS that do not meet these criteria are dealt with in an accompanying paper.[9]

Background: current MDS classification systems

Several classification systems have been developed to predict survival or transition to AML following MDS diagnosis. The first of these, the FAB system, was introduced in 1982 and is based on the percentage of blasts and morphological dysplastic features of blood and bone marrow.[4] According to this system, patients are diagnosed with MDS when dysplastic bone marrow hematopoiesis is present and/or myeloblasts are between 5 and 30% of all bone marrow cells. The FAB system served as the standard for MDS classification for two decades and provided considerable prognostic information. Nonetheless, the clinical outcomes of patients assigned to the same MDS subgroup remain too variable to accurately predict survival or transformation to AML in individual patients.

The International Prognostic Scoring System[5,6] provided a prospective risk assessment from the initial diagnosis but was dependent on having both an accurate bone marrow blast assessment and cytogenetic analysis. Increasing blast percentages[5-10,11-20,20-30] indicated an increase in the risk of leukemic transformation and of death from all causes. Therefore, an accurate definition of the blast percentage and separation of blast cells from promyelocytes is critical.

In 2001, the WHO[7] proposed a revision of the FAB morphological approach. The revisions included lowering the threshold for the percentage of blasts required to make the diagnosis of AML from 30% to 20%, thus elimination of the MDS subcategory of refractory anemia with excess blasts in transformation (RAEB-T). In addition, chronic myelomonocytic leukemia (CMML) was reclassified from a subcategory of MDS to a subcategory of myelodysplastic/myeloproliferative disorder.

Of considerable importance was the introduction of a new subtype: refractory anemia with multilineage dysplasia without ring sideroblasts (RCMD) or with ring sideroblasts (RCMD-RS) with ≥10% of dysplasia in at least two cell lines, and refractory anemia (RA) or refractory anemia with ring sideroblasts (RARS) with dysplasia restricted to the erythroid lineage. The precise qualitative features of the dysplastic cells were described with several illustrations, particularly of the erythroid dysplasia.

Developing a classification system

All of the classification systems described above depend on an assessment of dysplastic changes in the marrow. In addition, recognition and enumeration of blast cells is of critical importance both in the diagnosis of AML and MDS, and for stratifying MDS patients into prognostic groups.[10,11] According to the IPSS, for example, patients with ~10% bone marrow blast cells would be assigned to the intermediate 1 or 2 risk groups, and would have a worse prognosis than low-risk patients.[5]

It is often assumed that definitions of blast cells are applied uniformly by hematologists/pathologists worldwide, and that blast cells could be identified and counted very easily. Unfortunately this is not so. The FAB group defined type I and type II blast cells, both having a high nucleocytoplasmic ratio, a diffuse chromatin pattern and usually visible nucleoli; type I blast cells are agranular and type II have scanty granules.[4] Subsequently Goasguen and colleagues analyzed bone marrow smears obtained from 18 patients with MDS classified according to the FAB classification and defined a type III blast, with more than 20 fine azurophilic granules but otherwise with the characteristics expected of a blast cell.[12] In the FAB classification such cells were categorized as promyelocytes. The inclusion of type III blast cells in the blast cell count led to 7 patients (39%) being reclassified from refractory anemia with excess of blasts (RAEB) to RAEB-T and was found to give a better separation of survival curves of different FAB categories of MDS. Despite the ability of this classification system

to refine survival estimates for patients with MDS it was unclear how often type III blasts have been utilized in myelograms in typical clinical practice. Subsequent to this publication protocols for some clinical trials have included type III blast cells in the blast cell count but this has not been universal practice. The WHO classification does not give any specific recommendations for the definition of blast cells.[7]

In practice, although FAB type I and type II blasts can generally be readily distinguished from each other it has proved difficult to distinguish FAB type II blasts from type III blasts. In addition, the enumeration of promyelocytes, which are often abnormal in MDS, remains problematic and their separation from type II and type III blasts has remained imprecise.

Statistical analysis

Concordance was determined using the κ statistics.[13]

Results

The Working Group participants reviewed previous attempts to define blasts (agranular *vs.* granular) and promyelocytes. Each member of the group was asked to bring blood and bone marrow slides obtained from patients with various subtypes of MDS and/or AML that would serve as the basis for discussion of the identification of different types of blasts and promyelocytes. Myelograms were determined from these slides, and the data were captured and recorded electronically for subsequent statistical analysis. The starting point for developing definitions was the 1991 paper by Goasguen and colleagues.[12] In addition, criteria for separating granular blast cells from promyelocytes were developed.

Definition of myeloblasts

After a review of the literature, assessment of blood and bone marrow films individually and collectively, and much discussion, the participants arrived at a consensus regarding the definition of a myeloblast. Myeloblasts were defined in terms of several nuclear characteristics, including a high nuclear/cytoplasmic ratio, easily visible nucleoli and usually, but not invariably, fine nuclear chromatin. Nuclear shape is variable. Cytoplasmic characteristics include variable cytoplasmic basophilia; there may or may not be granules or Auer rods but no Golgi zone is detected (Figure 1). The exception to this last observation may be seen in cases of AML with t(8;21) where there may be blast cells with a small distinct Golgi, with or without an Auer rod, but with no other features of a promyelocyte. After reviewing all the available bone marrow smears, the IWGM group recommended that myeloblasts in MDS should be classified as agranular or granular. The agranular blasts correspond to the type I blasts of the FAB classification. Granular blasts are cells that have the nuclear features of blast cells but also have cytoplasmic granules. These cells will thus include type II blasts as defined by FAB, as well as type III blasts as defined by Goasguen *et al.*[12]

Granular blasts must be distinguished from promyelocytes (*see below*).

Promyelocytes

The group discussed the morphological features that define normal promyelocytes. Nuclear characteristics of normal promyelocytes included a central or eccentric nucleus and chromatin, which may still be fine or may be intermediate. The nucleolus is usually easily visible and prominent (Figure 1). The group determined that the principal distinguishing characteristic of the normal promyelocyte was the presence of a visible Golgi zone. Other cytoplasmic characteristics include uniformly dispersed azurophilic granules, and in most instances basophilic cytoplasm. Dysplastic promyelocytes have the recognizable features of a promyelocyte including a round, oval, or indented nucleus that is often eccentric, a Golgi zone (at least faintly visible) and a nucleus with fine or coarse chromatin and an easily visible nucleolus. Abnormal features that lead to recognition of promyelocytes as being dysplastic include reduced or irregular cytoplasmic basophilia, a poorly developed Golgi zone, hypergranularity, hypogranularity and irregular distribution (clumps) of granules.

The group agreed, therefore, on the following morphological categories: normal promyelocytes, blasts (which are differentiated as simply agranular or granu-

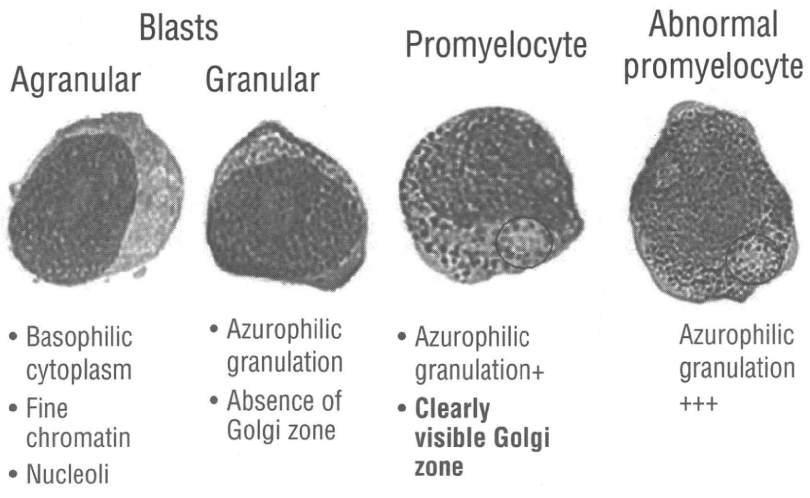

Figure 1. **Blasts, promyelocytes, abnormal promyelocytes.**

lar, irrespective of the number of granules) and dysplastic promyelocytes. To verify the reproducibility of these propositions five experts were asked to review 264 consecutive cells from one case of AML (FAB-M2). The pictures were captured utilizing a unique digital image, capable of merging multiple consecutive fields (600x800 pixels). Each observer performed the task on his/her own computer by downloading the file from a dedicated website of the MDS Foundation. A drop-down menu was provided with the following choices: blasts (agranular and granular), promyelocytes (normal or abnormal), mature granulocytes, others (Figure 2). Results were sent electronically to the MDS Foundation headquarters and analyzed by JG.[14]

Individual assessments can be seen in Table 1. If we consider that a very good concordance would be agreement of 5/5 or 4/5 experts, then an 89.4% concordance was achieved in separating blasts from promyelocytes. Examining the data with kappa statistics demonstrated a high concordance when viewing one observer versus another (Table 2).

It should also be noted that when performing a marrow differential count, the myeloblast percentage should be determined by counting at least 500 nucleated cells, with the total including at least 100 nonerythroid cells to improve precision. The Working Group emphasized the use of this number to be extremely important for correct classification of patients with MDS, especially when cells of the erythroid lineage exceed 50%. Other methods of determining the myeloblast percentage may result in some patients being classified incorrectly. Counting an adequate number of cells is of critical importance for the classification of patients whose blast counts fall near the boundary between MDS categories of different prognostic significance. It is similarly essential to perform a 500-differential count on the blood film of patients with circulating blast cells since relatively small differences in the percentage of blast cells are of prognostic significance; the 2008 WHO classification assigns patients to different MDS categories with a blast count of less than 1%, 1%, 2-4% or 5%.[15]

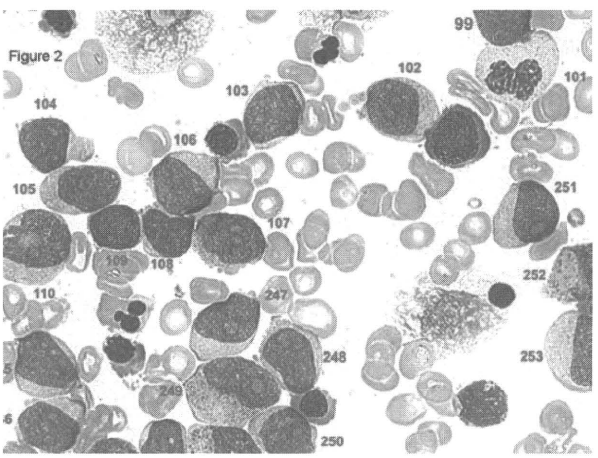

Figure 2. **Example of individual cells counted by the experts.**

Ring sideroblasts

The prognosis of patients with pure sideroblastic anemia may differ from that of patients with non-sideroblastic anemia; therefore, clear, standardized definitions of sideroblast types are necessary. Varying definitions of ring sideroblasts have led to confusion and controversy among clinicians. Early investigators defined ring sideroblasts as having iron granules in a perinuclear distribution surrounding the entire nucleus. Other investigators have required that perinuclear granules encircle at least one third of the perinuclear area, but not necessarily the entire nucleus.[16] Ringed sideroblasts were sometimes required to have a minimum of 5 granules and sometimes a minimum of 10 granules.

After a review of many cases of sideroblastic anemia, the group determined that ring sideroblasts should have at least 5 granules in a perinuclear distribution; that these granules could either surround the entire nucleus, be localized to portions of the perinuclear area or cover at least one third of the nucleus (Figures 3 and 4).

Table 1. **Agreement of the expert panel. (A) Light grey bar: Maturing Granulocytes. (B) Medium grey bar: Promyelocytes. (C) Black bar: Blast Cells.**

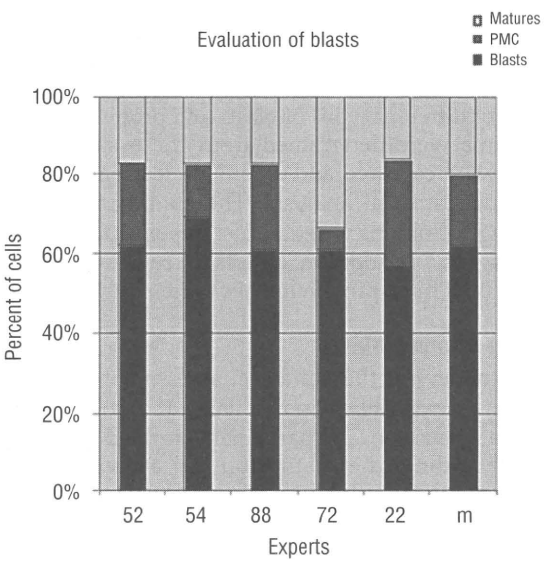

Table 2. **Degree of consistency among experts: percent agreement/unweighted κ coefficients for pairs of 5 readers based on 264 cells divided into 4 categories (blasts, promyelocytes, matures, others).**

Expert ref #	52	54	88	72	22
52	1	0.77/0.57	0.80/0.63	0.72/0.51	0.82/0.68
54	–	1	0.78/0.61	0.76/0.56	0.75/0.56
88	–		1	0.76/0.59	0.85/0.74
72	–	–	–	1	0.72/0.55
22	–	–	–	–	1

Consistency among readers is evaluated by percentage of agreement (first value) and the unweighted kappa coefficient (second value) for all pairs of readers. Conclusion: Percent agreement varies from 0.72 (pair (72×52) to 0.85 (pair 22×88) demonstrating a high concordance rate between experts. When adjusted for chance agreement, however, the κ values were somewhat lower indicating less than optimal agreement in some cases. The adjustment for chance agreement is influenced by small numbers of cells in two categories.

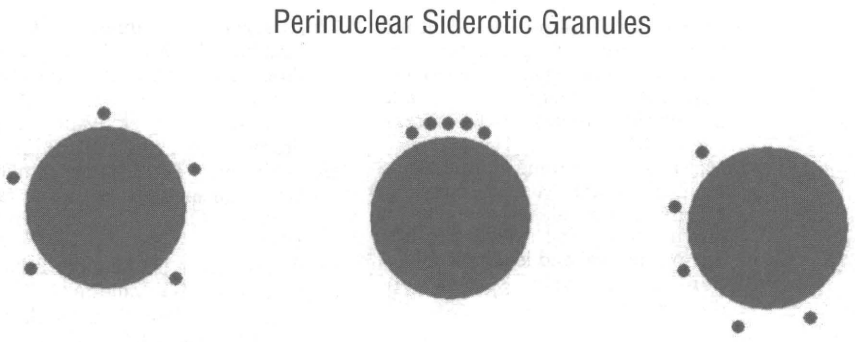

Figure 3. **Perinuclear siderotic granules (cartoon of potential examples).**

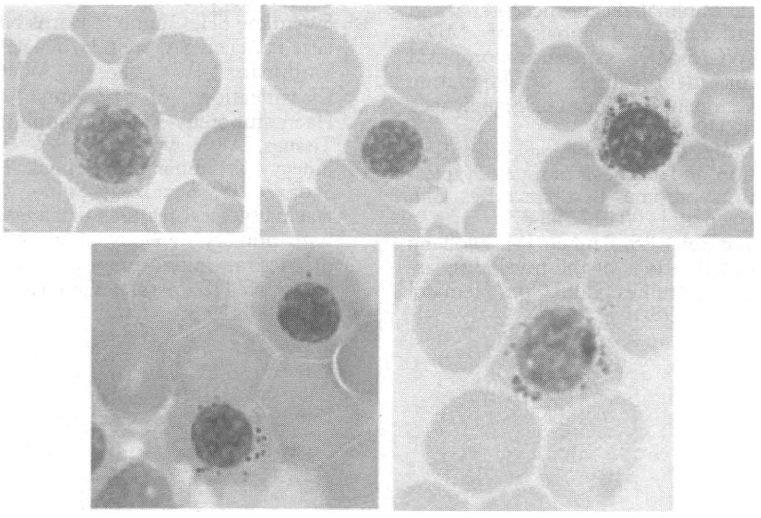

Figure 4. **Prussian blue reaction of erythroid precursors. a. Upper Panel (left to right): no siderotic granules; type 1 sideroblast (1 granule); type 3 sideroblast (numerous granules). b. Lower panel (left to right): type 1 sideroblast (upper cell), type 2 sideroblast (lower cell); type 3 sideroblasts (lower right); Hematoxylin counter stain; Prussian Blue reaction.**

The Working Group defined three types of sideroblast: Type 1 sideroblasts: fewer than 5 siderotic granules in the cytoplasm; Type 2 sideroblasts: 5 or more siderotic granules, but not in a perinuclear distribution; Type 3 or ring sideroblasts: 5 or more granules in a perinuclear position, surrounding the nucleus or encompassing at least one third of the nuclear circumference.

The group recommends that when counting ring sideroblasts all stages of erythroid precursors be counted and should include at least 100 nucleated erythroid precursors and for the definition of RARS the required number of ring sideroblasts remains at 15% as defined previously in the FAB and WHO classifications. The definition of a ring sideroblast proposed by the IWGMDS (an erythroblast with at least 5 siderotic granules covering at least a third of the circumference of the nucleus) has been incorporated into the 2008 WHO classification of Tumours of Haematopoietic and Lymphoid Tissues.[15]

The group also addressed the nuclear counterstain used to optimize the distinction of erythroid cells. The Working Group discussed the value of a number of stains such as neutral red, basic fuchsin, saffronin, hematoxylin, and light Giemsa, as well as staining for H-type ferritin and polyclonal antibody staining for siderotic granules. The group considered that further studies were needed to assess the value of these counterstains and methods but agreed that all had merits.

Only type 3 sideroblasts would qualify as *ring* sideroblasts to separate sideroblastic from non-sideroblastic anemia. This proposal will be tested in a similar manner to the blast definition by developing a web based digital image of multiple types of sideroblasts.

Discussion

In the absence of biological markers to stratify patients with MDS morphological assessment is essential for defining risk, regardless of which risk system is utilized. Because of the importance of determining the percentage of blasts as well as of ring sideroblasts, the IWGM-MDS focused on careful definitions that are illustrated and confirmed to be reproducible. The proposed definitions are intended to be used in conjunction with the WHO classification in order to make the categorization of patients with MDS more precise.

Authorship and Disclosures

GJM, JMB, JG, BJB, RB and TV reviewed the bone marrow preparations in the workshops and carried out the review of the digital images; GJM, JMB and BJB prepared the final manuscript; IB, MC, PF, UG, EH-L, IJ, AM, AkM, CMN, GS, MT and AY reviewed some of the material and contributed to the general discussions and all gave approval to the manuscript.

The authors reported no potential conflicts of interest.

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Cut-off value of red-blood-cell-bound IgG for the diagnosis of Coombs-negative autoimmune hemolytic anemia

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Direct antiglobulin test (DAT)-negative autoimmune hemolytic anemia (Coombs-negative AIHA) is characterized by laboratory evidence of in vivo hemolysis, together with a negative DAT performed by conventional tube technique (CTT) in clinically suspected AIHA patients. The immunoradiometric assay (IRMA) for red-blood-cell-bound immunoglobulin G (RBC-IgG) can be used to diagnose patients in whom CTT does not detect low levels of red cell autoantibodies. We investigated the diagnostic cutoff value of the IRMA for RBC-IgG in Coombs-negative AIHA and calculated its sensitivity and specificity. Of the 140 patients with negative DAT by CTT referred to our laboratory with undiagnosed hemolytic anemia, AIHA was clinically diagnosed in 64 patients (Coombs-negative AIHA). The numbers of Coombs-negative AIHA and non-AIHA patients changed with age and gender. The cutoff values were determined from receiver operating characteristic (ROC) curve according to age and gender. The IRMA for RBC-IgG proved to be sensitive (71.4%) and specific (87.8%) when using these cutoffs. Using these cutoffs for 41 patients with negative DAT referred to our laboratory in 2006, all the pseudonegative cases were treated with steroids before the test. The 31 untreated cases could be grouped using one cutoff value of 78.5 and showed 100% sensitivity and 94% specificity, independent of gender and age. Results indicate that RBC-IgG could become a standard approach for the diagnosis of Coombs-negative AIHA, when measured before treatment. Am. J. Hematol. 84:98–101, 2009. © 2008 Wiley-Liss, Inc.

Introduction

The detection of red-blood-cell-bound immunoglobulin G (RBC-IgG) and complement by direct antiglobulin test (DAT) remains the main serological assay in the diagnosis of autoimmune hemolytic anemia (AIHA) [1]. Several methodologies have been investigated for detection and evaluation of these autoantibodies. DAT by conventional tube technique (CTT) is the method most commonly used in the blood centers and is still considered a gold standard [2]. A positive DAT is almost always seen in association with AIHA [3] and forms the characteristic of the serological diagnosis of AIHA [4,5]. However, it has also been shown that a negative DAT does not exclude the diagnosis of AIHA [5,6] and 1–10% of patients with AIHA have been reported to show a negative DAT [7–9]. These patients, designated "Coombs-negative AIHA" patients, may carry lower numbers of IgG molecules per RBC, yielding a negative tube DAT and in vivo hemolysis [10]. Also, they may have only RBC-IgA autoantibodies or monomeric IgM molecules that induce the clinical and hematological features typical of AIHA [11]. The immunoradiometric assay (IRMA) [12], the complement fixation antibody consumption test [13] and the enzyme-linked antiglobulin test (ELAT) for RBC-IgG, as well as the enzyme-linked immunosorbent assay (ELISA) for IgG eluted from RBCs [14,15] are representative methods to quantitatively detect RBC-IgG. Flow cytometry [16] and the gel test [17,18] are semiquantitative methods. Despite the availability of these sensitive methods, there are no established standard tests for the diagnosis of Coombs-negative AIHA, which often makes it difficult for clinicians to diagnose AIHA in patients with DAT-negative hemolytic anemia. Therefore, the aim of the study was to assess the clinical utility of RBC-IgG levels in the diagnosis of Coombs-negative AIHA patients and to calculate the cutoff values, sensitivity, and specificity after a 1-year follow-up period.

Results

A total of 192 surveys were returned for analysis; a response rate of 78%. The mean age of participants was 51.0 ± 22.9 years (range 0.9–85) and 49% of participants were female. There were no significant differences in age and gender (49.7 ± 27.7 years old and 63% female) between responders and nonresponders to the survey. Of the responders, 144 were DAT-negative. Forty-seven percent of the DAT-negative hemolytic anemia patients (n = 68) were classified as AIHA; 64 had warm-type AIHA and four had cold-type AIHA. Significant differences were found in %Retic (P = 0.03), MCV (P = 0.01), LDH (P = 0.03), IDBIL (P = 0.03), and RBC-IgG (P < 0.0001) levels between the Coombs-negative AIHA and non-AIHA groups. There were no significant differences in age, gender, Hb, and Hp between the two patient groups.

The ROC curves for RBC-IgG levels, using clinical diagnosis as an indicator of AIHA, are shown in Fig. 1. Table I summarizes the AUCs, confidence intervals for AUCs, and likelihood ratios (LRs) for laboratory variables, as well as sensitivities and specificities, which were calculated at the

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optimal cutoff values (maximum point of efficiency curve). RBC-IgG showed the highest AUC values; the other three indices showed low AUC values. These data suggested that only RBC-IgG can effectively distinguish between the Coombs-negative AIHA and non-AIHA patients (see Fig. 2). Use of the cutoff point value (83) showed the sensitivity (70%), specificity (84%), and LR (4.8).

To discover the clinically significant cutoff values, the numbers of Coombs-negative AIHA patients were investigated according to age and gender. In females, there were two groups in the distribution of patients: one in the patients aged less than and one in those aged more than 45 years. In males, there was one peak in the patients aged more than 60 years. Together with the ROC curve for each group, the optimal cutoff values (maximum points of efficiency curve) were calculated for RBC-IgG levels. In females aged less than 45 years and those aged more than 45 years, the optimal cutoff values were 96 and 128, respectively. In males aged less than 60 years and those aged more than 60 years, the optimal cutoff values were 60 and 102, respectively.

Use of these cutoff points for 140 DAT-negative hemolytic anemia patients showed the slightly good sensitivity (71%), specificity (88%), and LR (5.9). Using these cutoff points, 41 cases of DAT-negative hemolytic anemia, which were referred to our laboratory in 2006, were categorized and showed slightly better sensitivity (78%), specificity (94%), and LR (14.1). Interestingly, all the pseudonegative cases had been treated with steroids (see Fig. 3). The 31 untreated cases could be grouped using one cutoff value as 78.5 (Fig. 3b) and showed high sensitivity (100%), specificity (94.1%) and LR [16]. Two patients with non-AIHA hemolytic diseases (drug-induced hemolytic anemia and myelodysplastic anemia) showed positive RBC-IgG, which might suggest the involvement of immunological hemolytic mechanisms [19]. In our laboratory, some patients with myelodysplastic anemia tended to show positive RBC-IgG (data not shown) mechanisms of which are analyzed by our collaborators.

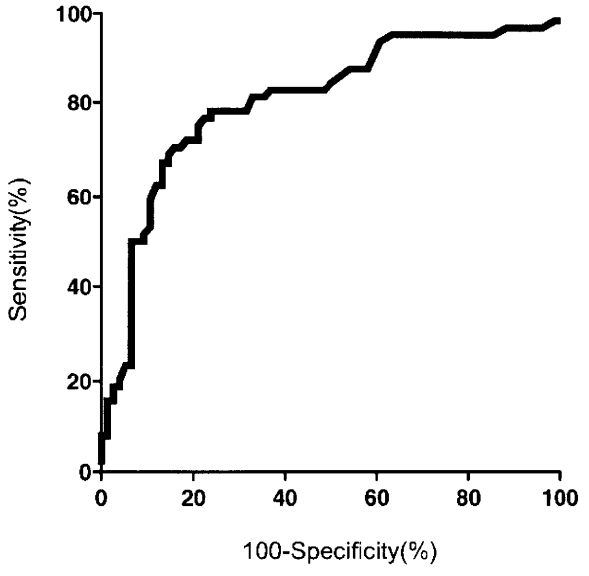

Figure 1. Receiver operating characteristic (ROC) curves for red-blood-cell-bound immunoglobulin G (RBC-IgG) (area under the ROC curves (AUC) 0.81), when the clinical diagnosis of AIHA by the attending doctors after 1-year follow-up was the gold standard.

Discussion

In the management of DAT-negative hemolytic anemia, it is important to distinguish Coombs-negative AIHA patients from other hemolytic anemia, because steroid treatment has major effects on AIHA [6], but steroids have also been associated with several serious side effects [20], which makes clinicians hesitate to use steroids to treat DAT-negative hemolytic anemia patients without diagnosis of AIHA.

In our laboratory, the immunoradiometric assay (IRMA) [12] is used to detect RBC-IgG quantitatively rather than semiquantitative methods such as flow cytometry [16] and gel column [17] for two reasons. First, in our laboratory IRMA has been used since 20 years ago as a central laboratory in Japan and its cost is supported by a grant for research on intractable diseases from the Ministry of Health, Labor and Welfare of Japan. Second, gel column method showed occasionally pseudopositive in some cases, the reason of which has remained unclear and flow cytometry requires normal RBCs as negative control in each measurement. Although the simple methods are desired, quantitative measurements should be used to guarantee their ability to measure subthreshold IgG.

Previously, we reported the value of RBC-IgG (33 ± 13) in 100 healthy Japanese adults and the RBC-IgG required to be DAT-positive (335 ± 72) [21]. Previous studies have reported very similar values [12,22]. The usefulness of RBC-IgG in diagnosis for Coombs-negative AIHA had been reported [23,24]. In previous studies, patients with Coombs-negative AIHA were reported to have abnormal levels of IgG, ranging from 70 to 434 [25] or from 76 to 350 [6]. There are, however, no reports referring to the IgG cutoff value, sensitivity, specificity, and LR. Practically, in our laboratory some non-AIHA samples from patients with hemolysis showed higher values than normal healthy individuals (Figs. 2 and 3), which might suggest the involvement of immunological hemolytic mechanisms [19]. In addition, some Coombs-negative AIHA patients had values very close to the normal range (see Fig. 2). So, in the practical diagnostic procedure, cutoff values must be calculated from RBC-IgG levels of DAT-negative patients with hemolysis rather than from normal RBC-IgG levels of healthy individuals. We have adopted the clinical diagnosis as the gold standard for the diagnosis of Coombs-negative AIHA because there are no established standards and many clinicians have previously clinically diagnosed Coombs-negative AIHA by the presence of hemolysis, denial of other

TABLE I. Area Under the ROC (Receiver Operating Characteristic) Curve (AUC), Confidence Intervals (95%) of AUCs, and Likelihood Ratio for Diagnosing AIHA in Patients With DAT-Negative Hemolytic Anemia (n = 140)

Parameter	AUC ± SE	Confidence interval (95%)	Cutoff point	Sensitivity	Specificity	Likelihood ratio
%Retic (%)	0.61 ± 0.05	0.51–0.71	6.8	54.9	82.8	1.9
MCV (fL)	0.65 ± 0.05	0.54–0.75	94.7	72.6	51.8	1.5
LDH (U/l)	0.62 ± 0.05	0.51–0.73	386	64.7	62.5	1.7
IDBIL (mg/dl)	0.62 ± 0.05	0.52–0.72	1.2	66.7	59.1	1.6
RBC-IgG	0.81 ± 0.04	0.73–0.88	83.0	70.3	84.2	4.8

Cutoff points, sensitivities, and specificities for each test are indicated in maximum points of the ROC curves. Calculated likelihood ratios are based on the cutoff points.

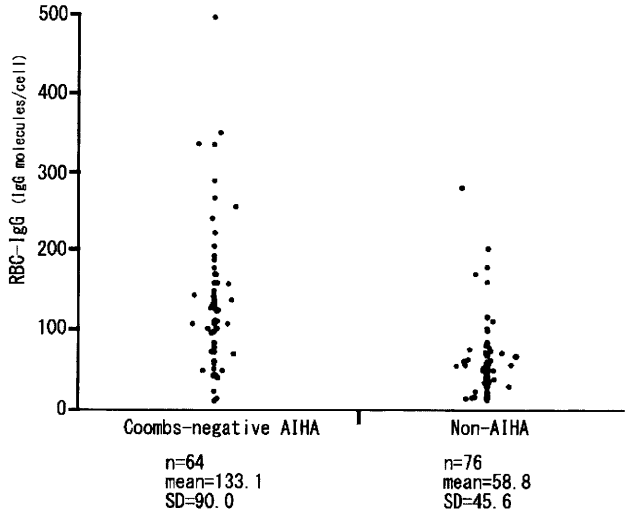

Figure 2. Red-blood-cell-bound IgG (RBC-IgG) of Coombs-negative AIHA and non-AIHA patients between 2003 and 2006. A significant difference was found in RBC-IgG levels ($P < 0.0001$) between the Coombs-negative AIHA and non-AIHA groups.

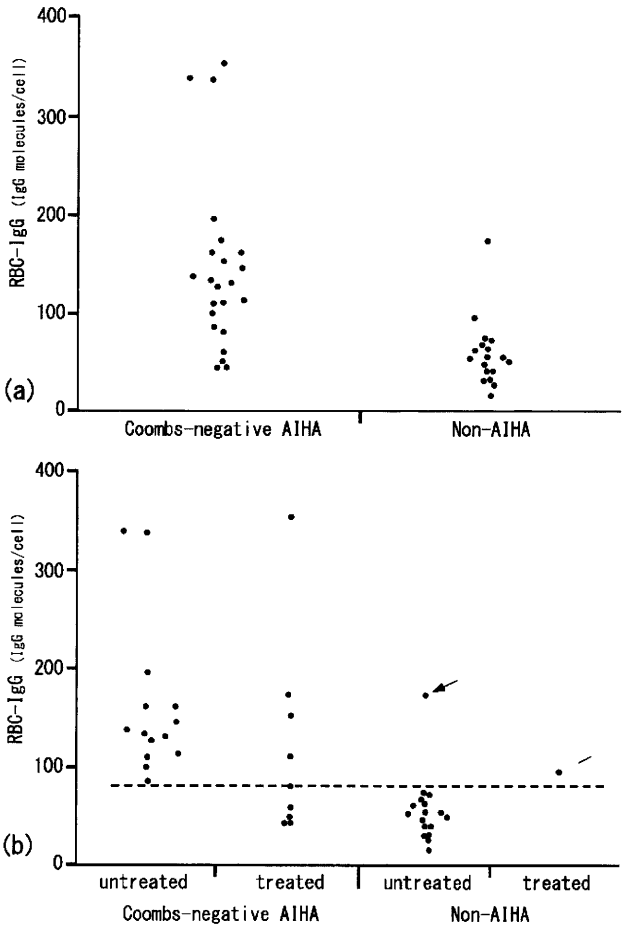

Figure 3. Red-blood-cell-bound IgG (RBC-IgG) of Coombs-negative AIHA and non-AIHA groups in 2006 (a). Each group consists of two subgroups treated or untreated with steroid drugs (b). A dotted line indicates 78.5 IgG molecules per cell. There were two non-AIHA patients with negative DAT, whose RBC-IgG levels were higher than 78.5. The black arrow represents a patient with drug-induced hemolytic anemia and the white arrow indicates a patient with hypoplastic myelodysplastic syndrome.

hemolytic diseases, and responsiveness to steroid treatments with respect of RBC-IgG value [26].

RBC-IgG levels of non-AIHA patients tended to increase with advanced age, especially in females. RBC-IgG levels of Coombs-negative AIHA in females also tended to increase with age and showed a gap at about 45 years of age, which gave two peaks at the ages of 25 and 65 years. In males, there was a smaller increase in RBC-IgG levels with advanced age, but Coombs-negative AIHA was most common in patients aged more than 60 years. These distribution trends were also reported in a previous report in Japanese patients [27]. The report suggested that these tendencies might be attributed to characteristics of AIHA, regardless of DAT-positive or -negative characteristics, and not to the population composition in Japan. In light of these trends, more effective cutoff values could be calculated using age- and gender-stratified analyses. Moreover, exclusion of the treated samples could increase the sensitivity and specificity of RBC-IgG and so it can be considered a standard approach for the diagnosis of Coombs-negative AIHA. Therefore, we propose that the RBC-IgG level should be measured for the diagnosis of Coombs-negative AIHA and the cutoff value used should be 78.5 if RBC-IgG is measured before treatment, and that after treatment the RBC-IgG level might range from the Coombs-negative value to as low as that seen in normal healthy individuals (see Fig. 3).

Materials and Methods

The study was performed over a period of 4 years from 2003 to 2006 at the laboratory of the Center for Community Medicine, Jichi Medical University, Tochigi, Japan, after approval by the Institutional Ethics Panel Committee.

Patients. During a 4-year period, 261 samples from 245 patients were referred to our laboratory for quantitation of RBC-IgG. Of these, 54 (22%) were DAT-positive and 191 (78%) were DAT-negative, as shown by analysis of polyspecific DAT by CTT (Ortho Diagnostics, USA) and monospecific DAT by CTT using anti-IgG and anti-C3d antibodies (Ortho Diagnostics, USA) following manufacturer's instructions.

Sample preparation. Heparinized whole blood (10 ml) samples were collected. The RBC layer was prepared by centrifuging the whole blood at 1,000 rpm for 20 min. The supernatant plasma and buffy coat were discarded. One milliliter samples of packed RBCs were diluted in 10 ml of phosphate-buffered saline (PBS), pH 7.0, 0.15 M. The diluted RBCs were passed through a cotton-wool column to exclude neutrophils and monocytes, according to the method of Jeje et al. [12]. The RBCs were washed four times with PBS, and the resulting RBCs (0.3 ml) were suspended in 0.4 ml of PBS.

Immunoradiometric assay (IRMA) for RBC-IgG. IRMA for RBC-IgG was performed according to the method of Jeje et al. [12] with some modifications. Samples of ^{125}I-labeled anti-human IgG antisera derived from goat (Du Pont, Wilmington, DE) were diluted in PBS containing 3% bovine serum albumin with a specific activity of ~10,000 cpm/200 μl (Wakojunyaku, Osaka, Japan). A volume of 400 μl of the washed RBCs was incubated for 1 h at 37°C with 200 μl of the diluted anti-human IgG. IgG beads were prepared using the methods described by Jeje et al. [12]. Human IgG and beads (SephasorbTM HP) were purchased from Sigma Chemical (St. Louis, MO) and Pharmacia Fine Chemicals (Uppsala, Sweden), respectively. Two hundred microliter samples of IgG beads (2×10^6) were added to the mixture of RBCs and ^{125}I-labeled anti-human IgG and incubated at 37°C for 30 min. The RBCs were lysed by the addition of 80 μl of 20% Triton X-100 (Sigma). The beads were washed four times with 20% Triton X-100-containing PBS, and the radioactivity was measured using a gamma counter (Aroka, Tokyo, Japan). A standard curve was generated using human IgG standards (10–10,000 ng IgG/ml; Sigma). The percent inhibition of binding was plotted against each concentration of IgG. Using the standard curve, RBC-IgG levels were calculated after counting the number of RBCs. Each attending doctor was informed of the RBC-IgG level within 3–10 days of ordering.

Clinical diagnosis questionnaire. At 1 year after referral to our laboratory, follow-up investigations were performed; the attending doctor used a questionnaire to assess the patient's clinical diagnosis. The bases of

clinical diagnosis of Coombs-negative AIHA were in vivo hemolysis (low hemoglobin (Hb) concentration, high percentage of reticulocyte (%Retic), high indirect serum bilirubin (IDBIL) level, high lactate dehydrogenase (LDH) level, low haptoglobin (Hp) level and/or high erythropoiesis level in bone marrow) and exclusion of other anemic icteric diseases without hemolysis (such as megaloblastic anemia, myelodysplastic syndrome, erythroid leukemia, congenital dyserythropoietic anemia, hepatobiliary diseases, and constitutional jaundice). AIHA was diagnosed by measuring the RBC-IgG level, steroid-reactivity and exclusion of alloimmune hemolytic anemia and drug-induced hemolytic anemia.

Statistical analysis. Patients who had negative DAT were divided into AIHA and non-AIHA groups on the basis of clinical diagnosis. The normality of the laboratory variables was analyzed using the Kolmogorov-Smirnov test with Lilliefors significance correction. As most variables were not normally distributed, a Mann-Whitney *U*-test was used to determine the differences between Coombs-negative AIHA and non-AIHA patients. The median range and interquartile range were also calculated for all variables.

The accuracy of the tests for diagnosis of AIHA in DAT-negative hemolytic anemia patients was evaluated using receiver operating characteristic (ROC) curves [28]. By this method, a test that is perfect has 100% sensitivity and no false-positives (1-specificity = 0) and will have an area under the curve (AUC) of 1.0, whereas a test that has no diagnostic value would have an AUC of 0.5. The 95% confidence intervals and LRs were also calculated.

JMP 7.0.1 for Macintosh (SAS Institute, Cary, NC) and GraphPad Prism 4.0c for Macintosh (GraphPad Software, San Diego, CA) were the statistical software used.

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