Introduction

Myelodysplastic syndromes (MDS) are clonal disorders of hematopoietic cells with variable clinical course and risk of evolution into acute myeloid leukemia (AML).[1,2] MDS appear to be the most common myeloid malignancy and their incidence increases steeply with age.[3] The diagnosis of MDS is based on a combination of clinical history, the morphological features of the peripheral blood (PB) and BM (e.g., percentages of blasts and dysplasia of cells), cytogenetic data, and ruling out other diseases.[4,5] The diagnosis is straightforward if clearly objective abnormalities, i.e., increase in blasts and/or ringed sideroblasts (RS) and/or the presence of chromosomal aberration, are detected. In other words, a diagnostic challenge exists in low-grade MDS that lack conventional, specific diagnostic markers, RS and karyotypic aberration. The diagnosis of this category (called *low-grade MDS without conventional markers* in this paper) largely relies on the presence of dysplasia, and therefore experienced examiners (hematologists/hematopathologists) are required to make the diagnosis. On the other hand, the dysplastic features of myeloid cells do not in themselves establish a diagnosis. Conditions other than MDS can induce dysplastic myeloid cells (e.g., deficiencies of vitamin B_{12} and folate, viral infections, ethanol or lead), and thus such conditions should be ruled out by careful history taking and physical and laboratory examinations.

Flow cytometry (FCM) has been established as a useful, routine diagnostic tool for acute leukemia and Non-Hodgkin's lymphoma but not for MDS. Recently, we and others have reported prototypes of diagnostic FCM for MDS.[6-10] Based on those recent developments, in a report from a recent international working conference on MDS, FCM was proposed as one diagnostic test.[11] To make diagnostic MDS FCM widely applicable, further studies on the parameters and analytical strategy usable in many laboratories are required.[12]

This study was conducted by two laboratories that have been working on diagnostic FCM for low-grade MDS.[7,9,10,13] We investigated the diagnostic utility of our protocol, which was designed to be applicable in many laboratories and to minimize variations among them, for prospective application in low-grade MDS patients, in particular low-grade MDS without conventional markers, in the two laboratories.

Design and Methods

Patients

This study included four cohorts of patients. The first was a cohort reported in our previous study, in which FCM data on BM cells from 27 low-grade MDS patients lacking RS and 90 non-MDS patients including 70 nonclonal cytopenic patients were collected.[10] The second cohort was made up of patients used for preliminary experiments, who were selected from patients who underwent BM aspiration in Nippon Medical School for diagnostic purposes. They consisted of 13 low-grade MDS patients lacking RS and 30 nonclonal cytopenic patients. Reference ranges (RRs) of parameters were determined using data from these cohorts before prospective FCM analysis (*see Results*). Patients in the prospective analysis were consecutive patients who underwent BM aspiration in our institutions in Japan (the third cohort) and Italy (the fourth cohort) and who were diagnosed with low-grade MDS or non-clonal cytopenia using conventional methods. These patients were either new patients with abnormal PB findings not ruling out low-grade MDS (e.g., cytopenia and/or macrocytosis without circulating blasts) for whom clinicians performed BM examination, or patients who had already been diagnosed with low-grade MDS and underwent BM examination to evaluate their disease status. Patients' BM samples were subjected to the present FCM analysis as well as to conventional laboratory tests, which included cytological and pathological examinations, and cytogenetic analysis using the standard G-banding technique.[14] Data on patients who underwent FCM analysis but had neither low-grade MDS nor non-clonal cytopenia, e.g., high-grade MDS, were excluded.

MDS was diagnosed according to the World Health Organization (WHO) criteria,[15] as described preiously.[16] In cases suspected to be low-grade MDS without conventional markers, observation was carried out for six months prior to making a diagnosis of MDS. Other diseases were also ruled out by repeated history taking and physical and laboratory examinations including follow-up PB data, for example, normalization of PB data after specific therapy for non-clonal cytopenia, and, if necessary, repeated BM examination. Karyotypes were interpreted using the standard criteria.[17] The International Prognostic Score System (IPSS) and WHO classification-based prognostic scoring system (WPSS) were applied to MDS patients according to previous reports.[18,19] Written, informed consent was obtained from all patients. This study protocol was approved by the Ethics Committee, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy, and by the Institutional Review Board of Nippon Medical School, Tokyo, Japan.

Flow cytometry

BM cells of the patients were aspirated into a heparinized syringe, immediately diluted with RPMI 1640 medium containing 10% fetal calf serum, and stored at 4°C overnight. The next morning (19-24 h after cell aspiration), nucleated cells were counted and stained with antibodies.

Then, samples were treated with the standard ammonium chloride method to lyse erythrocytes and washed with phosphate-buffered saline. The rationale for sample handling was described in our previous report.[10] Antibody staining was performed as follows: 100 μL of a cell aliquot containing $5–8\times10^5$ nucleated cells were placed into each tube and stained with three antibodies conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), or peridin chlorophyll (PerCP). Combinations of the three antibodies were CD10/CD34/CD45 (FITC/PE/PerCP), CD15/CD34/CD45, CD34/CD11b/CD45, and CD34/CD56/CD45. Antibodies were obtained from Becton Dickinson (BD, Franklin Lakes,

NJ, USA) (CD45-PerCP, CD34-PE, CD11b-PE, and CD15-FITC) and Beckman Coulter (Fullerton, CA) (CD34-FITC, CD10-FITC, and CD56-PE). The optimal volume of each antibody reagent for the staining was determined beforehand. Fluorescence minus one (FMO) controls were used as described previously.[13,20,21] Data were acquired using a FACSCalibur and FACSCanto flow cytometer (BD) in Tokyo and Pavia, respectively. At least 100,000 cell events were acquired.

Data were analyzed with CellQuest software (BD) by investigators who were blinded to the patients' clinical and laboratory data, including the diagnosis. First, four parameters were analyzed from a cell sample stained with the CD10/CD34/CD45 antibody combination. Analytical methods for three of these four parameters have been described previously.[13] On the forward scatter (FSC)-versus-side scatter (SSC) display, we defined *all nucleated cells* (R1, Figure 1A) and cells with relatively low SSC were gated (R2) and then plotted on a CD45-versus-CD34 display (Figure 1B).[13] Next, CD34$^+$ cells with intermediate CD45 expression were gated (R3) and then plotted on a CD45-versus-SSC display (Figure 1C), in which CD34$^+$ B-cell progenitors (stage I hematogones) formed an easily recognizable cluster (R5, arrow).[10,22,23] Other CD34$^+$ cells (R4) consist predominantly of myeloblasts, and thus these cells are called CD34$^+$ myeloblasts in this paper. Meanwhile, all cells were plotted on a CD45-versus-SSC display (Figure 1D) and granulocytic cells other than myeloblasts (R6, called granulocytic cells in this paper) and lymphocytes (R7) were gated. For granulocytic cells, the CD10-negative cell fraction was gated (R8, Figure 1E). Then we examined the following parameters: (i) the percentage of CD34$^+$ myeloblasts in all nucleated cells (called CD34$^+$ myeloblasts [%] in this paper); (ii) the percentage of CD34$^+$ B-cell progenitors in all CD34$^+$ cells (called CD34$^+$ B cells [%] in this paper); 3) Ly/Mbl CD45 ratio (mean fluorescence intensity [MFI] of CD45 on lymphocytes ÷ MFI of CD45 on CD34$^+$ myeloblasts); and 4) Gra/Ly SSC peak channel ratio (SSC channel number where the maximum number of CD10- granulocytic cells occurs ÷ SSC channel number where the maximum number of lymphocytes occurs). From cell samples stained with CD15/CD34/CD45, CD34/CD11b/CD45, and CD34/CD56/CD45 combinations, we analyzed the three other parameters. We gated CD34$^+$ myeloblasts as described above and their CD15, CD11b, and CD56 expression was quantified by determining the percentages of antigen-positive cells in all CD34$^+$ myeloblasts.

Determination of interlaboratory variability of flow data

Each of 10 BM samples obtained from the second patient cohort was divided into three aliquots and distributed to three laboratories in Tokyo. An FCM operator in each laboratory analyzed the above seven parameters according to the analysis manual written and distributed by Nippon Medical School. The manual contained all information described above, e.g, cell number, reagents for staining, and analytical methods.

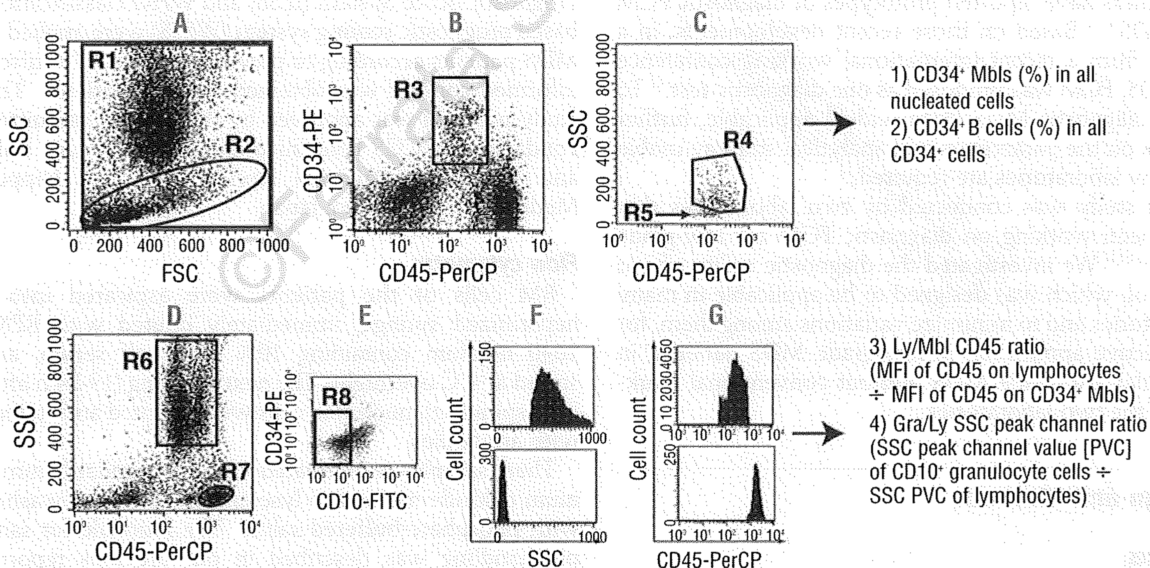

Figure 1. Analysis of four cardinal parameters from a single cell aliquot stained with CD10-FITC, CD34-PE, and CD45-PerCP antibodies. (A) All nucleated cells (R1) and cells with relatively low SSC (R2). (B) Cells in R2 in panel A were displayed on a CD34-versus-CD45 plot. CD34$^+$ cells with intermediate CD45 expression were gated (R3). (C) Cells in R3 in panel B were displayed on a CD45-versus-SSC plot. A cluster of CD34$^+$ B-cell progenitors was identified in the lower left region of CD34$^+$ cells (R5, arrow). The reliability of the R5 region was confirmed based on CD10 positivity. Cells in R4 were composed mainly of myeloblasts (Mbls) and thus simply called CD34$^+$ Mbls in this paper. (D) Granulocytic cells other than Mbls (R6, called granulocytic cells in this paper) and lymphocytes (R7) were gated on a CD45-versus-SSC plot. (E) Cells in R6 in panel D were displayed, and the CD10$^-$ fraction was gated (R8). (F) SSC of CD10$^-$ granulocytic cells (upper panel) and lymphocytes (lower panel). SSC peak channel values (SSC channel number where the maximum number of cells occurs) of both fractions were computed using the software. (G) CD45 expression of CD34$^+$ Mbls (upper panel) and lymphocytes (lower panel). Mean fluorescence intensity (MFI, GeoMean) of CD45 of both fractions was computed.

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Statistical analysis

Differences between two groups of data of continuous variables were analyzed using Student's t-test. Differences in categorical variables were evaluated using the χ^2 test. Interlaboratory variability of FCM data was determined based on the coefficient of variation (CV [%] = standard deviation [SD] ÷ mean x 100). To estimate the diagnostic power of the present FCM method, we calculated specificity, sensitivity, and the likelihood ratio according to the standard method,[24] and 95% confidence intervals (CI) of specificity and sensitivity were based on the binomial distribution.[25]

Results

Determination of reference ranges and their application to the preliminary cohort

Using data from the first patient cohort, we analyzed four parameters, CD34⁺ myeloblasts (%), CD34⁺ B cells (%), Ly/Mbl CD45 ratio, and Gra/Ly SSC peak channel ratio, and compared the data between 27 low-grade MDS patients and 70 non-clonal cytopenic patients (*Online Supplementary Figure S1*, upper panel, most data on CD34⁺ myeloblasts, CD34⁺ B cells, and Ly/Mbl CD45 ratio were

Table 1. **Patients in prospective cohorts.**

Diagnosis	Number (M/F)	Age (yr, mean ± SD)	IPSS	WPSS
Japanese cohort				
Non-clonal cytopenic patients	43 (18/25)	59 ± 18		
Immune thrombocytopenic purpura	17			
Anemia of chronic disease[1]	7			
Renal anemia	5			
Immune bicytopenia	3			
Iron-deficiency anemia[2]	3			
Others[3]	8			
Low-grade MDS patients	46 (26/20)	71.2 ± 14.4	10/28/4 (4)	6/21/2/5 (12)
RA	17			
RARS	2			
RCMD	17			
RCMD-RS	3			
MDS-U	7			
Patients without conventional markers[4]	26 (14/12)	68.9±16.3	7/16/0 (3)	4/14/1/0 (7)
Italian cohort				
Non-clonal cytopenic patients	63 (29/34)	60 ± 15		
Immune thrombocytopenic purpura	15			
Anemia of chronic disease[1]	12			
Anemia in liver disease	9			
Immune bicytopenia or pancytopenia	6			
Renal anemia	5			
Iron-deficiency anemia	2			
Autoimmune hemolytic anemia	2			
Others[3]	12			
Low-grade MDS patients	88 (49/39)	65.8 ± 12.5	49/32/3 (4)	27/33/17/7 (4)
RA	27			
RARS	13			
RCMD	37			
RCMD-RS	8			
5q– syndrome	3			
Patients without conventional markers[4]	55 (30/25)	63.4 ± 13.2	36/18/0 (1)	18/24/12/0 (1)

RA: refractory anemia; RARS: refractory anemia with ringed sideroblasts; RCMD: refractory cytopenia with multilineage dysplasia; RCMD-RS: refractory cytopenia with multilineage dysplasia and ringed sideroblasts; MDS-U: MDS-unclassified; 5q– syndrome: MDS associated with isolated del(5q); data for IPSS: low/intermediate-1/intermediate-2 (not applicable); data for WPSS: very low/low/intermediate/high (not applicable); [1]Japanese cohort: 1 congestive heart failure (CHF), 1 CHF with primary biliary cirrhosis, 1 rheumatoid arthritis, 1 systemic lupus erythematosus (SLE), 1 colon cancer, and 2 fever of unknown origin. Italian cohort: 5 systemic autoimmune disease (e.g., SLE), 4 carcinomas, 2 chronic bacterial infections, and 1 inflammatory bowel disease; [2]1 patient had complication with transient leukopenia; [3]Japanese cohort: 1 marginal anemia, 1 marginal leukopenia, 2 chronic idiopathic neutropenia, 1 hypomegakaryocytic thrombocytopenic purpura, 1 pernicious anemia, 1 alcoholic cytopenia, and 1 Sweet's syndrome with marginal anemia. Italian cohort: 7 chronic neutropenia (marginal to moderate), 2 drug-induced cytopenia, 1 transient cytopenia, 1 pernicious anemia, and 1 congenital sideroblastic anemia; [4]Patients lacked RS and karyotype abnormality. They included patients in whom a sufficient number of metaphases for karyotyping were not available (3 Japanese and 1 Italian). In other patients, more than 20 metaphases were analyzed and all were normal. Three Italian patients who did not undergo karyotyping were excluded.

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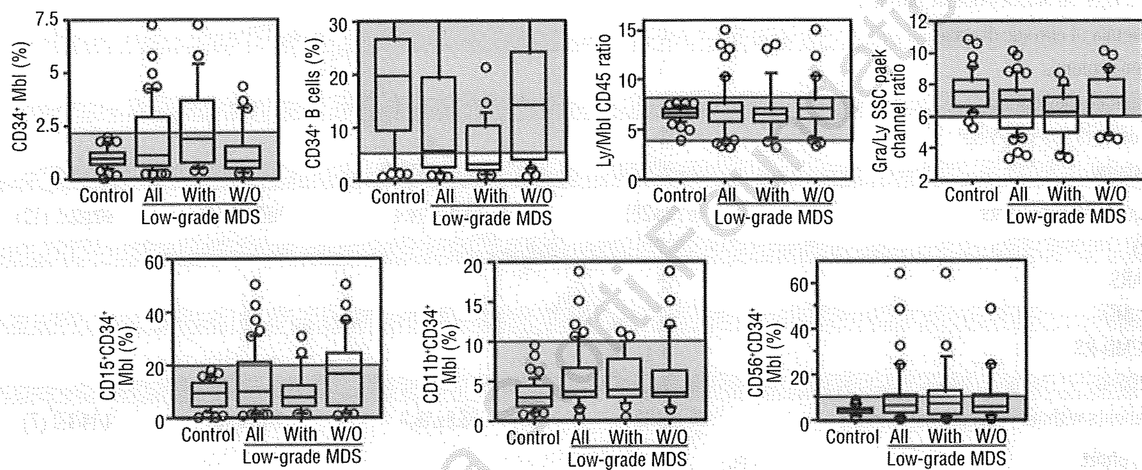
in our previous paper)[15]. Meanwhile, using BM samples from the second cohort, we determined CD15, CD11b, and CD56 expression on CD34+ myeloblasts (*Online Supplementary Figure S1*, lower panel) in addition to the above four parameters. The data suggest that these seven parameters might be useful in differentiating the two patient groups. Next, we examined the interlaboratory data variability of all seven parameters adopted (*Online Supplementary Table S1*). The data on CD34+ myeloblasts (%), CD34+ B cells (%), Ly/Mbl CD45 ratio, and Gra/Ly SSC peak channel ratio showed little interlaboratory variability, although data on CD11b, CD15, and CD56 expression (%) on CD34+ myeloblasts showed greater interlaboratory variability.

We then determined RRs for all parameters. For parameters showing little interlaboratory data variability, RRs were determined based on the receiver-operator characteristic curve using the data in the *Online Supplementary Figure S1*: CD34+ myeloblasts <2.4%, CD34+ B cells >5%, 4 <Ly/Mbl CD45 ratio <7.8, and Gra/Ly SSC peak ratio >6. For parameters showing substantial interlaboratory data variability, RRs were set to reduce/avoid events when nonclonal samples showed abnormal results (false-positive events), that is, CD11b expression on CD34+ myeloblasts <10%, CD15 expression on CD34+ myeloblasts <20%, and CD56 expression on CD34+ myeloblasts <10%. Using these RRs, the data from three laboratories shown in the *Online Supplementary Table S1* did not show any false-posi-



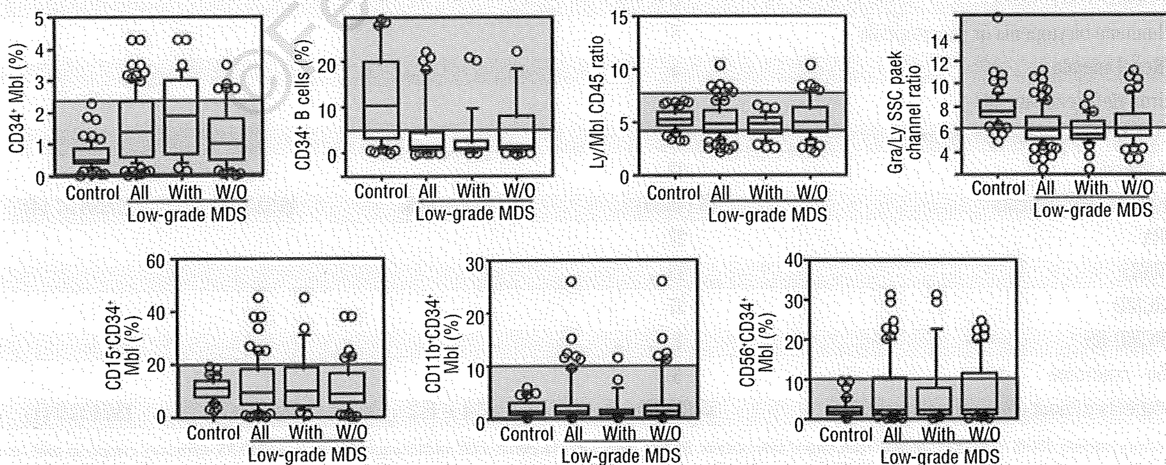


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Figure 2. **Analysis of seven flow cytometry parameters in the prospective cohorts. The horizontal lines in each boxplot represent the 90th, 75th, 50th, 25th, and 10th percentiles. Circles are outliers. Controls are nonclonal cytopenic patients. "All" indicates all low-grade myelodysplastic syndromes (MDS) patients, "With" indicates low-grade MDS with conventional markers, and "W/O" indicates low-grade MDS without conventional markers. Shaded areas are the predetermined reference ranges.**

tive results for all samples from non-clonal cytopenic patients (n=8).

Then, we applied FCM scoring for patients in the second cohort: one point was given for each parameter for which data were outside the RR. For example, if the data for two parameters were outside the RRs in an individual, the FCM score was 2. The results of scoring using four parameters (CD34⁺ myeloblasts [%], CD34⁺ B cells [%], Ly/Mbl CD45 ratio, and Gra/Ly SSC peak ratio) and all seven parameters are shown in *Online Supplementary Table S2*. An FCM score of 2 or more was not observed in any cytopenic control but observed in 46% and 69% of low-grade MDS patients in the scoring using four and seven parameters, respectively.

Application of diagnostic flow cytometry to the prospective cohorts

Using the same approach, we prospectively analyzed consecutive patients in Japan and Italy. Methodological details described in the Methods section and RRs were distributed to both laboratories beforehand. We defined CD34⁺ myeloblasts (%), CD34⁺ B cells (%), Ly/Mbl CD45 ratio, and Gra/Ly SSC peak ratio as cardinal parameters that must be analyzed for every sample, and defined CD11b, CD15, and CD56 expression on CD34⁺ myeloblasts as adjunctive parameters that should be analyzed in as many samples as possible. Patients analyzed are shown in Table 1. MDS subtypes in the Japanese cohort reflect the characteristics of Japanese MDS. That is, compared with MDS patients in Western countries, 5q- syndrome and MDS with RS are less frequent and hematopoietic cells are less dysplastic.[26] Among 134 low-grade MDS patients, 81 (26 Japanese and 55 Italians) had low-grade MDS without conventional markers. All seven parameters were analyzed in all Japanese patients and in 38 non-clonal cytopenic patients and 66 low-grade MDS patients in the Italian cohort. All data from the Japanese and Italian cohorts are shown in Figure 2. The data from both cohorts showed the same characteristics. When looking at all low-grade MDS patients in these cohorts (the second boxplot from the left in each panel of Figure 2), the data on CD34⁺ myeloblasts (%) deviated upward and those on CD34⁺ B cells (%) and Gra/Ly SSC peak ratio deviated downward compared with those in non-clonal cytopenic patients (the extreme left plot in each panel of Figure 2). Meanwhile, the Ly/Mbl CD45 ratio deviated upward or downward depending on the individual patient in MDS. Furthermore, CD34⁺ myeloblasts from a considerable proportion of low-grade MDS patients expressed higher levels of CD15, CD11b, and CD56 compared with those from non-clonal cytopenic patients. The above characteristics also held true when low-grade MDS patients who had RS and/or karyotype abnormality (low-grade MDS with conventional markers) and low-grade MDS without conventional markers were analyzed separately (the two right plots in each panel). When data from these low-grade MDS groups were compared, low-grade MDS patients with conventional markers had more *CD34⁺ myeloblasts (%)* and less *Gra/Ly SSC peak ratio* compared with low-grade MDS patients without conventional markers in both cohorts (*not shown in detail*).

The results of FCM scoring using the predetermined RRs are shown in Table 2. When all low-grade MDS patients were scored using data from the four cardinal parameters, 20 of 46 (43.5%) Japanese and 62 of 88 (70.5%) Italian patients scored 2 or more, among whom 13 of 46 (28.3%) Japanese and 25 of 88 (28.4%) Italian patients scored 3 or more. However, among non-clonal cytopenic patients, most scored 0 or 1, only a few

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Table 2. **Flow scores of patients in the prospective cohorts.**

	Flow score using 4 parameters						2 or more[1]			Flow score using 7 parameters							2 or more[1]		
	0	1	2	3	4	Cases positive/ cases examined	Sensitivity (%)	Specificity (%)	Likelihood ratio	0	1	2	3	4	5	Cases positive/ cases examined	Sensitivity (%)	Specificity (%)	Likelihood ratio
Japanese cohort																			
Non-clonal cytopenia	35	7	1	0	0	1/43				35	7	1	0	0	0	1/43	72	98	30.9
All low-grade MDS patients	12	14	7	11	2	20/46	44 (29-59)	98 (88-100)	18.7 (3.6-108.8)	2	11	15	12	5	1	33/46	(57-84)	(88-100)	(6.5-176.1)
Patients with conventional markers	4	4	5	6	1	12/20	60 (36-81)	98 (88-100)	25.8 (5.2-151.5)	0	4	7	7	1	1	16/20	80 (56-94)	98 (88-100)	34.4 (7.7-195.0)
Patients without conventional markers	8	10	2	5	1	8/26	31 (14-52)	98 (88-100)	13.2 (2.4-80.7)	2	7	8	5	4	0	17/26	65 (44-83)	98 (88-100)	28.1 (5.8-162.8)
Italian cohort																			
Non-clonal cytopenia	38	20	5	0	0		5/63			22	12	4	0	0	0	4/38			
All low-grade MDS patients	8	18	37	20	5	62/88	71 (60-80)	92 (82-97)	8.9 (4.2-20.4)	3	6	26	25	6	0	57/66	86 (76-94)	90 (75-97)	8.2 (3.9-19.4)
Patients with conventional markers	1	7	13	9	3	25/33	76 (58-89)	92 (82-97)	9.5 (4.6-20.7)	1	3	5	5	5	0	15/19	79 (63-89)	90 (75-97)	7.5 (3.3-16.2)
Patients without conventional markers	7	11	24	11	2	37/55	67 (53-79)	92 (82-97)	8.5 (4.0-19.5)	2	3	21	20	1	0	42/47	89 (77-97)	90 (75-97)	8.5 (4.1-18.4)

[1]*Data are the diagnostic power of the "flow score 2 or more." Data in parentheses are 95% CI.*

patients scored 2 (1/43 [2.3%] Japanese and 5/63 [7.9%] Italian patients), and none scored 3 or more. When data from all seven parameters were used to score all low-grade MDS patients, 33 of 46 (71.7%) Japanese and 57 of 66 (86.4%) Italian patients scored 2 or more, among whom 18 of 46 (39.1%) Japanese and 31 of 66 (47.0%) Italian patients scored 3 or more. In non-clonal cytopenic patients, most also scored 0 or 1, a few scored 2 (1/43 [2.3%] Japanese and 4/38 [10.5%] Italian patients), and none scored 3 or more. When low-grade MDS with conventional markers and low-grade MDS without conventional markers were scored separately, the results were similar between these two groups, except for the scoring using the four cardinal parameters in the Japanese cohort, among whom the number of patients who scored 2 or more was greater in low-grade MDS with conventional makers than in low-grade MDS without conventional markers (12/20 *vs.* 8/26, p=0.047).

Based on the above data, the diagnostic power of the present FCM scoring, i.e., sensitivity, specificity, and more importantly the likelihood ratio, was calculated (Table 2). When patients lacking excess blasts, RS, and karyotype abnormality scored 2 or more in the present FCM method, the likelihood ratio of MDS (low-grade MDS without conventional markers) was 13.2 (four-parameter method, sensitivity for positivity 31%) and 28.1 (seven-parameter method, sensitivity 65%) in the Japanese cohort and 8.5 (in both four-parameter and seven-parameter methods with sensitivities 67% and 89%, respectively) in the Italian cohort.

When the *FCM score 3 or more* is defined as positive, the FCM scoring is 100% specific to MDS in our cohorts, and thus the likelihood ratio is infinity. However, the sensitivity of the test is reduced. In detecting low-grade MDS patients without conventional markers, the sensitivity was 23% and 35% (four- and seven-parameter methods, respectively) in the Japanese and 24% and 45% (four- and seven-parameter methods, respectively) in the Italian cohorts. Other combinations of parameters, e.g., CD34+ myeloblasts (%) and CD11b, CD15, and CD56 expression (%) on CD34+ myeloblasts, did not improve the diagnostic power.

Analysis of clinical parameters in association with flow cytometry scores in patients in the prospective cohorts

Age and gender do not affect low-grade MDS detection using the FCM score, because they did not differ between non-clonal cytopenic patients with an FCM score of 1 or 2 and those with an FCM score of 0 in either cohort, and did not differ between low-grade MDS patients with an FCM score of less than 2 and those with an FCM score of 2 or more. The analysis of clinical parameters in association with FCM scores in MDS patients is summarized in the *Online Supplementary Table S3*. In the Italian cohort, the presence of multilineage dysplasia was associated with a high prevalence of FCM scores of 2 or more, but this was not the case in the Japanese cohort. A common phenomenon in both cohorts was that MDS patients for whom a poor prognosis was indicated by the IPSS or WPSS were associated with a high prevalence of FCM scores of 2 or more. Karyotype categories and transfusion dependency, which are components in the IPSS and/or WPSS, were not associated with the FCM score. The presence or absence of chromosomal aberrations as well as the presence or absence of RS (> 15% of erythroblasts) was not associated with the FCM score in either cohort (p>0.12 in all analyses).

Discussion

To enable this FCM protocol to be applied in many laboratories with acceptable data variation, our method has the following characteristics: (i) three-color FCM was adopted rather than four or more-color FCM, because numerous laboratories use the former and data reproduction is probably easier with this; (ii) four cardinal parameters, which can be analyzed from a single cell aliquot and show little interlaboratory variability, were adopted.

The rationale for choosing our seven parameters was as follows: two major components in marrow CD34+ cells behave in an opposing fashion in MDS: CD34+ myeloblasts increase while CD34+ B cells decrease when compared with marrow samples from control individuals.[10] Therefore, analyzing these two cell populations separately rather than analyzing all CD34+cells is more accurate and sensitive in detecting MDS-related changes. While other methods can be used to measure CD34+ B cells, such as examining CD34+CD19+ cells, we adopted the present method because data variability appears to be lower than with other methods.[23]

The Ly/Mbl CD45 ratio and the newly introduced Gra/Ly SSC peak channel ratio ensured the reproducibility of data by adjusting data on target cells with data on lymphocytes in the same sample. Although other groups reported that myeloblast CD45 expression decreases in a fraction of MDS patients,[27,28] by analyzing the Ly/Mbl CD45 ratio, we showed that myeloblast CD45 expression can increase or decrease in MDS. It was reported that the SSC of granulocytic cells decreases in a variable proportion of MDS patients.[6,27,29] In preliminary experiments, we found that data on the SSC peak channel were more useful in discriminating MDS from controls rather than other SSC-related data, such as mean SSC value. Also, we targeted CD10- granulocytic cells rather than all granulocytic cells in analyzing the Gra/Ly SSC peak channel ratio. There was no significant difference in the discriminating power between the Gra/Ly SSC peak channel ratios using CD10- granulocytic cells and using all granulocytic cells (*data not shown*). However, using the former might be beneficial to analyze samples contaminated significantly with PB, because most circulating neutrophils express CD10.[30]

In addition to the above cardinal parameters, we adopted three others, CD15, CD11b, and CD56 expression of CD34+ myeloblasts. MDS myeloblasts often show dysregulated expression of a variety of antigens.[10,31] Among these antigens, we selected CD15, CD11b, and CD56 based on the frequency of dysregulation and feasibility of discrimination between antigen-positive and antigen-negative cells. Finally, it should be noted that none of our parameters, except for *CD34+ myeloblasts*

(%), was affected by contamination with PB. Collecting data from a sufficient number of CD34+ cells provides necessary data on CD34-related parameters even if BM samples are diluted with PB. However, the dilution can influence *all nucleated cells* assessed by FCM and thus may cause falsely low *CD34+ myeloblasts (%)* data.

By applying these quantitative FCM parameters, we determined their RRs, which enabled us to judge the prospective FCM data objectively. We used non-clonal cytopenic patients as controls, rather than healthy individuals and patients with clonal diseases other than MDS, because non-clonal cytopenic patients are the main population that must be differentiated from MDS in clinical practice.[4,5] At the same time, our MDS patients included low-grade subtypes alone. In particular, the prospective cohorts included 81 low-grade MDS patients without conventional markers. This is the largest study analyzing this MDS category using diagnostic FCM. We found that our scoring discriminated low-grade MDS patients from non-clonal cytopenic patients with likelihood ratios suitable for diagnosis. Likelihood ratios with pre- and post-test probabilities, which are capable of refining clinical diagnoses,[24,32] may be underused in clinical practice.[33] If patients with a 50% pre-test probability of having MDS are positive for a diagnostic test with a likelihood ratio of 8–10, the post-test probability of having MDS is greater than 90%.[24] We would like to emphasize the importance of the diagnostic strategy using likelihood ratios, because FCM data in low-grade MDS are usually not absolutely specific, in contrast to other specific data in MDS, i.e., blast excess and cytogenetic aberrations.

There was a difference in the diagnostic power of the present FCM method between the Japanese and Italian cohorts. We think that although our parameters and their RRs have advantages for general use, fine-tuning of the RRs in each laboratory may further improve the diagnostic power. It should also be noted that FCM abnormalities may differ between ethnic groups as does cytomorphological dysplasia.[26] It is also noted that, in addition to the scoring that we used in this paper, the degree of abnormality of flow data might help diagnose low-grade MDS. Data from six parameters other than CD34+ B cells (%) showed Gaussian distribution in patients with non-clonal cytopenia (Figure 2). Therefore, it is expected that if data on these parameters deviate more from the RRs, the diagnosis of low-grade MDS is more certain. Furthermore, only one-parameter data would be diagnostic if they deviated profoundly from RRs. This approach is worthy of examination in future prospective studies.

Finally, FCM scores were associated with IPSS and/or WPSS but not with the karyotype categories or the presence or absence of karyotype abnormality in our cohorts. This suggests the possibility that our FCM protocol detects MDS-related abnormality that cannot be detected by chromosomal analysis and is linked with prognosis, as suggested by other studies.[31,34,35] Further study is required to verify whether the present FCM protocol has prognostic merit in MDS.

In conclusion, we showed a relatively simple, and thus applicable in many laboratories, FCM protocol for discriminating between low-grade MDS without conventional markers and non-clonal cytopenias. Further study is needed to improve the diagnostic power of this protocol.

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Authorship and Disclosures

KO conceived and designed the study, collected and analyzed data, and wrote the manuscript. MGDP designed the study, collected and analyzed data, and contributed to the writing of the manuscript. LM designed the study, collected and analyzed data, and edited the manuscript. CP, NY, AM, TY, HT, JT, and KD collected and analyzed data.

The authors reported no potential conflicts of interest.

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Pediatr Blood Cancer 2009;53:1011–1015

Treatment of Children With Refractory Anemia: The Japanese Childhood MDS Study Group Trial (MDS99)

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Background. Although hematopoietic stem cell transplantation (HSCT) is offered as a curative therapy for pediatric myelodysplastic syndrome (MDS), it may cause severe complications and mortality. Several reports have shown the efficacy of immunosuppressive therapy (IST) in adult patients with refractory anemia (RA), but its safety and efficacy remains to be fully elucidated in childhood RA. ***Procedure.*** Eleven children diagnosed with RA and enrolled on a prospective multicenter trial conducted by the Japanese Childhood MDS Study Group were eligible for analysis. If patients showed transfusion dependent or suffered from infection due to neutropenia, they received IST consisting of antithymocyte globulin (ATG), cyclosporine (CyA), and methylprednisolone (mPSL). ***Results.*** Eight children received IST, 2 received only supportive therapy, and one underwent HSCT without IST. Five (63%) of eight children who received IST showed hematological response. Of note, one patient showed the disappearance of monosomy 7 after IST. Responders were significantly younger than non-responders (29 months vs. 140 months; $P = 0.03$). No severe adverse events related to IST were reported in this study. Of 6 children with chromosomal abnormalities who received IST, four showed hematological response. The probability of failure-free and overall survival at 5 years was $63 \pm 17\%$ and $90 \pm 9\%$ respectively. ***Conclusion.*** IST is likely to be a safe and effective modality for childhood RA. Pediatr Blood Cancer 2009;53:1011–1015. © 2009 Wiley-Liss, Inc.

Key words: myelodysplastic syndrome; refractory anemia; children; immunosuppressive therapy

INTRODUCTION

Myelodysplastic syndrome (MDS) is a hematopoietic stem cell disorder and rarely occurs in childhood [1,2]. Refractory anemia (RA) is a subgroup of MDS with less than 5% of blasts in the bone marrow (BM) and little is known about childhood RA because of its rarity. European Working Group of MDS in Childhood (EWOG-MDS) retrospectively analyzed the clinical characteristics of children with RA [3]. They found that neutropenia and thrombocytopenia were more prominent than anemia [3,4] and karyotype had a strong impact on prognosis in children with RA [3]. Children with monosomy 7 were significantly more likely to progress to advanced disease and they recommended hematopoietic stem cell transplantation (HSCT) for this unfavorable group as early as possible, whereas, appropriate treatment for children with chromosomal abnormalities other than monosomy 7 and those with normal karyotypes remained to be determined.

Disturbance of the immune system may play a role in pathogenesis in some adults and children with RA [5–7]. Several reports have shown positive effects of immunosuppressive therapy (IST) in adult patients with RA [8–12]. The hematological response rate of IST was reported as 30–80% but IST could not restore the cytogenetic abnormalities or dysplastic features. Recently, EWOG-MDS reported the results of IST consisting of antithymocyte globulin (ATG) and cyclosporine A (CyA) in children with hypoplastic refractory cytopenia (RC) and normal karyotype or trisomy 8 who were thought as being at low risk of progression to advanced MDS [13]. However, the role of IST in children with RA has not been fully elucidated because the above study selected children with favorable predictive factors for a positive response to IST.

This study reports the outcome of 11 children with RA enrolled on a prospective multicenter trial (MDS99) conducted by the Japanese Childhood MDS Study Group, which applied IST with ATG and CyA to unselected patients who needed intervention.

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DOI 10.1002/pbc.22121
Published online 4 June 2009 in Wiley InterScience (www.interscience.wiley.com)

PATIENTS AND METHODS

Patients

Eleven children younger than 16 years of age were enrolled onto MDS99 from September 1999 to March 2004. They were diagnosed as having RA according to the French-American-British (FAB) classification [14] and diagnosis was confirmed by the central review of morphology by two independent investigators [15]. Cytogenetic analysis of the bone marrow cells was performed in each institution. There were no patients who had undergone previous chemotherapy or radiotherapy, nor patients with a history of congenital bone marrow failure syndrome or aplastic anemia in the analysis. The study was approved by the Steering Committee of the Japanese Childhood MDS Study Group and the institutional review boards of the participating institutions or the equivalent organization. Informed consent was obtained from the guardians of the patients.

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Received 4 February 2009; Accepted 27 April 2009

Treatment Protocol

Each patient with RA required repetitive bone marrow aspiration at 6–8 weeks intervals in order to confirm the diagnosis. If the disease was stable and blood transfusion was not required, patients were observed closely without any therapy. If patients showed transfusion dependent or suffered from infection due to neutropenia, IST was administered as follows: horse ATG (15 mg/kg/day) for 5 days as a slow intravenous infusion over 12 hr, CyA (6 mg/kg/day given orally as an initial dose, and the dose was adjusted to achieve a whole blood trough level of 100–200 ng/ml) was started on day 1 and continued until day 180, and methylprednisolone (mPSL; 2 mg/kg/day) was administered intravenously on days 1–7, then mPSL was administered orally and slowly tapered from day 8 to end on day 29. In this study, the use of G-CSF was not restricted. HSCT was recommended when a patient showed no response to IST and required further intervention because of cytopenia or progression to more advanced disease.

Evaluation and Statistical Analysis

Response to IST was evaluated at 6 months. Complete response (CR) was defined as a neutrophil count $>1.5 \times 10^9$/L, platelet count $>100 \times 10^9$/L, and hemoglobin (Hb) level of >11.0 g/dl. Partial response (PR) was defined as a neutrophil count $>0.5 \times 10^9$/L, platelet count $>20 \times 10^9$/L, and Hb level of >8.0 g/dl. When neither the CR nor the PR criteria were met, a patient was considered as no response (NR) to IST.

Mann–Whitney test and Fisher's exact test were applied to evaluate the differences between patients that responded to IST and those who did not. Failure-free survival (FFS) was calculated from the date of initiating IST to the date of treatment failure as follows; death, no response to IST at 6 months, HSCT, a second course of IST, acquisition of chromosomal abnormality, progression to advanced disease, or relapse. Overall survival (OS) was calculated from the date of diagnosis to the date of death or last follow-up. Both FFS and OS were estimated by the Kaplan–Meier method.

RESULTS

Patient Characteristics

Eleven children, 6 males and 5 females, were analyzed in this study (Table I). The median age at diagnosis was 67 months (range, 9 months to 15 years). Eight of 11 children had neutrophil counts of less than 1.5×10^9/L. All except 1 patient had Hb levels below 10 g/dl. Eight patients had platelet counts below 50×10^9/L. In total, one patient had anemia only, five had bi-cytopenia (anemia and neutropenia 2, anemia and thrombocytopenia 2, and neutropenia and thrombocytopenia 1), and five had pancytopenia at diagnosis. Since bone marrow biopsy specimen was available in only 6 of 11 cases, we determined cellularity by central pathological review from bone marrow smear rather than biopsy specimens and used a more suitable term, cell content, instead of cellularity in this report. Overall, there were only three patients in whom BM cell content was low. All patients showed dysplasia in multilineage series, which was compatible with the definition of refractory cytopenias with multilineage dysplasia (RCMD) in the World Health Organization (WHO) classification [16]. Data on the cytogenetic analyses at diagnosis were available for all patients. Karyotype was normal in three patients, and of the remaining eight patients, two had monosomy 7, two had trisomy 8, and four had other abnormalities; del (7)(q11), i(8)(q10), 20q-, and +der(1;19)(q10;q10). Of four patients in whom presence of paroxysmal nocturnal hemoglobinuria (PNH) cells was assessed by flow cytometry, none showed an expansion of PNH clone. Of five patients in whom data on HLA-DR was available, only one patient showed DR2 antigen, which is a broad antigen of DR15 and DR16.

TABLE I. Patients Characteristics

	Median (range)
Age	5y7m (9m to 15y5m)
Gender	M/F = 6:5
WBC ($\times 10^9$/L)	3.8 (1.1–12.5)
Neutrophil ($\times 10^9$/L)	0.94 (0.16–8.1)
PB blast (%)	0 (0)
Hb (g/dl)	6.2 (3.6–11.7)
Reticulocyte (%)	2 (1–44)
Reticulocyte ($\times 10^9$/L)	41.7 (12.3–572.0)
MCV (fl)	104 (84–123)
Plt ($\times 10^9$/L)	23.0 (3.0–117.0)
BM blast (%)	1.0 (0–4.8)
BM cell content	Low 3, normal 5, high 3
Chromosome	Normal/abnormal = 3:8
Cytopenia[a]	Anemia only 1, bi-cytopenia 5, pancytopenia 5

[a]Cut-off; neutrophils <1,500/μl, Hb < 10.0 g/dl, Plt < 50,000/μl.

Observation Without Intervention

Figure 1 shows the outcome of the 11 patients analyzed. Of 3 patients who initially received only supportive therapy, one with normal karyotype was still stable without therapy, one with trisomy 8 showed spontaneous improvement of anemia but the chromosomal abnormality remained. One with 20q- (UPN 046) showed stable disease for 2 years, but cytopenia deteriorated and IST was initiated at 968 days after diagnosis.

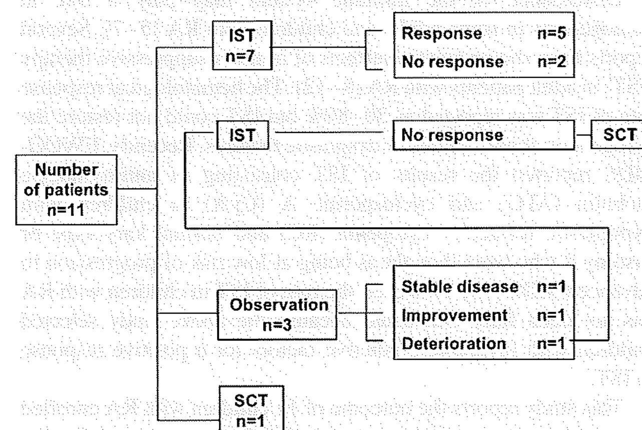

Fig. 1. Outcome of 11 patients with refractory anemia. SCT, stem cell transplantation; IST, immunosuppressive therapy.

Immunosuppressive Therapy

Seven patients received IST as the first-line treatment and one (UPN 046) received IST because of recurrence of cytopenia after 2-year observation. IST was given at a median of 42 (range 0–968) days after the diagnosis of RA. Five of eight patients showed response to IST at 6 months after the initiation of treatment (response rate was 63%; CR 2, PR3). Of five responders, three were able to successfully discontinue IST and remained disease-free, and the remaining two patients have been continuing therapy. Of note, the disappearance of a monosomy 7 clone after IST was observed in UPN 035 [17] and the patient is still in remission after 63 months. Of three non-responders, one was lost to follow up, one responded to a second course of IST, and one (UPN 046) underwent HSCT 3 months after initiating IST.

To address predictive factors for response to IST, the characteristics were compared between children who responded to IST and those who did not (Table II). The age at diagnosis was significantly younger in responders than in non-responders (median 29 months vs. 140 months; $P = 0.03$), whereas there was no statistically significant associations between response to IST and sex, neutrophil count, Hb level, platelet count, interval from diagnosis to IST, chromosomal abnormality, BM cell content, or number of cytopenia. Serious adverse events related to IST were not observed, including the progression to advanced disease. The most frequent adverse event in this study was pyrexia.

Hematopoietic Stem Cell Transplantation

Two children underwent HSCT in this series. One patient with 20q- (UPN 046) received bone marrow transplantation (BMT) from her human leukocyte antigen (HLA) 1-locus-mismatched father at 1,088 days after diagnosis because of non-response to IST. This patient suffered from adenoviral colitis, salmonella colitis, herpes zoster, and grade III acute GVHD of the skin, however, she is still alive without disease 23 months after BMT. One other patient with monosomy 7 (UPN 053) received BMT from a matched unrelated donor on 537 days after diagnosis without IST by physician's decision. His post-transplant course was uneventful, but disease relapsed 151 days after transplantation. A BM specimen at relapse showed severe fibrosis and progression to overt leukemia, and this patient died of disease at 656 days after transplantation.

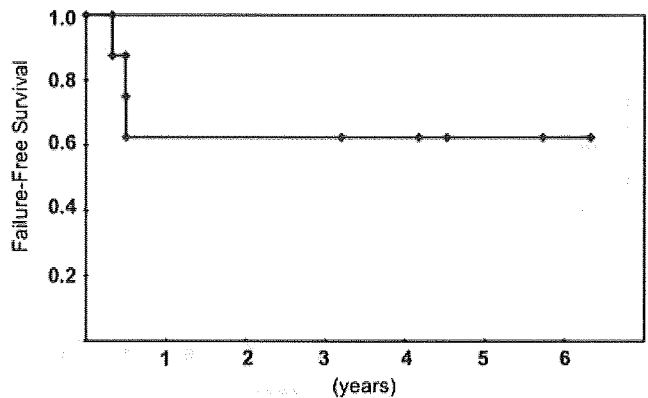

Fig. 2. Kaplan–Meier estimate of failure-free survival of patients who received immunosuppressive therapy. Failure-free survival was calculated from the date of initiating IST to the date of treatment failure as follows; death, no response to IST at 6 months, HSCT, a second course of IST, acquisition of additional chromosomal abnormality, progression to advanced disease, or relapse. The 5-year failure-free survival was $63 \pm 17\%$ (n = 8). Median follow-up was 1,346 days.

Chromosomal Abnormality

There were eight children with chromosomal abnormality in this study. Of those, six received IST and four showed responses to IST, including one with cytogenetic response (UPN 35).

Survival

Of eight children who received IST, three non-responders were considered as treatment failure. No patient died with IST after a median follow-up of 1,346 days; the 5-year FFS was $63 \pm 17\%$ (Fig. 2). Of total, 10 patients are alive after a median follow-up of 1,685 days; the 5-year OS was $90 \pm 9\%$ (Fig. 3).

DISCUSSION

Although HSCT is the curative modality for children with MDS, it may cause severe complications, mortality, and late sequelae. Several reports have shown encouraging results from the use of IST in adults with RA, and the hematological response rate to IST was 30–80% [8–12]. Yoshimi et al. [13] reported on 31 children with hypoplastic RC and normal karyotype or trisomy 8 treated with IST, which resulted in a response rate at 6 months of 71%, 3-year OS of

TABLE II. Comparison of Characteristics Between Responders and Non-Responders to IST

	Responder (n = 5)	Non-responder (n = 3)	*P*-value
Age[a]	2y5m	11y8m	0.03
Gender (male/female)	3:2	1:2	n.s.
Neutrophils[a] ($\times 10^9$/L)	1.27	0.63	n.s.
Hb[a] (g/dl)	8.0	6.2	n.s.
Plt[a] ($\times 10^9$/L)	31.0	20.0	n.s.
No. of cytopenia (tri-/bi-/anemia only)	2:2:1	2:1:0	n.s.
Decreased BM cell content	1/5	2/3	n.s.[b]
Time to IST[a] (day)	42	42	n.s.[b]
Chromosomal abnormality	4/5	2/3	n.s.[b]

[a]Median; [b]Evaluated by Mann–Whitney test and Chi-square test.

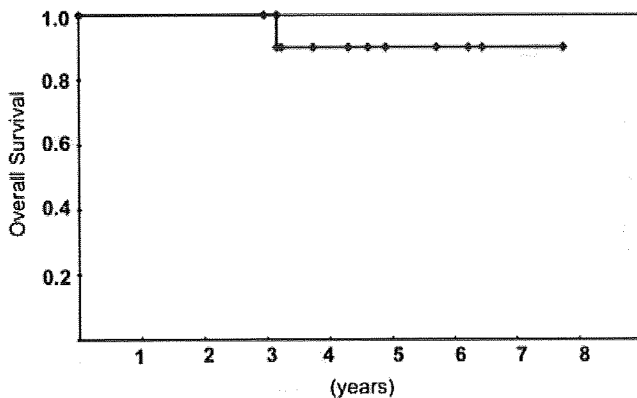

Fig. 3. Kaplan–Meier estimate of overall survival of all evaluable patients. Overall survival was calculated from the date of diagnosis to the date of death or last follow-up. The 5-year overall survival was 90 ± 9% (n = 11). Median follow-up was 1,685 days.

88%, and 3-year FFS of 57%. In contrast to the larger series by Yoshimi et al. children with RA and karyotypic abnormalities or non-hypoplastic marrow were also enrolled in this study. Overall, 5 of 8 patients (63%) responded to IST, and similar responses were observed in two-thirds of patients with chromosomal abnormalities. Patients whose BM cell content was not low also responded to IST (responder 4, non-responder 1); however, the significance of cellularity in pediatric RA still needs further study. No severe adverse events, disease progression, or death due to any cause after IST was reported. Only one death in this study was due to disease progression after HSCT, which was not related with IST. As a whole, the 5-year OS and FFS were 90% and 63%, which were comparable with the previous study in adult MDS and superior to our previous retrospective analysis of children with RA (4-year OS was 79%) [2]. Therefore, although the number of subjects was limited, we infer from these results that the IST is effective and safe for children with MDS.

The rationale for IST used as treatment of RA is based on previous studies, which suggested that alterations in the immune system might contribute to the pathogenesis in some subgroups of RA [5–7]. Dysregulated T cells are thought to destroy normal hematopoietic cells as bystanders as well as MDS clones [6]. IST can reduce MDS clone-specific T cells and improve normal hematopoiesis, but cytogenetic abnormalities and dysplastic features often persist [9,11,12]. However, in this study one patient showed the disappearance of karyotypic abnormalities. In addition, three of the responders were able to successfully discontinue IST. These results might be explained by the findings that the residual healthy stem cells can compensate for the loss of stem cells after the immune-mediated destruction is interrupted by IST in the setting of aplastic anemia [18,19]. Recovery of healthy hematopoiesis might outstrip MDS clones in these patients. In the patient with monosomy 7 who experienced cytogenetic response another mechanism could be speculated. The investigators from the EWOG-MDS reported that almost half of children with RA had monosomy 7 and they were likely to experience disease progression [3]. In contrast, anecdotal case reports described a decline or disappearance of a monosomy 7 clone [20]. Sloand et al. [21] reported paradoxical responses of monosomy 7 cells to G-CSF. Namely, high concentrations of G-CSF induced significant proliferation of monosomy 7 cells, but survival and proliferation of monosomy 7 cells were inferior to those of diploid cells at lower G-CSF levels. Thus, there is a possibility that the recovery of normal hematopoiesis after the administration of IST might affect the intrinsic level of G-CSF and survival of monosomy 7 cells. However, the interpretation of the present results still needs caution because most patients with RA and monosomy 7, including another case in this study, showed poor prognosis.

Previous studies on IST in adult RA found some factors that could predict good responders to IST, such as younger age, shorter duration of transfusion dependence, HLA-DR15, and presence of an expanded clone of PNH cells [8,10–12]. In this study, age was the only factor that showed a statistically significant difference between responders and non-responders to IST. The European study published by Yoshimi et al. [13] also contained older patients, but the proportion and treatment responses of older patients were not shown. Therefore, the effects of patient age on pathophysiology of pediatric RA and treatment response remain to be elucidated. Of the limited cases who were examined, no patient showed an expansion of PNH clone and only one patient had HLA-DR2 antigen, who responded to IST well. We did not systemically examine the immunological status such as TCR Vbeta repertoire [7] in this study. Clinical trials, including systematic studies on immunological status, are required to investigate prognostic factors more precisely in childhood RA because the sample size in this study was small.

Thus, a significant drawback of our study was small size of registered patients. We assumed that considerable number of patients with RA did not enter this study and might have received HSCT without IST. In fact, retrospective analysis of pediatric MDS in Japan showed that 52 patients with RA were diagnosed by the central morphological review between 1999 and 2006 [22]. Consecutive enrollment on both diagnostic and therapeutic trials would be essential for a future trial. It might allow the determination of biologic parameters that correlated with clinical characteristics.

In conclusion, the present results suggest the efficacy and safety of IST for children with RA. Disease-free status might be expected with IST in a subset of patients. Chromosomal aberration was not an absolute contraindication for IST, whereas using this approach for patients with monosomy 7 has not been substantiated. A larger prospective study including biological surrogate markers for therapeutic interventions would be important to elucidate the clinical characteristics of this rare disease as well as the prognostic factors and mechanism of IST.

ACKNOWLEDGMENT

The authors thank Dr. Y. Nagatoshi, Dr. M. Maeda, Dr. T. Kanazawa, Dr. N. Shiba, Dr. M. Nagasawa, Dr. T. Kusuda, Dr. K. Kokawa, Dr. T. Tsuruta, Dr. S. Oana, Dr. Y. Noguchi, Dr. S. Ikushima, and Dr. A. Watanabe for providing patients' information. We also thank Dr. C. Ogawa and Dr. A. Yoshimi for valuable advice.

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European Journal of Haematology 83 (328–333)

ORIGINAL ARTICLE

The impact of cytogenetic abnormalities on the prognosis of primary myelofibrosis: a prospective survey of 202 cases in Japan

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Abstract

Cytogenetic abnormalities were often observed in primary myelofibrosis patients. The presence of specific cytogenetic abnormalities, such as sole abnormalities of chromosome 13q−, 20q−, or −7/7q−, is reported to have the influence on the prognosis of primary myelofibrosis. We analyzed the data from the prospective survey of Japanese primary myelofibrosis patients which was conducted from 1999 to clarify the impact of cytogenetic abnormalities on the prognosis of primary myelofibrosis. A total of 202 primary myelofibrosis patients had the cytogenetic and the prognostic data. Eighty (40%) out of 202 cases had cytogenetic abnormalities, and an association was evident for platelet counts. Although the presence of an abnormal karyotype did not affect the prognosis, primary myelofibrosis patients with cytogenetic abnormalities other than 13q− and 20q− showed an inferior prognosis compared to patients with a normal karyotype or sole 13q− or 20q− abnormalities. Patients with an unfavorable cytogenetic profile (abnormal cytogenetics other than 13q− or 20q−) also had a greater tendency to transform to leukemia than patients with a favorable cytogenetic profile (normal cytogenetics, sole abnormalities of either chromosome 13q−, or 20q−). Abnormal cytogenetics other than 13q− or 20q− in primary myelofibrosis patients has the poor prognostic effect for both survival and the risk of leukemic transformation.

Key words primary myelofibrosis; cytogenetic abnormalities; 13q−; 20q−; −7/7q−

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Accepted for publication 12 June 2009

doi:10.1111/j.1600-0609.2009.01298.x

Primary myelofibrosis is a clonal stem cell disorder that is characterized by bone marrow histologic changes including collagen fibrosis, osteosclerosis, and angiogenesis (1), and is a type of myeloproliferative neoplasma along with polycythemia vera and essential thrombocythemia (2). Activated JAK2 mutation (V617F) or the activated myeloproliferative leukemia virus oncogene (MPL) (receptor for thrombopoietin) mutation was observed in about half or about 5% of primary myelofibrosis patients, respectively (3–9). This suggests that there is significant heterogeneity in the molecular basis of primary myelofibrosis, as the etiology remains unknown in the remaining 50% of patients without JAK2 or MPL mutations.

Cytogenetic abnormalities were often observed in primary myelofibrosis patients. Some studies suggested that the presence of cytogenetic abnormalities influenced the prognosis of primary myelofibrosis (10, 11), whereas other studies did not find such association (12, 13). Not only the presence or absence of cytogenetic abnormalities, some reports suggested that the specific cytogenetic

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abnormalities, such as sole abnormalities of chromosome 13q−, 20q− (14, 15), or −7/7q− (16), might have the influence on the prognosis of primary myelofibrosis. To clarify the impact of cytogenetic abnormalities on the prognosis in primary myelofibrosis patients, we analyzed the data from the prospective survey of Japanese primary myelofibrosis patients which was conducted from 1999. Cytogenetic result as well as clinical and laboratory evaluation were obtained from 202 primary myelofibrosis patients between 1999 and 2008. We found that primary myelofibrosis patients with cytogenetic abnormalities other than 13q− and 20q− had an inferior prognosis compared to patients with a normal karyotype or sole 13q− or 20q− abnormalities.

Materials and methods

The Research Committee for Idiopathic Hematopoietic Disorders in Japan conducted the prospective survey in collaboration with hematologists at 587 medical institutes throughout Japan, all of which were approved as designated institutes by the Japanese Society of Hematology. We sent a questionnaire to hematologists in December of each year, and collected information on patients with newly diagnosed primary myelofibrosis in the reference year. The diagnostic criteria for primary myelofibrosis were an increased number of atypical megakaryocytes, the presence of marrow fibrosis and osteosclerosis as determined by bone marrow biopsy, and the exclusion of secondary myelofibrosis, including other hematologic malignancies, cancer metastasis, and inflammatory or metabolic disorders (17). Myelodysplastic syndrome with fibrosis and secondary myelofibrosis preceding polycythemia vera or essential thrombocythemia were not included in this study. Cytogenetic studies were done at the time of diagnosis, and both direct technique and unstimulated 24-h culture methods were used to harvest 20 metaphases using bone marrow or peripheral blood cells. All data were analyzed using STATVIEW 5.0 (Abacus Concepts, Inc., Berkley, CA, USA) or SPSS software (SPSS Inc., Chicago, IL, USA). All *P* values were two-tailed and values $P < 0.05$ were considered statistically significant. Comparisons between categorical variables were performed by chi-squared statistics, and comparisons between categorical and continuous variables were performed using the Mann–Whitney U-test.

The mutational analysis for V617F JAK2 was approved by the Miyazaki University Institutional Review Board, and performed using DNA derived from peripheral blood granulocytes. Genomic DNA was purified, and exon 12 of JAK2 was amplified using primers 5′-TATAGTCATGCTGAAAGTAGG-3′ and 5′-TAACTGAATAGTCCTACAGTG-3′. The PCR products were sequenced directly using an ABI DNA analyzer.

The influence of karyotypic abnormalities on survival was examined. In addition, based on previous studies (14–16), cytogenetic findings of sole 13q− or 20q− abnormalities, abnormalities other than 13q− and 20q−, or −7/7q− were evaluated for an association with prognosis. Survival was estimated using Kaplan–Meier plots that analyzed the interval from the diagnosis date to death or last contact, and then examined for significance by the log-rank test. The comparison of leukemic transformation rates among different cytogenetic groups was estimated using a similar procedure. A Cox proportional hazards regression analysis was used to assess the following variables for the prognostic relevance of primary myelofibrosis patients; hemoglobin levels of <10 g/dL, a leukocyte count of either >30 or $<4 \times 10^9$/L, circulating blasts of $\geqq 1\%$ or the presence of constitutional symptoms in addition to the presence of cytogenetic abnormalities or specific cytogenetic abnormalities.

Results

The Research Committee for Idiopathic Hematopoietic Disorders began conducting a prospective survey of primary myelofibrosis in 1999, and a total of 202 cases had both cytogenetic and prognosis data during the 10-yr period until 2008. The median age of the patients was 65 yr. Males were 1.8 times more likely to be affected than females. During this period, 148 patients (73%) have received treatment for primary myelofibrosis including hydeoxyurea in 43 patients, melphalan in 11 patients, anabolic steroid in 51 patients, prednisolone in 34 patients, thalidomide in six patients and splenic irradiation in eight patients. Eighty-one patients received blood transfusions.

Eighty (40%) out of the 202 cases had cytogenetic abnormalities, including 31 patients with a complex abnormality or more than one abnormal chromosomes. Among 49 patients having a single abnormality, numerous chromosomes were affected, including del(20) (11 cases), del(13) (11 cases), +8 (3 cases) and del(12) (3 cases). Translocation, including chromosomes 12, 15, 8, 9, and others were seen in 9 cases. The V617F JAK2 mutation was analyzed only in 24 cases, and 12 out of them were positive for this mutation.

Cytogenetic abnormalities were examined for a significant association with other clinical and laboratory variables as shown in Table 1. After a univariate analysis, an association was only evident for platelet counts ($P < 0.05$). The presence of abnormal cytogenetics at the time of diagnosis had no significant impact on age, Hb level, WBC counts, the requirement of treatment, the ratio of leukemic transformation, or the prognosis.

Although the presence of abnormal cytogenetics did not influence survival of the patients, specific cytogenetic abnormalities might affect the prognosis. Sole

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Table 1 Comparison of clinical and laboratory features of patients with and without cytogenetic abnormalities

Characteristics	All patients (n = 202)	Normal cytogenetics (n = 122)	Abnormal cytogenetics (n = 80)	P value
Age in years	63.8 ± 12.1	64.0 ± 11.8	63.6 ± 12.6	0.7686
Median	65	64	65	
Range	13–95	36–95	13–84	
Sex, M/F	129/73	81/41	48/32	0.3549
Hemoglobin level (g/L)	8.9 ± 2.6	9.1 ± 2.7	8.6 ± 2.4	0.1285
White blood cell level (/μL)	16400.0 ± 32148.7	19675.4 ± 38944.2	11405.0 ± 16273.6	0.0688
Platelet count (×10^4)	29.0 ± 26.6	32.7 ± 26.2	23.4 ± 26.3	**0.0015**
Treatment (yes/no)	148/54	89/33	59/21	0.9001
Transformation to leukemia (yes/no)	15/187	6/116	9/971	0.0932
Outcome (alive/death)	117/85	72/50	45/35	0.6969
Follow-up years	2.3 ± 2.2	2.6 ± 2.3	2.0 ± 1.9	0.0712

Bold value indicates significant P values.

abnormalities of either chromosome 13q− or 20q− is reportedly associated with a favorable prognosis (14, 15). As shown in Fig. 1A, 22 cases with sole 13q− or 20q− abnormalities showed superior prognosis than the 58 cases with cytogenetic abnormalities other than 13q− and 20q− (P = 0.02), but not than patients with a normal karyotype (P = 0.17). The prognosis of primary myelofibrosis in patients having cytogenetic abnormalities other than 13q− and 20q− was inferior to all patients except for those having cytogenetic abnormalities other than 13q− or 20q− (P = 0.02) (Fig. 1B). Chromosome 7 deletions were previously reported to be associated with an unfavorable prognosis in myelofibrosis (16). In our study, 11 out of the 202 cases had del(7) or −7. The prognosis of primary myelofibrosis in patients with −7/7q− was same as that of patients having normal cytogenesis (P = 0.76).

We also examined the effect of extensively used adverse risk factor (hemoglobin level of <10 g/dL, a leukocyte count of either >30 or <4 × 10^9/L, circulating blasts of ≧1%, or the presence of constitutional symptoms) on the prognosis of primary myelofibrosis patients. Consistent with previous observation, univariate analysis confirmed the adverse prognostic relevance to overall survival of a leukocyte count of either >30 or <4 × 10^9/L (P = 0.033) and circulating blasts of ≧1% (P = 0.04) in addition to the presence of cytogenetic abnormalities other than 13q− or 20q− (P = 0.02). The presence of constitutional symptoms, hemoglobin level of <10 g/dL, or the requirement of therapy did not affect the prognosis of primary myelofibrosis patients in this cohort. On multivariate analysis, both leukocyte count (P = 0.02) and the presence of cytogenetic abnormalities other than 13q− or 20q− (P = 0.02) retained their significance. Circulating blasts of ≧1% lost its significance.

Fifteen patients converted to leukemia during the follow-up period. Six cases had normal cytogenetics, and 9 cases had the cytogenetic abnormalities other than 13q− or 20q−. None of the patients having sole abnormalities of either chromosome 13q− or 20q− converted to leukemia. As mentioned above, the presence of abnormal cytogenetics did not influence the tendency of leukemic transformation (Table 1). Patients with abnormal cytogenetics other than 13q− or 20q− had a greater tendency to transform to leukemia than patients with normal cytogenetics or sole abnormalities of either chromosome 13q− or 20q− (P = 0.002).

Discussion

In primary myelofibrosis patients, numerous chromosomes are affected; 40% of primary myelofibrosis patients had cytogenetic abnormalities in our study. The most frequently reported cytogenetic abnormalities include 13q−, 20q−, +8, and abnormalities of chromosomes 1, 7, and 9 (10, 12, 15, 18, 19). In our study, the total incidence of these major abnormalities is up to 71% in patients with a single chromosome abnormality, although the incidence of each individual lesion is less than 25%. The fact that there is no consistent cytogenetic abnormality in primary myelofibrosis patients might be because the range of abnormalities reflects the genomic instability present in primary myelofibrosis.

Cytogenetic abnormalities were only associated with platelet counts, and were not significantly associated with age, Hb level, WBC counts, leukemic transformation, or prognosis (Table 1). There is some discrepancy among previous reports regarding the prognostic relevance of cytogenetic findings in primary myelofibrosis (10, 11, 13, 15, 18). Some studies suggested that the presence of cytogenetic abnormalities influenced the prognosis of primary myelofibrosis (10, 11), whereas other studies did not find such association (12, 13). The idea that specific cytogenetic abnormalities have a differential effect on prognosis might account for this discrepancy. In reports that have linked specific chromosomal abnormalities to

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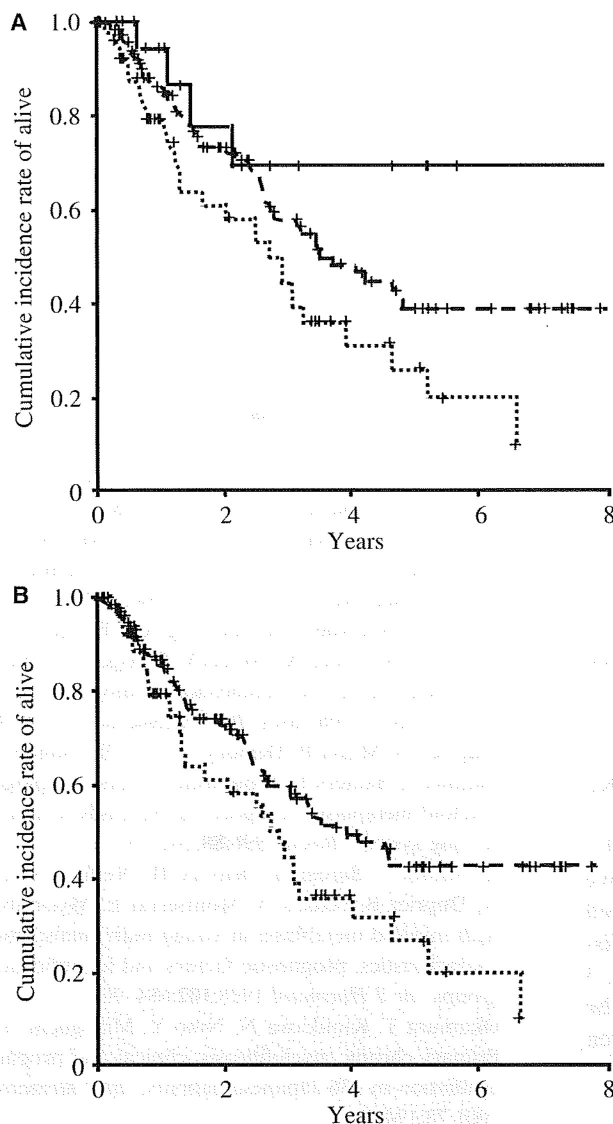

Figure 1 Survival rate curves for primary myelofibrosis patients according to cytogenetic groups. (A) ····→ Normal cytogenetics ($n = 122$). Either 13q− or 20q− lesion ($n = 22$). ⟶ cytogenetic abnormalities other than 13q− and 20q− ($n = 58$). ··→ The prognosis of primary myelofibrosis in patients having cytogenetic abnormalities other than 13q− and 20q− was inferior to patients having cytogenetic abnormalities with sole 13q− or 20q− ($P = 0.02$). (B) ····→ Normal cytogenetics, sole abnormalities of either chromosome 13q− or 20q− ($n = 144$). ··→ cytogenetic abnormalities other than 13q− and 20q− ($n = 58$). The prognosis of primary myelofibrosis in patients having cytogenetic abnormalities other than 13q− and 20q− was inferior to all patients except for those having cytogenetic abnormalities other than 13q− or 20q− ($P = 0.02$)

prognosis, sole abnormalities of either chromosome 13q− or 20q− was correlated with an indolent prognosis, and cytogenetic abnormalities other than 13q− and 20q− were associated with an inferior prognosis (14, 15). As in previous reports, the prognosis of patients with cytogenetic abnormalities other than 13q− and 20q− is poorer than those with sole 13q− or 20q− abnormalities (Fig. 1A). Tefferi *et al.* also reported that the sole abnormalities of either 13q− or 20q− was linked to a reduced risk of transformation in addition to the indolent nature (14). In our study, none of the 22 patients with sole abnormality of either chromosome 13q− or 20q−, 9 of the 58 patients with cytogenetic abnormalities other than 13q− and 20q−, and 6 of the 116 patients with normal cytogenetics converted to acute leukemia. Patients with an unfavorable cytogenetic profile (abnormal cytogenetics other than 13q− or 20q−) had a greater tendency to transform to leukemia than patients with a favorable cytogenetic profile (normal cytogenetics, sole abnormalities of either chromosome 13q−, or 20q−) ($P = 0.002$).

The genes located on 13q or 20q have not been identified. Although 13q− is also observed in multiple myeloma patients (20, 21), the gene located on deleted 13q may be different in these two diseases. In most myeloma patients with 13q−, RB1, the powerful tumor suppressor gene, is deleted (21), whereas the lesion on 13q in bcr/abl-negative chronic myeloid disorders does not involve RB1 (22). Differences in the involved gene may account for the prognostic value of 13q− in myeloma and primary myelofibrosis; 13q− is associated with an inferior prognosis for myeloma (20), but not for primary myelofibrosis (14, 15). In myelodysplastic syndrome, 20q− abnormalities have been associated with prolonged survival (23). The common deleted lesion on 20q− in myeloid malignancies is being investigated (24), and the h-l (3) mbt gene, which encodes a member of the polycomb group family of proteins and is the human homologue of a tumor suppressor gene in Drosophia (25), is a candidate for the responsible gene (24).

Strasser-Weippl *et al.* previously reported that Chromosome 7 deletions are associated with an unfavorable prognosis in myelofibrosis (16). Chromosome 7 deletions also tend to be associated with other chromosomal aberrations. Six of 7 patients in their report (16), and 9 of 11 patients harboring del(7) or −7 in our study had other chromosomal aberrations. Contrary to the previous report, −7/7q− is not associated with an unfavorable prognosis in our study. One possible reason for this discrepancy in the prognostic value of −7/7q− is that our survey is limited to primary myelofibrosis patients, while the report showing the unfavorable prognostic value of −7/7q examined secondary myelofibrosis (postpolycytemia vera or essential thrombocytosis) patients in addition to primary myelofibrosis patients (16). This possibility is unlikely, because it is very difficult to distinguish between a primary myelofibrosis and a secondary myelofibrosis in clinical practice. The other possibility might depend on the sensitivity of the assay used to

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detect the chromosome abnormality. We performed a G-banding stain in metaphase cells, whereas they adapted interphase fluorescence *in situ* hybridization (FISH) analysis to identify the chromosomal abnormality. To detect a specific chromosomal abnormality, FISH is much more informative than a G-banding stain because FISH can be used for interphase cytogenetic analysis. In fact, we detected abnormal metaphase in only 40% of patients using a G-banding stain, while they found cytogenetic abnormalities in 56% of patients using FISH analysis (16). In order to clarify these differences, a larger prospective survey is needed to determine the prognostic value of cytogenetic information and the role of involved genes in pathogenesis.

JAK2 mutation is observed in about half of primary myelofibrosis patients (3–7). We examined JAK2 mutation status in only 24 primary myelofibrosis patients, and 12 were positive for this mutation. This number is too small to evaluate the role of JAK2 mutation on survival or leukemic transformation in primary myelofibrosis. Hussein *et al.* examined the presence of JAK2 mutation in addition to the cytogenetic studies in 109 primary myelofibrosis patients, and found JAK2 mutations in 63(58%) patients (26). In their study, the presence of JAK2 mutation was inconsequential to survival. JAK2 mutation also may not be the disease-initiating lesion in transformation, because the emergence of JAK2V617F^{-} blast phase from previously positive myeloproliferative neoplasms was observed by Tam *et al.* (27), Campbell *et al.* (28) and Theocharides *et al.* (29), who reported disappearance of JAK2V617F positivity in 7 of 12 (58%), 3 of 4 (75%), and 7 of 17 (41%) patients, respectively. The presence or absence of JAK2 mutation or its allele burden does not appear to carry prognostic information (30).

Acknowledgements

This study was supported by Grants-in-Aid from the Ministry of Health, Labour and Welfare of Japan. We would like to thank Mayu Ota, Kazumi Sato, and Masami Dairaku for their excellent assistance in preparing this manuscript.

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Biochemical and Biophysical Research Communications 383 (2009) 411–416

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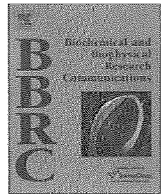

p27 deregulation by *Skp2* overexpression induced by the JAK2V617 mutation

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ARTICLE INFO

Article history:
Received 4 April 2009
Available online 11 April 2009

Keywords:
Myeloproliferative disease
JAK2V617F
Signal transducer and transcriptions (STATs)
p27[Kip1]
Skp2
Promoter analysis
EMSA
ChIP assay

ABSTRACT

Janus kinase 2 (*JAK2*) *V617F* mutation has been regarded as the major cause of myeloproliferative disorders (MPD). However, the mechanisms of abnormal cell growth by *JAK2V617F* have not been elucidated. In this study, cell cycle regulatory protein expression was analyzed using *JAK2V617F*-Ba/F3 and mock-Ba/F3. *JAK2V617F*-Ba/F3, but not mock-Ba/F3, showed IL-3 independent cell growth and constitutive STATs activation. Deregulation of p27[Kip1], the cell cycle regulator at the G1 to S transition, was observed in *JAK2V617F*-Ba/F3 but not in mock-control. p27[Kip1] deregulation was not due to *p27[Kip1]* mRNA level but due to high *Skp2* expression, a subunit of ubiquitin E3 ligase, through the STAT binding in the *Skp2* promoter. Like *JAK2V617F* overexpression, constitutively active *STAT5* or *STAT3* induced aberrant p27[Kip1] expression of Ba/F3 cells. Similar findings were observed in *BCR/ABL*-transfected Ba/F3. Our results elucidate the regulatory mechanism by which *JAK2V617F* modulates *Skp2* gene expression through the STAT transcription factors.

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Introduction

The Janus kinase (JAK)/signal transducers and activators of transcription (STATs) pathway plays an important role in hematopoiesis. Recently, a mutation that substitutes valine with phenylalanine at position 617 (V617F) of the JAK2 protein has been identified in patients with myeloproliferative disorders (MPD) [1]. A subset of patients, most commonly with polycythemia vera (PV), have homozygous *JAK2V617F* mutations, which are the result of mitotic recombination and duplication of the mutant allele [2]. Valine 617 locates in the pseudokinase domain (JH2) of JAK2. It is thought that the pseudokinase domain serves an auto-inhibitory role similar to the juxtamembrane domain of receptor tyrosine kinases such as FLT3 [3]. Biochemical evidence also supports the notion that *JAK2V617F* is a constitutively active tyrosine kinase [4]. When *JAK2V617F* was expressed in Ba/F3 cells with the erythropoietin (EPO) receptor, Ba/F3 cells resulted in EPO independent growth and in EPO hypersensitivity [5].

It has been suggested that elevated JAK2 tyrosine kinase activity contributes to transformation, probably in part through STAT transcription factors. However, the detailed molecular targets of JAK2 activation, which characterizes the MPD phenotype, are still obscure. In the current study, we compared profiles of cell cycle regulatory protein between *JAK2V617F*-Ba/F3 and mock-Ba/F3 cells. Based on these results, we focused on a cell cycle regulatory protein of p27[Kip1] [6]. Our current results suggested that aberrant expression of *p27[Kip1]* might be a cause of IL-3 independent cell growth of *JAK2V617F*. We further found that reduced p27[Kip1] expression was due to its increased degradation by SKP2, an F-box protein of the E3 ligase, SCF[SKP2].

We also observed that similar deregulation occurs in *BCR/ABL*-BaF3 cells, which has been recently reported by others [7,8]. Constitutively active STAT3 and STAT5 mimic the expression of p27[Kip1] observed in *JAK2V617F*-Ba/F3. The involvement of these proteins in MPD as well as CML pathogenesis was discussed.

Materials and methods

Cell lines and reagents. Mock, *JAK2* wild type (Wt) and *JAK2V617F* transfectants of Ba/F3 (*JAK2V617F-Ba/F3*) were reported previously [9]. Ba/F3, mock-Ba/F3 and Wt-Ba/F3 were maintained in RPMI 1640 medium supplemented with 10% FCS and 3% conditioned

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doi:10.1016/j.bbrc.2009.04.015