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A simple flow cytometric scoring system is useful for distinguishing myelodysplastic syndromes from non-clonal anemic disorders

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Myelodysplastic syndromes (MDS) are a myeloid neoplasm characterized by abnormal differentiation, ineffective hematopoiesis, and genetic instability with enhanced risk of transforming to acute myeloid leukemia (AML). The diagnosis of MDS is principally made based on the percentage of blasts in the bone marrow and peripheral blood, type and degree of dysplasia and the presence of ring sideroblasts. Recently, for making an accurate diagnosis of MDS, the aberrant antigen expression detection of hematopoietic cells by flow cytometry has been reported to be a useful. However, the diagnostic systems utilized in those studies are rather complicated. We modified an existing flow cytometric scoring system (FCMSS) based on aberrancies in the myeloid lineage and evaluated its usefulness in diagnosing various anemic disorders, including myelodysplastic syndromes (MDS). The flow cytometric score was significantly higher in MDS patients than in those with other anemic disorders, the exception being megaloblastic anemia (i.e., Vitamin B12 deficiency, folate deficiency). The data suggest that our FCMSS may provide useful information for making the diagnosis of MDS and other anemic disorders.

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Introduction

Myelodysplastic syndromes (MDS) constitute a heterogeneous group of myeloid neoplasms characterized by ineffective hematopoiesis, abnormal maturation of hematopoietic cells, chromosomal abnormalities, and an increased risk of evolution to acute myeloid leukemia (AML).[1] The diagnosis of MDS is based principally on morphological examination of bone marrow cells, i.e., the percentage of blasts, the type and degree of dysplasia, and the presence of ring sideroblast. However, because morphological diagnosis can be influenced by several factors, such as staining conditions and the skill of the examining physician, it is still considered to be difficult to make a definitive diagnosis. A consensus guideline for the diagnosis of MDS[2] recommends regular medical reviews with repeated blood counts and morphological assessments at appropriate intervals, if the morphological diagnosis remains uncertain. However, this strategy does not consistently contribute to solving diagnostic dilemmas.

Previous reports indicate that immuno-phenotypic characterization of bone marrow cells by flow cytometry (FCM) may be feasible for the diagnosis of MDS. Maturing cells as well as myeloblasts show immuno-phenotypic abnormalities. In light of the accumulated data regarding immuno-phenotypic aberrations in MDS, an international working conference redefined the minimum diagnostic criteria for MDS,[3] which included multidimensional FCM analyses as an important co-criterion for patients highly suspected to have MDS. The two subsequent international working conferences reported standardized FCM methods for application to the diagnosis of MDS.[4,5]

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The possible use of a flow cytometric scoring system (FCMSS) for the diagnosis of MDS was first explored by Wells et al.[6] They investigated the relationship between immuno-phenotypic aberrations of the myelomonocytic lineage and clinical courses. The FCMSS results correlated well with the established International Prognostic Scoring System (IPSS),[7] indicating the score to be an independent prognostic factor in MDS patients, including those who underwent allogeneic stem cell transplantation. Van de Loosdrecht et al[8] also examined the Wells scoring system and showed associations with transfusion dependence, disease progression and the World Health Organization (WHO) Classification-Based Prognostic Scoring System (WPSS).[9]

Several additional studies investigated the potential feasibility of flow-scoring systems for making a differential diagnosis of low-grade MDS from non-clonal cytopenias.[10-12] However, these systems were found to be too complex for utilization in the general clinical setting. The present study aimed to explore a simpler and more sensitive flow-scoring system focusing on immuno-phenotypes of myeloblasts and maturing granulocytes. Patients with low-grade MDS and other forms of non-MDS anemia were evaluated by 4-color FCM, and the usefulness of the flow-scoring system for making the differential diagnosis between these two disease categories was examined.

Methods

Patient characteristics

In total, 26 patients with MDS (15 males, 11 females) and 18 with definite non-MDS anemia (7 males, 11 females) were studied. All patients underwent bone marrow aspirations for diagnostic purposes prior to receiving supportive care and gave informed consent. The research protocol was approved by the Institutional Review Boards of Nagasaki University.

The MDS patients had a morphological diagnosis of refractory anemia (RA) (n=15) or refractory cytopenia with trilineage dysplasia (RCMD) (n=11), according to the WHO classification.[9] These patients were considered to have low-grade MDS.

The non-MDS anemia patients had anemia of chronic disorders (ACD) (n=5), pure red cell anemia (PRCA) (n=1), aplastic anemia (AA) (n=2), autoimmune hemolytic anemia (AIHA) (n=3), iron deficiency anemia (IDA) (n=1), anemia of chronic renal failure (n=3), pernicious anemia (n=2), or folate deficiency anemia (n=1).

Flow cytometric analysis

Four-color FCM was performed on a FACS Calibur (Becton Dickinson) using a combination of monoclonal antibodies that encompassed all differentiation stages of the granulocyte lineage (myeloid blasts and maturing myeloid cells) subpopulation. Seven panels of 4 monoclonal antibodies were selected based on previous studies[6] (Table 1). All monoclonal antibodies used in this study were obtained from BD (Biosciences, San Jose, CA, USA).

Bone Marrow was aspirated into a heparinized syringe, and total nucleated bone marrow cells were isolated after NH_4CI lysis of erythrocytes. Each sample was divided into 7 test tubes, and stained with a combination of monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridin chlorophyll (PerCP), and allophycocyanin (APC). The different cell compartments (progenitor cells and granulocytes) were identified using the CD45 vs Side Scatter (SS) gating method reported by Wells et al.[6] The granulocytic population was identified as $CD45^{dim/bright}/SSC^{int/high}$. Myeloid progenitor cells were identified as $CD45^{dim}/SSC^{dim}$.

Table 1. Combination of antigen used in the FCM

Tube	FITC	PE	PerCP	APC
1	blank	blank	CD45	blank
2	CD20	CD10	CD45	CD34
3	HLA-DR	CD11b	CD45	CD10
4	CD16	CD13	CD45	CD14
5	CD14	CD33	CD45	CD19
6	CD34	CD7	CD45	CD56
7	CD15	CD34	CD45	HLA-DR

Flow cytometric aberrancies

Aberrant expressions of certain antigens were defined as a more than 0.5 log difference from normal expression of that specific antigen. Aberrancies in myeloblasts and granulocytes were evaluated per subpopulation (Table 2). Flow cytometric analyses are summarized as follows: a single aberrancy is depicted in lower case letters representing a defined subpopulation of myeloid cells: b for blasts and g for granulocytes, multiple aberrancies are depicted in upper case letters (G).

Table 2. Flow cytometry aberrancies in myeloblast and granulocytes and the flow scores in MDS

Cell	Aberrancies
Myeloid blasts	
	Abnormal expression of CD13
	Abnormal expression of CD33
	Expression of CD15
	Abnormal expression of HLA-DR
	Expression of lineage infidelity markers (CD7,19,56)
Granulocytes (maturing myeloid cells)	
	Abnormal CD16/CD13 pattern
	Abnormal HLA-DR/CD11b
	Abnormal expression of CD15
	Expression of lineage infidelity (CD7,19,56)

Score point	Definition
0	No flow cytometric aberrancies in both cells
1	A single aberrancy in granulocytes
1	A single lineage infidelity markers on either granulocytes
2	2 aberrancies in granulocytes
2	2 lineage infidelity markers on granulocytes
2	A single aberrancy in myeloid blasts
2	A single lineage infidelity markers on myeloid blasts
3	3 or more aberrancies in granulocytes
3	3 or more lineage infidelity markers on granulocytes
3	2 or more aberrancies in myeloid blasts
3	2 or more lineage infidelity markers on myeloid blasts

Flow cytometric abnormal pathway

We analyzed the expression of granulocytic lineages and maturation-associated antigens of BM cells quantified in a previous study.[6] Relationships between CD11b and HLA - DR show in maturing myeloid cells that follow maturation stages. Relationships between CD13 and CD16 show in the maturing myeloid compartment. These relationships compared with normal pathway.

Flow cytometric scoring

Flow cytometric data are translated into a numerical flow-score from 0 to 3. We modified the scoring system of Wells et al[6] for simplification and greater sensitivity, by setting a higher point for abnormalities in myeloblast cells than mature granulocytes. Scores for the abnormalities generated for each cell type are summarized in Table 2. We then evaluated flow data by division into four categories: the presence of abnormal CD45 expression for myeloblasts, the presence of abnormal CD16/CD13 pattern for granulocytes (e.g. decrease of CD13 and CD16 double-positive population, overexpression of CD13 in CD16-positive cells), the presence of an abnormal HLA-DR/CD11b pattern for granulocytes (e.g. overexpression of HLA-DR in CD11b-positive granulocytes), and the presence of other abnormal expressions on granulocytes. Finally, we calculated the sum of scores to serve as our flow-score.

Statistical analysis

Proportions were compared between groups using either a chi-square test or Fisher exact test. The Mann-Whitney U test was used to compare flow-scores between the MDS and non-MDS groups. Correlation analysis was performed employing the Spearman rank correlation. Sensitivity, as evaluated among MDS patients, was defined as the proportion of patients with a flow-score above a specified cut-off point for positivity (true positive rate). Specificity was evaluated among patients with a diagnosis of non-MDS and defined as the proportion of subjects with a flow score below a certain criterion for positivity (also known as the true-negative rate). Sensitivity and specificity are reported for a range of cut-off values for the flow score to provide information about the trade-offs in these quantities as the cut-off value is shifted. *P*-values less than 0.05 were regarded as significant.

Results

Patient characteristics

Median age was 76.5 years (range, 34-87 years) in MDS patients and 61 years (range, 26-87 years) in non-MDS patients, i.e., the MDS patients were older than the non-MDS patients (Mann-Whitney, $P<0.01$). There was no difference in the male to female ratio between the two disease groups (chi-square, $P=0.22$). In non-MDS patients, adequate cytogenetic data were available for 8 patients, and all were normal. In MDS patients, adequate cytogenetic data were available for 24 patients, of whom 14 had a normal karyotype and 10 had an abnormal karyotype. IPSS scores were evaluable for 24 patients with MDS, all of whom showed IPSS less than 1.0. Age, sex, diagnosis category, hematological data, cytogenetic data, and IPSS score are presented in Table 3.

Table 3. Characteristics of patients with MDS

No	Age	sex	Diagnosis	cytogenetic	WBC (/μL)	Hb (g/dL)	Plt ($\times 10^4$/μL)	IPSS
Patients with MDS								
1	66	F	RA	normal	3800	7.8	11	0
2	34	M	RA	normal	3100	7.8	9	0.5
3	76	M	RA	del(20)(q11)	2800	8.2	7.4	0.5
4	48	M	RA	-Y	2200	8.4	9.2	0.5
5	85	M	RA	-Y	3400	9.2	15	0.5
6	74	M	RA	normal	3600	11	2	0
7	59	F	RA	normal	2300	5.3	0.8	0.5
8	82	M	RA	del(11)(q)	1900	9.3	9.3	1
9	77	M	RA	der(3)t(1;3)(q21;q29)	1100	9.3	6.6	1
10	77	F	RA	normal	6500	10.9	31.7	0
11	37	M	RA	normal	1700	12.5	3.6	0.5
12	85	F	RA	del(13)(q?)	3000	9.9	5	1
13	87	M	RA	normal	2700	9.8	2.2	0
14	72	F	RA	del(20)(q?)	5200	9.6	3.6	0
15	70	F	RA	normal	3100	9.1	7.9	0.5
16	55	M	RCMD	NA	3100	8.2	6.5	--
17	82	F	RCMD	NA	2800	7.8	7.2	--
18	82	F	RCMD	normal	2700	6.9	26.8	0
19	78	M	RCMD	5q-,+8	1100	7.8	35.6	1
20	78	M	RCMD	-Y	2500	11.7	8.5	0
21	70	M	RCMD	normal	4600	7.4	10.4	0
22	82	F	RCMD	+8	2500	8.8	36.7	0.5
23	84	M	RCMD	normal	3500	10.2	9.3	0
24	82	F	RCMD	normal	5300	6.5	22.2	0
25	76	F	RCMD	normal	2100	11.8	0.5	0.5
26	74	M	RCMD	normal	1800	9.2	0.3	0.5
Patients with non-MDS								
27	62	F	secondary	NA	3800	8.2	12.8	--
28	46	F	secondary	NA	2900	9.2	19.8	--
29	48	M	secondary	NA	3800	8.3	25.7	--
30	26	M	secondary	normal	2200	14.9	15.8	--
31	73	F	secondary	NA	2900	8.2	13.8	--
32	32	M	PRCA	NA	3800	7.5	12	--
33	54	F	CRF	normal	5400	8	30.3	--
34	48	F	CRF	NA	4800	9.2	28.5	--
35	60	M	CRF	normal	8700	7.4	33.8	--
36	74	M	AA	NA	2800	6.8	11.2	--
37	86	F	AA	normal	3000	8.9	16.2	--
38	65	F	AIHA	normal	4800	7.3	26.7	--
39	70	F	AIHA	NA	3900	6.4	18	--
40	70	F	AIHA	NA	3200	7.3	15.4	--
41	57	F	IDA	NA	2800	7.5	13.6	--
42	75	M	VitB12	normal	1800	6.7	7.5	--
43	87	F	VitB12	normal	2000	4.3	1.1	--
44	53	M	folate	normal	3300	10.7	6.2	--

NA: not available; IPSS: International Prognostic Scoring System; secondary: secondary anemia, PRCA:pure red cell anemia,AA: aplastic anemia, AIHA:autoimmune hemolytic anemia, IDA: iron deficiency anemia, CRF: anemia of chronic renal failure, VitB12: pernicious anemia, and folate: folate deficiency anemia.

Aberrant immuno-phenotype identified by flow cytometric analysis

Some examples of normal and aberrant granulopoiesis are shown in Figure 1. In the analysis of myeloid progenitor cells, a lineage infidelity marker was observed on myeloblasts in 11% of MDS cases (3 of 26), with co-expressed markers such as CD7, CD56 and CD19. Among non-MDS patients, 5.5% (1 of 18) had a lineage infidelity marker on CD34+ myeloid blasts. One patient with aplastic anemia showed expression of infidelity marker CD19 on myeloblasts in addition to abnormal granulocytic maturation. Other aberrant phenotypes on myeloid blasts observed only in MDS patients included low expressions of CD14 and CD11b, and

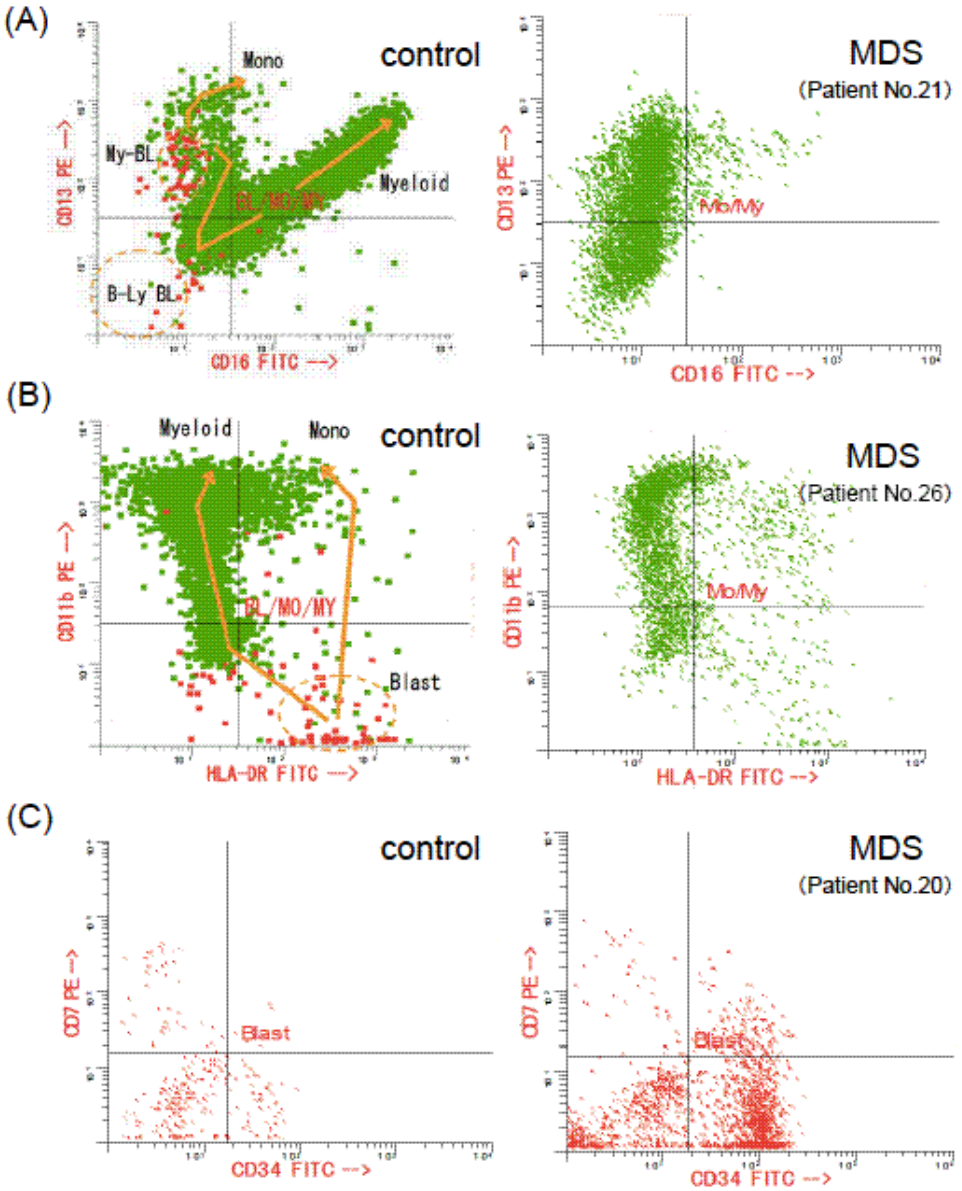

Figure 1. The pattern of normal and aberrant granulopoiesis. (A) The relationship between CD16 (x-axis) and CD13 (y-axis) for granulocytes. Development from immature to mature granulocytes is illustrated by a sickle-shaped yellow arrow in the graph of the control sample. The graph of patient with MDS shows aberrant antigen expression and maturation compared with the normal control. (B) The relationship between HLA-DR (x-axis) and CD11b (y-axis) for granulocytes. A yellow arrow in the control sample depicts normal maturation of immature blasts toward maturing granulocytes and monocytes. The granulocytic subpopulation in the MDS sample shows abnormal pathway of CD11b. (C) Expression of infidelity antigen CD7 (y-axis) on myeloid progenitors, defined by $CD45^{dim}SSC^{dim}$ and CD34 expression, is depicted.

over-expressions of CD13, CD33, CD34 and HLA-DR.

In the immuno-phenotype analysis of maturing myeloid cells (granulocytes), 50% of MDS (13 of 26), 53% of RA (8 of 15) and 45% of RCMD (5 of 11) patients showed abnormal expressions. Abnormal relations between CD16 and CD13 and between HLA-DR and CD11b were prominent in MDS patients. Other abnormalities of maturing myeloid cells included an abnormal pathway, lack of CD15, low stage 2 cells, and abnormal expression of CD38/CD56. Among patients with non-MDS anemia, immuno-phenotypic abnormalities of maturing myeloid cells (granulocytes) were found in 22% (4 of 18), with abnormal relations between CD16 and CD13, and also between HLA-DR and CD11b in 2 patients with pernicious anemia, one with folate deficiency, and one with anemia of chronic disorders. A patient with aplastic anemia showing expression of an infidelity marker (CD19) on myeloblasts also had abnormal granulocytic maturation, including an abnormal pathway with 3% of heterogeneous cells expressing CD38 strong positive/CD45dim/CD19positive. Flow cytometric results for individual patients are summarized in Table 4.

Table 4. Dyspoiesis of myeloblast and granulocytes detected by FCM

FCM abnormality	MDS (n=26) no (%)	non-MDS (n=18) no (%)
Abnormal myeloblasts	3 (11.5)	0 (0)
Abnormal maturing myeloid		
Abnormal CD16/CD13 pattern	11 (42.3)	5 (27.8)
decrease of CD13 and CD16 double-positive population	2 (7.7)	1 (5.6)
overexpression of CD13 in CD16-positive cells	9 (34.6)	4 (22.2)
Abnormal HLA-DR/CD11b (overexpression of HLA-DR in CD11b-positive granulocytes)	3 (11.5)	4 (22.2)
Abnormal expression of CD15	1 (3.8)	0 (0)
Expression of lineage infidelity markers(CD7,19,56)	0 (0)	0 (0)

Flow-score

Results of the flow-score are also summarized in Table 4. The scores were compared between MDS and non-MDS patients. Seventy-three percent of MDS patients (19 of 26), but only 28% of non-MDS patients (5 of 18), had flow-scores of 1 or greater. The relationships between the flow-score and patient groups are depicted in Figures 2 and 3. The median flow-score was 1 (range, 0-4) in MDS and 0 (range, 0-3) in non-MDS anemia, i.e., the former score was significantly higher than the latter (Mann-Whitney, P=0.03). Among MDS patients, there was no flow-score difference between RA and RCMD (Mann-Whitney, P=0.81). Among non-MDS patients, high scores were observed in one

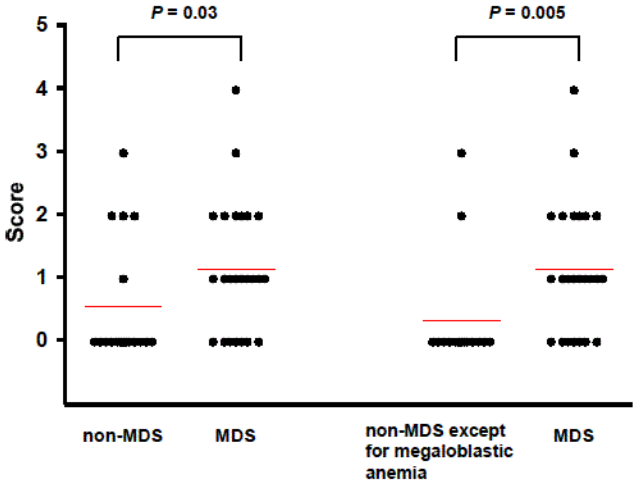

Figure 2. Flow-score comparison between MDS and non-MDS. Flow scores were calculated according to the scoring system shown in Table 2. Horizontal bars represent median values.

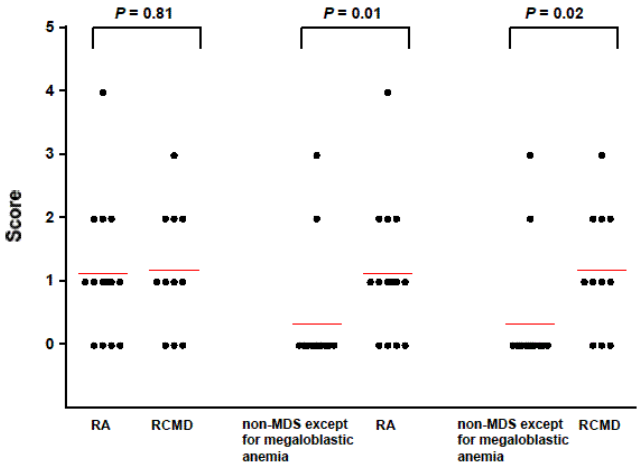

Figure 3. Flow-score comparisons between RA and RCMD, between RA and non-MDS, and between RCMD and non-MDS. Flow scores were calculated according to the scoring system shown in Table 2. Horizontal bars represent median values.

patient with anemia of chronic disorders, one with aplastic anemia, and 3 with megaloblastic anemia. Because all 3 patients with megaloblastic anemia had flow-scores of 1 or greater, we compared flow-scores between MDS and non-MDS cases excluding these 3 with megaloblastic anemia. The flow-score distribution was significantly higher in MDS than in non-MDS cases when the 3 megaloblastic anemia cases were excluded (Mann-Whitney, P=0.005). (Figure 3). Comparison of the scores between RA or RCMD and non-MDS excluding the 3 megaloblastic anemia cases, revealed these scores to also be significantly higher in RA (Mann-Whitney, P=0.01) or RCMD (Mann-Whitney, P=0.02) cases than in the non-MDS cases. There was no correlation between flow-score and IPSS score

Table 5. Immunophenotipic aberration on FCM and Flow-scoring

No	Diagnosis	Flow cytometry				Flow-scoring					
Patients with MDS		Lineage infidelity markers in blasts	Single aberrancy in blasts	Single aberrancy in granulocytes	Multiple aberrancies in granulocytes	Lineage infidelity marker	abnormal blasts	abnormal CD16/CD13 pattern	abnormal HLA-DR/CD11b	other abnormalities	Sum of flow-score
1	RA						0	0	0	0	0
2	RA			g			0	0	0	1	1
3	RA						0	0	0	0	0
4	RA			g			0	0	0	1	1
5	RA						0	0	0	0	0
6	RA		b				2	0	0	0	2
7	RA						0	0	0	0	0
8	RA			g			0	1	0	0	1
9	RA		b		G		2	1	1	0	4
10	RA			g			0	1	0	0	1
11	RA			g			0	1	0	0	1
12	RA						0	0	0	1	1
13	RA			g			0	1	0	0	1
14	RA				G		0	1	1	0	2
15	RA	CD19				2	0	0	0	0	2
16	RCMD	CD56				2	0	0	0	0	2
17	RCMD						0	0	0	1	1
18	RCMD						0	0	0	0	0
19	RCMD			g			0	1	0	0	1
20	RCMD	CD7				2	0	0	0	0	2
21	RCMD		b	g			2	1	0	0	3
22	RCMD						0	0	0	0	0
23	RCMD		g				0	1	0	0	1
24	RCMD						0	0	0	0	0
25	RCMD		g				0	1	0	0	1
26	RCMD				G		0	1	1	0	2
Pt with non-MDS											
27	ACD				G		0	1	1	0	2
28	ACD						0	0	0	0	0
29	ACD						0	0	0	0	0
30	ACD						0	0	0	0	0
31	ACD						0	0	0	0	0
32	PRCA						0	0	0	0	0
33	CRF						0	0	0	0	0
34	CRF						0	0	0	0	0
35	CRF						0	0	0	0	0
36	AA	CD19		g		2	0	0	0	1	3
37	AA						0	0	0	0	0
38	AIHA						0	0	0	0	0
39	AIHA						0	0	0	0	0
40	AIHA						0	0	0	0	0
41	IDA						0	0	0	0	0
42	VitB12				G		0	1	1	0	2
43	VitB12				G		0	1	1	0	2
44	folate			g			0	1	0	0	1

Results of flow cytometric analysis are summarized as follows; a single aberrancy is depicted in lower case representing a defined subpopulation of myeloid cells: b for blasts, g for granulocytes, multiple aberrancies are depicted in upper case (B and G), the lineage infidelity markers within normal blast percentages is depicted as nb, the lineage infidelity marker that is expressed on each definition is added to the marker.

(data not shown). The best diagnostic sensitivity and specificity of our flow-score system for low-grade MDS was observed when a cut-off score of 1 used, yielding a specificity of 73.1% with 72.2% sensitivity.

Discussion

In this study, we showed flow cytometric aberrancies to be frequent in the myeloid lineage of patients with low-grade MDS as compared to those with non-clonal forms of anemia. We also showed our flow-score to be useful for diagnosing MDS and other non-clonal anemic disorders, with the exception of megaloblastic anemia.

Flow cytometry is regarded as a potential new tool that may add significantly to diagnosis and prognosis determination for MDS patients. Herein, we studied the maturation pattern of myeloid cells by evaluating surface antigen expression in patients with anemia including MDS. Bone marrow samples from patients diagnosed as having MDS, AIHA, AA, PRCA or IDA were analyzed with a panel of antibodies targeting various differentiation stages of the granulocytic lineage. Consistent differentiation patterns were seen in most of the anemic disorders except for MDS, AA, Vitamin B12 deficiency and folate deficiency. Of note, patients with disorders other than MDS also exhibited aberrancies in maturing granulocytes. In 17 of 26 MDS patients diagnosed according to the WHO criteria, aberrant differentiation antigen expression was demonstrated in myeloid blasts and granulocytes.

In our study, aberrant immuno-phenotypes of maturing granulocytes were detected in about 40% of MDS patients, implying that this finding could provide a useful parameter for the differential diagnosis of various anemic disorders. However, since aberrancies in granulocytes were also detectable in AA, Vitamin B12 deficiency, folate deficiency and anemia of chronic disorders, careful interpretation is necessary. Therefore, at present, cytomorphology is still considered to be the mainstay for diagnosing MDS.

The present study has several limitations. First, the number of patients examined was too small to allow differences to be statistically confirmed. Second, we included a limited number of immuno-phenotypic markers in our flow-scoring system. Recently, two studies demonstrated the utility of their own FCMSS for diagnosing low-grade MDS based on abnormal multi-lineage antigen co-expression in CD34+ blasts, showing good sensitivity and specificity for distinguishing low-grade MDS from non-clonal cytopenia.[12,13] Another report suggested CD38 expression on CD34+ cells to be of value for diagnosing MDS based on a single marker.[14]

In conclusion, our simple FCMSS focusing on myeloblasts and granulocytes may be useful for distinguishing patients with low-grade MDS from those with other types of anemia. There is as yet no standard FCMSS that efficiently distinguishes MDS from other non-clonal cytopenias, and our simple scoring system may provide a basis for the development of standard protocol.

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