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Original Article

Utility of peripheral blood flow cytometry in differentiating low grade versus high grade myelodysplastic syndromes (MDS) and in the evaluation of cytopenias

Hooman H Rashidi[1], Xiangdong Xu[1], Huan-You Wang[1], Nelofar Q Shafi[2], Karuna Rameshkumar[3], Karen Messer[4], Brian R Smith[2], Michal G Rose[2]

[1]University of California San Diego School of Medicine & VA Medical Center San Diego, USA; [2]Yale University School of Medicine and Connecticut VA Medical Center, USA; [3]Department of Clinical Pathology, St. John's Medical College Hospital, Bangalore, India; [4]Division of Biostatistics, Moores UCSD Cancer Center, CA, USA

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Abstract: The diagnostic utility of flow cytometry in the evaluation of cytopenias and in the differential diagnosis of low -grade versus high-grade myelodysplastic syndrome (MDS) is not widely appreciated. In this report, we measured granulocyte CD10/control fluorescence ratio in 29 patients with MDS & chronic myelomonocytic leukemia (CMML) using peripheral blood (PB) flow cytometry (FC). We found a lower ratio in high-grade MDS and CMML (mean ratio of 2.2 ± 0.7) vs. low-grade MDS (3.65 ± 0.9) and 16 cytopenic controls without MDS (3.67 ± 0.65; $p<0.001$). The sensitivity and specificity of CD10 ratio <3 for the group that included the high risk MDS and CMML patients were 87.5% and 100%, respectively. Our data suggests that FC of PB may be helpful in the work-up of patients with cytopenias and in the differential diagnosis of low-grade vs. high-grade MDS.

Keywords: Myelodysplastic syndromes, flow cytometry, CD10, high-grade MDS, low-grade MDS, bone marrow, peripheral blood

Introduction

The myelodysplastic syndromes (MDS) are a heterogeneous group of clonal hematologic disorders of the elderly. They are characterized by peripheral blood cytopenias, and patients usually have a hypercellular bone marrow showing dysplastic morphologic features in at least one hematopoietic lineage. Their prognosis is variable, with some patients exhibiting minimal symptoms, while others succumb to symptomatic marrow failure and/or progression to acute myeloid leukemia (AML) [1, 2].

The diagnosis of MDS is established by correlating the clinical picture of refractory cytopenias with the morphologic abnormalities of the bone marrow aspirate and biopsy, along with cytogenetic abnormalities in the bone marrow cells. While flow cytometry (FC) of bone marrow aspirate is often performed, the main purpose/function of FC in the evaluation of bone marrow aspirate in patients with MDS at the present time focuses on the quantification and characterization of blasts. In 2001, Stetler-Stevenson et al performed bone marrow flow cytometric immunophenotyping of 44 patients with MDS and found that FC was useful in difficult diagnostic cases in which morphology and cytogenetics were non-conclusive [3]. A recent European Leukemia Net workshop sought to standardize methods and interpretations of FC of bone marrow in MDS [4] and discussed the role FC may play in the future in the diagnosis of some of the new World Health Organization (WHO) categories of good prognosis MDS, in which the morphologic abnormalities may be subtle [4, 5]. A number of other studies emphasizing marrow FC have come to similar conclusions [6-19], further establishing the value of this diagnostic modality.

Establishing the diagnosis of MDS using current guidelines requires a bone marrow aspirate and biopsy, which is an invasive procedure and not always definitively diagnostic, especially in low

grade MDS. Given the frequency in clinical practice of elderly patients with mild cytopenia(s) that in and of themselves would not demand therapeutic intervention, it would be desirable to have a blood assay that may help guide the need for furthermore invasive evaluation. The University of Pennsylvania group has previously explored the potential of peripheral blood FC analysis for the diagnosis of MDS [20, 21]. Using a four-antigen panel (CD10, CD11a, CD66, and CD116), their data showed that patients with MDS of any grade could be distinguished from normal controls with a sensitivity of 73% and a specificity of 90%. In the current study, we sought to (a) determine whether in patients with MDS a single flow cytometric abnormality in peripheral blood could aid in the diagnosis, for the purpose of further confirming, and perhaps simplifying, these prior observations, (b) determine whether MDS cytopenic patients could be distinguished from patients with non-MDS cytopenias and not just from normal individuals, with the idea that this was a common clinical conundrum that might benefit from a new peripheral blood based assay, (c) determine whether CD10 under-expression on blood granulocytes correlated with the severity of the disease and/or with established prognostic indices.

Materials and methods

Paired bone marrow aspirates and peripheral blood samples from 16 cytopenic controls without MDS and 29 cytopenic patients with established MDS and/or chronic myelomonocytic leukemia (CMML) diagnosed at the Veterans Affairs (VA) Connecticut Healthcare System were analyzed. Cytopenia was defined as hemoglobin < 10 g/dL, absolute neutrophil count < 1800 $x10^6$/L, and/or platelet count < 100,000 $x10^6$/L. The study was approved by the VA CT Institutional Review Board, and all patients gave written informed consent. Peripheral blood and bone marrow specimens of all patients and controls were reviewed by two hematopathologists (NQS and KS). The diagnoses of MDS were established according to the WHO classification [22] after review of clinical data, peripheral blood and bone marrow morphology, cytogenetic analysis, and flow cytometry of bone marrow aspirate. The cytopenic control group in this study consisted of 16 patients without MDS. Of note, however, the 16 cytopenic patients did not include any patients with neutropenia, whereas the MDS group included 43% (12/29) patients with neutropenia. Cytopenic patients without neutropenia were selected to minimize the possibility of an effect on CD10 expression from a low neutrophil count in the control group.

Four milliliter (ml) of acid-citrate-dextrose anticoagulated peripheral blood was collected for the flow cytometric studies and analyzed using standard techniques as previously published [16, 17]. The antibody panels for peripheral blood with associated fluorochromes included: FITC/PE/PECY5 (control), CD4/CD8/CD3, CD5/CD23/CD19, CD10/CD7/CD34, CD64/CD56/CD13, CD22/CD11b/CD33, CD57/CD8/CD3, CD45/glycophorin/CD34, and CD16/CD11b/CD13. All antibodies (Becton Dickinson, San Jose, CA) were appropriately titrated on normal human cells.

The mean channel number of 90° side scatter (SSC) and the percentage of positive cells for each marker were determined using 5-parameter, 3-colour flow cytometry with a FACScan flow cytometry equipped with a 15-mW argon laser (excitation at 488 mm; Becton Dickinson, CA). The sensitivity of fluorescence detectors was set and monitored using Calibrite Beads according to the manufacturer's recommendations. The amplifier voltage settings were kept constant and the variations of FSC and SSC were less than 2%. Cells were immunostained with relevant monoclonal antibodies with IgG1-FITC and IgG2-PE as negative isotypic controls. The data were analyzed with CellQuest software (Becton Dickinson, CA).

The FC data obtained from the blood was analyzed independently and blinded to the diagnosis of MDS. The expression of each marker was calculated as the ratio between the geometric mean fluorescence intensity of the specific marker and the geometric mean fluorescence of control antibody labeling (e.g. CD10 ratio). The CD10 ratio was calculated by using the Geometric mean fluorescent intensity (G-MFI) which was derived for the autofluorescence control and for the CD10 antibody. The G-MFI for CD10 was then corrected for the autofluorescence to generate the corrected G-MFI [21].

The diagnosis and classification of MDS were performed according to the World Health Organization criteria [22]. Cytogenetics studies were performed on all marrow aspirates. Pa-

tients with refractory cytopenia with multilineage dysplasia (RCMD), refractory cytopenia with multilineage dysplasia with ringed sideroblasts (RCMD-RS), and CMML were collectively considered in the same group (high-grade MDS) for the purpose of this study. No patients with refractory anemia with excess blasts (RAEB) were available for inclusion in the study. Low-grade MDS included refractory anemia (RA) and refractory anemia with ringed sideroblasts (RARS). International Prognostic Scoring System (IPSS) & World Health Organization Prognostic Scoring System (WPSS) were calculated on all patients with MDS as previously published [23, 24].

Results

Demographics

The mean age of the MDS patients was 75 years (range 47 to 96 years) and included 27 men and two women (consistent with the predominantly male patient population served by the VA). The mean age of the control group was also 75 years. Out of the 29 MDS patients, 16 were classified as high-grade MDS which included RCMD and CMML (although the new WHO criteria classifies CMML as MDS/MPN, these patients, due to their known clinical behavior, were also placed in the same prognostic category as high grade MDS). The remaining MDS patients were categorized as low-grade MDS which included RA and RARS. Sixty-two percent (18/29) of the MDS patients had a normal karyotype, of which 10/18 (56%) were in the high-grade group and 8/18 (44%) were in the low-grade group. **Table 1** summarizes the WPSS, IPSS, karyotype and the WHO classification of the 29 MDS patients.

Of the 29 MDS patients, 24 (82%) displayed abnormal peripheral blood morphology, which included macrocytosis (8 patients), microcytosis with hypochromasia (7 patients), teardrop cells (11 patients), neutropenia (12 patients), pseudo Pelger-Huet anomaly (3 patients), and giant platelets (8 patients).

Peripheral blood flow cytometry results

There were no significant differences in the granulocyte and monocyte expression of CD11b, CD13, CD14, CD16, CD33, CD 56, and CD64 between the cytopenic controls, the low-grade MDS and the high-grade MDS patients

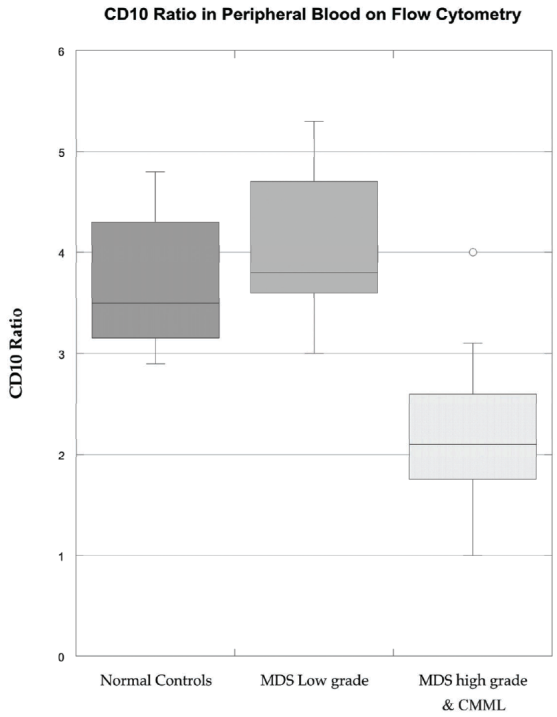

Figure 1. CD10 ratios in peripheral blood in patients with high grade MDS and CMML (n=16), low grade MDS (n=13), and cytopenic controls (n=16). Differences between the three groups are statistically significant (2.2 ± 0.7, 3.65 ± 0.9 and 3.67 ± 0.65 for high-grade MDS and CMML, low grade MDS, and control group, respectively, $p<0.001$ for comparison between high grade and low grade MDS, and high grade MDS with CMML and controls).

(data not shown). However, the mean CD10/control fluorescence ratio (CD10 ratio), the original hypothesis of the study, was significantly decreased in the high-grade MDS group [mean ± standard deviation (SD)] of CD10 ratio is 2.2 ± 0.7, compared with the low-grade MDS group (3.65 ± 0.9; $p < 0.001$) and also compared with the cytopenic control group of CD10 ratio 3.67 ± 0.65; $p<0.001$; (**Figure 1**).

The WPSS score was calculated for all patients based on Malcovati et al [24]. In that study, WPSS scores of 0 and 1 were found to be associated with low-risk MDS patients while scores of 2 or greater placed the patients at intermediate to high risk. Interestingly, these WPSS scoring profiles correlated well with the CD10 ratios calculated in our MDS patients, namely patients with high WPSS scores were found to have significantly lower CD10 ratios

Table 1. MDS Patient characteristics.

Patient No.	PBF CD10 Ratio	WHO Dx	WPSS	Age/Sex	IPSS	Dysplastic Lineage(s)	Cyto-genetics
1	1.0	RCMD	3	76/F	1	Tri	Tri 8
2	1.5	RCMD	2	96/M	0.5	Tri	-20q
3	1.7	RCMD	2	69/M	0.5	Tri	N
4	1.7	CMML	3	86/M	0	Tri	N
5	1.8	RCMD-RS	4	84/M	1.5	Bi (E&Me)	Mon 7
6	1.9	RCMD	2	73/M	0.5	Tri	N
7	2.0	RCMD	1	85/M	0	Tri	N
8	2.0	RCMD	2	80/M	0.5	Bi (E&Me)	N
9	2.2	CMML	3	71/M	0.5	Tri	N
10	2.2	CMML RCMD	4	71/M	1.5	Tri	-7q
11	2.5	RCMD	3	80/M	1.5	Bi: (E&Me)	Mon 7
12	2.6	RCMD	2	81/M	0.5	Tri	Tri 5
13	2.6	RCMD	1	47/F	0.5	Bi (E&Me)	N
14	2.6	RCMD	2	64/M	0.5	Tri	N
15	3	RA	1	72/M	0.5	Mono (E)	N
16	3.1	RA	1	71/M	1	Mono	N
17	3.1	RCMD-RS	2	89/M	0.5	Bi (E&Me)	N
18	3.2	RA	1	78/M	0.5	Mono (Me)	-Y
19	3.6	RA	1	77/M	1	Bi (Me & E)	-20q
20	3.7	RARS	1	88/M	0	Mono+RS (Me)	-Y
21	3.8	RA	1	58/M	1	Mono (Me)	N
22	3.8	RA	1	67/M	0.5	Mono (E)	N
23	4.0	RCMD-RS	2	77/M	1.5	Bi (E&Me)	N
24	4.1	RA	1	88/M	0	Mono (Me)	-Y
25	4.5	RARS	0	83/M	1	One+RS (E)	N
26	4.7	RARS	1	82/M	0.5	Mono (My)	N
27	4.7	RA	1		2	Bi (My&E)	N
28	5.1	RA	1	75/M	0	Mono (E)	N
29	5.3	RA	2	84/M	0.5	Mono (E)	Tri 15

PBF - peripheral blood flow cytometry; WHO - World Health Organization; WPSS - World Health Organization Prognostic Scoring System; IPSS - International Prognostic Scoring System; E - Erythroid, Me - Megakaryocytic, My - Myeloid; N - Negative; Tri - trisomy.

while the majority of the high CD10 ratio patients were found to have lower WPSS scores. The mean CD10 ratio for patients with WPSS scores 0 or 1 was 3.71 ± 0.88 SD versus 2.41± 1.07 SD for patients with WPSS scores of 2 or higher ($p=0.001$). A CD10 ratio of <3 correctly identified 14 out of 16 high grade MDS patient group for an observed sensitivity of 87.5%; All 29 of patients with a score of ≥3 for an observed specificity of 100% are either low-grade MDS patients (13 patients) or control group (16 patients). As oppose to the WPSS which showed a strong correlation to the CD10 ratio, IPSS did not correlate to the CD10 ratio in our study.

Discussion

In this study, the expression of CD10 on peripheral blood granulocytes was measured by FC, and significantly decreased CD10 ratio was observed in patients with high-grade MDS and CMML compared to both non-MDS cytopenic controls and to patients with low-grade MDS. Furthermore, the CD10 ratio correlated with an established prognostic index, the WPSS. The CD10 ratio was only noted to strongly correlate with the WPSS score and a CD10 ratio correlation to the IPSS score was not evident. The CD10 ratio correlation to WPSS score also correlated with more aggressive disease as noted in **Table 1**.

Our findings of decreased granulocyte CD10 expression in blood from MDS patients are consistent in part with a previous study on bone marrow, which showed lower granulocyte CD10 expression in MDS patients [25]. In that study, the investigators also found approximately one-

third the quantitative normal expression of CD10 on MDS granulocytes (extrapolating from the Figure in their paper) and also found no significant difference in CD13, CD33 or CD11b expression. CD10 has been shown to play an important role in the control of chemotaxis and the inflammatory response of neutrophils [26-28].

Although peripheral blood FC has been used to quantify and characterize circulating blasts in MDS [29, 30], the use of blood FC of granulocytic populations for MDS diagnosis has only been previously reported by Cherian et al [20, 21]. Our findings further support their observation that such an assay can be useful in the evaluation of patients with suspected MDS. The current study differs from theirs in that Cherian et al compared results to normal controls whereas we have chosen to analyze non-MDS cytopenic controls, thus extending the earlier results to further suggest that this can be a useful technique in distinguishing the cause of cytopenia in the elderly. Moreover, the current study suggests that the specific populations of MDS patients who have a normal granulocyte CD10 expression are those with low-grade disease and a better prognosis. Although further confirmatory studies are needed, our study nevertheless is consistent with the possibility that one might be able to gauge the importance of marrow examination in such patients, as opposed to watchful waiting, based on the flow cytometry assay. Although a "normal" granulocyte CD10 ratio (>3) would still include rare patients with high grade MDS (2 cases of RCMD), the CD10 ratio of <3 were only noted in the high grade MDS and CMML patients and were not seen in the low grade MDS or the control group. This data might be especially useful in the approximately one third of elderly people who have unexplained anemia, some of whom are thought to have undiagnosed MDS [31].

There are three limitations to the current study. First, the cut-off value used to determine predictive value of the assay was determined from the data. Second, although the study is consistent with and strengthens previous results, the total number of patients studied in this fashion remains relatively small. The prior study included patients with RAEB but the current study did not. While most patients with RAEB have rapidly worsening symptomatic cytopenias that would warrant a bone marrow evaluation regardless of the peripheral blood findings, this is still a relatively understudied group. Third, this retrospective study examined association of CD10 expression with risk score, not with actual clinical outcomes. We believe that the ideal next study would be a multi-institutional study of patients presenting with unexplained cytopenias, with aim to quantify the prognostic value of low peripheral blood CD10 expression against prospectively observed clinical endpoints.

In summary, our study demonstrates that a simple flow cytometric assay performed on peripheral blood may have a sensitivity as high as 87.5% to detect high risk MDS along with a very high specificity. If follow up prospective studies show similar results, potentially for those very elderly patients who diagnosis of MDS is contemplated, the clinician may opt to look at the CD10 ratio of peripheral blood as well. However, we do not foresee this to replace a bone marrow biopsy since it will not be able to distinguish the more common lower grade MDS from cytopenic patients without MDS. We think upon further validation studies, this approach may be incorporated in a workup of patients with suspected MDS and used in conjunction with the other studies which include a bone marrow biopsy, and the cytogenetics results. If confirmed in larger prospective studies, findings may help clinicians in determining further management of patients with otherwise unexplained cytopenias.

Address correspondence to: Dr. Hooman H. Rashidi, University of California San Diego School of Medicine & VA Medical Center San Diego, USA; Dr. Michal G. Rose, Yale University School of Medicine and Connecticut VA Medical Center, USA

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