Reduced CD38 expression on CD34+ cells as a diagnostic test in myelodysplastic syndromes

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ABSTRACT

Diagnosis of myelodysplastic syndrome can be difficult especially in cases with a low blast count and a normal karyotype. Flow cytometry has been used to distinguish myelodysplastic syndrome from non-clonal cytopenias. No one single simple flow cytometric parameter has been proposed to be diagnostic of myelodysplastic syndrome. We have studied samples from 100 myelodysplastic syndrome patients and as control samples; 70 non-clonal cytopenias, 5 subjects with normal hematology, 31 patients with acute myeloid leukemia and 11 with chronic myelomonocytic leukemia or myeloproliferative disorder. We show that reduced relative mean fluorescence of CD38 below a threshold value on CD34+ cells diagnosed low-grade myelodysplastic syndrome with 95% sensitivity (95% confidence interval, 87-99%) and 92% specificity (95% confidence interval, 82-97%). This simple flow cytometric test may be of value in the routine clinical diagnosis of myelodysplastic syndrome, especially in cases with a low blast count and normal karyotype.

Key words: myelodyplasia, immunophenotype, FACS diagnosis.

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Introduction

The clonal myelodysplastic syndromes (MDS) cause cytopenias that are more prevalent in an ageing population.[1,2] Low-risk MDS (blast count <5%) without ring sideroblasts and a normal karyotype can be difficult to differentiate morphologically from dysplastic non-clonal cytopenias, that are also common in the elderly. Several studies have used flow cytometry to try and distinguish low-risk MDS from non-clonal cytopenias by assessing the pattern of myeloid/erythroid/lymphoid maturation from progenitors and the immunophenotype of the CD34+ compartment.[3-13] However, no one simple flow-based marker reliably differentiates MDS from non-clonal cytopenias. This has given rise to a number of scoring systems combining multiple measurements. They have variable sensitivity/specificity in MDS diagnosis and can be complex, which detracts from their routine use in clinical non-specialist flow cytometry laboratories.

Perhaps the most common abnormality that we,[6] and others[9,11,12,14,15] have documented are reduced numbers of B-cell CD34+ progenitors in MDS. As B-cell progenitors have higher CD38 expression, and thus a higher CD38 relative mean fluorescent intensity (RFMI) as a population, we hypothesized that a lower number of B-cell progenitors would reduce the mean fluorescence intensity (MFI) of CD38 expression on CD34+ cells.

In addition, the MFI of CD38 on CD34+ cells would be further reduced in high-risk MDS as this condition has a higher number of immature CD34+CD38- hemopoietic progenitors.[8] Thus, we investigated whether reduced mean fluorescence intensity (MFI) of CD38 expression on CD34+ cells could be used as a surrogate marker for abnormalities in the MDS CD34+ compartment and whether this would provide a simple useful single flow cytometric measurement diagnostic of MDS.

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Design and Methods

Patients

Human adult bone marrow was obtained after informed consent. Two separate sample cohorts were analyzed in 2 different laboratories. Patients' characteristics are shown in *Online Supplementary Tables S1 and S2*.

Flow cytometry

All bone marrow samples were processed within 24-48 h of aspiration. For cohort 1 samples, mononuclear cells were isolated by layering cells on Ficoll (*Lymphoprep*, Axis-shield UK), followed by centrifugation at 1800 rpm for 30 min. Mononuclear cells were labeled with; FITC-conjugated anti-CD45 (clone HI30), PECy5-conjugated anti-IgG1, APC-conjugated anti-CD34 (581) (Becton Dickinson, San Josè, CA, USA); PECy5 anti-CD19 (J3-119), PECy5 anti-CD38 (LS198-4-3) (Beckman Coulter, Fullerton, CA, USA). In cohort 2, nucleated cells were analyzed after red cell lysis. They were labeled with combinations of; PERCP-conjugated anti-CD45 (clone 2D1); PE-conjugated anti-CD38 (HB7), APC-conjugated anti-CD34 (8G12), PERCP-conjugated anti-CD34 (8G12), PE-conjugated isotype control (CD56-MY31), APC-conjugated anti-CD33 (P67.6), PE-conjugated anti-CD117 (10452) (Becton Dickinson, San Jose, CA). In both cohorts cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San José, CA, USA). At least 100,000 nucleated cells (cohort 1) or 20,000 nucleated cells (cohort 2) were acquired for each antibody combination. All procedures were completed within 24-48 h of marrow aspiration for every patient in both cohort 1 and 2. This time period is less than the recommended upper limit of cell sample storage for FCM (72 h). Data was further analyzed using FlowJo software (Treestar, Ashland, OR, USA). $CD34^+$ cells were defined by standard serial gating ($CD34^+SSC^{lo}$ followed by $CD34^+CD45^{lo}$) (*Online Supplementary Figure S1*). Samples from cohort 2 with insufficient $CD34^+$ cells were excluded, as measurements from analysis would be unreliable. It is important that sufficient numbers of nucleated cells/$CD34^+$ cells are collected in hypocellular samples so that samples from hypoplastic MDS and aplastic anemia patients can be studied. RMFI was quantified as CD38 mean fluorescence intensity (MFI) divided by MFI of isotype control staining (*Online Supplementary Figure S1*). Samples from the second cohort were processed in a single clinical laboratory by at least 3 operators in rotation all using standardized operating procedures for instrument set up and compensation. Gating analysis was then checked by a further operator (*SF*). The intra-assay coefficient of variation (CV) of CD38 mean fluorescence intensity on $CD34^+$ cells in the clinical laboratory processing samples from the second cohort was 8.5% (10 replicates of a single sample) when collecting 20,000 nucleated bone marrow cells (~200 $CD34^+$ cells on average). This would be further improved by collecting sufficient nucleated cells to acquire more $CD34^+$ cells. Gating $CD34^+$ cells is routinely performed in many laboratories but in view of potential inter-operator and inter-laboratory variability, as well as variation of other flow cytometry parameters (instrument, fluorochrome, sample anticoagulation/ manipulation), other laboratories should establish their own reference ranges using the appropriate standardized analysis protocol when performing this assay.

Results and Discussion

We initially compared $CD34^+$ cells from low-grade MDS patients (n=10), normal controls (n=5), pathological controls and regenerating bone marrow samples post-allogeneic bone transplant (n=10) (*Online Supplementary Figure S2 and Online Supplementary Table S1*). In this cohort, essentially a *training cohort*, we specifically studied MDS cases where the diagnosis was unambiguous and controls that could not be confused with MDS. CD38 MFI was quantified by RMFI (see Methods) as this measurement is more

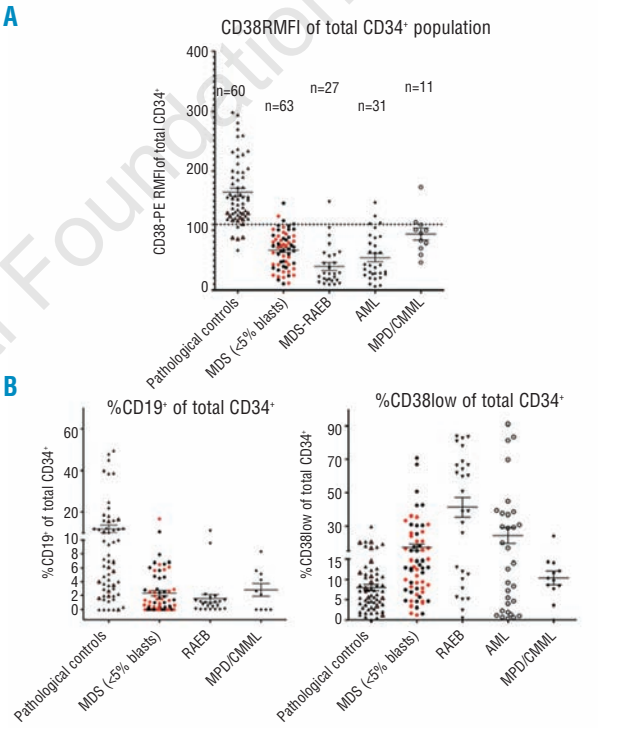

Figure 1. CD38 Relative Mean Fluorescence Intensity (RMFI) of $CD34^+$ cells and percentages of B cell and $CD38^{low}$ progenitors in MDS patients and control populations. Graphs show: (A) CD38-RMFI values (B) percentage values for $CD19^+CD34^+$ (left) and $CD34^+CD38^{low}$ cells (right) of total $CD34^+$ population in samples from patients with a range of conditions causing cytopenia including immune causes and/or splenomegaly (pathological controls, triangles) (n=60). Patients with immune thrombocytopenia, hemolytic anemia and liver disease are shown as black bordered red triangles; MDS with a blast count of <5% (n=63) (closed circles) including subgroup of MDS patients without ringed sideroblasts and/or an abnormal karyotype (n=32) (red circles); MDS-RAEB (n=27) (triangles); AML (n=31) (diamonds) and MPD/CMML (n=11) (open circles). The clinical characteristics are set out in the *Online Supplementary Table S2*. $CD34^+$ cells were defined by standard serial gating ($CD34^+SSC^{low}$ followed by $CD34^+CD45^{low}$). CD38-RMFI=CD38PE-MFI divided by MFI of isotype control-PE staining. Horizontal bars are means and the SEM for each sample group is shown. The number of samples (n=) in each group are shown in (A). Dotted line in (A) shows threshold value of CD38PE-RMFI defined by receiver-operator characteristic curve that in this cohort diagnoses low-grade MDS with 95% sensitivity (95% confidence interval, 87-99%) and 92% specificity (95% confidence interval, 82-97%).

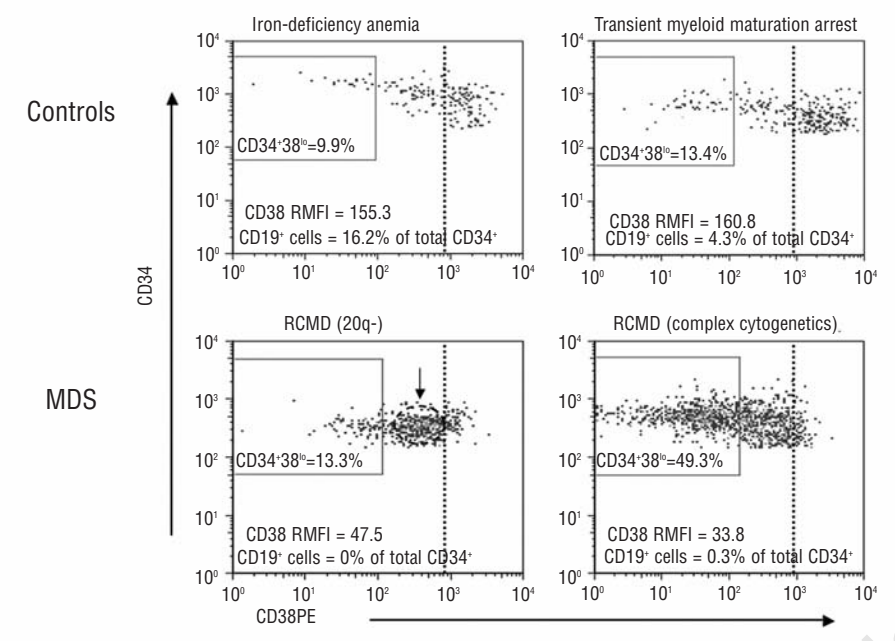

Figure 2. Examples of typical flow cytometric CD38 expression patterns of $CD34^+$ cells from pathological controls compared to MDS patients. $CD34^+$ cells were defined by standard serial gating ($CD34^+SSC^{lo}$ followed by $CD34^+CD45^{lo}$). CD38-RMFI (CD38PE-MFI divided by MFI of isotype control PE staining), as well as percentage of B-cell ($CD19^+$) and $CD38^{lo}$ progenitors of total $CD34^+$ population, is shown for each example. In the pathological control plots (top), $CD34^+$ cells are skewed towards a higher CD38 expression (to right of dotted line), despite no major shifts in frequency of $CD19^+$ or $CD38^{lo}$ progenitors. Plots of the MDS samples show that CD38-RMFI of $CD34^+$ cells can be reduced by a relative increase of $CD38^{moderate}$ cells or CD38lo cells and as well as a depletion of $CD38^{hi}$ cells. On the left MDS sample plot, the clustering of $CD38^{moderate}$ cells within $CD38^{moderate}$ region is shown by the dotted circle indicated by arrow.

reproducible. Large numbers of nucleated bone marrow cells (>0.5×10^6) were acquired to provide an accurate analysis of CD38 RMFI in low frequency $CD34^+$ subsets. CD38 RMFI in $CD34^+$ cells was lower in MDS patients (mean=27.6; range: 5.6-51.6) than normal controls (mean=58; range: 33.3-78.8). The difference in CD38 RMFI between MDS and pathological controls was even more striking, with no overlap in the range of values (MDS mean=27.6; range: 5.6-51.6 *vs.* pathological controls mean=194.5; range: 60.9-573). The low CD38 RMFI on $CD34^+$ cells does not simply result from an excess of immature $CD34^+CD38^{lo/-}$ progenitors, as when these were excluded from analysis by gating only the $CD34^+CD38^{moderate/high}$ cells (*Online Supplementary Figure S1*) CD38 RMFI values (of $CD34^+CD38^{moderate/high}$ subpopulations) remained reduced in MDS patients (mean=33.6; range: 16.1–62.9), compared to pathological (mean=210.1; range: 74.5–597.6) and normal controls (mean=65.1; range: 40.0–86.9) (*data not shown*).

We examined whether the relative frequencies of B-cell progenitors ($CD19^+CD34^+$) contributed to skewing of CD38 RMFI. Predictably, the percentage of $CD19^+$ $CD34^+$/total $CD34^+$ was reduced in MDS patients (mean=3.7%; range: 0.3-14.8%) compared to normal controls (mean=13.4%; range: 2.7-29.7%) and pathological controls (mean=12.9; range: 0.1-37.7%) (*Online Supplementary Figure S1B*). However, even in this small cohort, 4/10 pathological controls had reduced B-cell progenitors (<1%), suggesting that this single parameter is not specific to diagnose MDS.

In this initial MDS patient cohort, skewed decrease of CD38 expression on $CD34^+$ cells could not simply be attributed to decreased frequency of B-cell progenitors and/or $CD34^+CD38^{hi}$ precursors.

Since a reduction in the CD38 RMFI of $CD34^+$ cells might distinguish MDS patients from those with non-clonal cytopenias, we tested the clinical use of this parameter in a larger prospective patient cohort: 90 MDS patients (63 RA/RCMD and MDS-U; 27 RAEB-I and RAEB II) diagnosed by morphological review, clinical follow-up of at least six months and/or cytogenetic findings and 60 pathological control patients, the majority of whom had non-clonal cytopenias where the diagnosis was equivocal and MDS was considered as part of the differential diagnosis (e.g. anemia with dysplastic change but also mild renal impairment; pancytopenia with dysplasia in a patient with infection) (clinical details of these patients shown in *Online Supplementary Table S2*). Samples from AML patients (n=31, two thirds were >55 years) and CMML/MPD (excluding CML) (n=11) were also included (*Online Supplementary Table S2*). Samples in this cohort were routine clinical samples received and processed in a high throughput clinical laboratory using a four-color diagnostic antibody panel. Results from this cohort confirmed reduced CD38 RMFI of $CD34^+$ cells in MDS samples with a clear separation between the majority of MDS and control samples (Figure 1A), despite an overlap in percentage values for $CD19^+CD34^+$ and $CD34^+CD38^{lo/-}$ progenitors (Figure 1B). There was no discernable difference between the subgroup of MDS without an abnormal karyotype and/or ringed sideroblasts (n=32) compared to the subgroup with an abnormal karyotype and/or ringed sideroblasts (n=31) (Figure 1). This assay appears robust since it could be applied in a busy clinical laboratory that used different antibody clones/fluorochromes from those used by the independent laboratory analyzing cohort 1. In MDS samples without excess blasts the RMFI of CD38 (mean=67.2; range 10.6–146.2) was reduced compared to pathological controls (mean=163.9; range 67.6–299). Importantly, a threshold value of CD38 PE RMFI (Figure 1A) defined by receiver-operator characteristic curve diagnosed low-grade MDS with 95% sensitivity (95% confidence interval, 87-99%), 92% specificity (95% confidence interval, 82-97%), positive predictive value of 90.8% and negative predictive value of 91.5%. Five of 6 control samples with lowest CD38 RMFI values were from patients with immune

thrombocytopenia, hemolytic anemia, and severe liver disease. These diagnoses may, therefore, be more likely to produce false positives. Of the 3 MDS samples over threshold value, the highest value was from a MDS patient responding well to erythropoietin which may have affected the nature of $CD34^+$ cells in this patient. $CD34^+$ cells from RAEB patients displayed an even greater reduction in CD38 RMFI but over half of these patients also had a high frequency of $CD34^+CD38^{lo/-}$ progenitors (Figure 1). This is consistent with a previous study that specifically showed an increase in $CD34^+CD38^-$ cells in high-risk and not low-risk MDS.[8] Similar results were seen in AML samples (Figure 1).

The flow cytometric profile of CD38 expression on $CD34^+$ blasts in both MDS patient cohorts revealed several patterns. These include: (i) clustering of some $CD34^+$ cells in the CD38moderate/low region (Figure 2), occasionally aberrant expression of CD34; (ii) shift of a proportion of $CD34^+$ to $CD38^{low/-}$ region, most frequently seen in RAEB samples; and (iii) overall decrease in CD38 MFI without obvious clustering (*data not shown*). This contrasted with the pathological controls in which the $CD34^+$ population was often skewed towards higher CD38 expression (Figure 2).

This is the first study that has assessed the overall expression of CD38 on the total $CD34^+$ population. Similar to previous studies[4,5] we have not found an increased frequency of $CD34^+CD38^-$ progenitors in low-grade MDS, but despite this CD38 RMFI expression of the total $CD34^+$ population from MDS patients is clearly decreased. However, as RMFI data might be modified for a variety of technical reasons, our findings do need to be confirmed by other laboratories.

An explanation for this finding may be that MDS results in an altered composition of myeloid progenitors in the $CD34^+CD38^+$ population. For example, common myeloid and granulocyte-macrophage progenitors have a lower CD38 expression than megakaryocyte-erythroid progenitors.[16] Hence a relative imbalance in these progenitors could result in an overall decrease in the $CD34^+$ CD38 RMFI.

In summary, decreased CD38PE RMFI of the $CD34^+$ population below a threshold value provides a potential simple single diagnostic flow cytometric measurement that separates MDS cases from those with non-clonal cytopenia and other pathological cases. This assay needs to be further validated in other centers but may be of widespread clinical utility in the diagnosis of MDS, especially those cases with a low blast count and a normal karyotype where an objective quantitative diagnostic test does not exist.

Authorship and Disclosures

NG, AS, PV and SF designed the experiments. AS, CC, MD, GS and RB provided critical reagents and samples. NG, EM, W-KC, PR and SF performed the experiments. NG, PV and SF supervised the work. NG, EM, PV, and SF analyzed the results. SF made the figures. PV and SF wrote the paper and all the authors critically reviewed and revised it.

The authors reported no potential conflicts of interest.

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