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VARIATION OF BONE MARROW CD34+ CELL SUBSETS IN MYELODYSPLASTIC SYNDROMES ACCORDING TO WHO TYPES

S. C. REIS, F. TRAINA, K. METZE, S. T. O. SAAD, I. LORAND-METZE*

Faculty of Medicine, State University of Campinas, BR - 13081-970 Campinas – SP Brasil, e-mail: ilmetze@unicamp.br

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Bone marrow (BM) hematopoietic progenitor cells (CD34+) are a heterogeneous population with varying degrees of commitment and maturation to several cell lineages. In myelodysplastic syndromes (MDS), this population is increased. We examined the major cell types found in the blast gate by flow cytometry in newly diagnosed patients with MDS, compared them to normal BM and studied their variation according to WHO type. Two subsets defined by SSC were found both in normal BM and MDS, corresponding to myeloblasts and B-cell precursors. The number of B-cell precursors among all nucleated cells was equally low, independent of WHO type. However, the subset with an intermediate SSC, but CD117, CD13 and CD19 negative increased with the rise of myeloblasts. Concomitantly, the ratio between CD34+/CD117+/CD34-/CD117+ cells was increased. These two features are consistent with the maturation block occurring in the progression of the neoplastic clone. We conclude that the quantitative analysis of the cell types present in the BM blast gate by flow cytometry is not only important for the diagnosis of MDS in patients with peripheral cytopenias and a normal karyotype, but gives also important prognostic information of the patients

Key words: myelodysplasia, hematogones, blasts, SSC, WHO type, CD34+ cells

Flow cytometry has been used in MDS to examine not only the maturation abnormalities of the myelomonocytic lineage, but also to quantify CD34+ hemopoietic progenitors [1–7]. Several kinds of phenotypic abnormalities have been detected in these cells such as aberrant expression of cross-lineage antigens and asynchronous maturation, as it is also found in acute myeloid leukemia (LAIPs) [8–10]. Some of them have been associated with disease progression or a short survival [1, 2, 5–7].

A decrease of CD34+ B-cell precursors has also been repeatedly described [4, 11, 12]. Recently, Satoh et al [4] stated that the proportion of CD34+/CD19+ B-cell precursors among all CD34+ bone marrow (BM) cells is an easily reproducible parameter for the differential diagnosis between low-grade MDS and non-clonal disorders presenting peripheral cytopenias. However, the relative changes of the CD34+ cell subsets among the different WHO types of MDS and the change of the relation of these subsets during the progression of the disease have seldom been examined.

BM CD34+ cells are a heterogeneous precursor cell population with varying degree of lineage commitment, comprising mainly myelomonocytic precursors, very early erythroblasts, B-cell precursors (hematogones), and minor subsets such as immature dendritic cells, etc [13].

In a previous study we have shown that in MDS, a high SSC of the cells in the blast gate measured by flow cytometry is associated with a shorter survival of the patients [5].

Taking into consideration all these data, we examined the composition of cells in the blast gate in patients with MDS, compared it to normal BM and studied their variation according to WHO type.

Patients and methods

BM of consecutive newly diagnosed patients with MDS (45 cases) was examined and compared with normal BM (20 cases) from donors of allogeneic bone marrow transplantation. Diagnosis of MDS was based on peripheral blood (PB) counts, bone marrow (BM) cytologic smears stained with May-Grünewald Giemsa and Perls' stains as well as on karyotype evaluation. The patients were classified according to the WHO criteria [14]: 7 cases of refractory anemia, 1 5q- syndrome, 2 cases of

* Corresponding author

sideroblastic anemia, 19 of refractory cytopenia with multilineage dysplasia, 6 refractory cytopenias with multilineage dysplasia with ring sideroblasts, and 10 refractory anemias with excess of blasts (RAEB). According to the IPSS, 20 cases were low risk, 10 were intermediate-1, 6 were intermediate-2 and 5 were high risk. In 4 cases, no mitoses could be obtained for karyotyping. As the sample was small and IPSS could not be performed in all patients, the cases were grouped into MDS with <5% BM blasts (low risk) and MDS with >5% BM blasts (high risk) for analysis.

BM was analyzed by multiparameter flow cytometry using four-color combinations of monoclonal antibodies: CD19/CD34/CD45/CD117, CD16/CD34/CD45/CD13 and HLA-DR/CD14/CD45/CD33. Immunofluorescence staining was made using a standardized direct stain-and-then-lyse-and-wash technique [1]. A minimum of 50 000 events were acquired for each sample.

The CD34+ cells were separated in the CD34/SSC plot (Fig 1A). This cell population was then displayed on the CD45/SSC plot in order to examine their expression of CD45 and their localization in the several lineages separated by this second plot. In this gate, two subsets of cells could be separated according to SSC: subset 1 with a high and subset 2 with a low SSC (Fig. 1B) . The total number of CD34+ cells was determined in the SSC/CD34 plot. Hematogones were analyzed as percentage of all nucleated cells and of all CD34+ ones. The early maturation in the myeloid compartment was examined by the expression of CD34/CD117 (Fig. 2) and CD34/CD13 (Fig 1C) . In this last combination, 3 subsets could be detected by their expression of CD45 and SSC: lymphoid precursors (SSC ^{low}/CD13-), SSC ^{high}/CD13- and SSC^{high}/CD13+ cells. Data analysis was made using the PAINT-GATE software (BD-Biosciences). The mean fluorescence intensity of each antigen and the mean value for SSC was determined for each cell population. We also calculated the ratio between CD34+/CD117+ and CD34-/CD117+ cells (maturation ratio) as well as the ratio between CD34+/CD117+ cells and the total number of CD117+ cells as proposed by Pirrucello et al [6]. All patients gave informed consent, according to the local Ethics' Committee.

The differences of variables studied among normal BM and both groups of MDS were assessed by the Kruskal-Wallis' test. The correlations among them were studied by the Spearman rank order correlation. The Winstat software was used.

Results

Median age of the MDS patients was 59 (27 – 80) years. The features of the several types of cells in the blast gate are shown in Tables 1 and 2. In normal BM, two subsets of cells presenting a distinctive SSC were found in the blast gate. The subset with a higher SSC (subset 1) showed a positive correlation with the CD34+/CD117+ cells (r = 0.74; p< 0.005) but not the CD34+/CD13+ ones. Subset 2 had a positive correlation with the CD34+/CD19+ cells (r = 0.79; p = 0.0001) and the subset SSC^{low}/CD34+/CD13- (r = 0.62; p = 0.006). This subset, corresponding to B-cell precursors, was found in all normal BMs.

In MDS, both subsets could also be well separated by their different SSC, independent of WHO type and IPSS and presented similar values of SSC as in normal BM (Tab. 1). However, in RAEB a "smeared effect" could be observed (Fig 1B, Fig. 2C). Subset 1 was present in all cases, and was significantly correlated with the number of blasts in cytology (r = 0.88; p <0.0005), that of CD34+CD13- cells (r = 0.55; p <0.0005), that of CD34+CD13+ cells (r = 0.83; p <0.0005), CD34+/CD117- ones (r = 0.66; p <0.0005) and that of CD34+/CD117+ cells (r = 0.81; p <0.0005).

The ratio CD34+/CD117+ / CD34-/CD117+ cells increased with rise of total CD34+ cells (r = 0.44; p = 0.02), number of

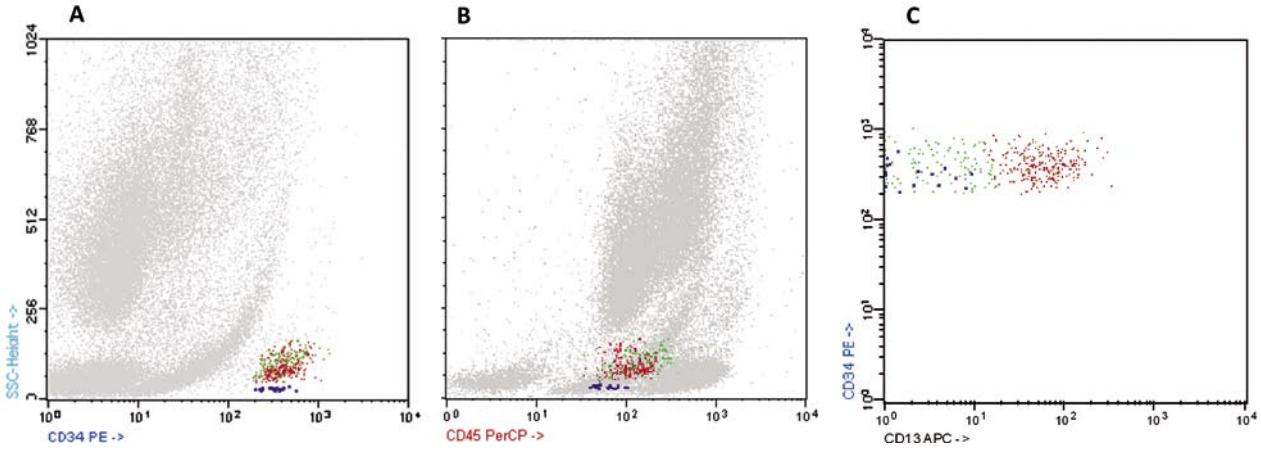

Figure 1: Analysis of the CD34+ cells in the combination CD16/CD34/CD45/CD13 (normal BM). A – separation of the CD34+ cells in the CD34/SSC bivariate plot. B - Localization of the CD34+ cells in the CD45/SSC bivariate plot. Subsets with a low and a high SSC. C – distribution of the 3 populations: SSC ^{low} /CD34+/CD13- (B-cell precursors) (cyan), SSC^{high}/CD34+/CD13- (immature non-lymphoid precursors) (green), and SSC^{high}/CD34+/CD13+ myeloblasts (red).

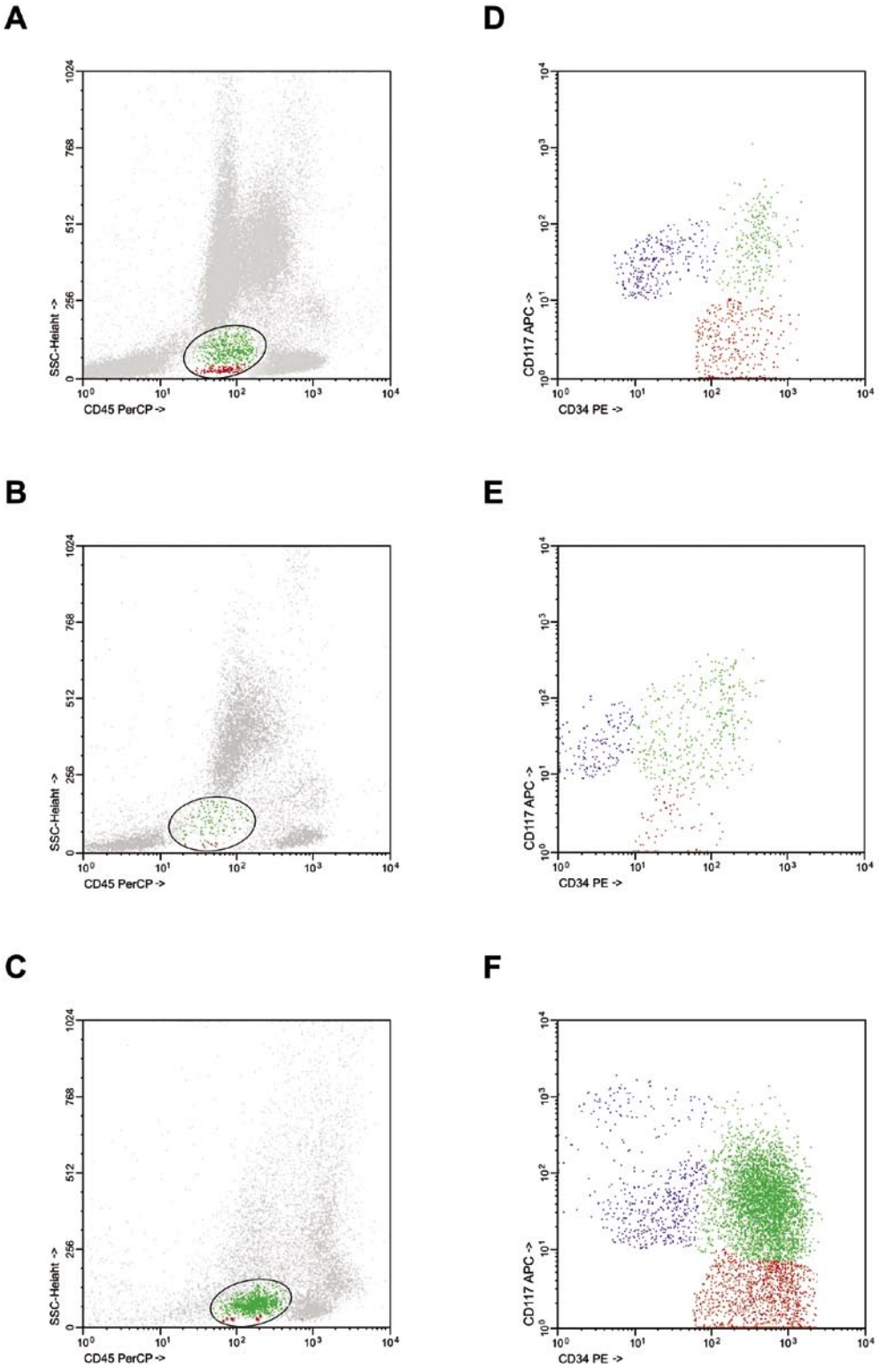

Fig. 2: A-C: subsets with a high and with a low SSC can be distinguished in the CD45/SSC plot in normal bone marrow (A) and patients with refractory anemia (B) or refractory anemia with excess of blasts (C). The proportion of cells in subset 2 is decreased in patients with MDS. D-E: early myeloid maturation analyzed in the CD34/CD117 combination. D – normal BM. E - refractory anemia (RA). F - refractory anemia with excess of blasts (RAEB). In RA, CD34+/CD117- cells are decreased, but in RAEB these cells increase.

Table 1: values of SSC in the several subsets of immature cells studied.

variable	Controls N = 20	MDS with blasts <5% n = 30	MDS with blasts >5% n = 9	P value*
% Blasts (cytology)	-	1.21 (0-4)	12.2 (5.5-19.0)	
SSC of all cells in the blast gate	110 (65-169)	106 (40-259)	113 (78-158)	0.66
SSC subset 1	113 (80-147)	117 (61-166)	107 (73-174)	0.86
SSC subset 2	38 (28-49)	36 (23-52)	47 (23-66)	0.15
SSC CD34+/CD117-	76	85	97	0.42
SSC CD34+/CD117+	109	109	107	0.96

Table 2: comparison of the variables studied between normal BM and patients with MDS (median and range)

variable	Controls N = 20	MDS with blasts <5% n = 35	MDS with blasts >5% n = 10	P value*
% Blasts (cytology)	-	1.21 (0-4)	12.2 (5,5-19.5)	<0.0005
% cells blast gate	1.26 (0.47-2.3)	1.63 (0.06-3.8)	8.04 (2.4-13.9)	<0.0005
% cells SSC^{high} (1)	0.69 (0.24-1.5)	0.81 (0.02-2.77)	6.8 (1.6-9.9)	<0.0005
% cells SSC^{low} (2)	0.16 (0.05-0.6)	0.03 (0-0.79)	0.31 (0-1.96)	0.001
Total CD34+ cells	0.78 (0.47-1.75)	1.18 (0.02-3.38)	6.8 (2.4-10.9)	<0.0005
% CD34+/CD19+ **	0.14 (0.04-0.53)	0.03 (0-0.42)	0.05 (0-0.30)	0.01
% CD34+/CD19+/total CD34+ cells	20.4 (6 -35)	11.6 (1-36)	1.7 (0.3 – 12.0)	<0.0005
Bl CD34+/CD13- low SSC	0.11 (0.02-0.32)	0.04 (0-0.44)	0.03 (0-0.51)	0.03
% CD34+/CD117+	0.41 (0-1.13)	0.55 (0.02 - 4.5)	4.86 (2.41-8.16)	0.01
Bl CD34+/CD13+	0.31 (0.11-0.85)	0.26 (0.04-1.63)	4.50 (2.36-6.43)	0.004
% CD34-/CD117+	0.21 (0.02-0.47)	0.42 (0.05-1.7)	1.43 (0.18-2.79)	0.009
% CD34+/CD117-	0.27 (0-0.72)	0.11 (0-0.48)	1.03 (0.19-2.82)	0.01
Bl CD34+/CD13- high SSC	0.20 (0-0.46)	0.21 (0-0.56)	1.67 (0.69-7.60)	0.003

* Kruskall-Wallis test; ** among all nucleated cells. Based only on the cases where the subset was found

cells in the subset 1 (r = 0.60; p = 0.001), CD34+/CD13+ (r = 0.65; p < 0.0005) and CD34+/CD117+ cells (r = 0.68; p < 0.0005).

The percentage of blasts in BM cytology correlated with the total CD34+ cells (r = 0.88; p <0.0005) but also with subpopulation 1 (r = 0.76; p < 0.0005), with the CD34+/CD117+ cells (r = 0.60; p = 0.002) and with the CD34+CD13+ ones (r = 0.52; p 0.001).

In MDS, subset 2 was observed only in 24/39 cases. Correspondingly, the number of B precursors was found in only 21 cases and the SSC^{low}/CD34+/CD13- cells were found in 29/39 cases. Their number was lower as in normal BM but similar in MDS with a low or a high BM number of blasts (Tab. 2). However, the percentage of CD34+/CD19+ cells among all CD34+ ones decreased with the rise of BM blasts, and was lower in high risk MDS.

As in normal BM, in MDS, subset 2 presented a correlation with CD34+/CD19+ cells (r = 0.62; p <0.0005) and with that of SSC^{low}/CD34+/CD13- cells (r = 0.87; p<0.0005). However, although CD34+/CD19+ cells and SSC^{low}/CD34+/CD13- cells were equally low in all types of MDS, the number of cells in subset 2, CD34+/CD117- cells and SSC^{high}/CD34+CD13- ones increased in the patients with RAEB. The percentage of BM blasts in cytology showed a correlation with subset 1 (r=0.76; p<0.0005) but also with subset 2 (r = 0.41; p 0.004).

The maturation ratio was correlated with the percentage of cells in the blast gate (r = 0.34; p = 0.06), the number of cells in subset 1 (r = 0.63; p = 0.001), total CD34+ cells (r = 0.44; p = 0.02), CD34+/CD13+ ones (r = 0.65; p < 0.0005) and CD34+/CD117+ ones (r = 0.68; p < 0.0005). They also were positively related to the CD34+/CD117- cells (r = 0.56; p = 0.003) the SSC^{high}/CD34+/CD13- ones (r = 0.63; p = 0.001) and IPSS (0.62; p = 0.001). The index proposed by Pirrucello showed a correlation only with the percentage of blasts in BM cytology (r = 0.50; p = 0.008) the total cells in the blast gate

(r = 0.48; p = 0.001) and the total number of CD34+ cells (r = 0.42; p = 0.028).

Discussion

CD34+ cells are increased in MDS, and are considered a hallmark in diagnosis and prognosis of this group of clonal disorders. Several phenotypic abnormalities have been described in these cells [1–4, 13, 15] which are more frequent in aggressive cases and progression to acute leukemia. Similar abnormalities have been found in acute myeloid leukemia, and have been useful for detection of residual disease after chemotherapy [8–10].

Bone marrow CD34+ cells are a heterogeneous precursor cell population comprising mainly myelomonocytic and B-cell precursors besides minor subsets [13].

Recent studies have shown that B-cell precursors are decreased in MDS [3, 4, 11–13]. This feature has been proposed as an easily reproducible parameter for diagnosis and useful for the differential diagnosis between low-grade MDS with a normal karyotype and non-clonal disorders presenting with peripheral cytopenias [4, 12]. These cells are found in the subset with a low SSC in the blast gate and have a CD34+/CD19+ phenotype. However, mainly low-grade MDS has been studied.

Our aim was to study the proportion of the major BM subsets of CD34+ cells in the blast gate in MDS, as compared to normal BM, as well as their variation according to the several WHO types and IPSS.

In normal BM we found two well-defined subsets in the blast gate: one presenting a high correspondence to myeloblasts (high SSC, CD34+/CD117+/CD13+ phenotype), and the other representing type I hematogones (low SSC, CD34+/CD19+ phenotype). In MDS both subsets could also be found. As in normal BM, subset 1 corresponded to myeloblasts, increasing in cases with a high BM blast count and a high number of CD34+ cells.

Subset 2 was absent in 39% of the cases with MDS. However, the number of these cells increased with the increase of BM blast count and that of CD34+ cells. But type I hematogones (CD34+/CD19+ cells) remained low, independent of WHO type and IPSS. The decrease in B-cell precursors is characteristic for all types of MDS, as has been already pointed out, confirming the hypothesis that an intrinsic defect of B-cell production is an early event in these clonal disorders [4, 11, 13]. Analyzing B-cell precursors in MDS, when their number was taken among all nucleated cells, they were equally low or absent in all WHO types. However, if they were calculated among total CD34+ cells, their number decreased with the rise of this last population. This may be the cause of discrepancies in the literature about the number of hematogones among the several WHO types [3, 4, 11, 13, 15].

The rise of cells in subset 2 in RAEB was due to CD34+/CD117-/CD13-/CD19- cells. In the CD34/CD13 plot we could separate 3 groups of cells: myeloblasts (high SSC, CD13+), hematogones (low SSC, CD34+/CD19+/CD13-) and immature CD34+/CD13- cells with a higher SSC. This last population increased with the rise of BM blasts, total CD34+ cells and IPSS. This speaks in favor that in MDS, unlike in normal BM, subset 2 may contain, besides B-cell precursors, early non-lymphoid precursors that increase together with myeloblasts. In cases with a higher number of blasts, both subsets are also more difficult to separate. These cells might be commited to the myelomonocytic lineage, or may be myeloblasts or monoblasts with a deficiency of CD13. Therefore, the decrease in subset 2 is a good variable for the differential diagnosis only in early MDS but not in more advanced cases.

It was also interesting to note that, with an increase of myeloblasts, the ratio between CD34+/CD117+ and CD34-/CD117+ cells decrease, speaking in favor of a maturation block in the myeloid series, associated with progression of MDS. Recently, Pirrucello at al [6] pointed out that the relative proportion of CD34+/CD117+ cells among all CD117+ cells was an important hallmark of disease progression, indicating a progressive maturation block. In our study, this index showed a correlation with the BM blasts in cytology and the total number of CD34+ cells. However, the ratio between CD34+/CD117+ and CD34-/CD117+ cells, although showing no correlation with blasts in cytology, had a significant correlation with IPSS, subset 1 and immature non-lymphoid precursors. Therefore we hypothesize that the increase of this last subset is another feature indicative of a maturation block occurring during progression of the abnormal clone. This may be due to accumulating genetic defects of the hemopoietic precursors or to the increasingly abnormal function of the BM microenvironment in high grade MDS leading to an inability to support maturation of uncommitted progenitors as observed in acute myeloid leukemia [13].

The SSC of each subset as well as of each phenotypically defined cell type in the blast gate was quite constant for normal and clonal hemopoiesis. However, the proportion of each of them varied between normal BM and MDS, as well as with the increase of CD34+ cells in MDS. Therefore, the SSC of the whole population in the blast gate was highly dependent of the proportion of CD34+/CD117+ myeloblasts in the gate. This confirms our earlier finding of the association of a high SSC of all cells in the blast gate with a shorter survival of the patients [5].

So we conclude that the quantitative analysis of the cell types present in the blast gate in MDS by flow cytometry is not only important for the diagnosis in patients with peripheral cytopenias and a normal karyotype, but also gives important prognostic information as it has also been shown for granulocyte maturation abnormalities [2, 6, 13]. This flow cytometric variable is easy to obtain and could give useful information for indication of several new treatments as well as timing of bone marrow transplantation. It would be interesting to know if the increase of immature non-lymphoid precursors is related to progression of the MDS clone during the evolution of the disease or to a shorter survival of the patients.

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