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Review article

Diagnostic Utility of Flow Cytometry in Myelodysplastic Syndromes.

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Abstract. The pathological hallmark of myelodysplastic syndromes (MDS) is marrow dysplasia, which represents the basis of the WHO classification of these disorders. This classification provides clinicians with a useful tool for defining the different subtypes of MDS and individual prognosis. The WHO proposal has raised some concern regarding minimal diagnostic criteria particularly in patients with normal karyotype without robust morphological markers of dysplasia (such as ring sideroblasts or excess of blasts). Therefore, there is clearly need to refine the accuracy to detect marrow dysplasia. Flow cytometry (FCM) immunophenotyping has been proposed as a tool to improve the evaluation of marrow dysplasia. The rationale for the application of FCM in the diagnostic work up of MDS is that immunophenotyping is an accurate method for quantitative and qualitative evaluation of hematopoietic cells and that MDS have been found to have abnormal expression of several cellular antigens. To become applicable in clinical practice, FCM analysis should be based on parameters with sufficient specificity and sensitivity, data should be reproducible between different operators, and the results should be easily understood by clinicians. In this review, we discuss the most relevant progresses in detection of marrow dysplasia by FCM in MDS

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The Diagnosis of Myelodysplastic Syndromes. Myelodysplastic syndromes (MDS) are a group of disorders clinically characterized by peripheral cytopenia, followed by a progressive impairment in the ability of myelodysplastic stem cells to differentiate and an increasing risk of evolution into acute leukemia.[1]

MDS represent one of the most common hematologic malignancies in Western countries. They typically occur in elderly people with a median age at diagnosis of 70 to 75 years in most series, and their annual incidence exceeds 20 per 100,000 persons over the age of 70 years.[1] The clinical course of the disease is very heterogeneous, ranging from indolent conditions spanning years to forms rapidly progressing to leukemia.[2] This heterogeneity reflects the complexity of the underlying genetic defects.[3]

According to the prevailing dogma, clonal transformation in MDS would occur at the level of a committed myeloid stem cell that can give rise to red cells, platelets, granulocytes and monocytes.[4] The biologic hallmark of these stem cells is, rather, dysplasia, which indicates a defective

capacity for self-renewal and differentiation and relies on various morphological abnormalities. Karyotypic aberrancies (involving loss of genetic material and less frequently balanced translocations) are detected in about 50% of primary MDS, and when present are a marker of clonal hematopoiesis.[5]

Important steps have recently been made in characterizing the molecular basis of MDS.[3] MDS del(5q) appears to derive from haplo-insufficiency of genes mapping to chromosome 5q32- q33, in particular from reduced expression of RPS14 and miR-145/-146a, and from mutations of Casein Kinase 1A1 and TP53 genes.[6] In addition, acquired somatic mutations have been detected in several genes, including TET2, ASXL1, CBL, ETV6, EZH2, IDH1, IDH2, KRAS, NPM1, NRAS, RUNX1, and TP53.[4] More recently, genes encoding for spliceosome components were identified in a high proportion of patients with MDS. These genes include SF3B1, SRSF2, U2AF35 and ZRSR2, and to a lesser extent, SF3A1, SF1, U2AF65 and PRPF40B.[7]

Although most of the mutated genes in MDS can be detected in different myeloid neoplasms and are not specific for MDS, they may be of value to provide evidence for a clonal disorder in patients with suspected MDS. In a recent comprehensive report,[7] a total of 52% of patients with normal cytogenetics had at least one point mutation. These figures are even higher when accounting for mutations of the genes encoding for splicing factors. Although the spread of massive genotyping methods will soon make possible for clinicians to detect a broad range of in peripheral blood at a reasonable cost, the screening of such molecular defects cannot be recommended at this stage on a routine basis.[7]

To date, the morphological evaluation of marrow dysplasia represents the basis of the World Health Organization (WHO) classification of these disorders.[8] This classification provides clinicians with a very useful tool for defining the different subtypes of MDS and determining individual prognosis. The combination of overt marrow dysplasia and clonal cytogenetic abnormalities allows a conclusive diagnosis of MDS. However, this combination is found only in some patients, who tend to be those with more advanced disease. In many instances, cytogenetics is not informative so that the diagnosis of MDS is based entirely and exclusively on morphological evaluation.[8]

The WHO proposal has raised some concern regarding minimal diagnostic criteria for formulating the diagnosis of MDS.[9] Morphology may be difficult to evaluate, because cellular abnormalities of bone marrow cells are not specific for MDS and may be found in other pathological conditions.[10,11] As a consequence, in clinical practice inter-observer reproducibility for recognition of dysplasia is usually poor, particularly in patients who do not have robust morphological markers such as ring sideroblasts or excess of blasts.[11] Moreover, poor technical quality of the specimen is a common obstacle in the accurate morphological diagnosis of MDS and also has an influence on the diagnostic yield of conventional cytogenetics. Finally, morphology may be difficult to evaluate in some patients either due to hypocellularity or fibrosis of the marrow.[12]

Rationale for the Application of Flow Cytometry in the Diagnostic Work-Up of Patients with Suspected MDS. Flow cytometry (FCM) immunophenotyping was introduced by WHO proposal for the classification of hematologic neoplasms as an indispensable tool for the diagnosis, classification, staging, and monitoring of several diseases, such as lymphoproliferative disorders and acute leukemias.[13] In addition, immunophenotyping has been proposed in last years as a tool to improve the evaluation of marrow dysplasia. Rationale for the application of FCM in the diagnostic work up of MDS is that: i) immunophenotyping is an accurate method for quantitative and qualitative evaluation of hematopoietic cells (in this context it should be underlined that however, the morphologic definition of bone marrow cells is not equal to and cannot be used in an exchangeable manner with flow cytometric nomenclature) and, ii) MDS have been found to have abnormal expression of several cellular antigens.[13-15] Flow cytometry immunophenotyping is able to identify specific aberrations in both the immature and mature compartments among different bone marrow hematopoietic cell lineages.[16-20] Although no single immunophenotypic parameter has been proven to be diagnostic of MDS, combinations of such parameters into scoring systems have been shown to discriminate MDSs from other

cytopenias with high sensitivity and acceptable specificity.

Flow cytometry was proven to be highly sensitive in identifying patients likely to be suffering from a clonal disease process (ie, an MDS lacking specific diagnostic markers such as excess blasts, ring sideroblasts or karyotypic aberrations) rather than cytopenia of undetermined significance, which includes cases of sustained cytopenias in one or more lineages that do not meet the minimal criteria for MDS and cannot be explained by any other hematologic or nonhematologic disease.[16-20] In addition, flow cytometry is useful for distinguishing refractory anemia from refractory cytopenia with multilineage dysplasia by identifying immunophenotypic abnormalities in myeloid and monocytic compartments.[16-20]

Although further prospective validation of markers and immunophenotypic patterns against control patients with secondary dysplasia and further standardization in multicenter studies are required, at present, flow cytometry abnormalities involving one or more of the myeloid lineages can be considered as suggestive of MDS.

Standard methods for cell sampling, handling, and processing, and minimal combinations of antibodies for flow cytometry analysis of dysplasia in MDS have recently been established by the International Flow Cytometry Working Group within the European LeukemiaNet.[21]

The integration of flow cytometry immunophenotyping following these standards is recommended in the workup of patients with suspected MDS by the European LeukemiaNet guidelines for diagnosis and treatment of primary MDS,[22] although the implementation of these procedures may not be immediately feasible in some hematologic centers.

In this report, we reviewed the most relevant advancements in the evaluation of marrow dysplasia by FCM in MDS.

Immunophenotypic Evaluation of Myeloid Dysplasia. Morphological granulocytic dysplasia as defined by WHO criteria is present in about 60% of MDS patients at diagnosis.[8,9] Most significant morphological alterations on granulocytic lineage included hypogranularity on myeloid cells, the presence of pseudo-pelger neutrophils and increased prevalence in bone marrow of myeloid cells in the earliest stage of maturation.[11] These abnormalities significantly affected the detection of physical parameters (i.e., side scatter, SSC and forward scatter, FSC) by FCM.[23] Defective capacity for self-renewal and differentiation by myelodysplastic stem cells also relies on various abnormalities of antigen expression on granulocytic cells, which may be easily detected by FCM due to a large availability of specific antibodies for myeloid lineage.[16-19] Reported aberrancies of granulocytic lineage include the presence of antigens that are not normally present, such as lymphoid antigens, and altered expression of myeloid antigens, either in a single population of cells or within a generation of maturing cells. Furthermore, monocytic compartment is also affected in MDS.[16-19]

Davis studied for the first time the pattern of CD16 and CD11b expression by maturing granulocytes in the bone marrow of patients with MDS and healthy controls.[15] There was a highly consistent normal pattern of CD11b and CD16 expression in the granulocytic series in healthy subjects, while in MDS patients an increased percentage of granulocytic cells with low CD16 or both low CD16 and low CD11b was noticed.15 In addition, an altered granulocytic maturation pattern can be demonstrated by plotting CD13 versus CD16.[16-19] During maturation; myeloid cells normally acquire increasing levels of CD16 that are initially accompanied by a decrease in CD13 expression as cells mature from blasts through the myelocyte and metamyelocyte stages of maturation, followed by intermediate levels of CD13 in band forms and high levels in segmented neutrophils. Several abnormalities on CD13/CD16 maturation pattern were described in MDS patients, including an increase of cells in myelocyte and metamyelocyte stages of maturation and a decrease of CD13+CD16+ neutrophils.[16-19]

Although these investigations defined immunophenotypic abnormalities in MDS, they did not address the potential contribution of FCM to the diagnosis of MDS. The study of Stetler-Stevenson et al. published in 2001 was the first to demonstrate that the identification of immunophenotypic abnormalities by FCM is useful in establishing a diagnosis of a MDS, especially when the results of the morphologic evaluation and cytogenetic studies are indeterminate.[16] In addition to maturation abnormalities, aberrancies in the expression of

several antigens on granulocytes such as CD64, CD10, and CD56 were described in MDS. Lymphoid antigens, such as CD2, CD5, CD7, and CD19 may be abnormally expressed on myeloid progenitors and maturing myeloid cells. Moreover, a common finding in these patients is the atypical expression of antigens on immature myeloid cells that are normally expressed on mature myeloid cells, such as CD11b and/or CD15.[17-19]

As far as monocytic compartment is concerned, most frequent abnormalities observed in MDS patients include altered expression of CD56, HLA-DR, CD36, CD33, CD15, CD14, CD13, and CD11b.[18,19,24] In general, the amount of abnormalities reported by FCM correlates with the degree of dysplasia assessed by morphology. Although most of the studies have evaluated bone marrow cells, there is some evidence that FCM analysis of peripheral blood could also assist in the diagnosis of MDS.[25] Scientific evidence suggests that aberrant antigen expression by myeloid cells is more frequent and carries more discriminant weight on detection of marrow dysplasia than altered expression of monocytic antigens.[24] A single myeloid immunophenotypic abnormality was reported in about 30–40% of patients affected with nonclonal cytopenia.[16-20] Therefore, a single myeloid immunophenotypic abnormality is not a definitive finding for MDS, and other abnormalities should be detected on granulocytic cells to conclude that myeloid dysplasia is present. Multiparametric evaluation of myeloid and monocytic maturation and antigen expression pattern leads to the identification of two or more aberrancies in the great majority of MDS cases (from 70% to more than 90% in different studies).[16-20,26] In general FCM is more sensitive in detection of myeloid dysplasia with respect to morphology, and immunophenotypic myeloid abnormalities are identified in a significant percentage of cases (from 20% to more than 90%) classified as refractory cytopenia with unilineage dysplasia or unclassifiable MDS.[16-20,26] In addition, FCM was found to be useful for detection of marrow dysplasia in a proportion of patients with marrow hypocellularity, fibrosis or inadequate specimen collection, suggesting that variables related to sample quality are less significant in immunophenotypic analysis than in morphological evaluation.[17]

The great variability on the percentage of reported immunophenotypic abnormalities in MDS patients reflect in part the biological heterogeneity within these disorders, but more likely, the lack of a standardized and reproducible procedure for the evaluation of these parameters.[21] The most largely used approach to evaluate myeloid dysplasia by FCM is pattern recognition analysis.[16] This is a qualitative method based on recognition of a deviation from normal antigen expression pattern. Although similarly to morphological evaluation this approach is a good tool for expert operators (i.e., people with extensive knowledge of changes in antigen expression in normal and pathological hematopoietic cell differentiation) pattern recognition analysis presents several weak points. The numerical description of the results is difficult, thus quantitative analysis is not possible; moreover, the precise definition of the normal pattern of reference may be complex.[13] Overall, FCM multiparametric approaches based on a quantitative evaluation of myeloid antigens allow to classify about 90% correctly of cases with suspected MDS.[16-20,26] The ELN working group for FCM in MDS started a consensus process on how to standardize sample collection/ preparation and data acquisition, that is expected to significantly improve the FCM accuracy in detection of marrow dysplasia.[21,27-30]

Immunophenotypic Analysis of Blast Cells. Clonal transformation in MDS occurs at the level of a myeloid committed stem cell which has a competitive advantage over normal stem cell compartment.[1] These hematopoietic precursors (blasts) are morphologically defined as ‘‘immature cells with uncondensed chromatin pattern, prominent nucleoli, low nuclear/cytoplasmic ratio, and no/few cytoplasmic granules’’.[11] The evaluation of blast compartment has diagnostic relevance in the WHO system, and the percentage of marrow blasts has recognized to have prognostic effect by all the currently available prognostic scores.8 In the WHO guidelines, despite inaccuracies inherent in manual differential counting, morphological analysis is actually the gold standard for determining blast percentage.[11] The first attempt of FCM immunophenotyping was to provide a quantitative estimation of bone marrow blasts with increased sensitivity and reproducibility with respect to morphological count. Unfortunately, the quantitative evaluation of marrow blasts in MDS

by FCM presents both technical and intrinsic limitations.[13] First, MDS blasts are not predominant cells in the bone marrow making their reliable analysis difficult, and in addition they are identified in the CD45 versus SSC dotplot as CD45lowSSClow cells; however, hypogranular more mature myeloid cells may have decreased SSC and fall in this region, and it may be difficult to distinguish monoblasts from more mature monocytes.[13] The percentage of CD34+ cells determined by FCM has been tested as a substitution for a visual blast count. However, although hematopoietic cells that express CD34 are blasts, not all blasts express CD34. It should be considered in addition that marrow samples for morphological evaluation can differ form that for FCM analysis in terms of cellularity . Hence, the percent of CD34+ cells determined by FCM as substitution for a visual blast count in MDS is discouraged by current WHO classification.[8,31]

More interesting results in the light of a diagnostic application of FCM in work-up of MDS patients derive from the analysis of immunophenotypic abnormalities of blast cell compartment. The proportion of CD34+ cells is significantly higher in MDS with respect to healthy subjects, and the great majority of cells are committed to the myeloid lineage (CD38+HLA-DR+CD13+CD33+).[14,32] In addition, a significant down-regulation of B-cell lineage-affiliated genes was observed in CD34+ hematopoietic precursors isolated from low-risk MDS with respect to healthy controls and patients with nonclonal cytopenia, and a reduction in stage I hematogones is one of most consistent immunophenotypic findings in MDS patients.[33,34] In different studies considering patients performing bone marrow evaluation for peripheral blood cytopenia, a significant decrease of CD34+ B cell progenitors was observed in 40–70% of subjects with a conclusive diagnosis of MDS and in 20–40% of patients with nonclonal cytopenia. The analysis of both percentages of CD34+ myeloblasts and CD34+ B cell precursors was found to have little interobserver variability.[33,34]

Several other immunophenotypic abnormalities on MDS blast cells were reported, including asynchronous co-expression of stem-cell and late-stage myeloid antigens (CD117, CD15, and CD11b) or abnormal expression of lymphoid markers (CD2, CD5, CD7, CD19, and CD56).[18,19,32,33,35] However, most of these parameters do not have adequate reproducibility in the MDS setting with the exception of lymphocytes- to-myeloblasts CD45 ratio that ensures acceptable interobserver variability by adjusting data on target cells with those on lymphocytes in the same sample.

The analysis of percentage of CD34+ myeloblasts, CD34+ B-cell progenitors and myeloblast CD45 expression by FCM has little interoperator variability and appears to be applicable in many laboratories.[36,37] When combined together with the evaluation of SSC on granulocytes, these parameters differentiate correctly the majority of MDS and pathological controls, sensitivity ranging from 30 to 70% and specificity ranging from 80% to more than 90% in different studies.[36-38] (**Figure 1**) All these findings strongly suggest that CD34-related parameters are good candidates for the identification of diagnostic markers that not only can be used for the diagnosis of MDS patients but also are relatively stable and result in acceptable between-operator data variation.

Immunophenotypic Evaluation of Erythroid Dysplasia. Erythroid dysplasia is the milestone of the morphological diagnosis of MDS. In fact, it is present in almost all patients with MDS and is the only morphological abnormality in those with refractory or sideroblastic anemia.[8,11]

The evaluation of erythroid dysplasia represents a challenge in the immunophenotypic analysis of myelodysplastic marrows: the precise identification of marrow erythroid precursors is problematic, and there is a limited availability of specific markers.[16]

The first critical issue of erythroid compartment immunophenotyping is the gating strategy to identify marrow erythroid precursors.[21,27,39] Nucleated erythroid cells are characterized by reduced/absent CD45 and low SSC. To gate CD45dim to negative/SSClow cells is certainly simple and seems likely to be reproducible. However, this region also contains mature (anucleate) red cells, cellular debris, and nonhematopoietic cells, which are not discriminable on the basis of CD45 or scatter proprieties. Alternatively, an immunological gate based on the antigens expressed by erythroid cells can be performed. During physiological development from the basophilic erythroblast to the erythrocyte, there is a progressive decrease in

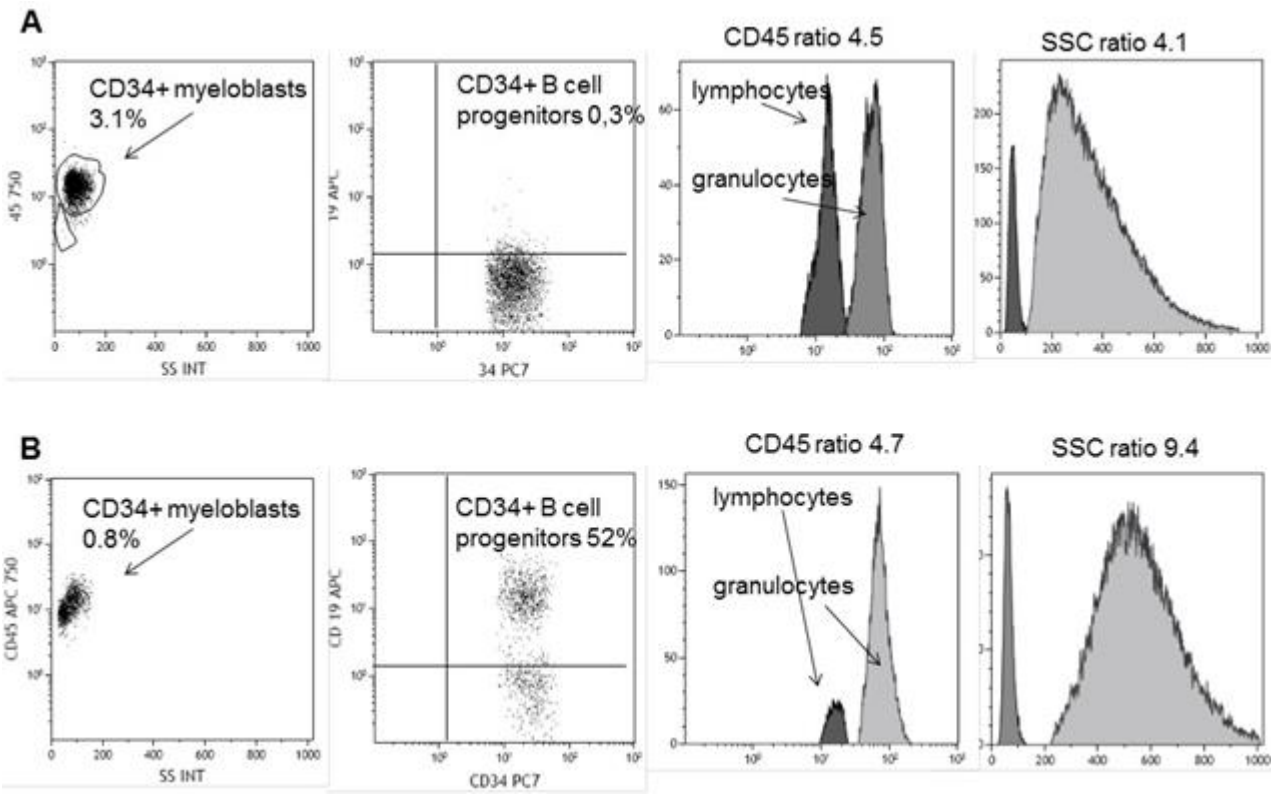

Figure 1. Detection of marrow dysplasia by analysis of four cardinal parameters of marrow dysplasia for from a single cell aliquot stained with CD34 and CD45 antibodies. A) bone marrow from representative MDS patients showing an increase of CD34+ myeloblasts, a decrease of CD34+ B cell progenitors, a reduced SSC in granulocytic cells and an aberrant expression of CD45 on myeloblasts; B) Healthy donor bone marrow.

CD45 expression.[21,27,39] An increase in glycophorin A (Gly A) is observed early upon differentiation from the basophilic erythroblast to the orthochromic erythroblast. Finally, CD71 is one of the earlier antigens expressed during erythroid maturation (which anticipates Gly A expression), remains on the reticulocyte after enucleation and then is lost prior to the loss of the RNA. From a theoretical point of view, gating erythroblast on the basis of CD71 expression would be preferable, Gly Aþ cells excluding a proportion of more immature erythroid precursors, which may be increased in MDS.[21,27,39] However, a dysregulation of CD71 expression is reported in MDS, and Gly A that has a very tight coefficient of variation of intensity from individual to individual should be preferentially adopted in gating erythroid precursors in the setting of MDS. The lysis process is also critical, affecting nucleated as well as mature red blood cells to an unknown variable degree.[27,30,39] Although a no-lyse, no-wash system would provide the most accurate estimate of the nucleated red cell, a lyse no-wash approach is certainly simpler and more easily implementable in the diagnostic workup of MDS patients. [21,27,39]

The study by Stetler-Stevenson et al. demonstrated for the first time the feasibility of the evaluation of erythroid dysplasia by FCM.[16] However, the only consistent erythroid abnormality in this study was a dys-synchronous expression of CD71 versus Gly A on red cell precursors.

In last years an increasing amount of studies addressed the issue of the immunophenotypic evaluation of erythroid compartment in MDS.[40-43] Flow cytometric aberrancies that have been reported to reflect MDS- related dyserythropoiesis are: a) an increased number of nucleated erythroid cells within total nucleated cells; b) an altered proportion of consecutive erythroid differentiation stages, such as an increased number of immature erythroid cells (CD117+ and/or CD105+) or, by contrast, a decrease in erythroid progenitors; c) an abnormal pattern of CD71 versus CD235a; d) a reduced expression of CD71 and/or CD36; and e) an overexpression of CD105. Most of these aberrancies are present in 70–80% of MDS cases.[40-43] The ELN working group for FCM in MDS recently reported the results of a multicenter study focused on defining those erythroid FCM parameters that enable distinction of dyserythropoiesis associated with MDS from non-

clonal cytopenias.[43] Analysis of the presence of aberrancies in the erythroid markers CD71 and CD36 (expressed as the coefficient of variation, CV), together with the MFI of CD71 and an abnormal percentage of CD117+ erythroid progenitor cells provided the best discrimination between MDS and non-clonal cytopenia. A weighted score based on these four parameters yielded a specificity of 90% and a sensitivity of 33%.

Addition of erythroid aberrancies to flow cytometric models based on the evaluation of myeloid abnormalities may significantly increase the sensitivity to detect myelodysplastic changes in bone marrow.[40-43]

Conclusions. The implementation of WHO classification of MDS in clinical practice compels a refinement of the accuracy to detect marrow dysplasia.8 FCM immunophenotyping has been proposed as a tool to improve the evaluation of marrow dysplasia.[13] To become clinically applicable,

FCM analysis should be based on parameters with sufficient specificity and sensitivity, data should be reproducible between different operators, and the results should be easily understood by clinicians.[13,15] With respect to this ideal situation, the results of the studies that pointed out the feasibility of immunophenotyping in diagnostic work-up of MDS patients raise some concerns: no single marker has proved able to discriminate accurately between MDS and other pathological conditions, no consensus exists on which diagnostic parameters are the most appropriate, and published protocols are mainly based on a qualitative analysis of cytometric variables thus limiting a wide clinical implementation.[21,27,29]

However, in recent years significant progresses were made. Clonal transformation in MDS occurs at the level of a CD34+ committed stem cell, and therefore CD34-related parameters are good candidates for identification of diagnostic markers for these disorders.[4,31,32] Consistent immunophenotypic aberrations reported in MDS CD34+ cell compartment are an increase of CD34+ myeloblasts, a decrease of B cell progenitors, expression of lymphoid antigens and abnormal CD45 expression. Increasing evidence suggests that these parameters have little interoperator variability and, when combined, are able in discriminating between MDS and patients with nonclonal cytopenia.[31,37]

Evaluation of erythroid dysplasia represents a challenge in the immunophenotypic analysis of myelodysplastic marrows due to a limited availability of specific markers.[16] Promising results are coming from recent studies, showing that the addition of erythroid aberrancies to flow cytometric models based on the evaluation of myeloid abnormalities may significantly increase the sensitivity to detect myelodysplastic changes in bone marrow.[40-43]

A standardized application of FCM in the diagnosis of MDS also requires a minimal variability in sample processing, antibody combinations, and data acquisition. The European LeukemiaNET (ELN) working group for FCM in MDS started a consensus process on how to standardize sample collection/preparation and data acquisition. It is expected to significantly improve the diagnostic accuracy of FCM in MDS.[21,27,28,29]

According to the available evidence and published diagnostic guidelines, in clinical practice immunophenotyping is strongly indicated in the screening evaluation of patients with peripheral blood cytopenia:[13,22] in this clinical situations, it can provide a sensitive screen for the presence of hematologic malignancy and/or assist in demonstrating the absence of disease. In addition, when morphology and cytogenetics are indeterminate, an abnormal phenotype determined by FCM can help to establish a definitive diagnosis of MDS.[13,22]

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