Abstract

Primary myelofibrosis (PMF) is a clonal hematopoietic stem cell disease characterized by abnormal proliferation of megakaryocytes with myelofibrosis, peripheral leukoerythroblastosis, and extramedullary hematopoiesis. Patients with PMF often exhibit anemia and variable degrees of dysgranulopoiesis with left-shifted maturation, hypogranulation, and abnormal nuclear condensation. In its early stages, the disease may manifest with thrombocytosis, while advanced PMF is associated with cytopenia and osteosclerosis.

At the molecular level, a gain-of-function mutation involving Janus kinase 2 (JAK2) is detected in approximately 50% of PMF cases. The JAK2 mutation status and allele burden seem to be prognostically inconsequential. Several reports have also documented a high incidence of cytogenetic abnormalities (in 33%-58% of cases); these abnormalities frequently involve chromosomes 1, 7, 8, 9, 12, 13, and 20, with trisomy 8 and del(12p) correlating with an unfavorable prognosis. The frequency of cytogenetic abnormalities increases as PMF progresses.

In patients with anemia, dysgranulopoiesis, and dysplastic megakaryocytic hyperplasia, the differential diagnosis of PMF often includes myelodysplastic syndrome (MDS) with fibrosis. Flow cytometric analysis is considered a useful ancillary tool for assessing myelodysplasia in MDS. Features such as low side scatter, aberrant expression of the neural cell adhesion molecule CD56, and abnormal maturation sequences are considered evidence of dysplasia. Aberrant expression of CD56 is also considered evidence of monocytic dysplasia. However, flow cytometric characteristics of myeloproliferative neoplasms, especially of PMF and its comparison with myelodysplastic syndrome with myelofibrosis (MDS-MF), are not well described. In this study, we
analyzed a large number of PMF cases by flow cytometry with the goal of identifying any features that could facilitate differential diagnosis from MDS-MF.

**Materials and Methods**

**Cases and Design**

By searching our database, we identified cases diagnosed as PMF with bone marrow specimens analyzed by flow cytometry at the time of initial evaluation at our institution during the period of January 2004 to August 2008. The diagnoses of PMF had been established by a combination of clinical features, laboratory findings, bone marrow biopsy results, peripheral blood smears, flow cytometric immunophenotyping, cytogenetic studies, and molecular analyses. Only cases with CD56 expression assessed by flow cytometry, known JAK2 V617F status, and cytogenetic studies were included. All cases were in the fibrotic stage. We also included cases of MDS-MF and cases of non-MDS, nonmyeloproliferative neoplasms (referred to henceforth as “normal”) undergoing staging bone marrow as control cases.

**Flow Cytometric Analysis**

Bone marrow aspirate materials were analyzed by 4-color flow cytometry (BD FACSCalibur, Becton Dickinson, San Jose, CA). The granulocyte and monocyte compartments were gated using side scatter vs CD45. CD3, CD4, and CD45 were frequently included in the panel to assist gating, and care was taken to exclude the natural killer/T-cell populations. To assess myeloid dysplasia, we analyzed multiple parameters. Intensity of CD56 expression in granulocytes and monocytes was measured, and the percentage of CD56+ cells was assessed vs isotype controls. The pattern of the granulocyte maturation sequence was assessed by using a combination of CD13-phycocerythrin and CD16-fluorescein isothiocyanate (FITC). Cytoplasmic granularity was assessed by measuring the median and the 10th percentile of side scatter (height). The size of the granulocytes was assessed by using the mean and geometric mean of the forward scatter (height). Data analysis and graphic generation were performed using FlowJo (Tree Star, Ashland, OR).

**Statistical Analysis**

We used the Student t test for statistical analysis to compare 2 groups of numeric data, with a P value less than .05 defined as statistically significant. Comparison of categorical data was performed by using χ² analysis. We generated graphs of the statistics with the Prism software package (GraphPad Software, La Jolla, CA) and Microsoft Excel (Microsoft, Redmond, WA) built-in programs.

**Results**

**Patient Characteristics**

The study group consisted of 70 PMF cases. An additional 17 MDS-MF cases and 20 other cases (including 18 malignant lymphoma and 2 nonhematopoietic malignancies) were also included as control cases.

As shown in **Table 1**, the ages of patients with PMF ranged from 31 to 88 years (median, 64 years). The median hematologic findings were as follows: WBC count, 8,800/μL (8.8 × 10⁹/L; range, 400-75,900/μL [0.4-75.9 × 10⁹/L]); hemoglobin, 10.2 g/dL (102 g/L; range, 5.9-16.9 g/dL [59-169 g/L]); and platelet count, 175 × 10³/μL (175 × 10⁹/L; range, 7.7-728 × 10³/μL [7.7-728 × 10⁹/L]). Based on the European Myelofibrosis Network Grading System, the cases were...
graded as follows: MF-1, 8; MF-2, 26; MF-3, 29; and MF-2-3, 7. Most were previously treated with various agents.

Of the 70 cases, 40 (57%) were positive for JAK2V617F, with the mutant allele burden ranging from 16% to 96% (median, 50%). Of the 70 cases, 34 (49%) showed 1 or more chromosomal abnormalities, including del(13q), del(20q), trisomy 8, trisomy 9, and del(7q). Table 2. The frequencies and types of the aberrancies observed in this study cohort are similar to those reported in the literature. It is noteworthy that among the JAK2V617F cases, 26 (65%) of 40 also had an abnormal karyotype, a rate twice that of cases without the mutation (30% [9/30]; P = .007). In addition, the proportion of cases positive for an abnormal karyotype increased with patient age (Table 3).

Flow Cytometric Findings

Aberrant Expression of CD56 in Granulocytes and Monocytes in PMF vs MDS

A high proportion of PMF cases displayed aberrant expression in myelocytes and monocytes. Table 1 summarizes these findings. Figure 1 and Figure 2 illustrate the distribution and percentage of CD56+ cells and representative histograms. CD56 was expressed in at least 10% of the myelocytes in 24 (34%) of 70 cases and in at least 20% of the myelocytes in 12 (17%) of 70 cases. The frequency of CD56 expression in monocytes was higher than in myelocytes: CD56 was positive in at least 10% of the monocytes in 48 (69%) of 70 cases and in at least 20% of the monocytes in 27 (39%) of 70 cases. Expression patterns in the MDS-MF group were similar to those in the PMF group. Conversely for the control group, none of the 20 cases showed significant CD56 expression in myelocytes and only 2 cases exhibited significant CD56 expression in the monocytes (Figure 1). It is important to note that the expression of CD56 did not correlate with the JAK2V617F status or the mutant allele burden (Figure 3).

Further study of 36 PMF cases that were labeled with an identical fluorochrome-conjugated antibody, CD56-FITC, for a direct comparison of the median fluorescence intensity (MFI) among different cases found that the MFI of CD56 correlated positively with the presence of cytogenetic aberrations (data not shown).

Granularity and Cell Size of the Granulocytes in PMF vs MDS

We compared the side scatter of granulocytes from PMF, MDS-MF, and control cases. As shown in Table 1 and Figure 4, the MDS-MF group as a whole showed substantially lower side scatter than the control group (P < .005). The side scatter of the PMF cases was also significantly lower than that of the MDS-MF cases and the control cases (P < .0001). The results indicate that granulocytes in a high proportion of the PMF cases were severely hypogranular.

During routine examination of bone marrow aspirate smears, we noticed that the granulocytes in the PMF cases appeared not only hypogranular but also smaller than those from the other 2 groups, with frequent pyknotic nuclei. We used forward scatter to assess and compare the size of the granulocytes from the PMF, MDS-MF, and control cases.
**Figure 2** Aberrant expression of CD56 in granulocytes and monocytes in a control vs 2 cases of primary myelofibrosis (PMF). APC, allophycocyanin; FITC, fluorescein isothiocyanate; SSC, side scattering.

**Figure 3** Lack of correlation between JAK2V617F mutation and percentages of monocytes (A) and granulocytes (B) positive for CD56. The correlation coefficients (r) are 0.049 between JAK2 mutant allele burden and the percentage of CD56+ monocytes and 0.112 between JAK2 and the percentage of CD56+ granulocytes. Note the similar distributions of the percentage of CD56+ cells between JAK2V617F+ cases and JAK2V617F– cases; the latter are shown as 0% of the JAK2V617F allele.
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The results are shown in Figure 5. The PMF cases showed significantly less forward scatter in a high proportion of cases as measured by the mean and the geometric mean ($P < .001$). This finding is unique to PMF, as no difference in forward scattering was identified in the MDS-MF and control cases.

Abnormal Maturation Sequence of Granulocytes in PMF (CD13/CD16)

We assessed 23 of the 70 PMF cases and all 17 MDS-MF cases using the CD13/CD16 panel. We observed abnormal granulocytic maturation/differentiation patterns for MDS-MF and PMF cases (Figure 6). The abnormal patterns of the CD13/CD16 histograms were indistinguishable between PMF and MDS-MF cases. Of the 23 PMF cases, 10 (43%) showed an abnormal maturation pattern, compared with 5 (29%) of 17 MDS-MF cases; this difference was not statistically significant.

Abnormal expression of CD56, low side scattering, and low forward scattering were independent findings with no significant correlation with each other in the PMF and MDS-MF groups. Furthermore, the aberrant patterns were observed in cases with and cases without morphologic evidence of dysplasia.

Figure 4: Median heights and 10th percentile heights of side scattering (SSC) of granulocytes. A, Examples of the SSC vs CD4-fluorescein isothiocyanate (FITC) of the control (Ctl) cases (left panel) and primary myelofibrosis (PMF) cases (right panel). B, Scatter graphs of the SSC from control, myelodysplastic syndrome with myelofibrosis (MDS-MF), and PMF cases. The median values are indicated by horizontal lines. MDS-MF, myelodysplastic syndrome with myelofibrosis.
Discussion

In this first comprehensive study of flow cytometric features of PMF, we found that the granulocytes and monocytes from PMF bone marrow exhibit multiple dysplastic features in frequencies comparable to or higher than those in MDS-MF. The dysplastic features include low side scattering, abnormal CD13/CD16 maturation patterns, and aberrant CD56 expression in granulocytes and monocytes. These findings highlight considerable overlap between PMF and MDS-MF cases as assessed by flow cytometry. However, a feature unique to PMF identified and confirmed in this study is significantly larger granulocytes seen in PMF compared with those in MDS-MF and normal bone marrow. Although the granulocytes from cases of MDS-MF show other features of dysplasia, the cell size remains stable and comparable to that of the control cases. This unique feature of PMF manifests mainly as significantly decreased forward scatter.

Despite different clinical manifestations and pathogenesis, striking similarities between PMF and MDS-MF in flow cytometric findings support that PMF, although a primarily myeloproliferative neoplasm, is a clonal hematopoietic stem cell disease with multiple defects in cell growth and cell death, as is MDS. MDS, as a group of diseases, is generally characterized by enhanced proliferation and increased apoptosis, whereas PMF is characterized by enhanced proliferation and impaired apoptosis. Nevertheless, similarities exist between the 2 entities. For example, both have a high frequency of cytogenetic abnormalities, and both carry the risk of progressing to acute leukemia, ie, the blastic phase. Furthermore, megakaryocytes in PMF show markedly dysplastic features, including large size, hyperchromasia, abnormal lobulation, and “naked” nuclei. At later fibrotic stage or “burnout” phase, PMF is dominated by peripheral cytopenia. It is, therefore, not completely unexpected to find that considerable dysplasia is also observed in PMF cases. The biologic implications of these findings remain unclear.

CD56 is a cell adhesion molecule normally expressed in natural killer cells and a subset of cytotoxic T cells. Aberrant expression of CD56 with varying frequencies has been recognized in MDS, chronic myelomonocytic leukemia, and chronic myelogenous leukemia. In addition, aberrant CD56 expression in myelocytes and monocytes can be seen in growth factor therapy. The pathologic significance of aberrant CD56 expression in PMF is unclear. Presumably, aberrant overexpression of CD56 might alter the adhesion properties of the cells, thereby affecting their tissue-homing and distribution pattern in the body. In MDS, a correlation was shown between the expression of CD56 in myeloblasts and the transfusion dependency of the patients. In our PMF cases, univariate analysis did not reveal significant correlation between patient survival and the percentage of CD56+ cells.
seen in MDS-MF cases, it seems reasonable to postulate that a phenotypically aberrant stem cell compartment is also likely to be present in PMF. While overlap between PMF and MDS-MF by flow cytometric analysis complicates differential diagnosis of the 2 entities, the results from our study suggest that decreased forward scatter identified as a unique feature of PMF could potentially aid in their distinction.

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References


