Diagnostic and prognostic values of S-phase fraction and aneuploidy in patients with bone marrow aplasia

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Abstract

Aim It is often difficult and challenging task to differentiate aplastic anemia (AA) from hypoplastic myelodysplastic syndrome (HMDS) among the patients with bone marrow aplasia. This is possibly because of the considerable clinical, cytological and histological similarities between these two disorders. As prognostic and therapeutic approach to AA and HMDS are different, it is imperative to differentiate them at the time of initial diagnosis. Various attempts have been made in the past to differentiate AA from HMDS. In the present study, we explored the value of certain new parameters i.e. S-phase fraction (SPF) and aneuploidy that could be used for this purpose.

Materials and methods The study included 46 consecutive patients with aplastic anemia, 15 patients with HMDS along with 32 age and sex-matched control subjects. S-phase fraction and aneuploidy analysis was carried out by flow cytometry using Mod Fit-LT V3.0 software.

Results The mean SPF value was significantly lower ($p=0.02$) in patients with AA and higher ($p=0.01$) in HMDS

as compared to that of the control. Aneuploidy was present in 15.2% patients with AA and in 33.3% HMDS cases. During follow-up, 4 patients with AA developed MDS, out of these, three patients had aneuploidy as well as high SPF value at the time of diagnosis. Two patients with HMDS who had aneuploidy and high SPF, converted into AML. Eleven patients died during the study, in whom 8 had aneuploidy and high SPF value.

Conclusion We conclude that high SPF value and presence of aneuploidy favour the diagnosis of HMDS rather than AA. SPF and aneuploidy may be important parameters in patients with AA to predict their propensity to evolve into myelodysplastic syndrome and acute myeloid leukemia. SPF value may also be useful in the early diagnosis of HMDS before morphologically evidence of dysplasia is apparent.

Keywords Aplastic anemia · Myelodysplastic syndrome · S-phase fraction · Aneuploidy · Prognosis · Diagnosis

Introduction

Pancytopenia is an important presentation in primary as well as in secondary hematological disorders and it is commonly classified into one of two distinct categories: pancytopenia with hypoplastic marrow, known as aplastic anemia (AA) and pancytopenia with normo- or hypercellular bone marrow is characteristic of myelodysplastic syndrome (MDS) [1]. Myelodysplastic syndrome is characterized by dysplastic and ineffective normocellular or hypercellular bone marrow with variable degree of peripheral blood cytopenia along with higher risk of transformation to acute myeloid leukemia (AML). However, 8–46% patients of

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MDS have hypoplastic bone marrow [2]. In this situation, it is often very difficult to determine whether the patient has AA or hypoplastic MDS (HMDS). Mistakes are frequently made and the patient with HMDS is often diagnosed as having AA. The differentiation between AA and HMDS is of great therapeutic and prognostic importance as the clinical course and management of these two entities differs. There is a higher risk of progression to AML in patients with HMDS in comparison to AA [1]. Both have probably same pathogenesis as they respond to immunosuppressive therapy (IST).

Various attempts have been made in the past to differentiate these entities using morphological, histological and conventional cytogenetics [1–11]. In addition, differences in the pattern of proliferation of bone marrow cells in AA and MDS have been reported [2,12–13] through immunohistochemistry techniques using several antigens (p53, proliferating cell nuclear antigen i.e. PCNA and CD34) but these are not reproducible and satisfactory. The distinction may be morphologically difficult even with careful marrow examination which may provide insufficient material due to extreme hypocellularity. Bone marrow findings such as the presence of bi- or trilineage dysplasia, increased bone marrow blasts, micromegakaryocytes, high numbers of megakaryocytes, slight marrow fibrosis and irregular distribution of megakaryocytes in the marrow biopsy are features favoring HMDS rather than AA [3, 4]. However, the problem is complicated by the fact that majority of HMDS cases have no increase in marrow blasts [5–7]. Some cases of HMDS may have dysplastic features confined to erythroid cell line which can be observed in AA as well [7–9]. Thus, using these criteria, morphological analysis may not enable a distinction to be made between AA and HMDS. Chromosomal abnormalities such as trisomy 8, deletion of 5, 7 and 20, are considered diagnostic of MDS [1]. The detection of a clonal chromosomal abnormality early in the course of the disease is an indication of MDS [3]. These abnormalities are stereotypically aneuploid rather than structural chromosomal alterations [14]. Cytogenetic studies are somewhat difficult to perform in patients with hypocellular bone marrow because of scanty cells in both peripheral blood and bone marrow. Therefore, undetected aneuploidy exists in a large percentage of patients with bone marrow hypoplasia. So, there remains a group of patients in which aneuploidy is not detected by conventional cytogenetics and for whom the differential diagnosis of AA and HMDS is in doubt.

Flow cytometric analysis of S-phase fraction (SPF) and aneuploidy have been studied in different hematological malignancies including myelodysplastic syndrome [15, 16], chronic myeloid leukemia [17], acute myeloid leukemia [18], non-Hodgkin's lymphoma [19] as well as in solid tumors such as prostate cancer [20] and breast cancer [21] for the purpose of classification, grading and prognosis. DNA histograms obtained by flow cytometry (FCM) provide two functional factors i.e. S-phase fraction and DNA

ploidy. SPF represents a continuous variable related to any proliferating cell population. SPF measures the percentage of a given cycling subpopulation between G_0/G_1 and G_2/M phase and it reflects cellular proliferation [15]. In contrast to SPF, aneuploidy is a discrete biomarker of malignancy. FCM can rapidly measure the cellular DNA content of large numbers of cells and estimate aneuploidy that is independent of cell proliferation. Cells with abnormal DNA content refer as aneuploidy. The evolution of aneuploidy can be an early phenomenon in leukemogenesis, which in course may affect the proliferation, differentiation and cell death [15]. Aneuploidy may be of value for differentiating “aplastic anemia” and the aplastic form of preleukemia or leukemia.

In this study, we used SPF and aneuploidy as novel tools to differentiate AA from HMDS. The findings of proposed study may provide a new marker for the early as well as correct diagnosis of patients with bone marrow hypoplasia along with the assessment of prognosis and thus allow us to choose appropriate treatment.

Materials and methods

The study group comprised of 61 consecutive patients with bone marrow aplasia out of which 46 patients were diagnosed as AA and 15 patients with HMDS. The diagnosis of bone marrow aplasia was made on the basis of complete blood counts (CBC), bone marrow aspiration and biopsy. The diagnostic criteria for AA were adopted from the International Agranulocytosis and Aplastic Anemia Study [22]: i) hemoglobin ≤ 100 g/L, ii) platelet count $\leq 50 \times 10^9/L$, iii) white blood cell count $\leq 1.5 \times 10^9/L$. Pathologic criteria for AA included the following: i) decreased cellularity ($< 25\%$) of the bone marrow biopsy specimen, ii) the absence of morphologic evidence of dysplasia in any cell lineage, iii) diploid karyotype. The diagnostic criteria for HMDS were [23]: i) $> 5\%$ of blasts in bone marrow smears, ii) ALIPs in bone marrow sections, iii) clonal cytogenetic abnormality or at least two of the following parameters: a) blasts in the peripheral blood, b) ring sideroblasts, c) atypical granulocytic precursors or megakaryocytes. Patients with HMDS ($n=15$) were subclassified according to World Health Organization (WHO) classification of MDS [24]. Most of the patients with HMDS had refractory anemia (RA) ($n=11$), 1 patient had refractory cytopenia with multilineage dysplasia (RCMD), 2 patients had refractory anemia with excess blasts-1 (RAEB-1) and 1 patient had refractory anemia with excess blasts-2 (RAEB-2). The median duration from the onset of symptoms and diagnosis in patients with bone marrow aplasia was 20 days with a range of 7–62 days. Thirty two age-sex matched control subjects were also included in the study. Patients who had received anti-lymphocyte globulin (ALG)/anti-thymocyte globulin (ATG), steroid or other IST were excluded from the study. All patients with bone marrow aplasia (AA & HMDS) were put on cyclospo-

rine (CsA) (5–10 mg/kg/day) as no one was able to afford ATG/ALG or bone marrow transplantation (BMT). The patients were followed up periodically and the dosage of CsA was adjusted according to clinicohematological parameters. Investigations were performed at the time of diagnosis prior to the treatment. SPF, aneuploidy analysis and bone marrow examinations (aspiration and biopsy) were repeated at 6 month intervals. An informed written consent was obtained from patients and control subjects. The study protocol was approved by the Institutional Ethics Committee.

Flow cytometric analysis

S-phase fraction (cell cycle analysis) and aneuploidy analysis in peripheral blood lymphocytes was carried out according to the method described by Krishnan [25]. Samples were analyzed on a Becton Dickinson flow cytometer (Becton Dickinson India Private Limited, Gurgaon, Haryana, India) with Mod Fit-LT V3.0 software (Verity Software House, Topsham, ME., U.S.A.) using 488 nm excitation wavelength. Cells acquired by FCM were plotted in side-scattered light (SSC) *versus* forward-scattered light (FSC), Fluorescence-2 Area (FL-2A) *versus* Fluorescence-2 Width (FL-2W) and FL-2A *versus* count. The threshold was adjusted to have the first peak at 200 channel of FL-2W plot and the lymphocyte cell population was gated from the plot of FL-2A *versus* FL-2W for analysis. The gated lymphocyte population was analyzed using the broadened rectangular model of the Mod Fit-LT V3.0 software, with a constant coefficient of variation of less than 6 and residual chi square (χ^2) value less than 5. This modal was also fitted with single cut debris subtraction and aggregate subtraction. Ten thousand cells were analyzed per sample. Human peripheral blood lymphocytes from hematologically normal individuals were used as the diploid reference standard.

Statistical data analysis

The findings were expressed as the mean $\pm$ standard deviation. The differences between the groups were compared with the use of Student-t test. A value of $p \leq 0.05$ was considered statistically significant.

Results

Sixty one patients having bone marrow aplasia, diagnosed on the basis of bone marrow aspiration and biopsy, were

included in the study. Fifteen patients (24.5%) showed the evidence of dysplasia in bone marrow examination, thus categorized as HMDS. The mean age of patients with AA and HMDS was 29.7 ± 15.9 years ($n = 46$, range 13–71 years, with a median age of 23.5 years) and 39.0 ± 20.2 years ($n=15$, range 15–75 years with median age of 35 years) respectively. The male to female ratio of patients was 3.1 to 1 and 2.7 to 1 in AA and HMDS groups respectively. The mean age of control subjects was 30.53 ± 15.28 years ($n=32$, range 14–67 years with median age of 25 years). The most common clinical presentation in patients with AA and HMDS was anemia. Other manifestations were bleeding, pyrexia, dyspnea on exertion, fatigue and pallor. No etiological association could be made in any of these cases.

Mean SPF value in patients with AA ($n = 46$) was $0.47 \pm 0.44\%$ (median value 0.30% with a range of 0.02 – 2.90%) and it was significantly lower ($p=0.02$) than that of control subjects. However, mean SPF value in patients with HMDS ($n = 15$) was $1.22 \pm 1.19\%$ (median value 0.64% with a range of 0.39–4.35 %) and was significantly higher ($p=0.0006$, 0.01) than in patients with AA as well as control subjects (Table 1).

At the time of diagnosis, 39 patients (84.8%) with AA had normal DNA histogram i.e. they did not had aneuploidy (Fig. 1). Aneuploidy was detected in seven patients (15.2%) of AA (Fig. 2) and five patients (33.3%) of HMDS (Fig. 3). Mean SPF value in patients with AA who had aneuploidy ($n = 7$) was $1.22 \pm 0.76\%$ and it was significantly higher ($p<0.0001$) than in AA patients who did not had aneuploidy ($n=39$). Similarly, mean SPF value in patients with HMDS who had aneuploidy ($n = 5$) was $2.56 \pm 1.28\%$ and it was significantly higher ($p = 0.0001$, 0.04) in comparison to those HMDS patients without aneuploidy ($n=10$) as well as patients with AA who had aneuploidy ($n = 7$).

Patients with AA were followed up for a median of 12 months and looked for the development of dysplasia as evident by morphological changes in repeat bone marrow examination and peripheral blood findings. Depending on these findings, AA patients were categorized into two groups: AA patients who developed dysplasia ($n = 4$) and AA patients who did not develop dysplasia ($n = 42$). Patients with AA, who developed dysplasia, had significantly higher ($p < 0.0001$) SPF value (1.47 ± 0.97) at the time of diagnosis than AA patients, who did not develop the dysplasia later. The incidence of aneuploidy (75%) was higher in AA patients with dysplasia. No patients were detected to have cytogenetic abnormalities.

Table 1 SPF value in control subjects, patients with aplastic anemia and Hypoplastic MDS at the time of diagnosis

S. No.	Subject	SPF %	p-Value
1.	Control Subjects ^a ($n=32$)	$0.67 \pm 0.22\%$	^{ab} 0.02, ^{ac} 0.01, ^{bc} 0.0006
2.	Aplastic anemia ^b ($n=46$)	$0.47 \pm 0.44\%$	
3.	Hypoplastic MDS ^c ($n=15$)	$1.22 \pm 1.19\%$	

Two patients (13.3%) with HMDS who had aneuploidy and high SPF, converted into AML within a period of 18 months. These HMDS patients who converted into AML, belonged to RAEB-1 and RAEB-2 subtypes. Eleven patients died during the study, in whom 8 had aneuploidy and high SPF value.

Discussion

Differentiating aplastic anemia (AA) from hypoplastic myelodysplastic syndrome (HMDS) at presentation can be problematic both clinically and pathologically. Although, majority of MDS patients have normo- or hypercellular marrow, a variable percentage have hypocellular marrow

Fig. 1 DNA histogram of AA patients at time of diagnosis

Fig. 2 DNA histogram of AA patients showing SPF and presence of aneuploidy

Fig. 3 DNA histogram of HMDS patients showing high SPF and presence of aneuploidy

[3]. The incidence of hypocellular MDS in patients with MDS is reported to be between 8% and 29% [5,6,8-11,26]. However, a recent study in Brazilian patients reports a much higher incidence (46%) of hypocellular MDS [7]. The difference in the reported incidence of cellularity among various studies may be related to case selection, marked topographic variation of hematopoietic cells in the marrow in MDS or the definition of hypocellularity. Most studies define hypocellularity as bone marrow cellularity <30% in patients with age of < 60 years [5,6,10,26]. Others used a criteria of <20% cellularity for patients above 60 years of age [9,11] or age-matched normal cellularity [5]. HMDS, in particular, can represent diagnostic difficulty since these can be confused with AA, perhaps as a result of considerable clinical, cytological, histological similarities. Presence of chromosomal abnormalities favors the diagnosis of MDS but due to profoundly hypocellular bone marrow in both conditions, it is often difficult to obtain sufficient material for adequate cytogenetic analysis. The differentiation between AA and HMDS is of great significance because of its therapeutic and prognostic impact, also because the patients with HMDS are at higher risk of developing AML in comparison to AA.

With immunosuppressive therapy (IST) such as antilymphocyte globulin (ALG)/antithymocyte globulin (ATG), patients with severe AA have long term survival of approximately 80% at 168 months [27]. Similarly, patients with moderate AA have median survival exceeding 174 months with androgen and supportive therapy [27]. On the other hand, several studies suggest that the median survival

for patients with HMDS is not significantly different from hypercellular MDS and varies from 22 to 33 months [5–7, 9–11]. Recent studies have revealed that the immune system plays an important role in the pathogenesis of both AA and MDS, hence some cases of HMDS may show transient response to IST thus adding further diagnostic difficulty [26, 28, 29].

Studies have demonstrated that the distinction between AA and HMDS may be morphologically difficult even with careful marrow examination. The problem is complicated by the fact that the majority of HMDS cases have no increase in marrow blasts and the dysplastic features are confined to erythroid cell line which can be observed in AA as well [7–9]. Chromosomal abnormalities including trisomy 8, deletion of 5, 7 and 20, are considered diagnostic of MDS [1]. In additions, there is a strong possibility that a proportion of cases of AA may be in fact the preleukemia [30]. The study of chromosomes offers the method of detecting the cases of preleukemia but the absence of frequent dividing cells to enable analysis of metaphase chromosomes is of the major limitation of conventional cytogenetics studies in patients with bone marrow aplasia. Besides this, apoptosis and proliferation patterns using immunohistochemistry, used to differentiate these entities do not yield reproducible and satisfactory results.

Flow cytometry (FCM) is a powerful tool which allows a fast and automated estimation of S-phase fraction (proliferative activity) and aneuploidy status (abnormal DNA content). It is also free from all encumbrances of karyotyping such as a small number of recognizable metaphases,

minimal chromosome spreading, poor banding quality and condensed or fuzzy appearance of chromosome. SPF has been studied in a number of hematological diseases including MDS and solid tumors for the purpose of classification, grading and prognosis [15–21].

There are few studies on flow cytometric analysis of cell cycle and DNA content in patients with MDS, however no study has been reported to our best knowledge on SPF and aneuploidy in patients with bone marrow aplasia for the differentiation between AA and HMDS. In the present study, we found that at the time of diagnosis, patients with AA had significantly lower ($p = 0.02, 0.0006$) SPF value as compared to control subjects and patients with HMDS. SPF value was significantly higher in patients with HMDS as compared to control subjects. Aneuploidy was detected in both the diseases but the incidence was higher in HMDS patients (33.3%) in comparison to AA (15.2%). Mean SPF value was significantly higher in those patients who had aneuploidy than the patients who did not have aneuploidy and it was applicable for both groups of patients i.e. AA or HMDS.

During the follow-up, 8.7% patients with AA developed dysplasia and these patients had significantly higher ($p < 0.0001$) SPF value than AA patients, who did not develop dysplasia later. The incidence of aneuploidy (75%) was also higher in AA patients with dysplasia. No patient was detected to have cytogenetic abnormalities. Two patients (13.3%) with HMDS who had aneuploidy and high SPF, converted into AML.

Conclusion

Our findings reveal that a higher SPF and the presence of aneuploidy in patients with bone marrow aplasia even without the morphological evidence of dysplasia and presence of cytogenetic abnormalities may place these patients distinct from aplastic patients with lower SPF and without aneuploidy. These patients with higher SPF and aneuploidy perhaps represent HMDS where morphological features of dysplasia have not yet appeared. In such cases, bone marrow biopsy should thoroughly be scrutinized for the evidence of dysplasia at the time of initial diagnosis. Furthermore, more sensitive tests such as Fluorescence In Situ Hybridization (FISH) may be used in order to detect cytogenetic abnormalities, which have otherwise been missed using conventional karyotyping.

We suggest that SPF and aneuploidy may be important parameters to differentiate AA from HMDS patients. SPF and the presence of aneuploidy in AA patients also suggest that these patients may represent a preleukemic state and evolve into MDS / AML in due course. An elaborate study with a larger sample size is needed to ascertain the precise role of SPF and aneuploidy in diagnosis and prognosis of bone marrow aplasia.

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