

Bone marrow cell cycle markers in inherited bone marrow failure syndromes

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Abstract

Patients with inherited bone marrow failure syndromes (IBMFS) are at increased risk of acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS), possibly related to cell cycle dysregulation. In a cross-sectional analysis of bone marrow from 77 IBMFS, 71 sporadic conditions (AML, MDS, acquired aplastic anemia) and 22 normal controls we found overexpression of p53 in IBMFS, AML, and MDS; of Ki-67 in IBMFS and AML; and of survivin in IBMFS compared with all other groups. The patterns of expression of cell cycle markers in IBMFS are thus distinct. Longitudinal studies will determine the diagnostic and prognostic significance of these findings.

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1. Introduction

The inherited bone marrow failure syndromes (IBMFS) comprise a group of genetic disorders characterized by single or multiple cytopenias, as well as distinctive clinical features and varied molecular pathways [1]. Affected individuals have an increased risk of myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) [2]. The most common IBMFS, Fanconi anemia (FA), Diamond-Blackfan anemia (DBA), Shwachman-Diamond syndrome (SDS) and Dyskeratosis congenita (DC), comprise at least 25% of all pediatric bone marrow failure cases (inherited and acquired) [3]. Since marrow cells from patients with sporadic MDS and AML have aberrant expression of cell cycle markers, we hypothesized that similar abnormal expression in IBMFS might

provide insight into the pathogenesis of these complications and be predictive of disease evolution. Normal regulation of hematopoiesis involves a comprehensive signaling system that maintains a balance between cell proliferation and apoptosis. Activation of the p53 tumor suppressor pathway leads to cell cycle arrest and initiates apoptosis [4]. Survivin, a member of the inhibitors of apoptosis proteins [5], inhibits apoptosis and regulates cell division. Ki-67 is essential in cell proliferation and is highly expressed during cell replication, in all active phases of the cell cycle (G₁, S, G₂, and mitosis), while absent in resting cells (G₀) [6]. Numerous cell cycle abnormalities have been reported in MDS and AML, reflecting disruption of normal regulators. Overexpression of p53 protein was reported in MDS and AML, including higher expression in high grade MDS (refractory anemia with excess blasts [RAEB]) and *de novo* AML than in normal controls, and even higher expression in MDS-derived AML [7]. Overexpression of p53 was also observed in low grade MDS (refractory anemia [RA]) when compared with acquired aplastic anemia [8], and in a proportion of AML and

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MDS marrows [9]. While some studies noted that the expression of Ki-67 was higher in high risk than low risk MDS, and in MDS than in normal controls [10], others found no difference in the expression of Ki-67 mRNA between AML, ALL and normal controls [11]. Survivin expression was increased in more than half of over 100 bone marrow aspirates from patients treated for *de novo* AML, while most of the remaining samples lacked any expression [12]; in another study, survivin expression was higher in the marrow of MDS and MDS-derived leukemia than normal controls [13]. The only study of cell cycle protein expression in IBMFS was in SDS, where we found overexpression of p53, comparable to MDS RA [14]. Two groups reported absence of mutations in *p53* genes in SDS and FA respectively [15,16].

Since the risk of MDS/AML is extremely high in patients with IBMFS, we hypothesized that overexpression of p53, Ki-67 and survivin in the marrows of patients with IBMFS would be similar to sporadic AML and MDS and different from marrows in other acquired disorders and normal individuals. Patients with IBMFS who have a high expression of cell cycle markers might be more likely to have or develop MDS or AML.

This study provides the cross-sectional results from the initial studies of patients, many of whom will be followed longitudinally to determine the association of the markers with outcomes. Clinical outcomes are not yet available, since the follow-up time has been short. However, we identified novel patterns of expression of cell cycle markers which have not been reported previously, which indicate that the inherited marrow disorders differ as a group from any of the acquired control conditions.

2. Materials and methods

2.1. Subjects

Bone marrow specimens were collected at multiple institutions and aspirate and biopsy slides were sent to the University of Texas Medical Branch (UTMB, Galveston, TX). The study was approved by the Institutional Review Boards (IRB) of UTMB and the National Cancer Institute. Data were entered into Microsoft Excel® and identifying information was removed. Analysis of the de-identified dataset was then granted an exemption from IRB review by the George Washington University Medical Center. Biopsies with less than 2000 nucleated cells were excluded, and the earliest sample was analyzed if sequential samples were available.

Bone marrow cellularity was defined as hypocellular if the proportion of area occupied by cells was less than expected for age [17,18]. MDS morphology required dyspoiesis in at least 10% of the cells in at least two lineages, as defined by the World Health Organization (WHO) classification schemes, but modification of the diagnostic criteria to require 20% dysplasia in at least two cell lines was applied [19]. WHO criteria of $\geq 20\%$ blasts were used to diagnose AML.

Immunohistochemistry for cell cycle markers was performed on biopsy slides from 77 patients with FA, DBA, SDS, or DC, and from 71 with sporadic AML, MDS, or acquired aplastic anemia (AA), as

well as from 22 normal controls (Table 1). Patients with an IBMFS or sporadic hematologic disease met standard diagnostic criteria. The normal control marrows were from individuals with a variety of localized, early stage lymphoproliferative disorders that did not involve the bone marrow, and whose blood counts and marrow morphology were normal.

2.2. Immunohistochemistry

Biopsies were fixed in formalin or B5 fixative and then rapidly decalcified in an aqueous solution of hydrochloric acid (RDO, Apex Engineering Corp., Aurora, IL). Four micrometer paraffin sections were mounted on poly-L-lysine-coated slides, which were subjected to a heat-based antigen retrieval method containing citrate buffer (pH 6) in a heated water bath (95–99 °C) for approximately 30 min. Using avidin-biotin-peroxidase complexes (ABC method), bone marrow biopsies were stained for p53 (DO-7, Dako, Carpinteria, CA), Ki-67 (Dako), survivin (Abcam Inc., Cambridge, MA), and caspase 3 (Cell Signaling Technology, Danvers, MA) according to the manufacturers' directions. Positive staining controls included a p53-positive colon adenocarcinoma, tonsils for Ki-67, pancreatic carcinoma for survivin, and tonsils for caspase 3. Any marrow nucleated cell with clear and unequivocal nuclear staining was scored as a positive cell for p53 and Ki-67, and cytoplasmic and/or nuclear staining for survivin (Fig. 1). All slides were reviewed by two pathologists (MTE and BJB) who were blinded to the source of the material; differences in readings were reconciled by simultaneous review using a double-headed microscope.

p53 and Ki-67 stains were nuclear, while survivin was both nuclear and cytoplasmic. p53 was expressed by immature cells with round nuclei and fine nuclear chromatin. Since double staining was not performed, these cells could have been myeloid or erythroid in origin. The type of cells expressing Ki-67 and survivin was difficult to identify because of the intensity of the staining. However, we presume that most stages of maturation and both erythroid and myeloid cells expressed these markers, due to the high proportion of cells with expression in most cases.

2.3. Analytical methods

Because of the small numbers of cases, we combined sporadic MDS cases into two groups, MDS-hi (RAEB 1 and 2), and MDS-lo (RC, RCMD, and RCMD with ringed sideroblasts), using the WHO classification [19]. Each of the cell cycle proteins was classified as overexpressed in a marrow sample if it was expressed in a higher portion of bone marrow biopsy cells than any of the normal controls for that marker (none of the normal marrows expressed p53, while the respective expression of Ki-67 and survivin in the normal marrows was 3 and 25% of the nucleated cells). We analyzed the numbers of patients in whom a marker was overexpressed, the percentage of positive cells in all individual marrows, and the percentage of positive cells among those in whom the markers were overexpressed. Statistical analyses were conducted using SAS Version 8.02 (SAS Institute Inc., Cary, NC). Chi-square or Fisher exact tests were used to compare frequencies and, due to the skewed distribution, a two-sided Wilcoxon rank sum test was used to compare continuous variables. Statistical significance was defined as $p \leq 0.05$. We also conducted subset analyses among the IBMFS according to the presence or absence of morphologic MDS.

3. Results

3.1. Subjects

The subjects and their bone marrow findings are summarized in Table 1. The apparent excesses of females in FA and of males in DC were not significant. Overall, there was no statistical difference in gender between the different marrow disorders (global chi-square $p=0.08$). Patients with an IBMFS were younger than those with acquired disorders and normal controls; this difference reached significance in each comparison ($p<0.05$). Within the IBMFS group, patients with SDS were significantly younger than those with FA, DBA or DC ($p<0.05$).

The frequency of age-adjusted bone marrow hypocellularity was similar among all the IBMFS and AA (75–100%). This frequency was significantly higher in IBMFS compared with AML, MDS-hi, MDS-lo and normal (0–29%; $p<0.001$). The frequency of morphologic MDS was similar among the different IBMFS (global chi-square $p=0.3$).

3.2. Immunohistochemistry

Bone marrows stained for p53, Ki-67, survivin and caspase 3 proteins were evaluated for the proportion of marrows in each disorder with overexpression, the percentage of marker positive cells in each sample, and the percentage of marker positive cells among those in which there was overexpression (Table 2 and Fig. 2). Caspase overexpression was seen with significantly increased frequency only in marrows from patients with MDS-hi, and not in any IBMFS nor in any other controls (data not shown).

3.3. p53

The frequency of overexpression of p53 in IBMFS was similar to AML, MDS-hi or MDS-lo ($p>0.2$); however, it

was significantly higher than in AA and normal, who did not express p53 ($p<0.0001$). The differences in frequencies of p53 overexpression among the individual IBMFS syndromes were not significant ($p>0.07$). The frequency of overexpression of p53 was similar in the IBMFS samples with and without MDS morphology ($p=0.4$).

These findings were further substantiated in a comparison of the proportions of p53 positive cells between IBMFS and the other bone marrow groups. When all patients were included, marrows from DC had a higher median percentage of p53 positive cells and were distinguished not only from AA and normal ($p<0.0001$), as was the case for the other IBMFS, but were also higher than those from FA, MDS-hi and MDS-lo ($p<0.04$). These results are driven by the inclusion of marrows in which there was no expression of p53. However, among only those marrows in which there was increased expression of p53, we found that DC, SDS, and the combined IBMFS group had significantly higher levels of expression than MDS-lo ($p<0.008$), and resembled MDS-hi and AML.

3.4. Ki-67

The frequency of overexpression of Ki-67 in IBMFS was not statistically different from AML ($p=0.5$); however, it was significantly higher in IBMFS than in MDS-hi, MDS-lo, AA and normal ($p<0.02$). More DBA marrows expressed Ki-67 than FA marrows ($p=0.03$), while other differences between IBMFS groups were not significant ($p>0.2$). DBA was also the only IBMFS with a significantly higher frequency of Ki-67 overexpression compared to MDS-hi ($p<0.001$); the other IBMFS groups were not significantly different from MDS-hi ($p>0.06$). As with p53, the presence or absence of MDS morphology was not related to the frequency of overexpression of Ki-67 ($p=0.8$). Expression of Ki-67 in sporadic MDS and AML was not increased, consistent with a prior report also using paraffin-embedded bone marrow biopsy slides [20].

Table 1
Subjects and bone marrow findings

Diagnosis	Number	Male:female	Age in years, median (range)	MDS morphology N (%)	Biopsy hypocellular N (%)
IBMFS	77	41:36	15 (1.4–48)	15 (19)	66/76 (87)
FA	25	8:17	16 (2–35)	6 (24)	22 (88)
DBA	21	14:7	15 (1.7–45)	2 (10)	15/20 (75)
SDS	20	11:9	10 (1.4–42)	6 (30)	18 (90)
DC	11	8:3	24 (3–48)	1 (9)	11 (100)
Sporadic	71	42:29	62 (1.7–88)	58 (82)	25 (35)
AML	12	8:4	53 (23–88)	12 (0)	0 (0)
MDS-hi	11	7:4	62 (6–80)	11 (100)	2 (18)
MDS-lo	35	20:15	66 (1.7–85)	35 (100)	10 (29)
AA	13	7:6	46 (7–75)	0 (0)	13 (100)
Normal	22	15:7	49 (2–84)	0 (0)	1 (5)

IBMFS, all inherited bone marrow failure syndromes combined; FA, Fanconi anemia; DBA, Diamond-Blackfan anemia; SDS, Shwachman-Diamond syndrome; DC, Dyskeratosis congenita; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; MDS-hi, high grade MDS; MDS-lo, low grade MDS; AA, acquired aplastic anemia; NL, normal; M, male; F, female. Denominator is shown where appropriate slides were not available for all patient samples. In all disorders, M = F (binomial probability test). No single group differed from the others in the sex ratio ($p=0.2$, global chi-square test). SDS were younger than the other IBMFS, and the entire IBMFS group was younger than any of the sporadic and normal control groups. The frequency of morphologic MDS was similar in all IBMFS ($p=0.3$, global chi-square test). IBMFS were more frequently hypocellular than the controls except AA ($p>0.1$).

Table 2
Expression of cell cycle markers

Diagnosis	Number (%) of patients with overexpression			% of positive cells, median (range)			% of positive cells among overexpressors, median (range)		
	p53	Ki-67	Survivin	p53	Ki-67	Survivin	p53	Ki-67	Survivin
IBMFS	47/77 (61)	44/64 (69)	42/56 (75)	3 (0–40)	33 (0–90)	80 (0–95)	10 (1–40)	50 (4–90)	90 (30–95)
FA	12/25 (48)	9/18 (50)	12/16 (75)	0 (0–40)	3 (0–90)	70 (4–95)	10 (1–40)	70 (4–90)	80 (50–95)
DBA	16/21 (76)	16/18 (89)	12/15 (80)	5 (0–40)	45 (0–60)	90 (15–95)	10 (1–40)	50 (8–60)	93 (30–95)
SDS	10/20 (50)	12/18 (67)	12/17 (71)	2 (0–40)	30 (0–80)	70 (0–95)	18 (3–40)	55 (4–80)	93 (60–95)
DC	9/11 (82)	7/10 (70)	6/8 (75)	10 (0–25)	35 (0–80)	85 (0–95)	15 (4–25)	60 (10–80)	93 (60–95)
Sporadic	34/71 (48)	14/70 (20)	5/67 (8)	0 (0–50)	2 (0–50)	5 (0–40)	3 (1–50)	5 (4–50)	35 (30–40)
AML	8/12 (67)	7/12 (58)	2/12 (17)	1 (0–50)	4 (1–10)	7 (1–40)	4 (1–50)	5 (4–10)	38 (35–40)
MDS-hi	4/11 (36)	3/11 (27)	0/10 (0)	0 (0–25)	2 (0–4)	14 (5–25)	11 (1–25)	4 (4–4)	0
MDS-lo	22/35 (63)	4/35 (11)	2/32 (6)	2 (0–40)	0 (0–50)	5 (0–40)	3 (1–40)	9 (4–50)	35 (30–40)
AA	0/13 (0)	0/12 (0)	1/13 (8)	0 (0–0)	0 (0–2)	0 (0–35)	0	0	35 (35–35)
Normal	0/21 (0)	0/22 (0)	0/19 (0)	0 (0–0)	0 (0–3)	5 (0–25)	0	0	0

Overexpression: expression in a higher proportion of cells in a patient biopsy than in any normal biopsy. Data show number of patients with overexpression, divided by the total number of patients studied. Percentage of patients shown in brackets.

The IBMFS had a higher median percentage of Ki-67 positive cells than all of the sporadic disorder groups ($p < 0.05$); however, FA, SDS and DC were similar to AML ($p > 0.08$) and FA was also similar to MDS-hi ($p = 0.6$). All of the IBMFS marrows, individually and combined, had much higher percentages of Ki-67 positive cells among the overexpressors when compared with the sporadic disorders ($p < 0.05$).

3.5. Survivin

A very high rate of overexpression of survivin distinguished IBMFS from the sporadic and normal groups ($p < 0.001$). Only two marrows were positive in the AML and MDS groups, and one in the AA group. Specific IBMFS syndromes were not different from each other in the frequency of survivin overexpression ($p > 0.3$). As with the other cell cycle markers, MDS morphology in the IBMFS marrows was not correlated with survivin expression ($p = 0.99$).

The median percentage of survivin-positive cells was much higher in all the IBMFS than in the other conditions or normals, and the percentage of positive cells among the survivin overexpressors with IBMFS was clearly above that of the others.

3.6. All markers

Fig. 3 shows the combined levels of overexpression of p53, Ki-67, and survivin. The control panel (left) indicates that p53 overexpression is frequent in MDS and AML, and that some of those patients who overexpress p53 also overexpress Ki-67; only one patient appears to overexpress all three markers. The IBMFS patients (right panel) separate into three groups. The first group resembles the controls, with low expression of survivin, and some overexpression of both p53 and Ki-67 in about half the patients. The second group all overexpress survivin, as well as p53 and Ki-67. The third group strongly

overexpress survivin (in more than 60% of their cells), as well as p53 and Ki-67.

4. Discussion

Overall, the patterns of expression of p53 and Ki-67 in the IBMFS marrows resembled the patterns in AML and MDS, and were higher in the inherited syndromes and the sporadic hematologic malignancies than in acquired aplastic anemia or normal control marrows.

p53 was not expressed in AA or normal bone marrows, as expected, and the previously reported increased expression in sporadic AML and MDS was confirmed. As a group, the IBMFS marrows also overexpressed p53 in frequencies similar to those in the sporadic conditions, and this overexpression was not associated specifically with MDS morphology in the IBMFS. In addition, the frequency of overexpression of p53 in patients with an IBMFS who expressed p53 was not only the same as or higher than the proportion of those with sporadic MDS and AML, but also the proportion of p53 positive cells in the IBMFS patients who did express p53 was higher than in the sporadic controls. The DC and SDS marrows in which p53 was expressed had the highest proportions of positive cells in any group.

The proportion of patients with an IBMFS who overexpressed Ki-67 was similar to the proportion with sporadic AML, and both were higher than those with MDS. Amongst the overexpressors of Ki-67, substantially more cells were positive in the IBMFS marrows than in the sporadic marrows.

Thus, the patterns of expression of p53 and Ki-67 in the four main types of IBMFS that we examined resemble the patterns described in the literature and confirmed here for MDS and AML. Although the frequency of MDS morphology was low (9–30%) among our IBMFS marrows, the frequency of overexpression of these two cell cycle markers ranged from 50 to 89%, and thus overexpression was clearly present in high proportions of patients who had nor-

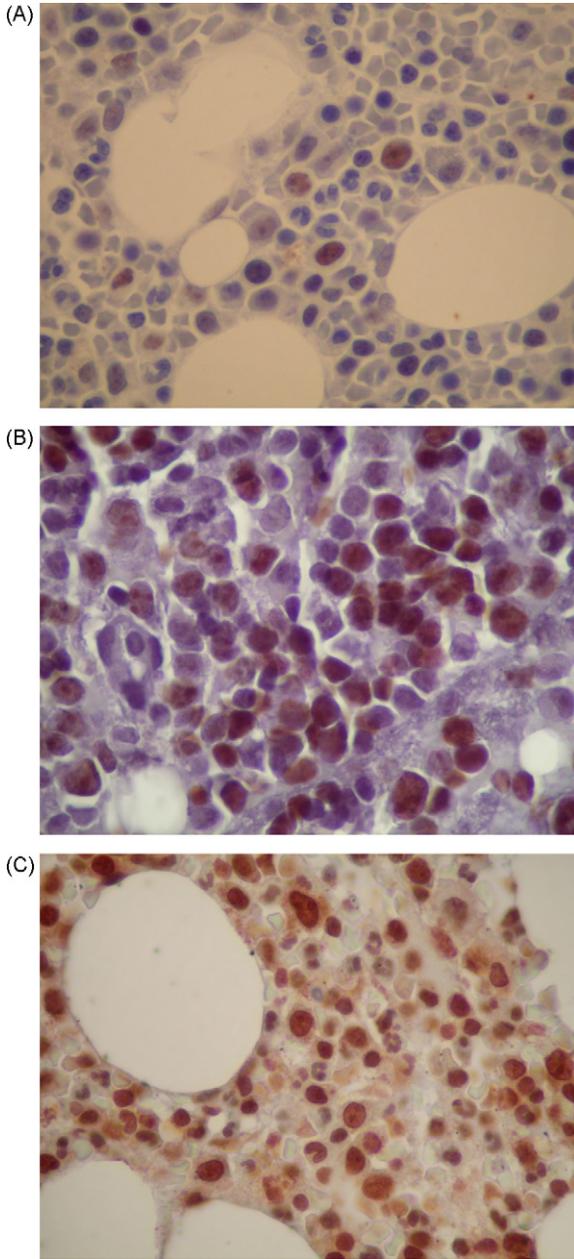


Fig. 1. Immunohistochemical stains in bone marrow biopsies. (A) p53, 7% positive, from a patient with DC. (B) Ki-67, 70% positive, from a patient with SDS. (C) Survivin, 70% positive, from a patient with DBA. Original magnification $\times 500$.

mal marrow morphology. The frequency of overexpression was generally similar in all four of the syndromes. The proportion of positive cells among the expressing marrows was somewhat higher in IBMFS than in sporadic AML and MDS. SDS and DC overexpressors had slightly more p53 positive cells than did FA and DBA, but the numbers of patients are small.

The results with survivin were unexpected. First, in contrast with previous reports, only one or two of the patients with sporadic disorders had any positive cells. Second, and related to the low expression in the controls, more than 75% of

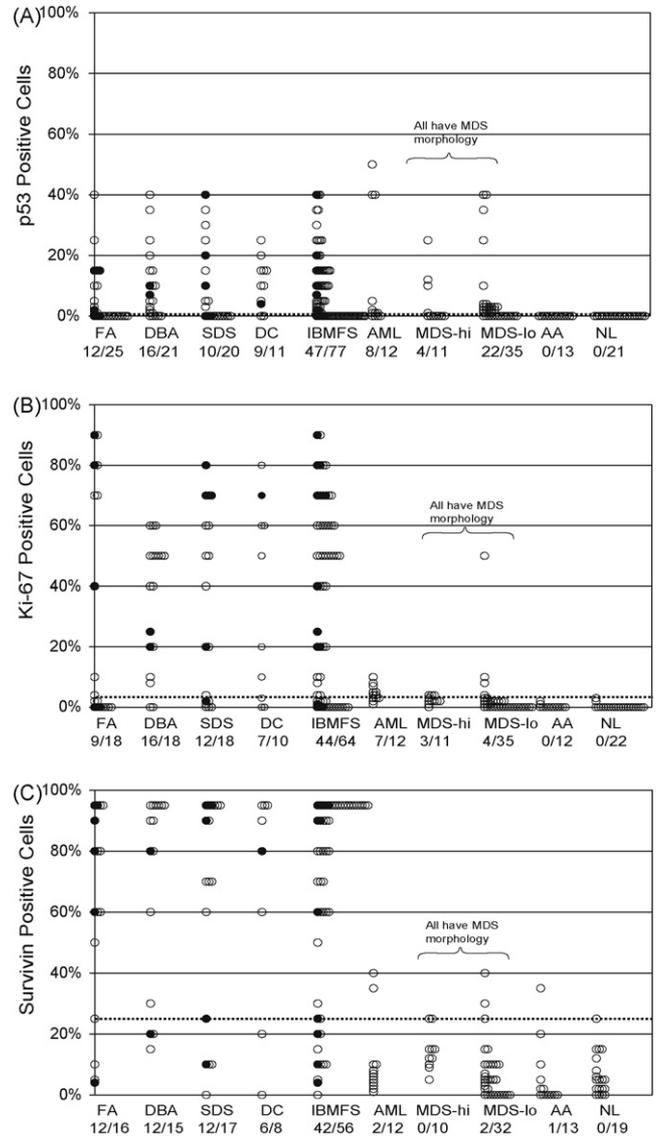


Fig. 2. Expression of cell cycle markers. Each circle represents a patient; a filled circle represents a patient with IBMFS whose bone marrow also shows morphologic myelodysplasia. The dotted line corresponds to the highest expression of that marker in the normal group. Numbers below the X axis represent the rate of overexpression of each marker in each disorder. (A) p53; (B) Ki-67; (C) Survivin. Abbreviations are defined in Table 1.

the IBMFS marrows had survivin-positive cells, and the proportions of positive cells in the overexpressors ranged from 30 to 95%, much higher than the 30–40% positive in the sporadic controls. This leads to the conclusion that, at least with our method, a high level of expression of survivin protein in a bone marrow biopsy may be helpful in distinguishing an inherited from an acquired bone marrow failure disorder. While it will not lead to specific syndromic diagnoses, it may indicate that an IBMFS work-up should be initiated. The results of a survivin immunohistochemistry assay can be available much more quickly than the results of chromosome breakage analysis for FA, telomere length for DC, and specific surrogate tests for DBA and SDS [2].

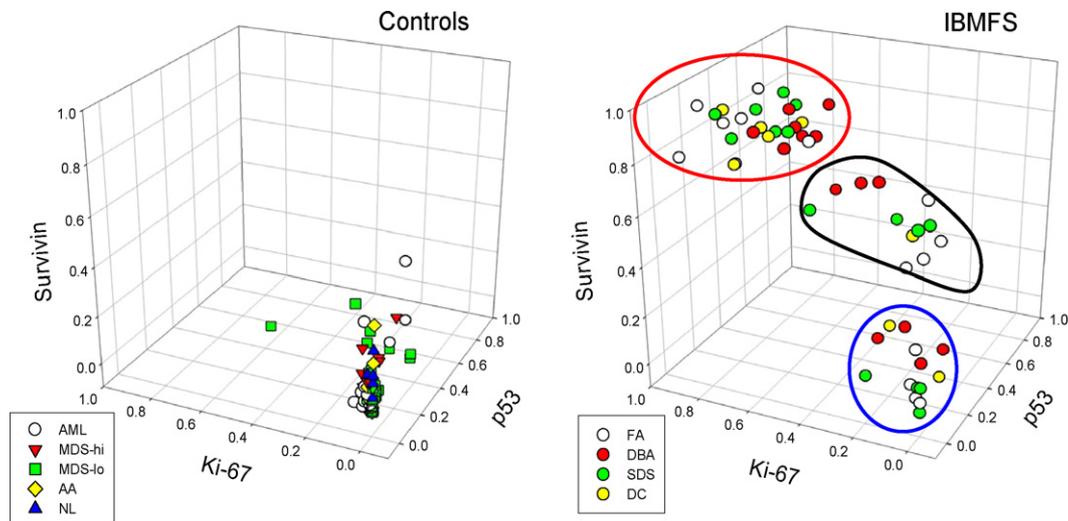


Fig. 3. Combined expression of all three cell cycle markers. Left, controls: open circle, AML; red inverted triangle, MDS-hi; green square MDS-lo; yellow diamond, AA; and blue triangle, normal. Right, IBMFS: white circle, FA; red circle, DBA; green circle, SDS; yellow circle, DC. Loops indicate those with low (blue), intermediate (black), and high (red) expression of survivin.

Our observations are in agreement with the published literature that indicates overexpression of p53 and Ki-67 in MDS/AML [5–7,10,16]; however, the close resemblance in overexpression of these markers between IBMFS and MDS/AML has not been reported previously. Similarities in the rate of overexpression of p53 and Ki-67 between IBMFS and MDS/AML may suggest a common cell cycle deregulation mechanism that is responsible for the preleukemic and leukemic processes in these marrow disorders. Moreover, the lack of association between p53 and Ki-67 in DBA and the negative association in DC in the face of a positive association of these two markers in AML and in FA and SDS, which have a significantly higher risk of MDS/AML [2], may be due to ineffective p53-dependent apoptosis or a cellular attempt to compensate by intensifying the apoptosis signal to balance out cell proliferation as indicated by Ki-67.

Unexpectedly, we found a significantly higher rate of overexpression of survivin in IBMFS compared with AML, MDS-hi and MDS-lo, and no difference in the expression of survivin in AML and MDS compared with normal. While the literature indicates overexpression of survivin in MDS/AML [12,13,17,18], a gradual increase followed by a rapid decrease in survivin expression has been described in association with MDS progression to AML in a limited number of patients [15]. While none of the acquired bone marrow disorder groups showed any correlation between the overexpression of survivin and Ki-67, we observed a strong positive correlation in the IBMFS marrow samples ($p < 0.008$, $r^2 = 0.5–0.7$). This observation is logical since survivin promotes cell proliferation and Ki-67 reflects its occurrence; however, the time sequence of overexpression of these markers was not examined in our study. A decrease in the expression of survivin in the immediate preleukemic or early leukemic period cannot be excluded due to the lack of samples from patients with

IBMFS who developed AML. While DC showed a negative association between p53 and survivin ($p = 0.02$, $r^2 = 0.6$) and FA and DBA showed no significant association ($p > 0.06$), only SDS showed a positive association ($p = 0.01$, $r^2 = 0.36$). This makes the theory of a reactive survivin elevation in an attempt by the marrow to overcome the p53-dependent apoptosis less likely and provides more support to the previous hypothesis of an ineffective p53-mediated apoptotic pathway in IBMFS. Survivin may provide the missing link for cell salvage from apoptosis by counteracting the p53 overexpression, rendering ineffective its apoptosis-mediated pathway. However, it is not clear from our study whether survivin overexpression is an inherent mechanism in IBMFS or is temporarily activated to maximize the proliferative activity of a smaller pool of marrow cells. Follow-up of IBMFS patients using these immunohistochemical stains may provide additional information regarding the risk of developing further bone marrow complications.

Our analysis included a relatively large number of samples from each disorder, which were analyzed in a single laboratory. The sample size and the standardized, masked analysis represent strengths of our study; however, the study is currently limited by its cross-sectional design. We have subsequent analyses on only half the patients; 11 had bone marrow transplantation for aplastic anemia, and 8 died from complications of their aplastic anemia. The median follow-up period is only 2 years (range 0.5–7 years), and none of the patients have progressed to full-blown MDS or AML. This study is the first systematic examination of cell cycle marker expression in all of the IBMFS, and we identified overexpression patterns which resemble those seen in patients with primary MDS or AML, even in patients with an IBMFS who did not have MDS/AML. A unique finding was the very high expression of survivin in the IBMFS and not in any of the acquired conditions.

Much longer follow-up and serial analyses of the expression of cell cycle regulation markers in IBMFS marrows are required to determine whether these markers serve only to distinguish inherited from acquired marrow disorders, or whether they identify patients with IBMFS who are at imminent risk of evolving into MDS and/or AML.

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