Clinical significance of *SF3B1* mutations in myelodysplastic syndromes and myelodysplastic/myeloproliferative neoplasms

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In a previous study, we identified somatic mutations of *SF3B1*, a gene encoding a core component of RNA splicing machinery, in patients with myelodysplastic syndrome (MDS). Here, we define the clinical significance of these mutations in MDS and myelodysplastic/myeloproliferative neoplasms (MDS/MPN). The coding exons of *SF3B1* were screened using massively parallel pyrosequencing in patients with MDS, MDS/MPN, or acute myeloid leukemia (AML) evolving from MDS. Somatic mutations of *SF3B1* were found in 150 of 533 (28.1%) patients with MDS, 16 of 83 (19.3%) with MDS/MPN, and 2 of 38 (5.3%) with AML. There was a significant association of *SF3B1* mutations with the presence of ring sideroblasts ($P < .001$) and of mutant allele burden with their proportion ($P = .002$). The mutant gene had a positive predictive value for ring sideroblasts of 97.7% (95% confidence interval, 93.5%-99.5%). In multivariate analysis including established risk factors, *SF3B1* mutations were found to be independently associated with better overall survival (hazard ratio = 0.15, $P = .025$) and lower risk of evolution into AML (hazard ratio = 0.33, $P = .049$). The close association between *SF3B1* mutations and disease phenotype with ring sideroblasts across MDS and MDS/MPN is consistent with a causal relationship. Furthermore, *SF3B1* mutations are independent predictors of favorable clinical outcome, and their incorporation into stratification systems might improve risk assessment in MDS. (*Blood*. 2011; 118(24):6239-6246)

Introduction

Myelodysplastic syndromes (MDS) are myeloid neoplasms (MPN) characterized by dysplasia in one or more cell lines, ineffective hematopoiesis, and variable risk of progression to acute myeloid leukemia (AML).[1] The World Health Organization (WHO) classification criteria for MDS diagnosis require evaluation of peripheral blood and bone marrow morphology combined with cytogenetic analyses, and define the following categories[2,3]: refractory cytopenia with unilineage dysplasia, refractory anemia with ring sideroblasts (RARS), refractory cytopenia with multilineage dysplasia (RCMD), refractory anemia with excess blasts (RAEB) type 1 (RAEB-1) and type 2 (RAEB-2), and myelodysplastic syndrome with isolated (del 5q) [MDS del(5q)].

MDSs are heterogeneous disorders ranging from indolent conditions with a near-normal life expectancy to subtypes very close to AML. To improve their prognostication, risk-based stratification systems have been developed. The International Prognostic Scoring System (IPSS) stratifies MDS patients into 4 risk groups by percentage of blasts in the bone marrow, type of cytogenetic abnormality, and number and degree of cytopenias at presentation.[4] The WHO classification–based prognostic scoring system (WPSS) takes advantage of the prognostic relevance of the WHO classification,[5] is able to classify MDS patients into 5 risk groups showing different survivals and probabilities of leukemic evolution, and can be used not only at diagnosis but also during follow-up.[6]

Increasing evidence indicates that chromosomal abnormalities play a major role in determining the heterogeneity of MDS.[7] This suggests that distinct molecular lesions probably contribute to the variable morphology and clinical outcome of these myeloid neoplasms. Correlating the presence of acquired mutations in MDS with clinical features and outcome will potentially provide a strong molecular basis for future classification and prognostic scoring systems.

Important steps have recently been made in characterizing the molecular basis of MDS. For example, MDS del(5q) appears to derive from haploinsufficiency of genes mapping to chromosome 5q32-q33, in particular from reduced expression of *RPS14*, miR-145, and miR-146a, and from *TP53* overexpression.[8-12] Acquired somatic mutations of *TET2* have been detected in different myeloid

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neoplasms and in approximately 25% of MDS patients.[13-16] Additional mutant genes, including *ASXL1*, *CBL*, *ETV6*, *EZH2*, *IDH1*, *IDH2*, *JAK2*, *KRAS*, *NPM1*, *NRAS*, *RUNX1*, and *TP53*, have been identified in smaller proportions of patients, particularly in those with high risk of leukemic evolution.[17] In a recent study, mutations in 5 of these genes have been found to be predictors of poor overall survival (OS), independently of established risk factors.[18]

Using massively parallel sequencing technology, we recently identified somatically acquired mutations in *SF3B1*, a gene encoding a core component of RNA splicing machinery, in diverse WHO categories but predominantly in MDS patients with ring sideroblasts.[19] This observation and the recent identification of somatic mutations in other key components of the spliceosome[20] strongly implicate abnormalities of mRNA splicing in the pathogenesis of MDS. In this work, we performed a comprehensive mutation analysis of *SF3B1* in patients with myelodysplastic neoplasms to define the clinical correlates of these mutations.

Methods

Patients

These investigations were approved by the Ethics Committee of the Fondazione Istituto di Ricovero e Cura a Carattere Scientifico Policlinico San Matteo, Pavia, Italy, and by other local institutional review boards. The procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 2000, and samples were obtained after subjects provided informed consent.

We studied 564 patients with MDS, 88 with MDS/MPN, and 40 with AML evolving from MDS (Table 1). The diagnostic criteria of the WHO classification of tumors of hematopoietic and lymphoid tissues were adopted.[2] Of 564 patients with MDS, 135 had refractory cytopenia with unilineage dysplasia presenting as refractory anemia (RA), 107 had RARS, 102 had RCMD without ring sideroblasts, 54 had RCMD with ring sideroblasts (RCMD-RS), 87 had RAEB-1, 57 had RAEB-2, and 22 had MDS del(5q). It should be noted that RCMD-RS was a separate MDS category in the 2001 WHO classification of myeloid neoplasms[21] but was incorporated into RCMD in the 2008 revision.[3] Of 88 patients with MDS/MPN, 67 had chronic myelomonocytic leukemia (CMML),[22] 18 had RARS associated with marked thrombocytosis (RARS-T),[23] and 3 had MDS/MPN unclassifiable (MDS/MPN, U). Forty patients had AML secondary to MDS.[2]

Patients were studied at diagnosis or during follow-up before any disease-modifying treatment (ie, allogeneic stem cell transplantation, aggressive chemotherapy, or hypomethylating agents). The *SF3B1* mutation status of 354 of the aforementioned 564 MDS patients, but not information on mutant allele burden or that on clinical significance of somatic mutation (impact on OS and contribution to the predictive power of IPSS or WPSS), was included in our previous report.[19] The *SF3B1* mutation status of the 67 patients with CMML, already reported in our previous study,[20] was included in this paper to compare CMML and RARS-T within MDS/MPN.

In a subgroup of 325 MDS patients, a quantitative enumeration of ring sideroblasts after Perls staining was performed using recently established consensus criteria[24] to study the relationship between *SF3B1* mutation status or mutant allele burden and the proportion of bone marrow ring sideroblasts.

Sample collection and cell separation

Mononuclear cells were separated from bone marrow samples by standard density gradient centrifugation, and granulocytes were isolated from peripheral blood as previously described.[23] Mononuclear cells were labeled with CD34 MicroBeads, and $CD34^+$ cells were isolated using MACS magnetic cell separation columns (Miltenyi Biotec) according to the manufacturer's recommendations.[25] $CD34^+$ cell purity was evaluated with FACS and was $> 90\%$. Genomic DNA was obtained from bone marrow mononuclear cells, $CD34^+$ cells, or peripheral blood granulocytes by following standard protocols for human tissue.

Mutation analysis of *SF3B1* and *SF3B1* mutant allele burden

The coding exons of *SF3B1* were screened using massively parallel pyrosequencing of DNA pools using the genome sequencer FLX system (Roche Diagnostics). Briefly, optimized primers for PCR (shown in supplemental Table 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article) were designed against all coding exons of *SF3B1* (CCDS33356, ENST00000335508).[19] Where appropriate, overlapping primer sets were used to allow for precise quantification of variant allelic burden estimates. On the 5′ end of each primer, oligonucleotide 8-mer indexes (shown in supplemental Table 2) were incorporated to allow effective separation of sample specific sequence information from the DNA pools.

High throughput sequencing of pooled PCR products was performed on the FLX 454, sequencing reads were mapped to the human genome (Build 37) with Burrows-Wheeler Aligner, and individual sample sequencing information was deconstructed as previously described.[19] All novel variants were verified using PCR-based Sanger sequencing.

For each reported *SF3B1* variant, the proportion of cells in a DNA sample that carry the variant can be directly estimated by calculating the proportion of sequencing reads reporting the mutant allele. Allelic burden estimates were derived for all variants with minimum sequence coverage of 25 reads and mutant allele representation of at least 10% of the total reads. Base sequence and mapping quality thresholds were both set to 25 (58 in Burrows-Wheeler Aligner).

JAK2 and *MPL* mutation analysis

Mutation analyses of *JAK2* and *MPL* were performed exclusively on patients with RARS-T.[23] A quantitative real-time RT-PCR–based allelic discrimination assay was used for detecting the *JAK2* (V617F) mutation and evaluating granulocyte mutant allele burden applying the standard curve method.[23] The sensitivity of this assay is approximately 0.2% of mutant alleles. *MPL* mutation scanning was performed using high resolution melting analysis,[26] and mutations were then verified using Sanger sequencing.

Statistical analyses

Numerical variables are summarized by median and range; categorical variable are described with count and relative frequency (percentage) of subjects in each category. Comparison of numerical variables between groups was carried out using a nonparametric approach (Mann-Whitney test or Kruskal-Wallis ANOVA). Comparison of the distribution of categorical variables in different groups was performed with either the Fisher exact test (when computationally feasible) or the χ^2 test (larger tables).

Survival analyses were performed with the Kaplan-Meier method. OS was defined as the time (in months) between the date of diagnosis and the date of death (for patients who deceased) or last follow-up (for censored patients). Leukemia-free survival (LFS) was defined as the time (in months) between the date of diagnosis and the date of leukemic transformation (for patients who progressed to AML) or last follow-up (for censored patients). Event-free survival (EFS) was defined as the time (in months) between the date of diagnosis and the date of first event (death or leukemic transformation for cases having at least 1 of these events) or last follow-up (for censored patients). Comparison between survival curves was carried out by the Wilcoxon test. Multivariate survival analyses were performed by Cox proportional hazards regression. All analyses accounted for left censoring of the observations at the time of mutation assessment.

Likelihood ratio tests were carried out to compare nested models with different covariates and parameterizations (eg, categorical vs continuous), and to test for interaction between covariates. To compare regression models with different covariate cut-off points, we used the Akaike information criterion.[27] This criterion provides a measure of the relative goodness of fit of a statistical model and a means for comparison among

Table 1. Proportion of patients carrying somatic mutations of *SF3B1* in the study population

WHO category	No. of patients studied	No. of sequencing failures*	No. of evaluable patients	No. (%) of patients carrying SF3B1 mutations
MDS				
RA	135	13	122	14 (11.5)
RARS	107	2	105	83 (79.0)
RCMD	102	6	96	6 (6.3)
RCMD-RS†	54	2	52	30 (57.7)
RAEB-1	87	4	83	7 (8.4)
RAEB-2	57	4	53	6 (11.3)
MDS del(5q)	22	0	22	4 (18.2)
MDS total	564	31	533	150 (28.1)
MDS/MPN				
CMML	67	5	62	4 (6.5)
RARS-T	18	0	18	12 (66.7)
MDS/MPN, U	3	0	3	0
AML secondary to MDS	40	2	38	2 (5.3)
All patients studied	692	38	654	168 (25.7)

*Failure was the result of insufficient sequence coverage.

†RCMD-RS was a separate MDS category in the 2001 WHO classification of myeloid neoplasms[21] but was incorporated into RCMD in the 2008 WHO classification.[2,3]

models, a lower Akaike information criterion value indicating a better trade-off between fit and complexity.

Analyses were performed using the Stata SE Version 11.2 software (StataCorp LP; http://www.stata.com).

Results

Somatic mutations of *SF3B1* in patients with myeloid neoplasms

Sufficient sequence coverage was obtained for 654/692 patients studied, and the main results of mutation analysis of *SF3B1* are reported in Table 1.

Overall, 150 of 533 (28.1%) MDS patients had a somatic mutation of *SF3B1*, and the proportion of positive patients was significantly higher in the RARS and RCMD-RS subgroups than in the remaining WHO categories (72.0% vs 9.8%, $P < .001$). Within patients with MDS/MPN, 16 of 83 (18.3%) carried a somatic mutation in *SF3B1*, and the proportion of positive patients was significantly higher in RARS-T than in the other WHO categories (66.7% vs 6.2%, $P < .001$). Only 2 of 38 patients (5.3%) with AML evolving from MDS carried an *SF3B1* mutation.

Clinical and hematologic features of patients with *SF3B1* mutation were compared with those of patients without mutation in a subgroup of 325 subjects whose data at the time of molecular evaluation were available. Overall, the prevalence of *SF3B1* mutations was higher in females than in males (27% vs 18%, $P = .02$), and patients with *SF3B1* mutation showed higher platelet count (median value, 257×10^9/L vs 104×10^9/L, $P < .001$), higher proportion of ring sideroblasts (40% vs 0%, $P < .001$), and marrow erythroblasts (40% vs 27%, $P < .001$), and lower proportion of bone marrow blasts (2% vs 3%, $P < .001$; supplemental Figure 1).

SF3B1 mutation types and mutant allele burden

Twenty-three mutations were identified mapping in 15 different codons. As shown in Table 2, the most frequent mutation involved codon 700 (57.7% of all mutated cases), whereas lower frequencies were observed for mutations in codons 666 (10.7%), 662 (10.1%), 625 (6%), and 622 (7.1%). All the mutations identified were missense substitutions. No significant difference was found among different types of mutations as regards WHO subgroup, clinical features, or hematologic parameters.

The *SF3B1* mutant allele burden could be accurately assessed (as defined under "Mutation analysis of *SF3B1* and *SF3B1* mutant allele burden") in 161 of 168 mutated cases. The overall median value was 39.5% (range, 5.4%-70.3%), and frequencies of observations are reported in Figure 1. The histogram of mutant allele burden was compatible with a bimodal distribution that resulted from a mixture of 2 normal distributions with different variance and means. The first normal distribution accounted for 22% of observations with a mean value for mutant allele burden equal to 15%, whereas the second distribution accounted for 78% of observations with a mean value of 41%. Seventeen patients showed a mutant allele burden higher than 50%, but only in 6 cases this value was higher than 60%. In none of these 6 cases, however, was there sufficient coverage that the 95% confidence interval (CI) excluded 50%. These findings are consistent with the presence of a dominant clone with heterozygous *SF3B1* mutation in most of the cases, and with the existence of a minority mutant clone in a small fraction of patients.

To account for potential bias of DNA source in the evaluation of *SF3B1* mutation status and mutant allele burden, we studied potential differences between these subgroups (supplemental Figure 2). Median values for *SF3B1* mutant allele burden were 40.6% (24.6%-52.1%) in the $CD34^+$ cell DNA subgroup, 40.9% (7.5%-70.3%) in the granulocyte DNA subgroup, and 35.7% (10.1%-51.1%) in the bone marrow DNA subgroup. Kruskal-Wallis 1-way ANOVA by ranks showed no significant difference in *SF3B1* mutant allele burden between these subgroups ($P = .077$).

No significant relationship was found between mutant allele burden and clinical or hematologic features, including clinical outcome. However, the proportion of WHO categories defined by ring sideroblasts (RARS, RCMD-RS, and RARS-T) was higher within patients with a mutant allele burden $\geq 25\%$ (indicating a dominant clone) than in those with a burden $< 25\%$ (indicating a minority clone, $P = .022$)

Relationship between *SF3B1* mutations and ring sideroblasts

An accurate quantitative enumeration of ring sideroblasts using consensus criteria[24] was performed in 325 MDS patients. Ring

Table 2. *SF3B1* mutation type and *SF3B1* mutant allele burden

SF3B1 mutations	No. of patients with mutation	Proportion of patients with mutation as a percentage of all mutated cases	Mutant allele burden, %, median (range)
K700E	97		
Codon 700	97	57.7	38.4 (7.5-70.3)
K666R	9		
K666N	3		
K666Q	3		
K666T	2		
K666M	1		
Codon 666	18	10.7	44.6(17.1-62.7)
H662Q	15		
H662D	1		
H662Y	1		
Codon 662	17	10.1	39.1 (7.0-52.1)
E622D	12		
Codon 622	12	7.1	39.4(20.2-45.3)
R625L	6		
R625C	3		
R625G	1		
Codon 625	10	6.0	37.5(10.6-45.1)
D781G	3		
E592K	2		
A744P	2		
E491G	1		
R590K	1		
N626D	1		
V701F	1		
V701I	1		
G740R	1		
A1188V	1		
Miscellaneous codons	14	8.4	36.4 (5.4-50.0)

sideroblasts were identified at variable percentages (range, 1%-78%) in 74 of 212 patients assigned to WHO categories that are not defined by this morphologic feature, including MDS del(5q), RA, RCMD, RAEB-1, and RAEB-2. Of these, 44 subjects (10 RA, 22 RCMD, 5 RAEB-1, and 7 RAEB-2) showed a proportion of ring sideroblasts below the diagnostic threshold of 15% for assignment to a sideroblastic subtype (RARS or RCMD-RS), whereas 30 patients had a proportion of ring sideroblasts ≥ 15% [7 MDS del(5q), 23 RAEB].

Of the aforementioned 325 patients, 101 (31%) carried a mutation in *SF3B1*. A strong association was found between *SF3B1* mutation and disease phenotype with ring sideroblasts. Of 101 patients with an *SF3B1* mutation, indeed, 91 had more than 15% ring sideroblasts, 7 patients had 1% to 14%, and only 3 patients did not show any ring sideroblasts in the bone marrow (Kendall Tau-b correlation coefficient = 0.54, $P < .001$). Based on these data, the *SF3B1* mutation status had a positive predictive value for disease phenotype with ring sideroblasts of 97.7% (95% CI, 93.5%-99.5%), whereas the absence of ring sideroblasts had a negative predictive value for *SF3B1* mutation of 97.8% (95% CI, 93.8%-99.5%).

Significant relationships were found between *SF3B1* mutant allele burden and percentage of ring sideroblasts ($P = .002$, Figure 2), or percentage of bone marrow erythroblasts ($P = .01$).

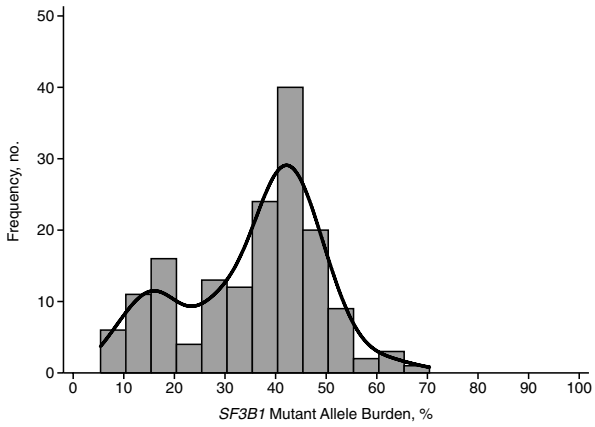

Figure 1. Histogram of *SF3B1* mutant allele burden. The bimodal distribution has been highlighted by adding a Gaussian kernel density plot to the histogram. The first normal distribution (on the left) accounts for 22% of observations, with a mean value for *SF3B1* mutant allele burden equal to 15%. The second normal distribution (on the right) accounts for 78% of observations, with a mean value equal to 41%.

Relationship between *SF3B1* mutations and clinical outcome in MDS

Survival data were available for 323 of the 533 patients with MDS successfully analyzed for *SF3B1* mutation. Median observation time from diagnosis was 23 months (range, 1-267 months), whereas median time from diagnosis to mutation analysis was 4.5 months (range, 0-182 months). Patients carrying an *SF3B1* mutation showed a significantly better OS compared with those without mutation (median survival, 90 vs 50 months, $P = .001$; Figure 3A).

To further define the prognostic relevance of *SF3B1* mutations, we performed univariate and multivariate analyses using a Cox proportional hazards regression model. In univariate analysis, *SF3B1* mutation positively affected OS (HR = 0.35, 95% CI, 0.17-0.72, $P = .009$). The significant value of *SF3B1* mutation was retained in a multivariate analysis, including age, sex, hemoglobin level, absolute neutrophil count, platelet count, cytogenetic risk,

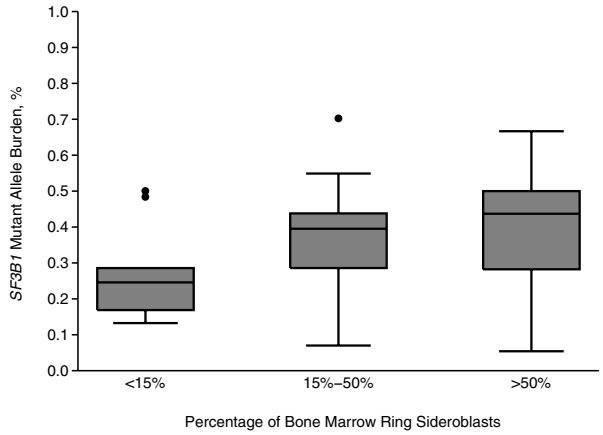

Figure 2. Relationship between *SF3B1* mutant allele burden and proportion of ring sideroblasts. Values for percentage of ring sideroblasts are grouped here in 3 arbitrary categories: < 15% (n = 183), 15% to 50% (n = 85), and > 50% (n = 57). Data are shown in a box plot depicting the smallest and largest observation (lowest and highest horizontal line, respectively), lower and upper quartile with median value (box), and outliers (dots).

bone marrow blasts, and ring sideroblasts (HR = 0.15, 95% CI, 0.03-0.78, $P = .025$).

Patients with the *SF3B1* mutation showed a significantly better LFS than those without mutation (HR = 0.32, 95% CI, 0.11-0.91, $P = .032$; Figure 3B). This result was confirmed in a multivariate analysis, including age, sex, hemoglobin level, absolute neutrophil count, platelet count, cytogenetic risk, bone marrow blasts, and ring sideroblasts (HR = 0.33, 95% CI, 0.11-0.99, $P = .049$).

EFS was calculated by combining the 2 previous outcomes (death or leukemic transformation). Patients with the *SF3B1* mutation showed a significantly better EFS compared with those without the *SF3B1* mutation in both univariate (HR = 0.33, 95% CI, 0.19-0.62, $P < .001$) and multivariate analysis, adjusted for demographic and hematologic variables (HR = 0.29, 95% CI, 0.10-0.82, $P = .019$; Figure 3C).

We then evaluated OS and LFS in the subgroup of patients with RARS and RCMD-RS specifically. Univariate analysis of *SF3B1* mutation status showed a significant effect on OS (HR = 0.28, 95% CI, 0.09-0.94, $P = .039$) but no effect on LFS (HR = 0.62, 95% CI, 0.1-3.78, $P = .6$) in this subgroup. The protective effect of *SF3B1* mutation on OS was lost in multivariate analysis because of significant colinearity among predictor variables.

Relative contribution of *SF3B1* mutation status to OS as predicted by IPSS or WPSS

Both IPSS and WPSS are currently used for clinical prognostication of MDS. To establish whether the *SF3B1* mutation status can add to the predictive power of IPSS or WPPS, we fitted multivariable Cox models, including age, sex, IPSS or WPSS, and *SF3B1* mutation status.

Age was initially modeled as a continuous covariate but was then parameterized as a binary variable with respect to a cut-point. This was set at 70 years (ie, the round value closest to the median age). Its optimal fit compared with other cut-points (ie, 55, 60, 65, and 75) was confirmed by the Akaike information criterion.[27] Both IPSS and WPSS were modeled as continuous covariates, as the likelihood ratio test of each model compared with the respective one having a categorical parameterization showed no significant difference between these alternatives.

As shown in Table 3, the IPSS risk group was strongly associated with OS, but the *SF3B1* mutation status remained an independent predictor of better survival even after adjustment for IPSS. This was true also in patients with low or intermediate-1 IPSS risk. Indeed, within these risk groups, patients carrying an *SF3B1* mutation showed significantly better OS and EFS compared with those without mutation ($P = .038$ and $P = .026$; Figure 4).

The WPSS risk group was strongly associated with OS (Table 3), whereas the significance of *SF3B1* mutation status as an independent predictor of favorable outcome was borderline ($P = .05$). The likelihood ratio test performed on the inclusion of *SF3B1* mutation status into the model based on WPSS was nonetheless significant ($P = .018$), indicating that such inclusion

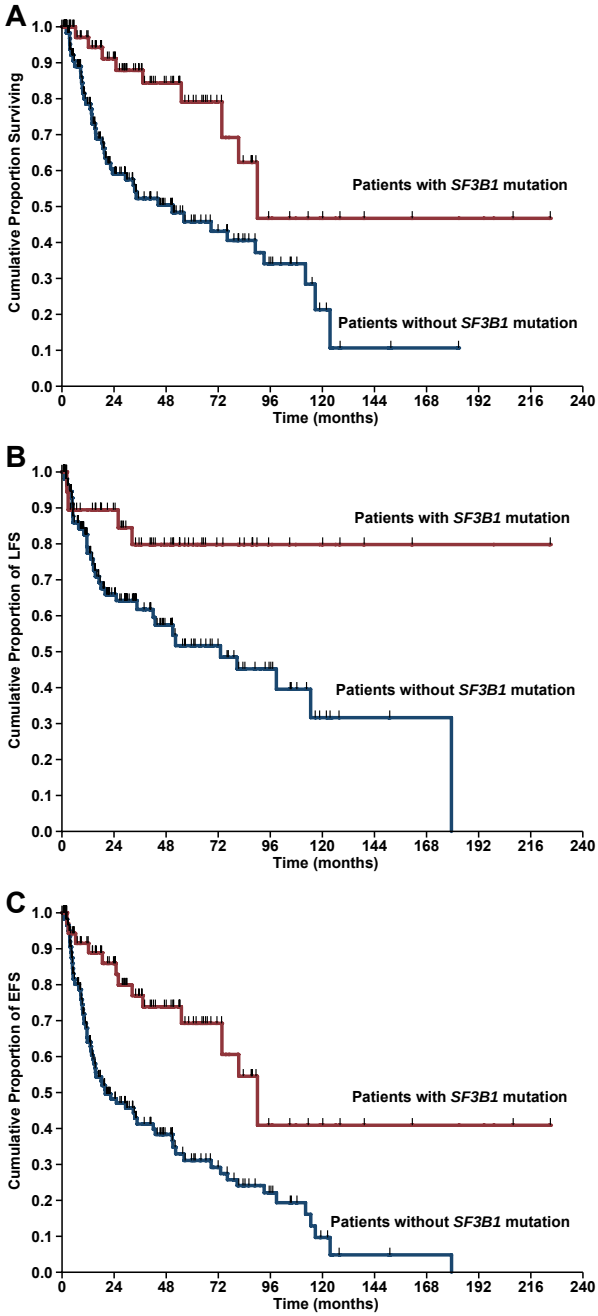

Figure 3. Kaplan-Meier analysis of survival in MDS patients stratified according to *SF3B1* mutation status. (A) OS. (B) LFS. (C) EFS. Vertical tick-marks indicate right-censored patients.

Table 3. Relative contribution of *SF3B1* mutation status to the ability of IPSS or WPSS to predict OS in MDS patients*

Risk factor	Hazard ratio	*P*
Multivariate analysis including IPSS		
Age (< 70 y vs ≥ 70 y)	1.52	.224
Sex (male vs female)	0.69	.292
IPSS risk group	2.16	< .001
SF3B1 mutation status (mutation present vs absent)	0.21	.038
Multivariate analysis including WPSS		
Age (< 70 y vs ≥ 70 y)	1.39	.324
Sex (male vs female)	0.86	.668
WPSS risk group	1.96	< .001
SF3B1 mutation status (mutation present vs absent)	0.23	.050

*Multivariate analysis of OS was performed using a Cox proportional hazards regression model that included age, sex, IPSS risk group or WPSS risk group, and *SF3B1* mutation status. IPSS and WPSS were modeled as a continuous covariate.

added to the model's predictive power. It should be noted that the RARS category is present as a favorable variable in the WPSS[6]; therefore, the close association of RARS with *SF3B1* mutations reduces the independent predictive power of the latter in a multivariable Cox model that includes both variables.

Somatic mutations of *SF3B1* in patients with MDS/MPN

In RARS-T patients, the mutant allele burden ranged from 17% to 62% (median, 41%), and was not significantly different from that of other WHO categories with ring sideroblasts. No difference was found in clinical and hematologic variables between mutated and unmutated RARS-T patients.

Four RARS-T patients had the *JAK2* (V617F) mutation, whereas an additional one had the *MPL* (W515L) mutation. Two patients with the *JAK2* (V617F) mutation also carried an *SF3B1* mutation, and the *SF3B1* mutant allele burden was greater than that for *JAK2* mutation (57% vs 13% in one case, and 17% vs 10% in the other one). The RARS-T patient carrying both an *SF3B1* mutation and *MPL* (W515L) had a fully dominant clone that was heterozygous for both mutations.

Discussion

The findings of this study indicate that there is a close relationship between *SF3B1* mutations and ring sideroblasts across a variety of myelodysplastic neoplasms and clearly demonstrate that this molecular lesion is associated with a favorable clinical outcome in these disorders.

Ring sideroblasts are erythroblasts with iron-loaded mitochondria visualized by Prussian blue staining (Perls reaction) as a perinuclear ring of blue granules.[28] The iron of ring sideroblasts is stored in mitochondrial ferritin,[28] encoded by the *FTMT* gene, which is typically overexpressed in RARS.[29,30]

The sideroblastic anemias include both hereditary and acquired conditions, and the most common acquired forms are RARS and RARS-T.[31] RARS is characterized by erythroid dysplasia and ineffective erythropoiesis, and its clinical course is stable for years in most cases with a low risk of leukemic evolution.[32,33] $CD34^+$ cells from patients with RARS have a particular gene expression profile characterized by overexpression of mitochondria-related genes and, in particular, genes involved in heme synthesis (eg, *ALAS2*),[34] and reduced expression of *ABCB7*, a gene encoding a protein involved in the transport of iron/sulfur clusters from mitochondria to the cytoplasm.[35] RARS-T is a myeloid neoplasm with both myelodysplastic and myeloproliferative features at the molecular and clinical levels[23,36] that may develop from RARS through the acquisition of somatic mutations of *JAK2*, *MPL*, or other as-yet-unknown genes.[23]

In this study, 79% of patients with RARS, 57.7% of those with RCMD-RS, and 66.7% of those with RARS-T carried somatic mutations of *SF3B1* (Table 1). A recent independent report describes similar proportions of mutated patients: 19 of 23 (82.6%) cases of RARS and 39 of 53 (73.6%) cases of RCMD-RS.[20] There was a significant association of *SF3B1* mutations with the presence of ring sideroblasts, and the mutant gene had a positive predictive value for this morphologic feature of 97.7% (95% CI, 93.5%-99.5%). These findings strongly support a causal relationship between *SF3B1* mutations and ring sideroblasts, and more generally between abnormalities of mRNA splicing, abnormal gene expression profiles, and mitochondrial iron overload.[19,34,35] Of note, similar values for *SF3B1* mutant allele burden were found in $CD34^+$ cells and circulating granulocytes (40.6% vs 40.9%; supplemental Figure 2), indicating that somatic mutations occur in multipotent hematopoietic stem cells and are then transmitted to their myeloid progeny. It should also be noted that patients with *SF3B1* mutation had a higher percentage of bone marrow erythroblasts than those without mutation. This establishes a relationship between *SF3B1* mutations and expanded but ineffective erythropoiesis, which represents a distinctive feature of RARS within MDS.[32,33,37]

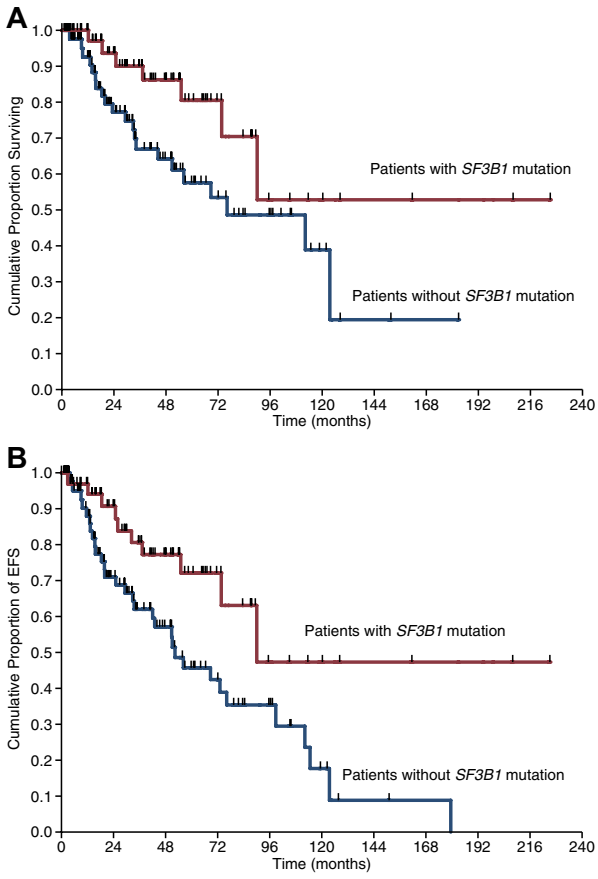

Figure 4. Kaplan-Meier analysis of survival in MDS patients with low or intermediate-1 IPSS risk stratified according to *SF3B1* mutation status. (A) OS. (B) EFS. Vertical tick-marks indicate right-censored patients.

The median *SF3B1* mutant allele burden was approximately 40% (Figure 1), indicating that in most patients hematopoiesis was sustained by a dominant clone heterozygous for the mutation. This was particularly true for patients with WHO categories defined by ring sideroblasts (RARS, RCMD-RS, and RARS-T), suggesting that somatic mutations of *SF3B1* might be an early pathogenetic event in these patients. In none of these mutated patients, however, was the mutant allele burden significantly higher than 50%. This excludes chromosomal defects, such as copy number variation or copy-neutral loss of heterozygosity involving chromosome 2q33.1, where *SF3B1* maps. In approximately one-fifth of cases, moreover, the relatively low *SF3B1* mutant allele burden was more compatible with a minority clone, suggesting that myelodysplastic transformation had been driven by other molecular mechanisms in these subjects and that the *SF3B1* mutation represented a secondary genetic event.

The RCMD-RS category was created in the 2001 WHO classification of myeloid neoplasms[21] but was then incorporated into RCMD in the 2008 WHO classification.[2] The rationale for this incorporation was that multilineage dysplasia represents a strong negative prognostic factor per se irrespective of the presence or absence of ring sideroblasts.[3] Our findings suggest that RCMD and RCMD-RS should be instead considered as separate conditions, or at least that a distinction should be made between patients with wild-type *SF3B1* and those with a somatic mutation of this gene.

Within MDS/MPN, a remarkable difference in the incidence of *SF3B1* mutations was found between CMML and RARS-T (Table 1). In the last few years, several mutant genes have been detected in CMML patients, including *TET2*, *ASXL1*, *CBL*, *RUNX1,* and *EZH2*.[38] Rarely, however, ring sideroblasts are observed in these patients, and this is reflected in the low incidence of *SF3B1* mutations observed in this study. It should be noted, however, that somatic mutations in genes encoding other key components of the spliceosome (*SRSF2*, *USAF35*, *ZRSR2*, *U2AF65*, and *SF3A1*) have been recently reported in 50% of CMML patients, clearly indicating that abnormalities of splicing machinery play a major role in the pathophysiology of this myelodysplastic/myeloproliferative neoplasm.[20] Ring sideroblasts represent one of the morphologic hallmarks of RARS-T,[39] and in the current study most of these patients indeed carried a somatic mutation of *SF3B1*. Our observations suggest that RARS-T may result from a combination of *SF3B1* and *JAK2* (or *MPL*) mutations, responsible for myelodysplastic and myeloproliferative features,[23] respectively.

Approximately one-fourth of patients with ring sideroblasts (RARS, RCMD-RS, and RARS-T subgroups) had a wild-type *SF3B1*. This suggests that additional mutant gene(s) may be involved in the pathogenesis of ring sideroblasts in myeloid neoplasms. Indeed, somatic mutations of *SRSF2* or *ZRSR2* have been recently identified in 7% of cases of MDS with ring sideroblasts that had wild-type *SF3B1*.[20] Nonetheless, approximately 15% of these patients did not carry any mutation in genes encoding key components of the spliceosome.

The fact that a small proportion of patients with WHO categories not specifically associated with ring sideroblasts carried an *SF3B1* mutation was expected. Indeed, clonal evolution of MDS is probably a multistep process in which several genetic events occur.[19] A somatic mutation of *SF3B1* may therefore represent a second genetic event: for instance, it may occur in a patient with MDS del(5q) in whom del(5q) was the initiating event. Alternatively, a RARS patient carrying a mutant *SF3B1* gene may acquire another somatic mutation that involves excess of blasts and modifies his/her clinical phenotype from RARS to RAEB.[18] However, this latter progression appears to be relatively unlikely, and this is particularly true with respect to progression from RARS to AML. Indeed, previous studies clearly indicated that the risk of leukemic evolution is low in patients with RARS,[5,33] a notion indirectly confirmed also by our observation that only 2 of 38 (5.3%) patients with AML evolving from MDS carried a somatic mutation of *SF3B1*.

So far, individual risk assessment in MDS has been based on the use of scoring systems that include clinical, morphologic, and cytogenetic parameters.[40] We and others propose that assessment of somatic mutations probably improves both diagnosis and prognostication in patients with MDS,[41] although a few simple parameters (eg, the degree of anemia[42]) will continue to be of pivotal importance for risk assessment. In a recent study,[18] mutations in 5 genes (*TP53*, *EZH2*, *ETV6*, *RUNX1*, and *ASXL1*) were found to be independently associated with decreased OS in MDS. This study, however, was mainly focused on somatic mutations that are associated with unfavorable outcome and disease progression toward AML. On the other hand, somatic mutations of *TET2* represent a marker of clonal proliferation in MDS[14,43] but are not associated with any WHO category nor have prognostic relevance.[16,18]

In the current study, we show that *SF3B1* is the first mutated gene in MDS to be strongly associated with a specific disease phenotype (ie, ring sideroblasts). From a clinical viewpoint, *SF3B1* mutations are independent predictors of favorable clinical outcome in terms of better OS and lower risk of leukemic evolution. As shown in Table 3, incorporation of *SF3B1* mutations may add relevant information to the risk stratification systems currently used in clinical practice. Incorporating additional mutant genes, in particular those associated with unfavorable outcome,[18] might further refine clinical decision making in MDS.[44]

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Authorship

Contribution: M.C., L.M., E.P., D.T.B., J.B., J.S.W., P.A.F., M.R.S., P.J.C., and E.H.-L. designed research; L.M., E.P., M.G.D.P., E.T., M.J.G., A.L.G., I.A., A.G., M.C.D.V., S.C., S.T., N.K., A.H., J.H., and L.J.M. performed research; M.C., L.M., E.P., and C.P. analyzed and interpreted data; M.C., L.M., E.P., and P.J.C. wrote the

manuscript; and all authors critically revised the manuscript and approved the final version of the manuscript.

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