SF3B1 mutation identifies a distinct subset of myelodysplastic syndrome with ring sideroblasts

Luca Malcovati,1,2 Mohsen Karimi,3 Elli Papaemmanuil,4 Ilaria Ambaglio,2,5 Martin Jädersten,3 Monika Jansson,3 Chiara Elena,1,2 Anna Galli,2 Gunilla Walldin,3 Matteo G. Della Porta,2,5 Klas Raaschou-Jensen,6 Erica Travaglino,2 Klaus Kallenbach,7 Daniela Pietra,2 Viktor Ljungström,8 Simona Conte,3 Emanuela Boveri,9 Rosangela Invernizzi,5,10 Richard Rosenquist,8 Peter J. Campbell,4 Mario Cazzola,1,2* Eva Hellström Lindberg3*

1Department of Molecular Medicine, University of Pavia, Pavia, Italy & 2Department of Hematology Oncology, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Policlinico San Matteo, Pavia, Italy; 3Center for Hematology and Regenerative Medicine (HERM), Karolinska University Hospital, Stockholm, Sweden; 4Cancer Genome Project, Wellcome Trust Sanger Institute, Hinxton, UK; 5Department of Internal Medicine, University of Pavia, Pavia, Italy; 6Department of Hematology, Copenhagen University Hospital, Denmark; 7Department of Pathology, Copenhagen University Hospital, Denmark; 8Department of Immunology, Genetics and Pathology, Uppsala University, Uppsala, Sweden; 9Department of Pathology, Fondazione Istitutodi Ricovero e Cura a Carattere Scientifico (IRCCS) Policlinico San Matteo, Pavia, Italy; 10Department of Internal Medicine, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Policlinico San Matteo, Pavia, Italy;

*MC and EHL are equal senior authors

Running title: SF3B1-mutant myelodysplastic syndrome
Key points

- Within MDS with ring sideroblasts, SF3B1 mutation defines a homogeneous subgroup with isolated erythroid dysplasia and favorable prognosis.

- MDS with ring sideroblasts and wild-type SF3B1 is mainly characterized by multilineage dysplasia and unfavorable prognosis.

Abstract

Refractory anemia with ring sideroblasts (RARS) is a myelodysplastic syndrome (MDS) characterized by isolated erythroid dysplasia and 15% or more ring sideroblasts in the bone marrow. Ring sideroblasts are found also in other MDS subtypes, such as refractory cytopenia with multilineage dysplasia and ring sideroblasts (RCMD-RS). A high prevalence of somatic mutations of SF3B1 has been reported in these conditions. To identify mutation patterns that affect disease phenotype and clinical outcome, we performed a comprehensive mutation analysis in 293 patients with a myeloid neoplasm and 1% or more ring sideroblasts. SF3B1 mutations were detected in 129/159 cases (81%) of RARS or RCMD-RS. Among other patients with ring sideroblasts, lower prevalence of SF3B1 mutations and higher prevalence of mutations in other splicing factor genes were observed (P<.001). In multivariable analyses, patients with SF3B1 mutations showed significantly better overall survival (HR =. 37, P=.003) and lower cumulative incidence of disease progression (HR=0.31, P=.018) compared with SF3B1-unmutated cases. The independent prognostic value of SF3B1 mutation was retained when the analyses were restricted to MDS subgroups without excess blasts, as well as to sideroblastic categories (RARS and RCMD-RS). Among SF3B1-mutated patients, coexisting mutations in DNA methylation genes were associated with multilineage dysplasia (P=.015) but had no effect on clinical outcome. TP53 mutations were frequently detected in patients without SF3B1 mutation, and were associated with poor clinical outcome. Thus, SF3B1 mutation identifies a distinct MDS subtype that is unlikely to develop detrimental subclonal mutations and is characterized by indolent clinical course and favorable outcome.
Introduction

Ring sideroblasts characterize a group of MDS first described in 1956 by Bjorkman. These disorders were categorized as idiopathic acquired sideroblastic anemia in the FAB classification, and then as RARS or RCMD-RS in the World Health Organization (WHO) classification, according to the presence of 15% or more bone marrow ring sideroblasts and dysplasia in one or more myeloid lineages.

A high prevalence of somatic mutations in SF3B1 was reported in MDS with ring sideroblasts. Mutations of SF3B1 were found in about 30% of patients with MDS and 20% of patients with myelodysplastic/myeloproliferative neoplasm (MDS/MPN), but a significantly higher mutation prevalence was noticed in sideroblastic categories, including RARS, RCMD-RS and the provisional entity defined as RARS associated with marked thrombocytosis (RARS-T).

However, in patients with MDS or MDS/MPN, the association between ring sideroblasts and SF3B1 mutations is even stronger than suggested by the higher prevalence of these mutations in WHO categories with ring sideroblasts. In fact, ring sideroblasts can be detected at variable percentages also in patients assigned to WHO categories that are not defined by this morphological feature. When accounting for these cases, SF3B1 mutation status had a positive predictive value for disease phenotype with ring sideroblasts of 97.7%.

In a group of myeloid disorders classified on the basis of morphological criteria, identifying specific associations between genotype and disease phenotypes is essential to define disease entities according to their distinctive genetic profiles. Recently, based on a comprehensive mutation analysis, we sought for genotype-phenotype correlations adopting unsupervised hierarchical clustering analyses including the WHO classification criteria and somatic mutations. The results of these analyses suggested that mutation patterns may improve the classification of these neoplasms, and two disease subsets were recognized within MDS with ring sideroblasts according to SF3B1 mutation status. However, further investigations are needed to identify the sources of variability of disease features and clinical outcome in MDS with ring sideroblasts.
In this study, we performed a comprehensive mutation analysis of genes implicated in myeloid disorders in a large and well-characterized cohort of myeloid neoplasms with 1% or more ring sideroblasts with the aim to identify mutation patterns that affect disease phenotype and clinical outcome.
Patients and Methods

Patients and clinical procedures

These investigations were approved by the Ethics Committee of the Fondazione IRCCS Policlinico San Matteo, Pavia, Italy, the Karolinska Institutet, Stockholm, Sweden and 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 patients with myeloid neoplasms with 1% or more ring sideroblasts followed at the Department of Hematology Oncology, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy; the Department of Medicine, Division of Hematology, Karolinska University Hospital, Stockholm, Sweden; and the Department of Hematology, Copenhagen University Hospital, Denmark. The study population consisted of 293 patients, including 243 patients with MDS, of whom 159 assigned to sideroblastic categories (RARS, RCMD-RS) and 84 to other WHO categories, and 50 with MDS/MPN (Table 1).

Diagnostic procedures were performed according to the recommendations of the European LeukemiaNet. In order to classify patients, peripheral blood and bone marrow specimens were analyzed by two independent hematopathologists who were blinded to clinical data. The diagnostic criteria of the WHO classification of tumors of hematopoietic and lymphoid tissues were adopted. Quantitative enumeration of myeloblasts, ring sideroblasts and monocytes and their precursors was performed using recently established consensus criteria.

Patients were studied at diagnosis or during follow-up before any disease-modifying treatment (i.e., allogeneic stem cell transplantation, intensive chemotherapy, or hypomethylating agents). A portion of these patients were included in our recent studies of targeted gene sequencing in myelodysplasia.
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.\textsuperscript{19} Genomic DNA was obtained from bone marrow mononuclear cells or peripheral blood granulocytes by following standard protocols for human tissue.

Targeted gene sequencing

A core panel of 42 genes selected on the basis of prior implication in the pathogenesis of myeloid disease was analyzed in the whole study cohort (Supplemental Table 1).

One-hundred-eighty-three patients were studied using a panel of 111 genes selected on the basis of prior implication in the pathogenesis of myeloid disease using 2 lanes of Illumina HiSeq (Illumina Inc., CA, USA), as previously reported.\textsuperscript{12}

One-hundred-ten patients were analyzed for 42 genes recurrently mutated in myeloid disorders using Illumina HiSeQ 2000 system at the Sci-Life lab, Stockholm, Sweden. Additional information is provided in Supplemental Material. Variant allele frequency (VAF) was calculated for each mutation identified as number of variant reads divided by total reads.

\textbf{JAK2 and CALR mutation analysis}

\textit{JAK2} (V617F) mutation was analyzed using a quantitative real-time polymerase chain reaction (qRT-PCR)-based allelic discrimination assay in order to obtain a precise quantification of mutant allele burden.\textsuperscript{19} Mutations of \textit{CALR}, not included in the original panel of 111 genes, were analyzed by sequencing, as previously described.\textsuperscript{20}

\textbf{Hematopoietic progenitor colony assay}

Hematopoietic progenitors colonies (CFU-E, BFU-E, CFU-GM) were obtained from bone marrow mononuclear cells and individual colonies were isolated and genotyped, as previously reported.\textsuperscript{19}
Statistical analysis

Comparison of numerical variables between groups was performed using a nonparametric approach (Mann-Whitney test or Kruskall Wallis ANOVA). Comparison of the distribution of categorical variables in different groups was performed with either the Fisher exact test or the $\chi^2$ test. VAF estimates were used to evaluate clonal and subclonal variant relationships within each sample, as previously reported.\textsuperscript{12}

Survival analyses were performed by means of Cox proportional hazards regression. The cumulative incidence (CI) of disease progression was estimated with a competing risk approach, considering death for any cause as a competing event. The comparison of cumulative incidence curves was carried out using the Pepe-Mori test,\textsuperscript{21} whereas the effect of quantitative covariates was estimated by applying the Fine-Gray regression model.\textsuperscript{22} All analyses accounted for left censoring of the observations at the time of mutation assessment. Analyses were performed using the Stata SE 12.1 (StataCorp LP, College Station, TX, \url{http://www.stata.com}) software.
Results

Spectrum of mutation in MDS with ring sideroblasts

Within the whole cohort of MDS with ring sideroblasts (number of patients: 243), the median number of mutation per patient was 1, ranging from 0 to 7. Overall 18 MDS patients did not have evidence of genetic or cytogenetic abnormality, including 4 with RARS and with 3 RCMD-RS. No significant difference in mutation prevalence was observed among MDS categories, whereas a significantly lower median number of mutations per patient was observed in MDS compared with MDS/MPN (1, range 0-7, versus 2, range 0-7, P=.001).

The most frequently mutated gene categories in the whole cohort of MDS with ring sideroblasts were splicing factors (181/243, 74.5%), DNA methylators (81/243, 33%), chromatin modifiers (35/243, 14.4%), and transcription factors (28/243, 11.5%). The most frequent mutations involved SF3B1 (151/243, 62.1%), TET2 (49/243, 20.1%), DNMT3A (26/243, 10.7%), SRSF2 (19/243, 7.8%), ASXL1 (19/243, 7.8%), RUNX1 (12/243, 4.9%), EZH2 (10/243, 4.1%), TP53 (10/243, 4.1%), U2AF1 (7/243, 2.9%), ZRSR2 (6/243, 2.5%) (Supplemental Figure 1A).

A significantly higher incidence of mutations in splicing factors was observed in sideroblastic categories (RARS, RCMD-RS) (139/159, 87.4%) compared to RA and RCMD with <15% ring sideroblasts (12/34, 35.3%) or RAEB (23/43, 53.5%) (P<.001). In detail, a significantly higher rate of SF3B1 mutations was noticed in sideroblastic categories (129/159, 81.1%) compared to RA/RCMD (4/34, 11.8%) or RAEB (13/43, 30.2%) (P<.001). Conversely, a significantly lower prevalence of mutations in splicing factors other than SF3B1 (namely, SRSF2, U2AF1, ZRSR2) was observed in RARS/RCMD-RS (11/159, 6.9%) compared with RA/RCMD (8/34, 23.5%) (P=.008) or RAEB (11/43, 25.6%) (P=.001) (Supplemental Figure 1B). In addition, a significantly higher rate of SF3B1 mutations was noticed in MDS with isolated del(5q) (5/7, 71%) compared to RA/RCMD (P=.027).

A significantly higher prevalence of mutations in genes involved in DNA methylation was observed in RARS/RCMD-RS compared with RAEB (54/159 vs 5/43 respectively, P=.004). Conversely, significantly lower rate of mutations in chromatin modifiers and transcription factors was found in sideroblastic categories compared with RAEB (17/159 vs 11/43, P=.022, and 9/159 vs 13/43,
P<.001, respectively). In addition, a significantly higher prevalence of mutations in TP53 was found in RA/RCMD (4/34, 11.8%) and RAEB (4/43, 9.3%) compared with RARS/RCMD-RS (2/159, 1.2%) (P=.01). Finally, a significantly higher prevalence of mutations in genes involved in signaling was observed in MDS/MPN with ring sideroblasts compared with RARS/RCMD-RS (28/50 vs 7/159 respectively, P<.001).

Clinical and genetic correlates of SF3B1 mutation in MDS with ring sideroblasts

Within sideroblastic categories, SF3B1 mutation was found in 81/90 cases of RARS (90%), and 48/69 RCMD-RS (70%). Among patients classified in other MDS categories, SF3B1 mutations were observed in 22 of 84 (26.2%). In detail, SF3B1 mutations were detected in 4 of 27 cases of RCMD showing a proportion of ring sideroblasts ranging from 3 to 9%, 5 of 7 MDS with isolated del(5q) (ring sideroblasts from 21 to 69%), and 13 of 43 with RAEB-1 or -2 (ring sideroblasts from 7 to 78%).

When analyzing the whole MDS study population, patients with MDS carrying SF3B1 mutation showed significantly lower incidence of multilineage dysplasia (46.3% vs 82.6%, P<.001), lower proportion of dysplastic myeloid cells and megakaryocytes (median values 14% vs 27% and 3% vs 30% respectively, P<.001), higher absolute neutrophil (2.62 x10^9/L vs 1.6 x 10^9/L, P<.001) and platelet counts (269 x 10^9/L vs 123 x 10^9/L, P<.001), higher proportion bone marrow ring sideroblasts (40% vs 15%, P<.001) and serum ferritin level (untransfused patients: 408 vs 223 ng/mL, P<.001), as well as a significantly lower proportion of bone marrow blasts (2.6% vs 4.9%, P<.001) and incidence of chromosomal abnormalities (43/151 vs 43/92, P=.006) compared with SF3B1 unmutated cases.

These associations were confirmed when the analyses were restricted to patients with less than 5% bone marrow blasts (multilineage dysplasia: 40% vs 75%, P<.001; proportion of dysplastic myeloid cells: 12% vs 20%, P=.04; proportion of dysplastic megakaryocytes: 0% vs 25%, P<.001; absolute neutrophil count: 2.64 x 10^9/L vs 1.6 x 10^9/L, P<.001; platelet count: 275 x 10^9/L vs 148 x 10^9/L, P<.001; bone marrow ring sideroblasts: 40% vs 15%, P<.001; serum ferritin level: 412 vs 220 ng/mL, P<.001; bone marrow blasts: 1.8% vs 2.3%, P<.001; incidence of chromosomal abnormalities (32/133 vs 22/60, P=.08) (Supplemental Figure 2).
We then focused on patterns of co-occurring mutations in SF3B1-mutated patients with MDS, and found that SF3B1 mutation showed a limited pattern of recurrently co-mutated genes including those involved in DNA methylation (39%), chromatin modification (10%), and RUNX1 (5%) (Figure 1). Conversely, mutual exclusivity was observed between SF3B1 mutation and other splicing factors (SRSF2 and U2AF1) (P<.001) and TP53 (P=.001).

We then tested the prognostic value of SF3B1 mutations. When analyzing the whole MDS study population, in univariable analysis SF3B1 mutations showed a significant effect on OS (HR=.26, P<.001) and on cumulative incidence (CI) of disease progression accounting for death as a competing event (HR=.27, P=.001) (Figure 2A and 2B). The independent prognostic value was retained in multivariable analyses including demographic and disease-related factors (OS: HR .37, P=.003; CI of disease progression accounting for death as a competing event: HR=0.31, P=.018) (Table 2). Next, we performed uni- and multivariable analyses in patients stratified into WHO categories. When the analyses were focused on sideroblastic categories (RARS, RCMD-RS), the independent prognostic value of SF3B1 mutations was confirmed in both univariable (OS: HR=.25, P=.001; CI of disease progression: HR=.28, P=.032) and multivariable analyses (OS: HR=.27, P=.007; CI of disease progression: HR=.22, P=.026) (Table 2; Figure 2C and D). This independent value was retained when including in the multivariable analysis patients with RA or RCMD (OS: HR=.38, P=.009; CI of disease progression: HR=.33, P=.049). By contrast, within MDS with excess blasts (RAEB-1 and -2), the mutation did not retain significant effect on OS (P=.17) and CI of disease progression (P=.28).

Genetic Determinants of Disease Phenotype and Clinical Outcome in MDS associated with SF3B1 mutation

We investigated whether the current WHO classification criteria may identify distinct subsets within SF3B1-mutated MDS. We first evaluated the impact of bone marrow blasts, and found that a percentage of bone marrow blasts equal/higher than 5% significantly affected survival and CI of disease progression (HR=4.31, P=.02, and HR=3.86, P=.05).

Next, we assessed the effect of the proportion of bone marrow ring sideroblasts (equal/higher or lower than 15%) among SF3B1-mutated patients, and found that this threshold did not have significant impact on OS (P=.83) and CI of disease progression (P=.85). Finally, we evaluated the
impact of uni- or multilineage dysplasia within SF3B1-mutated patients, and no significant effect of multilineage dysplasia was found on OS (P=.5) and CI of disease progression (P=.94) (Figure 3C, D).

Therefore, we focused on MDS associated with SF3B1 mutation, defined by SF3B1 mutation, no excess blasts or del(5q) irrespective of the proportion of ring sideroblasts (81 RARS, 48 RCMD-RS, 4 RA or RCMD) with the aim to identify genetic determinants of disease phenotype variability and clinical outcomes. Overall survival and CI of disease progression of MDS associated with SF3B1 mutation as defined above are shown in Figure 3 (A, B). Interestingly, DNA methylation gene mutations (TET2, DNMT3A) were significantly associated with multilineage dysplasia (P=.015) but neither number of dysplasias nor presence or absence of methylation gene mutations were associated with hematological parameters, overall survival (P=.78) or CI of disease progression (P=.51) (Supplemental Figure 3).

A significant correlation was observed between SF3B1 mutant allele burden and proportion of bone marrow ring sideroblasts (P=.009). VAFs analysis showed that in most cases (91%) SF3B1 mutation was in the dominant clone.

We then used Cox regression models in SF3B1-mutated MDS with the aim to identify mutation patterns associated with relevant clinical outcomes, including survival, risk of disease progression and RBC transfusion-dependency. We found that mutations in RUNX1 were significantly associated with worse OS (HR=9.02, P=.004) and higher CI of disease progression (HR=19.12, P=.002) (Figure 3E, F). The prognostic value of RUNX1 mutations was confirmed in multivariable analyses adjusted for demographic and disease-related variables (P=.018 and P=.037 for OS and CI of disease progression, respectively). Unambiguous statistical evidence of subclonality of RUNX1 mutations was obtained in 50% of cases. In addition, we found that mutations in RUNX1 and EZH2 were associated with a significantly higher CI of RBC transfusion-dependency estimated with a competing risk approach (P=.002 and P=.007, respectively) (Supplemental Figure 4).

Mutation patterns in SF3B1-negative MDS with ring sideroblasts

We then focused on SF3B1-unmutated MDS with ring sideroblasts, no excess blast or del(5q) (7 RA, 23 RCMD, 9 RARS, 21 RCMD-RS). A significantly higher prevalence of mutations in splicing factors other than SF3B1 (SRSF2, U2AF1, ZRSR2) was observed in SF3B1-unmutated MDS with ring
sideroblasts compared with MDS associated with \textit{SF3B1} mutation (18/60 vs 1/133, \(P<.001\)) (Supplemental Figure 1B). In detail, 13 patients carried mutation of \textit{SRSF2}, 4 of \textit{U2AF1} and 1 in \textit{ZRSR2} (Figure 1).

Within \textit{SF3B1}-negative MDS with ring sideroblasts, a significantly higher prevalence of mutations in \textit{TP53} was found (9/93, \(P=.001\)), 6 of 9 cases showing disease phenotype with multilayer dysplasia and no excess blasts. No frame-shift or nonsense mutations were observed. All missense mutations found in these patients were located outside of p53 DNA binding surface in β-sandwich region.

**Clonal analysis in MDS/MPN with ring sideroblasts**

Fifty patients with ring sideroblasts met WHO criteria for classification as MDS/MPN. Nine cases were classified as CMML; 4 out of 9 patients had mutations in \textit{SF3B1}, which was associated with mutation in \textit{TET2} in 3 of 4. Among the remaining ones, four had a mutation in \textit{SRSF2}, 3 having co-mutation of \textit{TET2}. All CMML with ring sideroblasts showed a myelodysplastic phenotype according to FAB criteria, whereas a higher proportion of ring sideroblasts was observed in \textit{SF3B1}-mutated vs \textit{SRSF2}-mutated patients (50\% vs 6.5\%).

Forty-one patients were classified as RARS-T according to WHO criteria. Thirty patients out of 41 (73.2\%) had mutation of \textit{SF3B1}, 20 of \textit{JAK2} (V617F) (48.8\%), 4 of \textit{MPL} (9.8\%) and 6 of \textit{CALR} (14.6\%). Six additional patients classified in different WHO categories showed \textit{JAK2} (V617F): one patient had a slight increase of bone marrow blasts, one had proportion of ring sideroblasts lower than 15\%, whereas 4 showed at the time of diagnosis a platelet count lower than 450x10^9/L precluding the diagnosis of RARS-T. Overall, the occurrence of \textit{JAK2}, \textit{MPL} or \textit{CALR} mutations in patients with ring sideroblasts had a positive predictive value for myelodysplastic/myeloproliferative phenotype of 82\%, whereas the absence of mutations in these genes had a negative predictive value of 95\%.

\textit{SF3B1} median mutant allele burden was 43\% (range 14.7-48.7\%), consistent with the presence in the majority of patients of clonal hematopoiesis characterized by a dominant clone carrying an heterozygous \textit{SF3B1} mutation (Supplemental Figure 5). Patients carrying the \textit{JAK2} (V617F) mutation had a median allele burden of 7.1\% (range 0.1-27.7\%), whereas median allele burden of
*MPL* (W515L) and *CALR* mutations were 27.5% (range 25-50%) and 46.2% (range 10-59%), respectively.

We genotyped individual colonies from peripheral blood in 4 patients with multiple mutations. In three cases data were consistent with the existence of a dominant hematopoietic clone carrying the *SF3B1* mutation with the subsequent emergence of a *JAK2*-mutated subclone (Supplemental Figure 6A). The other patient, who was initially *SF3B1*-positive and *JAK2*-negative, had at the time of colony assay a mutant allele burden equal to 50% and 1% for *SF3B1* and *JAK2*, respectively.

Forty-three of 45 colonies were heterozygous for *SF3B1* (K700E) and wild type for *JAK2* mutation. The opposite pattern (*JAK2*-positive and *SF3B1*-negative) was observed in the remaining 2 colonies. All 45 colonies were found to carry a mutation of *ARHGAP26* (R367Q), a GTPase activating protein that mediates the activity of the GTP binding proteins RhoA and Cdc42. These data indicate the coexistence of two distinct subclones originating from a founding clone, a dominant one carrying the *SF3B1* mutation and a minority *JAK2*-positive clone (Supplemental Figure 6B).


Discussion

In this study, we performed targeted gene sequencing in a large and well-characterized cohort of myeloid neoplasms with 1% or more ring sideroblasts. Our findings indicate that irrespective of current WHO criteria, SF3B1 mutation defines a distinct subset of MDS with homogeneous molecular/clinical features and favorable prognosis. Conversely, MDS patients with ring sideroblasts and wild-type SF3B1 have more heterogeneous features and worse outcome.

The studies published so far on the prognostic relevance of SF3B1 mutations in MDS have provided conflicting results. In a study including 107 MDS patients with equal or more than 15% ring sideroblasts, SF3B1 mutation was associated with better overall and leukemia-free survival in univariable analysis, while this prognostic value was lost in multivariable analysis. Conversely, a recent study found that SF3B1-mutated cases had a significantly better survival compared with unmutated cases in both uni- and multivariable analysis. In our study, we analyzed a large cohort of 243 MDS with 1% or more ring sideroblasts, and found that SF3B1 mutations had a positive prognostic value on survival and risk of disease progression, and that this independent value was retained when the analysis was restricted to WHO subtypes without excess blasts or to sideroblastic categories.

We found that patients with MDS carrying SF3B1 mutation showed a homogeneous phenotype characterized by high prevalence of isolated erythroid dysplasia and high proportion of bone marrow ring sideroblasts. Notably, current criteria adopted by the WHO to classify MDS subtypes without excess blasts failed to recognize distinct subsets within SF3B1-mutated MDS. In fact, in SF3B1-mutated patients multilineage dysplasia was sustained by mild myeloid dysplasia without significant effect on peripheral cytopenia and outcome. Occasional patients in our study had SF3B1 mutation and a proportion of ring sideroblasts below the threshold of 15%, and no significant difference was observed in disease phenotype and outcome compared with patients having 15% or more ring sideroblasts, supporting the classification of these cases according to their SF3B1 mutation status. Conversely, the development of excess blasts significantly worsened the prognosis of SF3B1-mutated patients, suggesting that clonal evolution may overcome the positive prognostic value of the mutation. Taken together, these results suggest that SF3B1 mutation is the major determinant of disease phenotype, irrespective of current WHO
classification criteria, and support the recognition of MDS associated with SF3B1 mutation as a distinct MDS subtype.

Bone marrow ring sideroblasts and SF3B1 mutation were also reported in a fraction of patients with MDS with isolated del(5q).10 In our cohort, only seven patients with MDS with isolated del(5q) and ring sideroblasts were identified, precluding any further inference about the most appropriate classification of these cases. A recent study by Woll et al.25 showed that del(5q) usually precedes recurrent driver mutations with the exception of SF3B1-mutated cases, in which the initiating event may be either del(5q) or SF3B1 mutation. Thus, additional studies of distinct myelodysplastic stem cells are needed to determine unambiguously the phylogenetic relationship among these driver lesions as a basis for a genetic ontogeny-based classification.

When focusing on MDS associated with SF3B1 mutation, defined by SF3B1 mutation, no excess blasts or del(5q), irrespective of ring sideroblasts percentage, we found that mutations in genes involved in DNA methylation were associated with multilineage dysplasia. Mutations in DNA methylators were reported to be early events in myeloid neoplasms,26-28 and to skew hematopoietic precursors toward myeloid differentiation.29 Consistently with these findings, mutations in TET2 and DNMT3A were reported to be associated with multilineage dysplasia in MDS.13

The recognition of a disease subset with a unique molecular basis may have potential clinical implications. We previously showed that patients with SF3B1 mutation have a high degree of ineffective hematopoiesis resulting in inappropriately low hepcidin levels and propensity to parenchymal iron loading.30 Recently, a transforming growth factor-β superfamily ligand was found to correct ineffective erythropoiesis in mice,31,32 and preliminary results from a phase 2 study in MDS showed higher a response rate in patients with SF3B1 mutation.33 Furthermore, several compounds were reported to bind to the SF3b complex and to inhibit mRNA splicing,34,35 and preliminary results showed that SF3B1 modulators may induce tumor regression and increase survival in SF3B1-mutant xenografts.36

A high incidence of mutations in JAK2, as well as in MPL and CALR, was previously reported in patients with RARS-T in combination with SF3B1 mutations.10,19,37 In this study, we combined VAFs analysis with genotyping of individual hematopoietic colonies in order to clarify the hierarchical
relationship between mutations in these patients. Our results suggest that the occurrence of SF3B1 mutation represents an early event in patients with RARS-T, whereas the subsequent occurrence of a somatic mutation of JAK2 typically involves the emergence of minority subclones of the dominant SF3B1-positive clone. Taken together, these results suggest that the occurrence of mutations activating JAK-STAT pathway is a recurrent pattern of evolution of SF3B1 positive clones, in accord to the hypothesis of genetic predisposition that early mutations shape the future trajectories of clonal evolution through constraints on the repertoire of cooperating genetic lesions. 

Compared with MDS associated with SF3B1 mutation, SF3B1-unmutated MDS with ring sideroblasts, showed a higher incidence of disease phenotype with multilineage dysplasia, and a significantly worse prognosis, suggesting that these are indeed a different subset with clinical and hematological features more similar to the WHO category of RCMD. In these patients, we observed a high prevalence of mutations in TP53, which are conversely mutually exclusive with SF3B1 mutations. Interestingly, all the mutations found in these patients were missense and located outside of p53 DNA binding surface in β-sandwich region. The most characterized mutation in this region is Y220C, which forms a cavity on p53 surface causing protein instability and aggregation, and several small molecules were reported to reactivate Y220C mutated p53 with potential clinical application. This finding should be investigated in more details, and may open up a new diagnostic and therapeutic approach in treatment of these patients.

In conclusion, this study showed that SF3B1 mutation is a major determinant of disease phenotype and clinical outcome in MDS with ring sideroblasts. SF3B1-mutated MDS are characterized by homogeneous hematological features, favorable prognosis, and restricted patterns of co-mutated genes and clonal evolution. Overall, these results strongly support the recognition of MDS associated with SF3B1 mutation as a distinct MDS subtype. Conversely, SF3B1-negative MDS with ring sideroblasts represent a subset with high prevalence of TP53 mutations and worse outcome that should be taken into consideration in clinical decision-making.
Acknowledgments

These studies were supported by grants from Associazione Italiana per la Ricerca sul Cancro (AIRC), Fondo per gli investimenti della ricerca di base (FIRB, project no. RBAP11CZLK), and Ministero dell’Istruzione, dell’Università e della Ricerca (MIUR, PRIN 2010-2011) to MC; from Fondazione Veronesi and Regione Lombardia/Fondazione Cariplo to MGDP; from Associazione Italiana per la Ricerca sul Cancro (AIRC) (IG 15356) to LM.

Authorship

Contribution: LM, MC and EHL designed the study, performed statistical analysis and wrote the manuscript. EP, MK, AG, DP, RR and PJC analyzed sequencing data and performed bioinformatic analysis; MaJ, IA, MoJ, CE, GW, MGDP, KRJ, KK, VL, SC collect clinical data; ET, RI, EB performed blinded revision of diagnostic specimens.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Luca Malcovati, MD, Department of Hematology Oncology, Fondazione IRCCS Policlinico San Matteo, Viale Golgi 19, 27100 Pavia, Italy; e-mail: luca.malcovati@unipv.it, or Mario Cazzola, MD, Department of Hematology Oncology, Fondazione IRCCS Policlinico San Matteo, Viale Golgi 19, 27100 Pavia, Italy; e-mail: mario.cazzola@unipv.it
References

Table 1. Clinical features of patients with myeloid neoplasms and ring sideroblasts included in the study.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>N. of Patients</td>
<td>293</td>
</tr>
<tr>
<td>Age (median, range)</td>
<td>70 yrs (18-96)</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>161/147</td>
</tr>
<tr>
<td>Diagnosis:</td>
<td></td>
</tr>
<tr>
<td>Myelodysplastic syndrome</td>
<td></td>
</tr>
<tr>
<td>RARS</td>
<td>243</td>
</tr>
<tr>
<td>RCMD-RS</td>
<td>90</td>
</tr>
<tr>
<td>RCUD (RA)</td>
<td>69</td>
</tr>
<tr>
<td>RCMD</td>
<td>7</td>
</tr>
<tr>
<td>MDS with isolated del(5q)</td>
<td>27</td>
</tr>
<tr>
<td>RAEB-1</td>
<td>7</td>
</tr>
<tr>
<td>RAEB-2</td>
<td>20</td>
</tr>
<tr>
<td>Myelodysplastic/myeloproliferative neoplasm</td>
<td>50</td>
</tr>
<tr>
<td>CMML</td>
<td>9</td>
</tr>
<tr>
<td>RARS-T</td>
<td>41</td>
</tr>
<tr>
<td>Hemoglobin (g/dL) (median, range)</td>
<td>9.9 (5.6-15.8)</td>
</tr>
<tr>
<td>RBC transfusion-dependency</td>
<td>108/293 (37%)</td>
</tr>
<tr>
<td>WBC count (x 10^9/L) (median, range)</td>
<td>4.9 (1.0-13.9)</td>
</tr>
<tr>
<td>Absolute neutrophil count (ANC, x 10^9/L) (median, range)</td>
<td>2.5 (0.1-12.9)</td>
</tr>
<tr>
<td>Platelet count (x 10^9/L) (median, range)</td>
<td>263 (7-1130)</td>
</tr>
<tr>
<td>Serum Ferritin (ng/mL) (median, range)</td>
<td>541 (30-5745)</td>
</tr>
<tr>
<td>Bone marrow blasts (%) (median, range)</td>
<td>2 (0-19)</td>
</tr>
<tr>
<td>Bone marrow ring sideroblasts (%) (median, range)</td>
<td>32 (1-95)</td>
</tr>
<tr>
<td>Chromosomal abnormalities</td>
<td>83/277 (30%)</td>
</tr>
</tbody>
</table>
Table 2. Results of uni- and multivariable analyses in MDS with 1% or more ring sideroblasts and in sideroblastic categories (RARS, RCMD-RS).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Overall Survival</th>
<th>Cumulative Incidence of Disease Progression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Univariable</td>
<td>Multivariable</td>
</tr>
<tr>
<td></td>
<td>analysis</td>
<td>analysis</td>
</tr>
<tr>
<td></td>
<td>HR</td>
<td>P value</td>
</tr>
<tr>
<td><strong>MDS with ≥1% ring sideroblasts</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SF3B1 mutation</td>
<td>.26</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Age</td>
<td>1.02</td>
<td>.082</td>
</tr>
<tr>
<td>Bone marrow blasts†</td>
<td>1.17</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Multilineage dysplasia§</td>
<td>3.7</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>RBC transfusion dependency°</td>
<td>1.89</td>
<td>.02</td>
</tr>
<tr>
<td>Absolute neutrophil count*</td>
<td>2.96</td>
<td>.015</td>
</tr>
<tr>
<td>Platelet count*</td>
<td>1.76</td>
<td>.006</td>
</tr>
<tr>
<td>Karyotype*</td>
<td>2.28</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

**Sideroblastic Categories (RARS, RCMD-RS)**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Overall Survival</th>
<th>Cumulative Incidence of Disease Progression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Univariable</td>
<td>Multivariable</td>
</tr>
<tr>
<td></td>
<td>analysis</td>
<td>analysis</td>
</tr>
<tr>
<td></td>
<td>HR</td>
<td>P value</td>
</tr>
<tr>
<td>SF3B1 mutation</td>
<td>0.25</td>
<td>.001</td>
</tr>
<tr>
<td>Age</td>
<td>1.03</td>
<td>.11</td>
</tr>
<tr>
<td>Bone marrow blasts†</td>
<td>1.23</td>
<td>.099</td>
</tr>
<tr>
<td>Multilineage dysplasia§</td>
<td>3.06</td>
<td>.005</td>
</tr>
<tr>
<td>RBC transfusion dependency°</td>
<td>2.07</td>
<td>.046</td>
</tr>
<tr>
<td>Absolute neutrophil count*</td>
<td>1.19</td>
<td>.86</td>
</tr>
<tr>
<td>Platelet count*</td>
<td>2.20</td>
<td>.011</td>
</tr>
<tr>
<td>Karyotype*</td>
<td>2.11</td>
<td>.029</td>
</tr>
</tbody>
</table>

*Bone marrow blasts were analyzed as a percentage of bone marrow nuclear cells. §§ Multilineage dysplasia was analyzed as uni-versus multilineage dysplasia according to WHO criteria. ° RBC transfusion dependency was defined according to Malcovati et al.40 and Such et al.41. *Cytogenetic abnormalities, absolute neutrophil count and platelet count were categorized according to the revised international prognostic scoring system.42
Legends to Figures

**Figure 1.** Mutation patterns in MDS with ring sideroblasts. Diagnosis of each sample according to WHO criteria is shown by indicated colors.

**Figure 2.** Overall survival and cumulative incidence of disease progression of patients with MDS with ring sideroblasts classified according to SF3B1 mutation status. Plots A and B report respectively overall survival and cumulative incidence of disease progression of the whole cohort of MDS with ring sideroblasts according to SF3B1 mutation status (total number of patients = 243; SF3B1 mutated = 151, SF3B1 unmutated = 92) (P values from multivariable analyses, OS: P=.003; CI of disease progression: P=.018). Plots C and D report respectively overall survival and cumulative incidence of disease progression of patients with MDS classified into sideroblastic categories (i.e. RARS and RCMD-RS) (total number of patients = 159; SF3B1 mutated = 129, SF3B1 unmutated = 30) (P values from multivariable analyses, OS: P=.007; CI of disease progression: P=.026).

**Figure 3.** Overall survival and cumulative incidence of disease progression of patients with MDS associated with SF3B1 mutation. Plots A and B report respectively overall survival and cumulative incidence of disease progression of MDS associated with SF3B1 mutation, defined by SF3B1 mutation, no excess blasts or del(5q) (81 RARS, 48 RCMD-RS, 4 RA or RCMD). Plots D and E report respectively overall survival and cumulative incidence of disease progression of patients with MDS associated with SF3B1 mutation according to uni- or multiligneage dysplasia (P values from multivariable analyses, OS: P=.5; CI of disease progression: P=.94). Plots E and F report respectively overall survival and cumulative incidence of disease progression of patients with MDS associated with SF3B1 mutation according to RUNX1 mutation status (RUNX1 unmutated = 128, RUNX1 mutated = 5) (P values from multivariable analyses, OS: P=.018; CI of disease progression: P=.037).
Figure 2

A) Cumulative Probability of Survival

B) Cl of Disease Progression

C) Cumulative Probability of Survival

D) Cl of Disease Progression

P = 0.03

P = 0.018

P = 0.007

P = 0.026
SF3B1 mutation identifies a distinct subset of myelodysplastic syndrome with ring sideroblasts

Luca Malcovati, Mohsen Karimi, Elli Papaemmanuil, Ilaria Ambaglio, Martin Jädersten, Monika Jansson, Chiara Elena, Anna Galli, Gunilla Walldin, Matteo G. Della Porta, Klas Raaschou-Jensen, Erica Travaglino, Klaus Kallenbach, Daniela Pietra, Viktor Ljungström, Simona Conte, Emanuela Boveri, Rosangela Invernizzi, Richard Rosenquist, Peter J. Campbell, Mario Cazzola and Eva Hellström Lindberg