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Plenary paper

Mutations affecting mRNA splicing define distinct clinical phenotypes and correlate with patient outcome in myelodysplastic syndromes

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A cohort of MDS patients was examined for mutations affecting 4 splice genes (*SF3B1, SRSF2, ZRSR2*, and *U2AF35*) and evaluated in the context of clinical and molecular markers. Splice gene mutations were detected in 95 of 221 patients. These mutations were mutually exclusive and less likely to occur in patients with complex cytogenetics or *TP53* mutations. *SF3B1*^{mut} patients presented with lower hemoglobin levels, increased WBC and platelet counts, and were more likely to have *DNMT3A* mutations. *SRSF2*^{mut} patients clustered in RAEB-1 and RAEB-2 subtypes and exhibited pronounced thrombocytopenias. *ZRSR2*^{mut} patients clustered in International Prognostic Scoring System intermediate-1 and intermediate-2 risk groups, had higher percentages of bone marrow blasts, and more often displayed isolated neutropenias. *SRSF2* and *ZRSR2* mutations were more common in *TET2*^{mut} patients. *U2AF35*^{mut} patients had an increased prevalence of chromosome 20 deletions and *ASXL1* mutations. Multivariate analysis revealed an inferior overall survival and a higher AML transformation rate for the genotype *ZRSR2*^{mut}/*TET2*^{wt} (overall survival: hazard ratio = 3.3; 95% CI, 1.4-7.7; *P* = .006; AML transformation: hazard ratio = 3.6; 95% CI, 2-4.2; *P* = .026). Our results demonstrate that splice gene mutations are among the most frequent molecular aberrations in myelodysplastic syndrome, define distinct clinical phenotypes, and show preferential associations with mutations targeting transcriptional regulation. (*Blood*. 2012;119(14):3211-3218)

Introduction

Myelodysplastic syndromes (MDSs) are a heterogeneous group of myeloid neoplasms showing clonal hematopoiesis, aberrant differentiation, peripheral cytopenias, and risk of progression to acute myeloid leukemia (AML).[1] Although cytopenias are the major clinical challenge in low-risk disease, transformation to AML can be observed in a significant number of high-risk MDS patients. The broad range of individual genes affected by mutations indicates that a variety of molecular mechanisms are involved in the pathogenesis of these disorders. Like *TET2*,[2,3] the most commonly affected gene in MDS, several others, such as *ASXL1*,[4] *EZH2*,[5,6] and *DNMT3A*,[7] are involved in epigenetic regulation of transcription. Additional genes known to be mutated in MDS include *RUNX1*,[8] *IDH1/2*,[9] *TEL/ETV6*,[8] *TP53*,[8] and *NRAS*.[8] As MDS patient outcome remains dismal, identification of novel molecular markers in MDS that allows further subclassification and possibly risk-directed therapeutic intervention remains of major interest.[8,10] Next-generation sequencing approaches have identified mutations in genes encoding multiple components of the RNA splicing machinery. The most frequently documented mutations affect splice genes *U2AF35*, *ZRSR2*, *SRSF2*, and *SF3B1*. Collectively, these mutations were observed in up to 85% of different myeloid neoplasms with myelodysplastic features,[11,12] thus suggesting an important contribution of genetic alterations involving splicing components to the pathophysiology of MDS.

The detailed splicing mechanism is complex and relies on 5 small nuclear ribonucleoproteins (snRNP) and their associated

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proteins, which form the spliceosome. It processes premessenger RNA (pre-mRNA) into mature mRNA and controls the diversity of splice variants.[13] Each snRNP is composed of a single uridine-rich small nuclear RNA (snRNA) accompanied by multiple proteins and has a specific function. The recently discovered mutations in the splicing machinery of patients with myeloid neoplasms are predicted to affect core components of initial steps, such as the recognition of the 3′ splice acceptor site of the pre-mRNA target intron (U2AF35 and SRSF2) or the recruitment of the U2 snRNP to the branch point proximal to the 3′ splicing site that contains SF3B1. Although abnormal splicing was described to be associated with *U2AF35*[11,14] and *SF3B1*[12] mutations, the biologic mechanisms linking the splicing machinery to cellular transformation and leukemogenesis remain elusive. Since the initial report on *SF3B1* mutations in MDS, other groups have confirmed the high frequency of *SF3B1* mutations in MDS patients and the association of the mutations with the presence of ring sideroblasts (RSs).[15-17] However, little is currently known about the clinical course, morphologic features, prognostic impact, and concomitant molecular aberrations of MDS patients harboring *SRSF2*, *ZRSR2*, or *U2AF35* mutations.

Therefore, we examined genomic DNA of 221 MDS patients at diagnosis for the presence of *SF3B1*, *SRSF2*, *ZRSR2*, and *U2AF35* mutations by direct sequencing and evaluated their prognostic impact with regard to other mutations, including *ASXL1, CBL, DNMT3A, ETV6, EZH2, IDH1/2, JAK2, NRAS*, *RUNX1, TET2*, and *TP53*.

Methods

Patients

The 221 MDS samples were collected at time of enrolment in multicenter clinical trials in France between 1999 and 2011 at Paris Cochin (n = 134) and Marseille Institute Paoli-Calmette (n = 87). Clinical and hematologic data were recorded after informed consent in accordance with the Declaration of Helsinki, and the analysis of the samples was approved by the institutional review boards of Paris Centre and Marseille. The distribution of WHO subtypes, International Prognostic Scoring System (IPSS) risk groups, and cytogenetic risk groups (according to WHO 2008[18]) is shown in Table 1. Follow-up information was available for 198 of the 221 MDS patients. The follow-up information was updated by means of clinic visits. Treatment details for both cohorts have been published earlier[4,19] and are shown according to mutation status in supplemental Table 1 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

Cytogenetic and mutation analysis

Cytogenetic analysis was performed by G- and R-banding analysis. Mononuclear cells from diagnostic bone marrow samples were enriched by Ficoll density gradient centrifugation and were stored in liquid nitrogen until use. Genomic DNA was extracted from samples using the All Prep DNA/RNA Kit (QIAGEN) according to the manufacturer's recommendations. Genomic DNA was amplified by linear whole genome amplification using the REPLI-G Kit (QIAGEN) to perform a first identification screen. All candidate mutations were subsequently analyzed in an independent experiment using nonamplified genomic DNA. The genomic regions that span exon 12 of *ASXL1*,[4] exons 8 and 9 of *CBL*,[20] exons 15 to 23 of *DNMT3A*,[21] the entire coding regions of *ETV6* and *EZH2*,[6] exons 4 of *IDH1* and *IDH2*,[22] exon 14 of *JAK2*,[23] exons 1 and 2 of *NRAS*,[24] exons 3 to 8 of *RUNX1*,[25] the entire coding region of *TET2*,[19] and exons 5 to 8 of *TP53*[25] were analyzed as previously reported. *SF3B1* (exons 12-16), *SRSF2* (exon2), *ZRSR2* (exons 1-11), and *U2AF35* (exons 2 and 6) were amplified using intron-flanking primers tagged with M13 universal primers at the 3′ or 5′ ends. PCR fragments were directly sequenced in both directions and were analyzed using the Mutation surveyor Version 3.97 software (Softgenetics). Nontumoral tissue was analyzed when available (DNA from buccal swab or $CD3^+$ T cells; n = 20).

Statistical analysis

Overall survival (OS) end points, measured from the date of diagnosis, were death (failure) and alive at last follow-up (censored). Time to AML progression was measured from the date of MDS diagnosis to the time of AML diagnosis. Progression to AML was defined according to the 2008 WHO classification. The median follow-up time for patients alive was calculated according to the method of Korn.[26] Primary analysis was performed on OS and time to AML progression. The Kaplan-Meier method, log-rank test, and Cox proportional hazards models were used to estimate the distribution of OS and time to AML progression and to compare differences between survival curves, respectively. Pairwise comparisons were performed by Mann-Whitney test for continuous variables and by 2-sided Fisher exact or χ^2 tests for categorical variables, and are provided for exploratory purposes.

For multivariate analysis, a Cox proportional hazards model was constructed for OS and time to progression to AML, adjusting for potential confounding covariates.[27] Variables considered for model inclusion were IPSS risk group, transfusion dependence, age (below vs above median), and mutation status of all 16 analyzed genes (mutated vs wild-type). Variables with $P \leq .1$ in univariate analysis for OS were included in the model. The statistical analyses were performed with the statistical software package SPSS Version 19.0 (SPSS). Genomic alterations were considered as a mutation when the variation was not listed in dbSNP database (build 131 and 132), shown to be acquired or had been identified as such in other studies.

Results

Mutation status of *SF3B1, SRSF2, ZRSR2*, and *U2AF35* in MDS patients

Among the 221 patients with MDS, 37 had *SF3B1* mutations (16.4%), 25 had *ZRSR2* mutations (11.1%), 25 had *SRSF2* mutations (11.1%), and 12 patients harbored mutations affecting *U2AF35* (5.4%). In total, 99 mutations affecting one of the 4 genes were detected in 95 patients (42.2%). Splice gene mutations were mostly mutually exclusive (91 of 95, 97%; $P < .001$), with concomitant mutations affecting 2 splice genes detected in only 4 patients. Two patients harbored mutations in both *SRSF2* and *ZRSR2*, one patient had *SF3B1* and *SRSF2* mutations, and another patient had *SF3B1* and *ZRSR2* mutations.

The 37 mutations in *SF3B1* were all heterozygous missense mutations affecting 6 mutational hotspots that are located in the HEAT domains 3 to 6 (Figure 1). The most common recurrent *SF3B1* mutation affected amino acid residue K700 (21 of 37, 56.7%). The somatic nature of *SF3B1* mutations was confirmed by sequencing nontumoral $CD3^+$ cells in 8 patients (6 patients, K700E; 1 patient, D781G; and 1 patient, R625L). Except for detection of Y44H in one patient, all mutations observed in *SRSF2* affected amino acid residue P95 and were predominantly heterozygous missense mutations. Notably, a 24-bp deletion starting at amino acid residue P95 was found in 3 patients. The somatic nature of this deletion was verified by sequencing $CD3^+$ cells from one patient. Except for one frameshift mutation starting at Q157, mutations in *U2AF35* affected positions S34 and Q157 and were heterozygous missense mutations. The acquired nature of substitutions S34F and Q157R was verified by sequencing DNA from $CD3^+$ cells (n = 2) or buccal swab (n = 1). In contrast to *SF3B1*, *SRSF2*, and *U2AF35,* mutations in *ZRSR2* were spread

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Table 1. Clinical characteristics of 221 MDS patients according to *SF3B1*, *SRSF2*, *ZRSR2*, and *U2AF35* mutation status

Characteristic	All	*SF3B1* mut (n = 37)	*SF3B1* wt (n = 184)	P	*SRSF2* mut (n = 25)	*SRSF2* wt (n = 196)	P	*ZRSR2* mut (n = 25)	*ZRSR2* wt (n = 196)	P	*U2AF35* mut (n = 12)	*U2AF35* wt (n = 209)	P
Age, y	217			.17			.25			.7			.71
Median	71.9	73	71		70	72		71.9	71.9		73.5	71.9	
Range		51-95	35-95		49-87.8	35-95		57.9-95	35-95		56-88	35-95	
Sex	221			.88			0.86			< .001			.016
Male, no. (%)	129	22 (59)	107 (58)		15 (60)	114 (58)		24 (96)	105 (54)		11 (92)	118 (56)	
WHO subtype	221			< .001			.19			.048		.48	.48
RA, no. (%)	34	2 (5)	32 (17)		2 (8)	32 (17)		1 (4)	33 (17)		4 (33)	30 (14)	
RARS, no. (%)	27	20 (54)	7 (4)		1 (4)	26 (13)		0 (0)	27 (14)		1 (8)	26 (13)	
RCMD, no. (%)	31	0 (0)	31 (17)		3 (12)	28 (14)		3 (12)	28 (14)		1 (8)	30 (14)	
RCMD-RS, no. (%)	8	6 (16)	2 (1)		0 (0)	8 (4)		0 (0)	8 (4)		1 (8)	7 (3)	
RAEB-1, no. (%)	55	1 (3)	54 (29)		12 (48)	43 (22)		11 (44)	44 (23)		1 (8)	54 (26)	
RAEB-2, no. (%)	45	3 (8)	42 (23)		6 (24)	39 (20)		9 (36)	36 (18)		4 (33)	41 (20)	
5q− syndrome, no. (%)	2	0 (0)	2 (1)		0 (0)	2 (1)		0 (0)	2 (1)		0 (0)	2 (1)	
RARS-T, no. (%)	6	5 (14)	1 (1)		0 (0)	6 (3)		0 (0)	6 (3)		0 (0)	6 (3)	
MDS-U, no. (%)	13	0 (0)	13 (7)		1 (4)	12 (6)		1 (4)	12 (6)		0 (0)	13 (6)	
RSs				< .001			.047			.011			.86
Present, no. (%)	41	31 (84)	10 (5)		1 (4)	40 (20)		0 (0)	41 (21)		2 (17)	39 (19)	
Karyotype risk	213			.71			.21			.25			.99
Low, no. (%)	155	28 (76)	128 (70)		15 (60)	141 (72)		20 (80)	136 (69)		9 (75)	147 (70)	
Intermediate, no. (%)	38	7 (19)	31 (17)		7 (28)	31 (16)		5 (20)	33 (17)		2 (17)	36 (17)	
High, no. (%)	19	2 (5)	17 (9)		1 (4)	18 (9)		0 (0)	19 (10)		1 (8)	18 (9)	
Bone marrow blasts	217			< .001			.022			.011			.77
Median, %	4	2	5		5	4		6.5	4		3	4	
Range, %		1-19	0-19		1-18	0-19		1-18	0-19		1-16	0-19	
Hemoglobin	211			< .001			.13			.7			.54
Median, g/dL	9.8	9	10.1		10.9	9.8		9.4	9.9		10.1	9.8	
Range, g/dL		6-11.8	6-15		7.1-15	6-15		7.7-15	6-15		7.5-14.5	6-15	
WBC count	207			.001			.046			.008			.44
Median, $\times 10^9$/L	4.2	6.1	4		3.2	4.3		2.9	4.3		5.2	4.2	
Range, $\times 10^9$/L		1.8-36.7	0.9-18.4		1.2-13	0.9-36.7		0.9-6.8	1.2-36.7		1.8-8.5	0.9-36.7	
Neutrophil count	205			.001			.006			.009			.31
Median, $\times 10^9$/L	2.1	3.4	1.9		1.1	2.3		1.1	2.2		2.8	2.1	
Range, $\times 10^9$/L		0.7-23.1	0.3-15.8		0.3-9.7	0.3-23.1		0.3-4.9	0.3-23.1		0.5-6.5	0.3-23.1	
Platelet count	212			< .001			< .001			.4			.47
Median, $\times 10^9$/L	156	271	141		88	165.5		149	157.5		134.5	156	
Range, $\times 10^9$/L		23-1398	5-714		6-359	5-1398		48-290	5-1398		32-500	5-1398	
IPSS, no.	216			.036			.042			.017			.07
Low risk, %	74	19 (51)	55 (30)		3 (12)	71 (36)		3 (12)	71 (36)		5 (42)	69 (33)	
Intermediate-1, %	91	15 (41)	76 (41)		11 (44)	80 (41)		16 (64)	74 (38)		2 (17)	89 (43)	
Intermediate-2, %	26	1 (3)	25 (13)		6 (24)	20 (10)		5 (20)	21 (11)		4 (33)	22 (11)	
High, %	25	2 (5)	23 (13)		4 (16)	21 (11)		1 (4)	24 (12)		1 (8)	24 (11)	
Cytology													
Multilineage dysplasia, no. (%)	115/204	14 (41)	101 (59)	.050	17 (71)	98 (54)	.13	21 (84)	94 (53)	.003	5 (45)	110 (57)	.45
Dyserythropoiesis, no. (%)	137/197	30 (94)	107 (65)	.001	16 (70)	121 (70)	1	17 (68)	120 (69)	.63	8 (73)	129 (69)	.81
Dysgranulopoiesis, no. (%)	140/201	15 (47)	125 (74)	.002	23 (92)	117 (66)	.009	22 (88)	118 (67)	.033	7 (64)	133 (70)	.66
Dysmegakayopoiesis, no. (%)	131/200	14 (44)	114 (68)	.008	21 (84)	110 (63)	.038	20 (80)	108 (62)	.038	7 (64)	121 (64)	.96
Transfusion dependence	193			.024			.036			.38			.27
Yes, no. (%)	91	22 (59)	69 (38)		5 (20)	86 (44)		8 (32)	83 (42)		3 (25)	88 (42)	

over the entire coding region and were mainly frameshift, splice site, or nonsense mutations (17 of 25, 68%). A 6-bp insertion starting at amino acid residue R446, resulting in the duplication of the amino acids arginine and serine, was observed in 9 patients (9 of 221, 4.1%). This duplication was also detected in nontumoral $CD3^+$ cells of 2 patients and therefore classified as a polymorphism. A detailed overview of mutation sites is shown in Figure 1.

Clinical phenotype of patients harboring splice gene mutations

Patients with *SF3B1* mutations presented with a distinct blood count at diagnosis. There was no significant difference in age, sex, or karyotype between *SF3B1*^{mut} and *SF3B1*^{wt} patients. Hemoglobin levels were significantly lower (median: 9 vs 10.1 g/dL; $P < .001$), while white blood cell counts (WBC; $P = .001$) and platelets ($P < .001$) were higher in *SF3B1*^{mut} compared with *SF3B1*^{wt}

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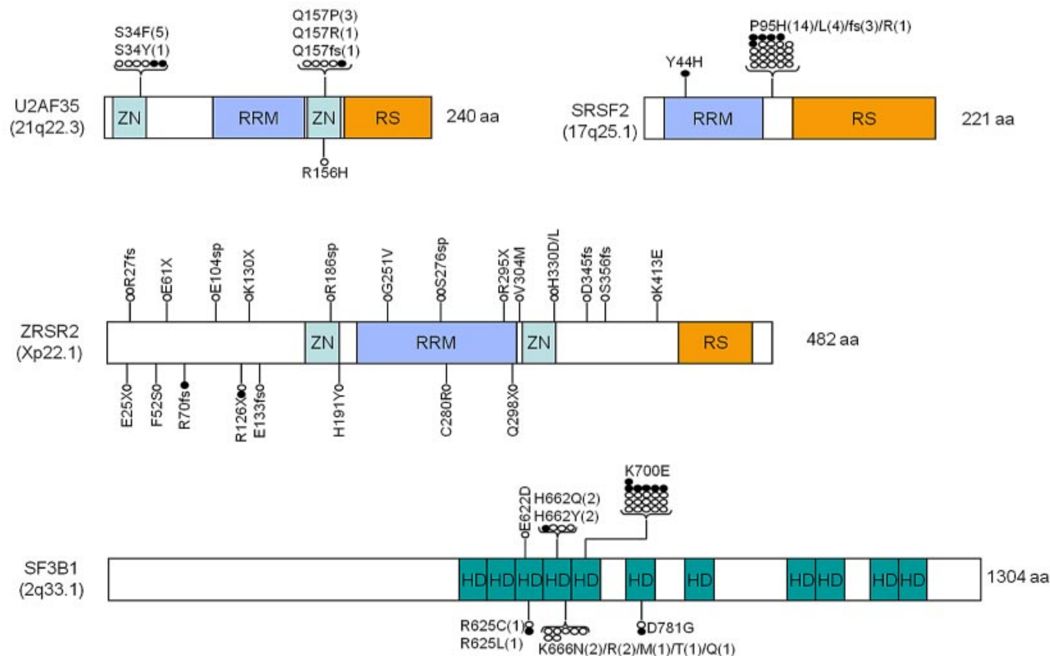

Figure 1. Localization of mutations identified in splice genes. Each mutation is shown with a circle. ● represents confirmed somatic mutations; and colored boxes, known domain structures. ZN indicates zinc finger; RRM, RNA recognition motif; RS, arginine-serine-rich domain; and HD, HEAT domain.

patients. Cytologic evaluation revealed a higher proportion of dysmorphic features in the erythroid lineage (94% vs 65%, $P = .001$), whereas multilineage dysplasia, dysgranulopoiesis, and dysmegakaryopoiesis were less often observed (Table 1). The percentage of bone marrow blasts was also lower in *SF3B1*^{mut} patients (median: 2% vs 5%; $P < .001$). A strong association between the presence of RSs and *SF3B1* mutation was observed: 31 of 41 patients with RS harbored a mutation in *SF3B1* (84% vs 5%; $P < .001$). A bivariate regression analysis, including RS and *SF3B1* mutation status, revealed an independent association for *SF3B1*^{mut} patients with lower hemoglobin levels (odds ratio = 0.21, 95% CI, 0.06-0.7, $P = .01$). These findings resulted in a higher proportion of transfusion dependence for *SF3B1*^{mut} compared with *SF3B1*^{wt} patients (59% vs 38%; $P = .024$).

In contrast, *SRSF2*^{mut} patients presented with substantially decreased numbers of neutrophil granulocytes (median: 1.1 vs 2.3×10^9/L; $P = .006$) and platelets (median: 88 vs 165×10^9/L; $P < .001$), whereas no differences for hemoglobin levels were observed. Dysplastic features affected predominantly granulopoiesis and megakaryopoiesis. Eighteen of 25 *SRSF2*^{mut} patients were classified according to the WHO classification as refractory anemia with excess of blasts (RAEB-1) or RAEB-2 (72% vs 42% in *SRSF2*^{wt} patients; $P = .004$). RSs were observed in one single *SRSF2*^{mut} patient, and this case was the only one with a concomitant *SF3B1* mutation. The transfusion dependence rate was significantly lower for *SRSF2*^{mut} patients than for *SRSF2*^{wt} patients (20% vs 44% in *SRSF2*^{wt} patients; $P = .036$).

Patients harboring *ZRSR2* mutations were almost exclusively male (24 of 25, 96%) and often presented with isolated neutropenias (median: 1.1 vs 2.2×10^9/L; $P = .009$). RSs were not observed in any of the 25 *ZRSR2*^{mut} patients ($P = .011$). Twenty of 25 *ZRSR2*^{mut} patients were classified according to the WHO classification as RAEB-1 or RAEB-2 (80% vs 41% in *ZRSR2*^{wt} patients; $P < .001$, Table 1; supplemental Table 2). *ZRSR2*^{mut} patients mainly clustered into the IPSS intermediate-1 and intermediate-2 risk groups (84% vs 49% in *ZRSR2*^{wt} patients; $P = .017$).

Almost all *U2AF35*^{mut} patients were male (11 of 12 = 92%; $P = .016$), but no significant differences in age, WHO classification, bone marrow blasts, hemoglobin, WBC, platelets, transfusion dependence, or IPSS score were observed. Interestingly, patients with a del20q were more likely to have *U2AF35* mutations than patients with no del20q (30% vs 4.5%; $P = .014$).

Molecular associations of splice gene mutations in MDS

In our MDS patient cohort, *TET2* mutations were the most common (54 of 221, 24.4%), whereas mutations in *ASXL1* (37 of 221, 16.4%), *DNMT3A* (26 of 221, 11.6%), *RUNX1* (16 of 221, 7.1%), *TP53* (13 of 221, 5.8%), *IDH1/2* (12 of 221, 5.3%), *CBL* (11 of 221, 4.9%), *JAK2* (9 of 221, 4%), *EZH2* (7 of 221, 3.1%), *ETV6* (2 of 221, 0.9%), and *NRAS* (2 of 221, 0.9%) were less frequent. At least one mutation in any of the 16 analyzed genes was found in 154 of 221 patients (69.7%; Figure 2). Mutations in splice genes (n = 95 patients) were mutually exclusive from the presence of a complex karyotype (1.1% vs 12.6% in patients wild-type for the 4 splice genes; $P = .001$) and were significantly less often detected in patients with *TP53* mutations (2% vs 9% in patients wild-type for the 4 splice genes; $P = .04$). To identify interactions of splice genes with other mutations, we performed Fisher exact test. Interestingly, each splice gene was significantly associated with one particular gene involved in epigenetic regulation. *DNMT3A* mutations were more often found in *SF3B1*^{mut} patients than in *SF3B1*^{wt} patients (35% vs 7%, $P < .001$; Table 2). *TET2* mutations occurred more often in *SRSF2*^{mut} and *ZRSR2*^{mut} patients compared with *SRSF2*^{wt} and *ZRSR2*^{wt} patients (44% vs 22%; $P = .02$ and 56% vs 20%; $P < .001$, respectively). A trend was observed for *RUNX1* mutations to be enriched in patients concomitantly mutated for *SRSF2* (16% vs 6%; $P = .09$). Finally, *ASXL1* mutations were more often seen in *U2AF35*^{mut} patients than in *U2AF35*^{wt} patients (45% vs 17%; $P = .03$).

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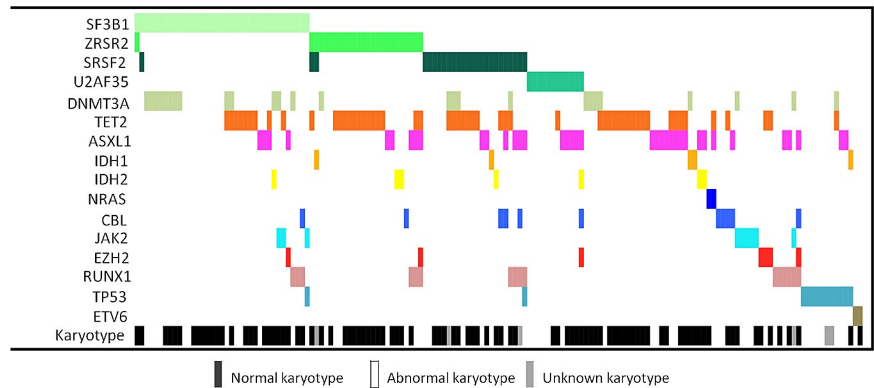

Figure 2. Distribution of molecular aberrations in 154 MDS patients with at least 1 identified mutation in the 16 investigated genes.

Prognostic impact of splice gene mutations

The prognostic impact of splice gene mutations was evaluated in MDS patients for whom follow-up information was available (n = 198). The median follow-up of patients alive was 31 months. In univariate analysis, OS and AML transformation rates according to the mutation status of the 4 different splice genes were similar (Figure 3A-H). Next, we evaluated these 2 endpoints in low- and high-risk MDS patients defined by their IPSS score. Except for a higher AML transformation rate of *ZRSR2*^{mut} patients compared with *ZRSR2*^{wt} patients in the IPSS-low or intermediate-1 subgroups (P = .022), no differences on patient outcome according to the splice gene mutation status were observed (supplemental Figures 1-2). As specific molecular associations between mutations targeting epigenetic and splice activities were identified, we analyzed the prognostic impact of these molecularly defined genotypes. No difference in patient outcome was observed when analyzing the genotypes *SF3B1*^{mut}/*DNMT3A*^{mut} or *SF3B1*^{mut}/*DNMT3A*^{wt} (data not shown). In contrast, *SF3B1*^{wt}/*DNMT3A*^{mut} patients had an inferior OS (hazard ratio [HR] = 2.35; 95% CI, 1.09-5.46; P = .033) and a higher risk of AML transformation (HR = 2.7; 95% CI, 1.05-7.68; P = .04; Figure 4A-B) compared with all other genotypes (*SF3B1*^{mut}/*DNMT3A*^{mut} or *SF3B1*^{mut}/*DNMT3A*^{wt} or *SF3B1*^{wt}/*DNMT3A*^{wt}). An inferior OS and a higher transformation rate to AML was also seen in patients defined by the genotype *SRSF2*^{mut}/*TET2*^{wt} in univariate analysis (OS: HR = 2.32; 95% CI, 1.05-5.11; P = .031; AML transformation: HR = 2.94; 95% CI, 1.13-7.61; P = .02; Figure 4C-D). Patients with the genotype *ZRSR2*^{mut}/*TET2*^{wt} had a shorter OS (HR = 2.21; 95% CI, 1.01-4.85; P = .042) and tended to have a higher rate for AML transformation (HR = 2.4; 95% CI, 0.94-6.81; P = .077; Figure 4E-F) compared with all other genotypes.

In multivariate analysis, including age, IPSS risk groups, transfusion dependence, mutation status for *ASXL1*, *RUNX1*, *TP53*, *CBL*, and the genotypes *DNMT3A*^{mut} /*SF3B1*^{wt}, *SRSF2*^{mut}/*TET2*^{wt}, and *ZRSR2*^{mut}/*TET2*^{wt}, the genotype *ZRSR2*^{mut}/*TET2*^{wt} was found to be an independent unfavorable prognostic factor for OS (HR = 3.3; 95% CI, 1.4-7.7; P = .006) and *ZRSR2*^{mut}/*TET2*^{wt} independently associated with a higher AML transformation rate (HR = 3.6; 95% CI, 2-4.2; P = .026; Table 3).

Discussion

Identification of novel targets to direct new treatment approaches remains a major challenge in MDS. Recent discovery of novel pathway mutations affecting spliceosome core components prompted us to investigate the 4 most recurrently mutated genes in a large cohort of 221 MDS patients.[11] Our present study confirms the high prevalence of mutations affecting splicing activity in this heterogeneous disorder.[11] Almost half of investigated patients presented a mutation in *SF3B1* (16.4%), *SRSF2* (11.1%), *ZRSR2* (11.1%), or *U2AF35* (5.4%). We identified distinct clinical phenotypes and molecular association patterns for each splice gene. While *SF3B1*^{mut} patients were likely to present with RSs and reduced hemoglobin levels leading to a higher transfusion dependence rate, patients harboring *SRSF2* mutations clustered in RAEB-1 and RAEB-2 subtypes and had pronounced thrombocytopenias. *ZRSR2*^{mut} patients had a similar clinical phenotype, clustered in IPSS intermediate-1 and intermediate-2 risk groups, had higher bone marrow blast percentages, and often exhibited isolated neutropenias. The association between a splice gene mutation and a specific clinical phenotype remained independently significant when analyzed together with IPSS-risk group and WHO disease subtype in a linear regression model (eg, *SF3B1* mutation and reduced hemoglobin levels [P = .011], or *SRSF2* mutation and thrombocytopenia [P = .025]). This suggests that splice gene mutations play a causal role in the disease course. Interestingly, each splice gene mutation was associated with one concomitant

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Table 2. Molecular associations of splice gene mutations (Fisher exact test)

	TET2^{mut} (n = 54)	*ASXL1*^{mut} (n = 37)	*DNMT3A*^{mut} (n = 26)	*RUNX1*^{mut} (n = 16)	*TP53*^{mut} (n = 13)	*IDH1/2*^{mut} (n = 12)	*CBL*^{mut} (n = 11)	*JAK2*^{mut} (n = 9)	*EZH2*^{mut} (n = 7)	*ETV6*^{mut} (n = 2)	*NRAS*^{mut} (n = 2)
SF3B1^{mut} (n = 37)	9	4	13 (P < .001)	3	1	1	1	3	1	0	0
SRSF2^{mut} (n = 25)	11 (P = .02)	6	4	4 (P = .09)	1	3	3	0	0	0	0
ZRSR2^{mut} (n = 25)	14 (P < .001)	5	1	3	0	3	1	0	1	0	0
U2AF35^{mut} (n = 12)	1	5 (P = .03)	0	0	0	1	1	0	1	0	0

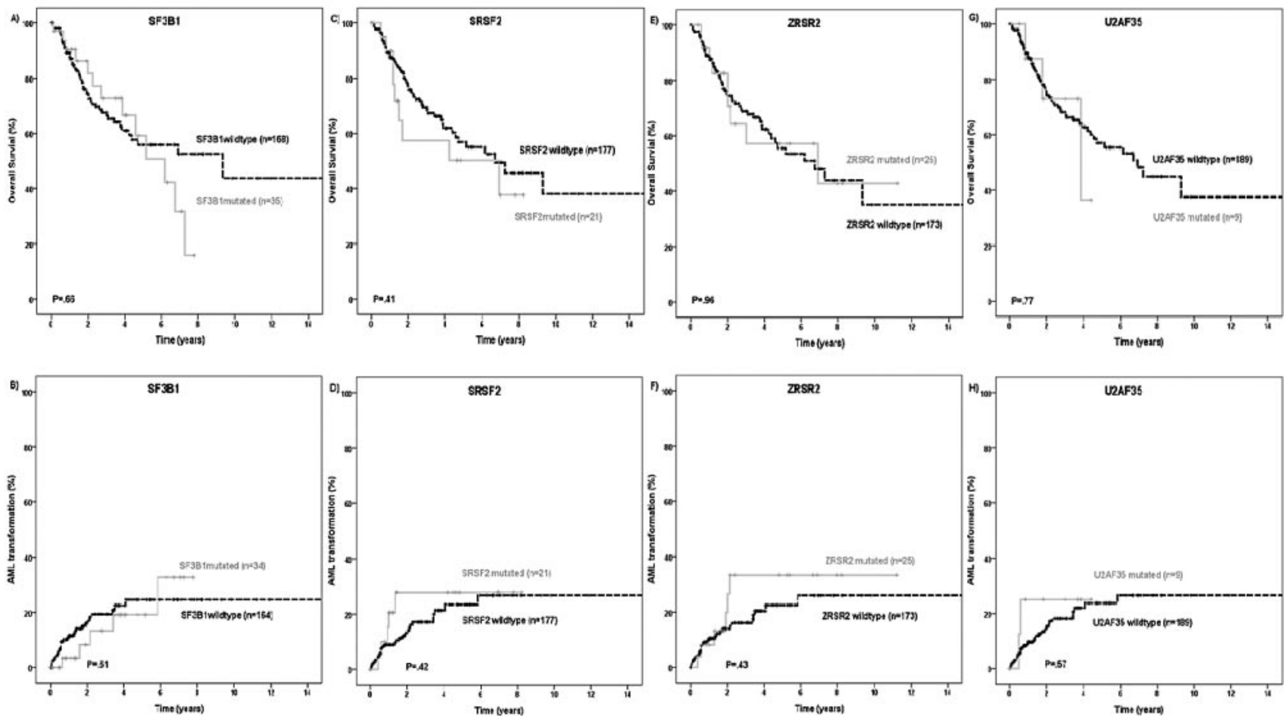

Figure 3. Kaplan-Meier curves for OS and time to AML transformation. (A-B) The *SF3B1* mutation status. (C-D) The *SRSF2* mutation status. (E-F) The *ZRSR2* mutation status. (G-H) The *U2AF35* mutation status in 198 MDS patients (log-rank test).

mutation in a gene involved in epigenetic regulation of transcription. Although half of the patients with *DNMT3A* mutations also had *SF3B1* mutations, *TET2* mutations were significantly enriched in both *SRSF2* and *ZRSR2* mutated patients. Our results demonstrate *U2AF35* mutations to be enriched in patients with *ASXL1* mutations and confirm an earlier report that a deletion of chromosome 20 is often found in *U2AF35*^{mut} patients.[14] However, in contrast to Graubert et al,[14] whose analysis of 150 MDS patients

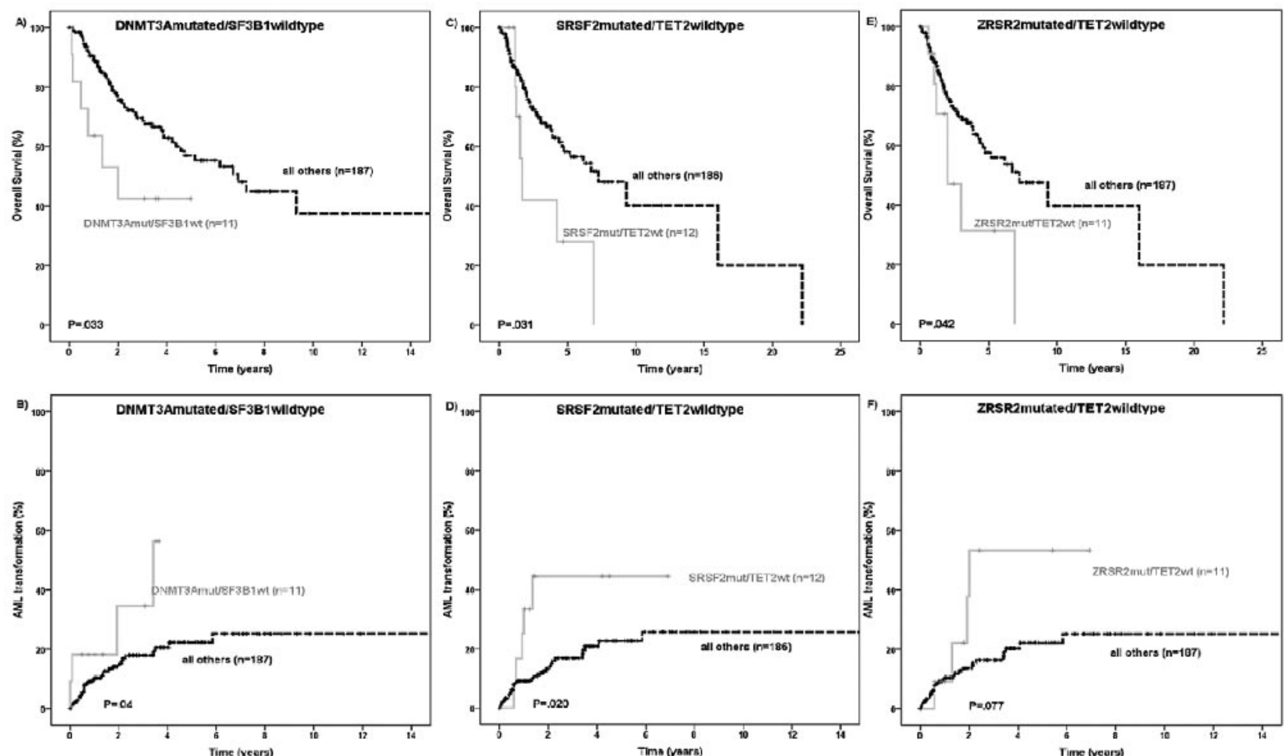

Figure 4. Kaplan-Meier curves for OS and time to AML transformation. (A-B) The *DNMT3A*/*SF3B1* mutation status. (C-D) The *SRSF2/TET2* mutation status. (E-F) The *ZRSR2/TET2* mutation status in 198 MDS patients (log-rank test).

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Table 3. Univariate and multivariate analysis for OS and time to AML transformation in MDS patients

	OS (univariate analysis)			OS (multivariate analysis)			AML transformation (univariate analysis)			AML transformation (multivariate analysis)		
	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*
Age, y (above vs below the median)	1.9	1.1-3.2	.013	1.7	0.9-3.0	.065			NS			
IPSS risk groups* (high vs low)	2.2	1.7-2.8	< .001	2.1	1.6-2.8	< .001	2.9	2.1-4	< .001	3.2	2.1-4.7	< .001
Transfusion dependence (yes vs no)	2.9	1.7-5.1	< .001	2.3	1.3-4.2	.005	2.4	1.1-5	.023	2.2	1-4.9	.042
$ASXL1^{mut}$ vs $ASXL1^{wt}$	2.0	1.2-3.5	.007			NS	2.8	1.4-5.6	.002			NS
$RUNX1^{mut}$ vs $RUNX1^{wt}$	2.0	1-4.1	.044			NS	4.6	2.2-9.9	< .001	3.1	1.3-7.4	.009
$TP53^{mut}$ vs $TP53^{wt}$	4.4	2.1-9.1	< .001	2.0	1-4.7	.041	3.9	1.5-10.2	.006			NS
CBL^{mut} vs CBL^{wt}	2.6	1.1-6.0	.027	4.4	1.7-11.6	.002	4.3	1.7-11.3	.003	4.1	1.4-12.4	.01
$DNMT3A^{mut}$ / $SF3B1^{wt}$ vs all other	2.4	1.1-5.5	.033			NS	2.7	1.1-7.7	.04			NS
$SRSF2^{mut}$ /$TET2^{wt}$ vs all other	2.3	1.1-5.1	.031			NS	2.9	1.1-7.6	.02			NS
$ZRSR2^{mut}$ /$TET2^{wt}$ vs all other	2.2	1.0-4.9	.042	3.3	1.4-7.7	.006	2.4	0.9-6.8	.077	3.6	2-4.2	.026

NS indicates not significant.
*IPSS-low indicates IPSS-low risk or intermediate-1; and IPSS-high, IPSS-high or intermediate-2.

suggested an increased probability of secondary AML progression for patients with *U2AF35* mutations, we observed no association between *U2AF35* mutations and transformation to AML. Whether the suggested higher transformation rate is because of *U2AF35* mutations or confounded by the presence of an *ASXL1* mutation, which was consistently shown to be associated with worse OS and higher AML transformation rates in myeloid malignancies,[8,10,28] must be verified in even larger cohorts of MDS patients. Of the 7 patients who carried a *U2AF35* mutation and were wild-type for *ASXL1*, one had progression to AML and died 10 months after MDS diagnosis. It is currently debated whether the recurrent *ASXL1* variation (c.1934dupG;p.Gly646TrpfsX12), which accounts for the majority of patients who we classified as mutated for *ASXL1,* is a bona fide somatic mutation or an artifact.[29] This alteration was found in the majority of patients who we classified as mutated for *ASXL1*. In accordance with previous reports, we did not detect this alteration in genomic DNA from $CD3^+$ cells of the same patients, when available.[10,30,31]

In contrast to some recent reports, we observed no favorable impact on OS for patients with *SF3B1* mutation.[16,32] This may be at least partially related to the heterogeneity of investigated cohorts: our study cohort includes a lower number of refractory anemia with RSs (RARS)/refractory cytopenia with multilineage dysplasia (RCMD)-RS or RARS-T patients compared with others (19% vs 29%)[16,32] and a higher number of advanced MDS or RAEB-1 or RAEB-2 subtypes (45% vs 26%).[32] However, when restricting survival analysis to patients with RSs (RARS, RCMD-RS or RARS-T; n = 36 with available clinical information), no difference on OS or AML transformation rate was observed (data not shown). These results are in accord with 2 recent reports investigating the prognostic importance of *SF3B1* mutations in larger cohorts of MDS patients with RSs, which did not find an independent value within this subgroup of patients where the highest prevalence of *SF3B1* mutations is found.[32] We identified a negative prognostic impact on OS and higher rate of AML transformation for $SF3B1^{wt}$/ $DNMT3A^{mut}$ patients. This finding is in accord with recent reports in AML and MDS, which demonstrated a strong association of *DNMT3A* mutations with adverse outcome.[7,33-35] Furthermore, we show that the prognostic impact of *SRSF2* and *ZRSR2* mutations depends on the mutation status of *TET2*, the gene most commonly mutated in MDS. These findings are of major interest as they link two pathways involved in the pathophysiology of MDS. Whether the identified genotype combinations are restricted to MDS or are also found in other myeloid or lymphoid malignancies needs to be established. However, the molecular crosstalk between these mutations is difficult to predict because loss of Tet2 has been demonstrated to endow cells with a growth advantage,[36-38] whereas overexpression of mutated *SRSF2* or *U2AF35* has antiproliferative effects[11] and will need to be investigated in appropriate settings. Our data support the idea that the mutations in the splice genes contribute to the clinical/biologic phenotype of the MDS clone.

In conclusion, we identified mutations of *SF3B1, SRSF2, ZRSR2,* and *U2AF35* in 42.2% of MDS patients and found a strong association between the clinical phenotype and the different splice gene mutations. A distinct molecular pattern involving the epigenetic regulation of transcription and the splicing machinery was identified and associated with patient outcome. Although confirmation of these results in larger cohorts is necessary before redefining risk-guided therapy strategies in MDS, integration of molecular analysis of the splice genes at diagnosis may improve classification of MDS patients.

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Authorship

Contribution: F. Damm, O.K., O.A.B., and M.F. designed the research; F. Damm, O.K., V.G.-B., A.R., C.H.-C., V.D.V., L.C., L.S., V.C., and N.C. performed the research; A.G.-B., B.S., O.B.-R., A.S.-T., A.S.-B., F. Dreyfus, T.P., S.d.B., N.V., D.B., and

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C.P. contributed patient samples and clinical data; F. Damm, O.K., V.G.-B., A.R., M.A.M., N.C., and N.C.P.C. analyzed the data; F. Damm, O.K., O.A.B., and M.F. wrote the manuscript; and all authors read and agreed to the final version of the manuscript.

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