Spliceosome mutations exhibit specific associations with epigenetic modifiers and proto-oncogenes mutated in myelodysplastic syndrome

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ABSTRACT

The recent identification of acquired mutations in key components of the spliceosome machinery strongly implicates abnormalities of mRNA splicing in the pathogenesis of myelodysplastic syndromes. However, questions remain as to how these aberrations functionally combine with the growing list of mutations in genes involved in epigenetic modification and cell signaling/transcription regulation identified in these diseases. In this study, amplicon sequencing was used to perform a mutation screen in 154 myelodysplastic syndrome patients using a 22-gene panel, including commonly mutated spliceosome components (*SF3B1*, *SRSF2*, *U2AF1*, *ZRSR2*), and a further 18 genes known to be mutated in myeloid cancers. Sequencing of the 22-gene panel revealed that 76% (n=117) of the patients had mutations in at least one of the genes, with 38% (n=59) having splicing gene mutations and 49% (n=75) patients harboring more than one gene mutation. Interestingly, single and specific epigenetic modifier mutations tended to coexist with *SF3B1* and *SRSF2* mutations (P<0.03). Furthermore, mutations in *SF3B1* and *SRSF2* were mutually exclusive to *TP53* mutations both at diagnosis and at the time of disease transformation. Moreover, mutations in *FLT3*, *NRAS*, *RUNX1*, *CCBL* and *C-KIT* were more likely to co-occur with splicing factor mutations generally (P<0.02), and *SRSF2* mutants in particular (P<0.003) and were significantly associated with disease transformation (P<0.02). *SF3B1* and *TP53* mutations had varying impacts on overall survival with hazard ratios of 0.2 (P<0.03, 95% CI, 0.1- 0.8) and 2.1 (P<0.04, 95% CI, 1.1- 4.4), respectively. Moreover, patients with splicing factor mutations alone had a better overall survival than those with epigenetic modifier mutations, or cell signaling/transcription regulator mutations with and without coexisting mutations of splicing factor genes, with worsening prognosis (P<0.001). These findings suggest that splicing factor mutations are maintained throughout disease evolution with emerging oncogenic mutations adversely affecting patients' outcome, implicating spliceosome mutations as founder mutations in myelodysplastic syndromes.

Introduction

Myelodysplastic syndromes (MDS) comprise a heterogeneous group of clonal hematopoietic stem-cell disorders with diverse phenotypes, characterized by varying severity of ineffective hematopoiesis, bone marrow dysplasia, rate of progression to acute myeloid leukemia (AML), overall survival (OS) and response to therapy.[1-3] Cytogenetic abnormalities are detected in up to 60% of patients, in whom the type and complexity of these aberrations correlate with progression, leukemia transformation and response to therapy.[2,4] Furthermore, application of high-density single nucleotide polymorphism arrays has led to the enhanced detection of smaller chromosomal aberrations, including micro-deletions or uniparental disomy with an overall loss of heterozygosity.[5,6]

Recent studies have found mutations involving multiple components of the mRNA splicing machinery including *SF3B1*, *SRSF2*, *U2AF1*, *ZRSR2*, *PRPF40B*, *U2AF65* and *SF1* in patients with MDS, myeloproliferative disorder (MPN) and AML.[7-18] Moreover, the most frequently mutated spliceosome component in MDS, *SF3B1* (30% of cases), is aberrant in 70-85% of cases of refractory anemia with ringed sideroblasts (RARS) and is highly associated with the presence of ringed sideroblasts.[7,11] Fundamentally, however, the influence of such *SF3B1* mutations is not just in myeloid tissue and RARS, but has now been observed in chronic lymphocytic leukemia and lymphoid tissue,[19,20] suggesting that genetic background plays an important role in the functional manifestation of spliceosome aberrations.

Over the past decade a number of novel gene mutations that are associated with MDS have been identified, including genes involved in epigenetic regulation (*TET2*,[21] *DNMT3A*,[22] *IDH1/2*,[23] *ASXL1*[24] and *EZH2*[25]), suggesting an underlying genomic instability or aberrant transcription regulation in the evolution of this disease. Moreover, the occurrence in MDS of known oncogenic mutations or mutations in genes involved in cell signaling/transcription regulation has also been extensively studied in recent years, including mutations in *TP53* (8%),[26] *NRAS/KRAS*,[27] *RUNX1* (9%),[28] *FLT3* (6%),[29,30] *ETV6* (3%)[28] and *CCBL* (2.3%).[28] In fact, around 80% of MDS patients have defects in one or more of these 'epigenetic' or 'oncogenic' factors. A recent study by Bejar *et al.* showed that mutations in

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five genes (*TP53*, *RUNX1*, *EZH2*, *ASXL1* and *ETV6*) are independent predictors of poor OS in patients with MDS.[28] Data from this study and elsewhere have shown that *TP53* remains the only gene with a statistically robust prognostic impact in MDS. However, aberrations in *DNMT3A*[31] and *FLT3*,[29] which have previously been attributed prognostic significance, were not analyzed in this study. In another study, Thol *et al.* investigated various epigenetic, cell cycle/apoptotic genes and spliceosome components in 193 patients with MDS, and found *SRSF2* mutations were associated with *RUNX1* and *IDH1* mutations while *U2AF1* mutations were associated with *ASXL1* and *DNMT3A* mutations. In addition to this, *SRSF2* mutations were associated with poor OS and more frequent progression to AML.[10] However, several genes, including *FLT3*, *CCBL*, *JAK2*, *TET2* and *EZH2*, which are frequently mutated in MDS were not analyzed in these patients. Therefore, to gain a better understanding of spliceosome aberrations and how they interact with other coexisting mutations, as well as to determine their prognostic significance in isolation or in combination with other mutations, we performed a comprehensive mutation screen in 154 MDS patients.

Design and Methods

Clinical data and patients' samples

Eight MDS patients [5 with RARS, 1 with RARS in transformation (RARS-T), 1 with refractory cytopenia with multilineage dysplasia and ringed sideroblasts (RCMD-RS) and 1 with therapy-related MDS] with >50% ringed sideroblasts were initially selected for whole-exome sequencing. An additional 146 MDS patients were selected for mutation analysis of the splicing pathway genes, epigenetic modifiers and other cell signaling/transcription regulator genes, followed by validation of the candidate mutations. Patients with MDS seen at King's College Hospital from June 2004 to June 2011 were enrolled in this study. All patients provided written informed consent in accordance with National Research Ethics Protocol (KCLPR060 PR029). The demographic and clinical characteristics of the studied patients are detailed in Table 1. All patients were risk stratified according to International Prognostic Scoring System (IPSS) categories. The clinical variables, French-American-British classification, World Health Organization (WHO) subtype and the prognostic risk of all patients were ascertained at the time of sample collection. The median follow-up was 21.4 months (range, 1-83 months). The cohort was followed up to January 2012

Table 1. Clinical characteristics of the patients studied. Patients were stratified by the presence or absence of splicing factor mutations *SF3B1*, *SRSF2* and *U2AF1*. Cytogenetics failed in four patients in this cohort. *P* values with statistical significance are highlighted in bold. **Epigenetic *modifiers*-*TET2*, *ASXL1*, *DNMT3A*, *IDH2*, *EZH2* and *IDH1*; **Cell signaling/transcription regulators -*FLT3*, *RUNX1*, *NRAS*, *CKIT*, *CCBL*, *JAK2* and *MPL*; ***TP53*. n- represents number of patients; % - represents percentage of patients.

Patients' characteristics	Overall	*SF3B1* mutant	*SF3B1* wild type	*P* value	*SRSF2* mutant	*SRSF2* wild type	*P* value	*U2AF1* mutant	*U2AF1* wild type	*P* value
	154	24 (16%)	130 (84%)		20 (13%)	134 (87%)		15 (10%)	139(90%)	
Age, years				0.42			0.16			0.8
Median	65.5	65.2	63.1		66.9	63.1		62.8	63.5	
Range	17-85	35-83	17-85		51-82	17-85		48-75	17-85	
Sex				0.98			0.5			0.38
Male [n (%)]	104 (67%)	16 (66%)	88 (68%)		12 (60%)	92(69%)		12 (80%)	92(66%)	
WHO category *				**<0.001**			**<0.001**			0.2
RA/RCMD [n (%)]	40 (26%)	0 (0%)	40 (31%)		6 (30%)	34 (25%)		4 (27%)	36 (26%)	
RARS/RCMD-RS [n (%)]	24 (16%)	20(83%)	4 (3%)		0 (0%)	24 (18%)		0 (0%)	24 (17%)	
RAEB -1/2 [n (%)]	49 (32%)	1 (4%)	48 (37%)		7 (35%)	42 (32%)		5 (33%)	44 (32%)	
s AML [n (%)]	15 (10%)	2 (8%)	13 (10%)		0 (0%)	15 (11%)		4 (27%)	11 (8%)	
t MDS/AML [n (%)]	12 (8%)	0 (0%)	12 (9%)		0 (0%)	12 (9%)		1 (7%)	11 (8%)	
CMML & MPD/ MDS-U [n (%)]	14 (9%)	1 (4%)	13 (10%)		7 (35%)	7 (5%)		1 (7%)	13 (9%)	
Bone marrow blasts				0.19			0.39			0.27
Median (%)	5	1	6		8.5	4		9	4	
Range	0-80	0-19	0-72		0-19	0-80		0-59	0-80	
IPSS cytogenetic risk group	151			**<0.001**			0.15			0.46
Good [n. (%)]	90	22 (96%)	68(53%)		14 (70%)	76 (58%)		7 (50%)	83(61%)	
Intermediate [n. (%)]	17	1 (4%)	16 (13%)		4 (20%)	13(11%)		3 (21%)	14 (11%)	
Poor [n. (%)]	44	0 (0%)	44 (34%)		2 (10%)	42 (31%)		4 (29%)	40 (28%)	
Transfusion dependency				**0.04**			0.3			0.19
Yes [n (%)]	80 (51%)	17 (71%)	63 (47%)		8 (40%)	72 (52%)		10 (66%)	70 (49%)	
Progression to AML				**0.02**			**0.02**			0.4
Yes [n. (%)]	44 (28%)	2 (8%)	42 (31%)		10 (50%)	34 (24%)		3 (20%)	41 (29%)	
Co-existing mutations **										
Epigenetic Modifier mutations [n. (%)]	80 (52%)	14 (58%)	66 (51%)	0.5	14 (70%)	66 (49%)	0.07	9 (60%)	71 (54%)	0.6
Cell signaling/transcription regulator mutations [n (%)]	28 (18%)	3 (12%)	25 (19%)	0.5	10 (50%)	18 (13%)	**0.03**	2 (13%)	26 (19%)	0.9
TP53 mutations [n (%)]	19(12%)	0 (0%)	19 (15%)	**0.04**	0 (0%)	19 (14%)	0.07	3 (20%)	16 (12%)	0.4

for disease progression, and survival. The survival data for patients who underwent allogeneic hematopoietic stem cell transplantation (HSCT) (n=35, 22%) were censored on the day of the transplant and the treatments received by other patients are annotated in *Online Supplementary Table S1*.

DNA was extracted from CD34$^+$ cells (n=8), CD34$^-$CD3$^+$ (n=18), CD34$^-$CD235$^+$ (n=1), CD71$^+$CD235$^+$ (n=3), CD34$^-$CD235$^-$ (n=3), CD34$^-$CD3$^+$CD4$^+$ (n=3), CD34$^-$CD19$^+$ (n=3), skin (n= 27), buccal swab (n=3) and bone marrow total nucleated cells (n=154) using QIAamp DNA extraction kits (Qiagen) according to the manufacturer's protocol. Out of 154 cases, whole-genome-amplified DNA was used in 40 cases for mutation screening.

Amplicon sequencing

Mutation screening for *SF3B1*, *SRSF2*, *U2AF1*, *ZRSR2*, *TP53*, *FLT3*, *DNMT3A*, *ASXL1*, *EZH2*, *NRAS*, *KRAS*, *JAK2*, *CCBL*, *RUNX1*, *CEBPA*, *BRAF*, *MPL*, *NPM1*, *IDH1*, *IDH2*, *C-KIT* and *TET2* was performed on bone marrow total nucleated cell DNA using the Roche GS FLX platform as described previously[21] (see *Online Supplementary Methods* for details; *Online Supplementary Tables S2* and *S3*).

Exome sequencing

Eight patients with more than 50% ringed sideroblasts were selected for whole-exome sequencing, using DNA from CD34$^+$ cells. The exomic regions of the genome were enriched using Agilent SureSelect Human All Exon Kit and paired end sequencing was performed using Illumina HiSeq 2000 (version 3 chemistry) (see *Online Supplementary Methods* for details).

Statistical analysis

Statistical calculations were performed using SPSS version 17.0 (SPSS Inc.) as described in the *Online Supplementary Methods*. A *P* value of ≤0.05 was considered statistically significant.

Results

Somatic mutations in myelodysplastic syndromes

Whole-exome sequencing (Illumina) using CD34$^+$ cells from eight RARS patients initially revealed mutations in *SF3B1* in seven cases (*Online Supplementary Results; Online Supplementary Tables S4* and *S5*).

We next utilized a 22-gene amplicon sequencing panel for genes known to be mutated in MDS for an additional 146 MDS patients comprising: splicing factor genes *SF3B1*, *SRSF2*, *U2AF1* and *ZRSR2*; genes implicated in epigenetic regulation *TET2*, *IDH1/2*, *ASXL1*, *EZH2* and *DNMT3A*; and known oncogenes/genes involved in cell signaling/transcription regulation: *TP53*, *FLT3*, *NRAS*, *KRAS*, *RUNX1*, *CCBL*, *C-KIT*, *JAK2*, *MPL*, *CEBPA*, *BRAF* and *NPM*. Acquired mutations in any one or more of these genes were detected in 76% (n=117) of the cohort, with 38% (n=59) of the patients having splicing factor mutations and 49% (n=75) of patients harboring more than one mutation (Figure 1 and *Online Supplementary Table S6*). Known single nucleotide polymorphisms and insertion/deletion variants listed in the International Center for Biotechnology Information Single Nucleotide Polymorphism database (dbSNP, build 135) and previously reported as germ line were excluded from further analysis. Novel mutations present in 48/54 cases which had not been previously reported in the literature were confirmed as acquired by their absence in paired constitutional DNA. The remaining seven novel variants (3 stop codons, 1 frame-shift mutation, 1 in-frame-shift deletion and 2 splice-site mutation) detected in six patients were also included in the analysis although a constitutional source of DNA was not available (*Online Supplementary Table S6*). The nature of these mutations makes it unlikely that they are benign inherited variants. The remainder of the mutations identified in this study had previously been reported in the literature as acquired mutations.

Spliceosome gene mutations: frequency and clinical correlates

Sequencing of the splicing factor genes revealed 61 somatic mutations in 38% (59 of 154) of MDS patients (Table 1 and *Online Supplementary Table S6*), comprising *SF3B1* 16% (n=24), *SRSF2* 13% (n=20), *U2AF1* 10% (n=15) and *ZRSR2* 1% (n=2). WHO subgroups for mutations of splicing factor genes included, RARS/RCMD-RS (20/24, 83%), chronic monomyelocytic leukemia (CMML) or MDS/MPN (9/14, 64%), secondary AML (6/15, 40%), refractory anemia with excess blasts (RAEB)-1/2 (13/49, 27%), and refractory anemia/RCMD (10/40, 25%), but were uncommon in therapy-related MDS (1 of 12). Importantly, splicing factor mutations were more common in patients in low/int-1 IPSS categories (36/68, 53%) than in those in int-2/high risk IPSS categories (12/63, 19%, P<0.002). Furthermore, with the exception of one case, all patients with isolated splicing factor mutations and no additional coexisting mutations (n=21) were in the low/int-1 IPSS categories. Patients with complex karyotypes were less likely than those in good and intermediate risk IPSS cytogenetic groups to harbor any splicing factor mutation (13 % *versus* 47% and 44%, P<0.002).

Overall, 24 of 154 (16%) MDS patients had a somatic mutation of *SF3B1*; however, the frequency of *SF3B1* mutation was significantly higher in patients with RARS/RCMD-RS (20/24, 83%) than in patients in other WHO categories (4/130, 3%, P<0.001). Although there were no significant differences in patients between the groups with respect to age, sex, blast percentage and neutrophil count, *SF3B1* mutations correlated strongly with lower hemoglobin concentration (median-8.9 *versus* 10.1 g/dL, P<0.006), higher platelet count (median-296 *versus* 102x10^9/L, P<0.001), low/int-1 risk IPSS score (31% *versus* 0%, P<0.001), normal cytogenetics (24% *versus* 0%, P<0.002), transfusion dependency (21% *versus* 9%, P<0.03) and a decreased likelihood of leukemic progression (4% *versus* 15%, P<0.02) when compared with wild-type *SF3B1*.

Among the 154 MDS patients, 20 had *SRSF2* mutations (13%) and showed a significantly higher neutrophil count (median 11 *versus* 2.8x10^9/L, P<0.001) and higher hemoglobin levels (median 10.9 *versus* 9.8 g/dL, P<0.03) compared to patients with wild type *SRSF2*. There was no difference in platelet count or transfusion dependency rate between the groups. *SRSF2* mutations were more frequently seen in patients with MDS/MPN or CMML (50%) and RAEB-1/2 (14%), but were absent in patients with low-risk IPSS, including those with ringed sideroblasts. Interestingly, both patients with isochromosome 17q (n=2) had mutations of the *SRSF2* gene which maps to 17q25.1. There was a significant difference in rates of leukemic progression between patients with mutant or wild-type *SRSF2* (50% *versus* 24%, P<0.02), with two-thirds of patients with co-existing *SRSF2* and cell signaling/transcription regulator mutations progressing to AML.

U2AF1 mutations were detected in 15 (10%) patients

with clustering in male patients (12/15, 80%) and was also associated with lower hemoglobin (median 9 *versus* 10 g/dL, $P<0.05$) when compared to wild-type *U2AF1*, but no significant differences in age, platelets, neutrophils, transfusion dependence, IPSS score or WHO subtype were observed between the two groups. *ZRSF2* mutations were detected in only two (1%) MDS patients.

Interestingly, splicing factor mutations were largely mutually exclusive to each other, with only two patients having two separate spliceosome gene mutations, one with mutations in *SF3B1* and *U2AF1* and the other with mutations in *SF3B1* and *ZRSR2* genes.

Splicing factor mutations: type, site and allele burden

All *SF3B1* mutations were non-synonymous amino acid substitutions with an average mutant allele burden of 41% (n=24), indicative of a heterozygous state. Amino acids affected were K700E (n=11), H662Q (n=4), K666Q/R (n=2), E622D (n=2), D781G (n=1) and R625C (n=1) and clustered in the protein c-terminal HEAT motifs implicated in snRNP stabilization within the U2 snRNP complex of the major spliceosome.[32]

Similarly, the majority of *SRSF2* mutations were non-synonymous amino acid substitutions, with a heterozygous profile and an average mutant allele burden of 37.5% (n=20), comprising P95H/L/R (n=16) changes as previously reported.[8] Importantly, a novel 24-base-pair deletion in *SRSF2* causing the frameshift mutation Y93fsX121 was detected in four patients: this deletion could be predicted to cause loss of protein function.

In contrast, *U2AF1* mutations had a lower average mutant allele burden of 26.7% (n=15) and were exclusively S34F (n=4) or Q157P/R/H (n=11) amino acid changes, found within the amino- and the carboxyl-terminal zinc finger motifs, respectively, flanking the U2AF homology motif (UHM) domain. On the other hand, *ZRSR2* mutations had a much higher average mutant allele burden of 60.5% (n=2), were distinct in nature and did not cluster in the same protein domain, comprising an in-frame deletion (S439_R440del) and a frame-shift deletion E133Gfs11X.

Prevalence of mutations in epigenetic modifiers and cell signaling /transcription regulators

The overall frequency of mutations of genes involved in epigenetic regulation (*TET2*, *ASXL1*, *DNMT3A*, *IDH2*, *EZH2* and *IDH1*), cell signaling/transcription regulators (*FLT3*, *RUNX1*, *NRAS*, *C-KIT*, *CCBL*, *JAK2* and *MPL*) and mutations in tumor suppressor gene *TP53* were 52% (n=80), 18% (n=28) and 12% (n=19), respectively (Table 1 and *Online Supplementary Tables S6 and S7*). Interestingly, mutations predicted to effect epigenetic regulation were detected in nearly half of the MDS patients, with *TET2* mutations being the most frequent in 22% (n=34) of the cohort. The frequency of other mutations was *ASXL1* (17%, n=26), *DNMT3A* (10%, n=15), *IDH2* (8%, n=13), *EZH2* (7%, n=11) and *IDH1* (1%, n=2). Although mutations in epigenetic modifiers clustered in female patients (64% *versus* 25%, $P<0.05$), there were no differences in WHO subtypes, IPSS score, transfusion dependency or leukemic transformation rate when compared with cases wild-type for genes involved in epigenetic regulation. Furthermore, mutations in genes involved in cell signaling/transcription

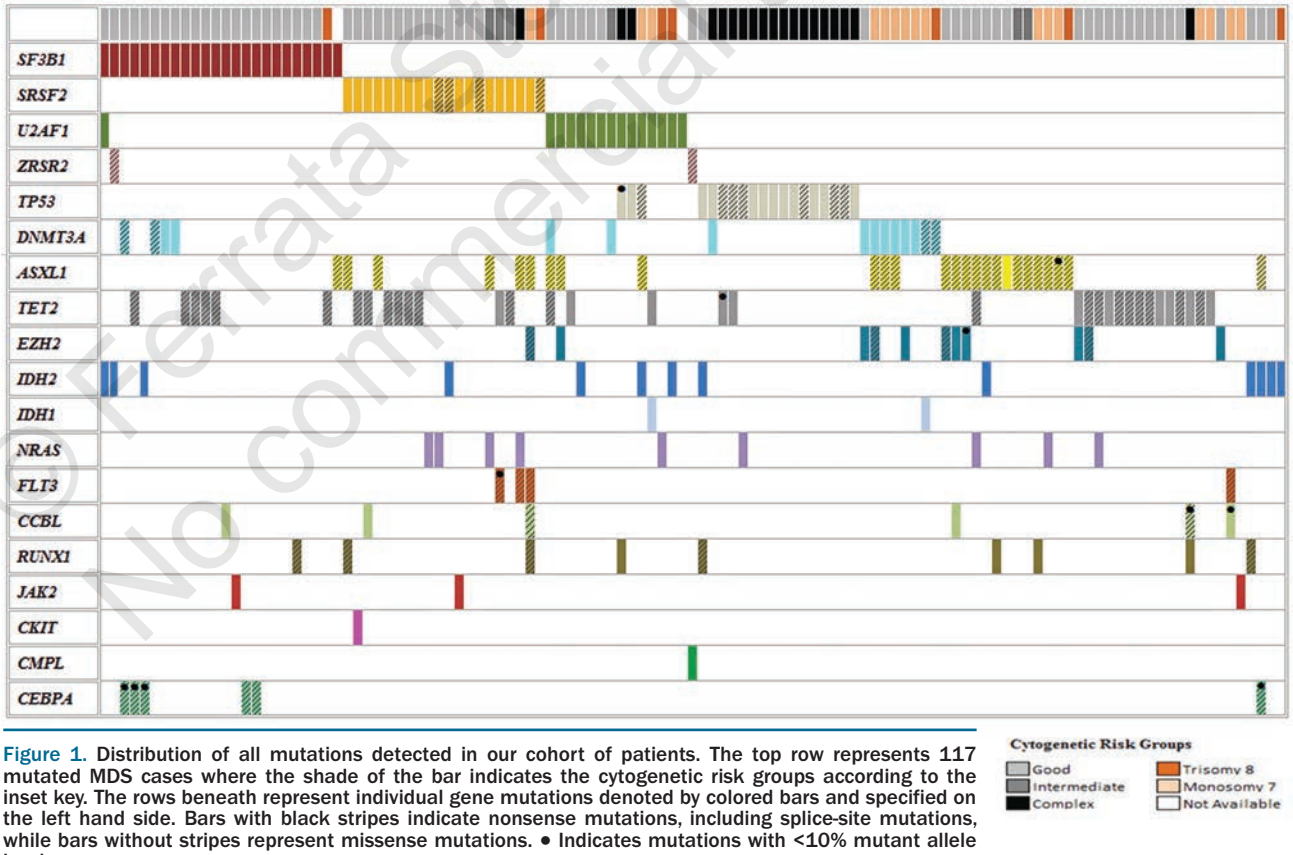

Figure 1. Distribution of all mutations detected in our cohort of patients. The top row represents 117 mutated MDS cases where the shade of the bar indicates the cytogenetic risk groups according to the inset key. The rows beneath represent individual gene mutations denoted by colored bars and specified on the left hand side. Bars with black stripes indicate nonsense mutations, including splice-site mutations, while bars without stripes represent missense mutations. • Indicates mutations with <10% mutant allele burden.

regulation were detected in 18% of MDS patients with mutations of *NRAS* and *RUNX1* each present in 6% (n=9 each), *CCBL* (4%, n=6), *FLT3* (3%, n=4) and *JAK2* (2%, n=3). *C-KIT* and *MPL* mutations were detected in one patient each. Overall these mutations, with the exception of *JAK2* and *MPL*, were associated with high-risk MDS, increased blast count, transfusion dependency and increased likelihood of leukemic transformation when compared to their wild-type counterparts (P<0.01). No *BRAF*, *NPM* and *KRAS* mutations were found in our cohort of patients.

Mutual exclusivity of TP53 with spliceosome components

TP53 mutations were detected in 12% (19/154) of cases (*Online Supplementary Table S7*). *TP53* mutations were infrequent in patients with splicing factor mutations (5%, 3/59) compared to in patients with wild-type splicing factor genes (17%, 16/99, P<0.04). However, among patients with splicing factor mutations, all three *TP53* mutations were observed exclusively in those who had mutations in the spliceosome component *U2AF1* (20% *versus* 0%, P<0.01).

Coexistence and exclusivity of splicing factor mutations with commonly mutated epigenetic modifiers and cell signaling/transcription regulating genes

Of the 59 patients with spliceosome mutations, 16 (27%) had isolated splicing factor mutations, while 28 (48%) and 15 (25%) had mutations in epigenetic modifiers and cell signaling/transcription regulator mutations, respectively, including eight patients with coexisting mutations from all three mutation classes (Figure 1; *Online Supplementary Figure S1A,B*, *Online Supplementary Table S7*). Regardless of disease subtypes, MDS cases with non-*SF3B1* splicing factor mutations had significantly more mutations in other genes screened here (mean 2.35 mutations/case) than did patients with *SF3B1* mutations (mean 1.85 mutations/case, P<0.03).

Furthermore, mutations of epigenetic modifiers were associated with mutant *SRSF2* compared to wild type (70% *versus* 50%, P<0.07), which was predominantly due to the presence of more *TET2* mutations with mutant *SRSF2* than wild-type (40% *versus* 25%, P<0.04). Patients with splicing factor mutations were less likely to have multiple epigenetic modifier mutations (14%, n=5) compared to patients with wild-type splicing factors (33%, n=15) (P<0.03), although if present, multiple mutations of epigenetic modifiers more often coexisted with *U2AF1* (n=4) than with *SF3B1* or *SRSF2* (n=1) mutations. *DNMT3A* mutations were less likely to be seen with *SRSF2* mutations compared with other spliceosome mutations (0% *versus* 15%, P<0.08), while *ASXL1* mutations were more likely to occur together with *SF3B1* than with other splicing factor mutations (23% *versus* 4%, P<0.07), indicating non-random mutation associations and tolerances. A trend towards an association between *IDH2* and *U2AF1* was observed, when compared to other splicing mutations (P<0.06).

Significantly, for all MDS cases, mutations of genes involved in cell signaling/transcription regulation (*FLT3*, *NRAS*, *CCBL*, *RUNX1*, *JAK2*, *MPL* and *C-KIT*) clustered with splicing factor mutations (27% *versus* 13%, P<0.02). When splicing factor mutations were looked at individually, these non-*TP53* mutations co-existed frequently with *SRSF2* mutations (50% *versus* 13%, P<0.003), compared to MDS patients with wild-type *SRSF2*. Furthermore, when considering all 59 cases of splicing factor mutants alone, mutations of *FLT3*, *NRAS*, *CCBL*, *RUNX1*, *JAK2*, *MPL*, *C-KIT* and *TP53* were significantly less often associated with SF3B1 mutations than with other splicing factor alterations (8% *versus* 34%, P<0.009). *SRSF2* mutations co-existed with mutations of cell signaling/transcription regulation genes, especially with alterations of *NRAS* (P<0.04) and *FLT3* (P<0.03), compared with non-*SRSF2* splicing factor mutations (40% *versus* 13%, P<0.02). Furthermore, *NRAS* mutations (n=9) were mutually exclusive to aberrations of epigenetic modifiers *IDH2* (n=13) and *EZH2* (n=11). *CEBPA* mutations co-existed significantly with mutant *SF3B1* compared with other splicing factor mutations (21% *versus* 0%, P<0.008).

The average mutant allele burden for *SF3B1* and *SRSF2* mutations was 41% (24 cases) and 37.5% (20 cases), respectively. The average mutant allele burden of coexisting point mutations present alongside these splicing factor mutations was 35% and 37.5%, respectively, with the mutant allele burden of epigenetic modifiers (38% and 39%) being higher than cell signalling/transcription regulator mutations (27.5% and 28.75) for *SF3B1* and *SRSF2*, respectively. In contrast, *U2AF1* mutations had a lower average allele burden of 26.7% (15 cases) with 38.8% average mutation allele burden of other coexisting mutations where the burden of the cell signaling/transcription regulator mutations (45%) was higher than that of epigenetic modifier mutations (33%). This seemed to be due to the *U2AF1/TP53* mutant cases which had a relatively higher *TP53* than *U2AF1* mutant allele burden in 2/3 cases. *ZRSR2* mutation allele burden was, on average, 60.5% (2 cases).

Sequential acquisition of cell signaling/transcription regulating gene mutations in SF3B1 mutant clones with disease transformation

Only two of 24 patients with RARS/RCMD-RS and *SF3B1* mutations developed AML at different times after diagnosis (patient UPN RC060337 at 4 months; patient UPN RC090006 at 36 months). To investigate the contributions of *SF3B1* and coexisting mutations in disease evolution we screened relevant sequential samples for both these cases. Sequencing analysis of the samples taken from patient UPN RC060337 (A) and UPN RC090006 (B) at the time of diagnosis (A) and at presentation at our institute (B, 24 months after diagnosis) revealed the *SF3B1* mutation with a mutant allele burden of 39% and 42%, respectively (example for patient A in Figure 2 and *Online Supplementary Figure S2*). A *TET2* mutation was detected at diagnosis, with a mutant allele burden of 60%, in patient A (Figure 2) and a *RUNX1* mutation in patient B. However, patient B developed AML and underwent allogeneic HSCT in morphological remission, following induction chemotherapy. He relapsed with AML and lost his donor chimerism shortly after the transplant. The *SF3B1* and *RUNX1* mutation burdens were maintained at the same levels during the RARS stage and also during the AML phase after HSCT in patient B. Likewise, the allele burdens of *SF3B1* and *TET2* mutations remained the same at transformation in patient A. Interestingly, at the time of transformation to AML, patient A also acquired a mutation in *RUNX1* (F163Y) with a mutant allele burden of ≈30%, and a *FLT3*-ITD (F590_W603dupInsP) with a mutant allele burden of ≈50% (Figure 2 and *Online Supplementary Figure S2*). Following intensive chemotherapy, patient A attained a transient morphological remission but relapsed promptly with *FLT3*-ITD and *RUNX1* mutant allele burdens increasing to ≈80% and ≈45%, respectively. Importantly, the mutation allele burden of *SF3B1* and *TET2* genes remained constant at around 40%

to 50% throughout the disease, which as a heterozygous mutation would occur in a majority of sample cells. As such, *RUNX1* and *FLT3* mutations seemed to have evolved from the *SF3B1/TET2* clonal population and coexist in the same cells (*Online Supplementary Figure S2*).

Prognostic significance of mutations

The median OS of the entire cohort was 34.4 months [(95% confidence interval (CI): 16.9 to 51.9 months]. Univariate analysis revealed that patients with *SF3B1* mutations had a better OS [not reached (NR) *versus* 24.2 months, *P*<0.003] (Figure 3A) and progression-free survival (PFS) (NR *versus* 40.3, *P*<0.02) than patients with wild-type *SF3B1* (*Online Supplementary Figure S3A*), while none of the other splicing factor gene mutations had an impact on either outcome measure. The median OS of patients with any splicing factor mutation was significantly better than those with a wild-type spliceosome (NR *versus* 24.2 months, *P*<0.03) (Figure 3B), but no difference in PFS was seen between the groups (*Online Supplementary Figure S3B)*.

In a univariate model, there was no significant difference in either OS or PFS in patients with other individual mutations compared to patients with wild-type genes, except for *TP53* and *NRAS* (*Online Supplementary Figure S4A-D*). Among patients with epigenetic modifier mutations (n=80), a proportion also had coexisting splicing factor mutations (n= 36) and 44 patients had epigenetic modifier mutations alone. A trend towards a better survival was seen in the group of patients with epigenetic modifier mutations with splicing gene mutations compared with the rest (*P*<0.06), although the mutations of genes involved in cell signaling/transcription regulation and *TP53* mutations were evenly distributed in both groups (*Online Supplementary Figure S5A,B*).

Interestingly, patients harboring both mutations of splicing factor and of genes involved in cell signaling/transcription regulation or *TP53* (n=15) had an extremely poor OS (15.8 months *versus* NR, *P*<0.009) and PFS (12.5 months *versus* NR, *P*<0.001) when compared to patients with spliceosome mutations without mutations of genes involved in cell signaling/transcription regulation (n=44), (Figure 4A and 4B) although this group had only three patients with a *TP53* mutation.

Multivariable analysis using variables: age at sampling, WHO category, bone marrow blast count, IPSS cytogenetic group, transfusion dependency status, and *SF3B1*, *NRAS*, *TP53* mutations, revealed that *NRAS* mutations did not affect either OS or PFS. *SF3B1* and *TP53* mutations had varying impacts on OS with hazard ratios (HR) of 0.2 (*P*<0.03, 95% CI, 0.1- 0.8) and 2.1 (*P*<0.04, 95% CI, 1.1-4.4), respectively. None of the analyzed genotypes, including *SF3B1* and *TP53*, affected PFS in the multivariable model (*Online Supplementary Table S8*).

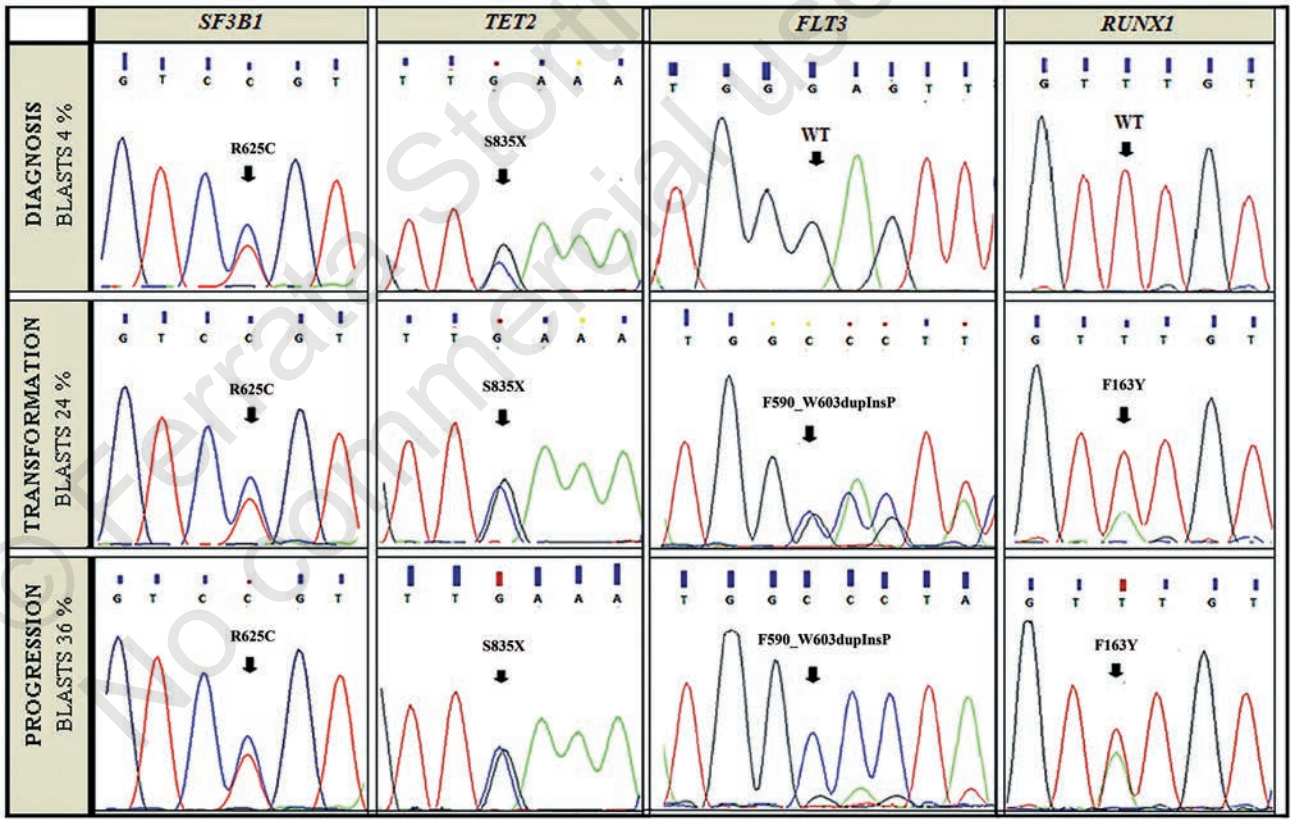

Figure 2. Clonal evolution and disease progression in a patient with RARS. Sequencing analysis of sequential samples in patient A (UPN RC060337) with an *SF3B1* mutation. The three horizontal rows represent samples collected at different time points: diagnosis, transformation to AML and disease progression after a short-lasting remission. Accumulation of the oncogenic mutations and changes in mutant allele burden levels are seen through disease progression along with an increase in the blast count. Sanger sequencing was used to confirm/determine the mutation status throughout the experiment. 454 sequencing confirmed the mutation level differences at start and end points of the experiment. WT-wild type.

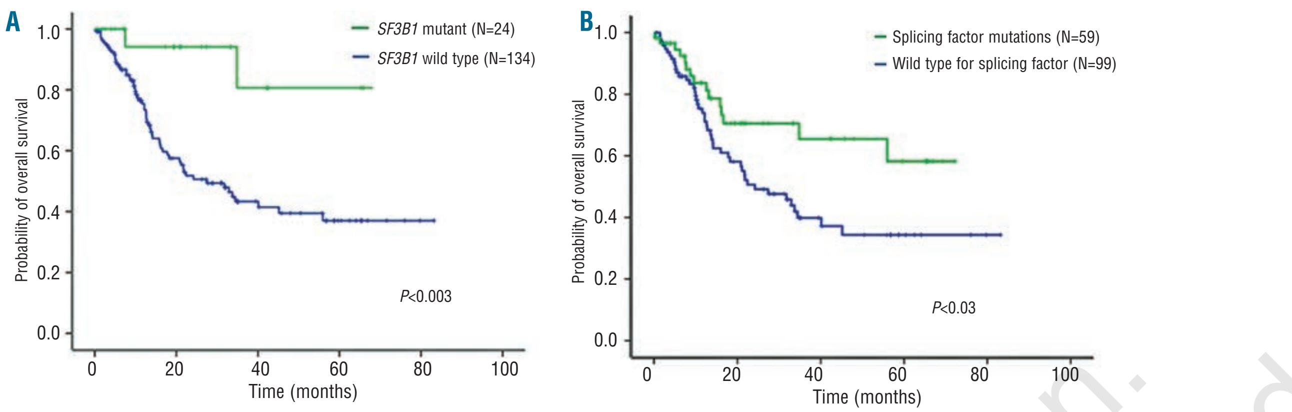

Figure 3. **Overall survival for patients with *SF3B1* mutations (n=24) compared to those with wild type *SF3B1* (n=130) (A) and overall survival for patients with spliceosome mutations (n=59) compared to patients without splicing factor mutations (n=99) (B).**

Discussion

In our study, 76% of MDS patients had mutations of the genes screened in this study, with nearly 50% of patients having more than one mutation. Splicing factor genes, *SF3B1*, *SRSF2*, *U2AF1* and *ZRSR2* were mutated in 38% of patients. Although collectively these mutations were found in a wide spectrum of MDS subtypes, some were strongly associated with specific disease features. For example, *SF3B1* mutations were strongly correlated with high levels of ringed sideroblasts and were, therefore, found in 80% of RARS/RCMD-RS cases, as reported in other studies.[7] Conversely, *SRSF2* and *U2AF1* mutations were often seen in advanced forms of MDS such as RAEB and CMML respectively, which fitted well with a higher number of coexistent mutations in genes involved in cell signaling/transcription regulation (e.g. *NRAS*, *FLT3* and *RUNX1*) with known oncogenic functions.

Occurrence of *SF3B1* mutations has been linked to significantly better OS and in some instances longer, leukemia-free and event-free survival in RARS.[7] Similarly, we show a beneficial, independent prognostic impact for *SF3B1* mutations on outcome, especially OS. In accordance with this observation is an absence of coexistent *TP53* aberrations, which is a strong predicator of poor OS according to data presented both here and elsewhere.[28] Furthermore, data from a cohort of 317 MDS patients indicated no influence of *SF3B1* mutations on OS or time to leukemic progression.[18] In addition to this, previous studies have also linked mutations of *U2AF1*[13,15] and *SRSF2*[13] with an increased risk of progression to AML and/or shorter OS. However, a study by Thol *et al.* demonstrated that only *SRSF2* mutations were associated with shorter OS as well as time to AML progression, whereas mutations in *U2AF1* were not.[10] We could only demonstrate a correlation of *SRSF2* mutations with progression to AML, but no impact on OS. *U2AF1* mutations similarly did not have any impact on the outcome in our study. Although *NRAS* and *TP53* mutations had an impact on outcome, this remained statistically significant in a multivariate model only for *TP53*.

Within the spliceosome mutant group, *SF3B1* seemed to be the strongest driver of a beneficial effect and the only spliceosome factor independently associated with better OS and PFS. Such a model possibly suggests that splicing factor mutations are early disease events and define a 'founder' disease clone that subsequently develops additional, increasingly deleterious mutations during the natural course of the disease, clonal evolution and disease progression. Furthermore, as particular splicing factor mutations differ in their patterns of association with other mutations, this suggests a hierarchy of tolerated mutational load and limitation to particular paths of disease evolution. For example, a dearth of co-existent *TP53* mutations throughout spliceosome mutant cases and mutual exclusivity of *TP53* mutations with *SF3B1* and *SRSF2* mutants at the time of diagnosis and disease transformation further supports a restrictive pattern of genetic insults. Furthermore, a restrictive pattern of additional coexisting mutations previously linked with disease transformation, such as *FLT3* and *RUNX1*, are seen alongside *SF3B1* mutations, although this might be expected in low-risk diseases such as RARS/RCMD-RS, which make up the majority of cases with *SF3B1* mutants. However, it is important to note that *SF3B1* mutant cases which gained such oncogenic mutations during transformation maintained a constant *SF3B1* mutant allele burden, indicating evolution within the same disease clone population.

Interestingly, *SRSF2* mutants, which are present in cases of both low- and high-risk disease, similarly did not coexist with *TP53* mutations here, but rather *FLT3*, *NRAS* or *RUNX1* mutant oncogenes, as was the case for transformed *SF3B1* mutants. Conversely, *U2AF1* mutations were found to coexist with *TP53* mutations here, although the *U2AF1* mutation burden was significantly lower than that of *TP53* in 2/3 of these cases, where TP53 mutation burden was high and consistent with loss of heterozygosity on chromosome 17p confirmed by metaphase cytogenetics in one of these cases. Furthermore, *FLT3* and *NRAS* mutations were independently more likely to occur with *SRSF2* mutations, accentuating the rate of leukemic transformation in these patients, but were less likely to occur with *SF3B1* mutant cases. The questions therefore remain: are such mutual exclusivities driven at a molecular level and due to a lethal combination of genetic lesions and do mutations in different spliceosome components carry different weights in terms of biological importance and different thresholds of additional insults that a particular mutant clone can endure?

Significantly, mutations of epigenetic modifiers also seemed to fall in with particular splicing factor mutations. For example, *ASXL1* mutations were less likely to coexist with *SF3B1* mutations than with *SRSF2* and *U2AF1* muta-

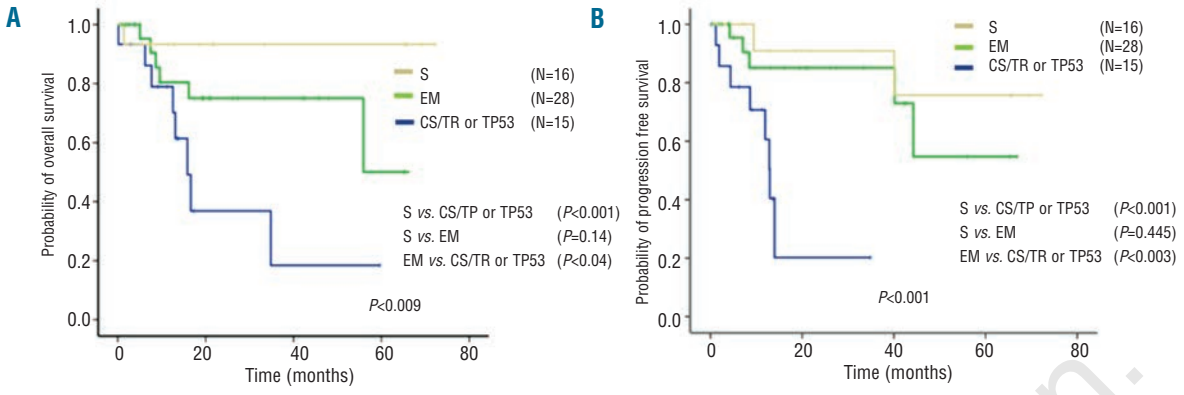

Figure 4. Overall survival (A) and progression-free survival (B) for patients with spliceosome mutations (n=59), stratified according to patients with splicing factor mutations (S) co-existing with mutations of genes involved in cell signaling/transcription regulation (CS/TR) or *TP53* mutations *versus* splicing factor mutations co-existing with epigenetic modifier (EM) mutations *versus* patients with splicing factor mutations alone.

tions, while *DNMT3A* mutations were not found with *SRSF2* but coexisted with *SF3B1* mutants (4/24). Again this highlights the importance of genetic background and how particular mutation combinations may attain an acceptable biochemical equilibrium and stable disease. It is of particular interest that only single epigenetic modifier mutations seemed to occur together with *SF3B1* and *SRSF2* mutations, which would fit the theory that such mutations represent early events in disease when numerous clonal genetic lesions or separate disease clones have not yet evolved. This is again highlighted in the case of *TET2* mutations, considered as early disease events that do not add prognostic value in MDS[21], but which were more likely to be found in MDS cases with a mutant spliceosome, especially *SRSF2* mutations, in our cohort of patients.

Mutation analyses performed on serial samples in patients who were initially diagnosed with RARS and subsequently transformed to AML, also provided us with clear evidence that *SF3B1* mutations are an early ancestral event. In these cases, the *SF3B1* mutant clone survives various treatments during the course of the disease, acquiring additional oncogenic mutations such as *FLT3* and/or *RUNX1*, enabling the disease to evolve. Supporting this theory, MDS patients with splicing factor mutants in isolation have a better OS generally, which seemingly worsens as additional mutant genes are added to the genetic makeup. Hence, the contribution of *SF3B1* mutations to disease transformation or progression into AML may be limited, but the mutations may instead provide a favorable environment or sufficient pressure for other more destabilizing mutations to occur.

We initially performed whole-exome sequencing on $CD34^+$ cells as such progenitors are likely to form the reservoir of myeloid mutations, as has been previously reported.[21,33] The fact that *SF3B1* mutations are present in $CD34^+$ and in differentiated $CD235^+CD71^+$ cells, but not in T or B cells in MDS, further reinforces the importance of these mutations and relate to a clonal advantage through the course of myeloid disease, particularly in RARS.

Functional characterization of spliceosome mutations and their contribution to myelodysplasia is still unclear.[7] However, a recent study by Visconte *et al.* indicated the role of *SF3B1* aberrations in the formation of ring sideroblasts in MDS.[34] Furthermore, a majority of splicing factor mutations are heterozygous and often clustered in particular protein functional domains, indicating an altered gain-of-function and underlining biological significance. In *in-vitro* knockdown experiments of spliceosome components, *SF3B1* and *U2AF1* have been linked to aberrant cell cycle characteristics, cell cycle arrest and increased apoptosis, where aberrant splicing of cell-cycle genes has been noted.[8,35] Furthermore, a study by Yoshida *et al.* showed that overexpression of mutant *U2AF1* gene in mice leads to reduced reconstitution capacity of hematopoietic stem cells.[8] However, heterozygous knockout of *SF3B1* in mice elsewhere was not linked to altered splicing activity itself but rather altered interactions with polycomb proteins leading to deregulation of gene expression.[36] This finding adds further weight to the fundamental role that aberrations of the splicing machinery and epigenetic modifiers play in MDS. It is noteworthy that functional studies to date have not as yet arrived at a consensus in identifying definitive functional pathways affected by splicing factor mutations, even in RARS in which *SF3B1* mutations dominate. This again implies interplay of several factors driving the MDS clone, where spliceosome mutations are perhaps fuelling subtle biochemical changes which can be built upon in the course of disease evolution.

It is now becoming clear that splicing regulation and global genomic epigenetic marks are intricately linked, where epigenetic 'annotation' of intron/exon boundaries influences the rate of transcription or recruitment of splicing effector proteins to particular histone modifications.[37-40] It therefore seems likely that as our knowledge grows of how the splicing machinery and cellular epigenetic modifiers communicate in the control of gene expression patterns, we shall move towards a fuller understanding of the functional consequences of their dysregulation in leukemia.

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