Clinicopathologic characterisation of myeloid neoplasms with concurrent spliceosome mutations and myeloproliferative-neoplasm-associated mutations

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ABSTRACT

Aims Spliceosome genes (*SF3B1*, *SRSF2*, *U2AF1* and *ZRSR2*) are commonly mutated in myeloid neoplasms, particularly in myelodysplastic syndromes (MDS). *JAK2*, *MPL* and *CALR* mutations are associated with myeloproliferative neoplasms (MPN). Although *SF3B1* and MPN-associated mutations frequently co-occur in the rare entity MDS/MPN with ring sideroblasts and thrombocytosis (MDS/MPN-RS-T), myeloid neoplasms with concurrent spliceosome and MPN-associated mutations encompass many disease entities and are not well characterised.

Methods Specimens from 2016 to 2019 with concurrent spliceosome and MPN-associated mutations were identified, and the clinicopathologic features were assessed.

Results The 36 cases were divided into mutational categories based on their spliceosome mutation. At diagnosis, cases with concurrent *U2AF1* and MPN-associated mutations had lower leucocyte counts and platelet counts than did the other groups. Cases with mutant *SRSF2* were more likely to have *ASXL1* and *IDH2* mutations, while *U2AF1*-mutated neoplasms were more likely to have an abnormal karyotype. The most common *SF3B1* K700 and *U2AF1* S34 mutational hotspots were underrepresented in our cohort of myeloid neoplasms with concurrent spliceosome and MPN-associated mutations, as *SF3B1* and *U2AF1* mutations tended to involve other codons. Numerous WHO-defined disease entities were represented in each spliceosome gene category; although MDS/MPN-RS-T were only identified in the group with *SF3B1* mutations, they constituted only 1/4 of the neoplasms in the category.

Conclusions Myeloid neoplasms with different mutant splicing factor and concurrent MPN-associated mutations demonstrate somewhat different clinical and pathologic features, but t he association between genotypes and phenotypes in these overlapping neoplasms is not straightforward.

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INTRODUCTION

The WHO classification[1] categorises the myeloid neoplasms into several broad categories including myelodysplastic syndromes (MDS), myeloproliferative neoplasms (MPN), myelodysplastic/myeloproliferative neoplasms (MDS/MPN) and acute myeloid leukaemia (AML). MDS are characterised by ineffective haematopoiesis, morphologic dysplasia, increased haematopoietic cell turnover and cytopenia(s).[2–4] MPNs, in contrast, show increased effective haematopoiesis, cytosis, frequent organomegaly and occasional myelofibrosis. MDS/MPNs exhibit a mixture of MPN-like and MDS-like features, presenting with varying degrees of cytoses, cytopenias and dysplasia.

The advent of high-throughput sequencing has led to a greater understanding of the genetic composition of the myeloid neoplasms, with different classes of mutations enriched in different WHO categories. Mutations in genes encoding components of the spliceosome (*SF3B1*, *SRSF2*, *U2AF1* and *ZRSR2*) are frequently seen in myeloid neoplasms. Mutant splicing factors can lead to alternative splicing that affect specific target genes.[5] The spliceosomal mutations jointly have the highest frequency in MDS but are also identified in a sizeable portion of MDS/MPN, including chronic myelomonocytic leukaemia (CMML).[6–8] Individually, *SF3B1* mutation has the highest incidence in MDS, especially in entities associated with ring sideroblasts, but *SF3B1* mutations can also be found in AML, MDS/MPN and MPN.[9–11] The frequency of *SRSF2* mutation is the highest in CMML and is estimated to be 5%–15% in MDS.[6 12 13] The incidence of *U2AF1* mutation is 5%–15% in a variety of myeloid neoplasms, including MDS, AML, CMML and MPN.[6 7 13 14] *JAK2*, *MPL* and *CALR* mutations (collectively referred to as MPN-associated mutations) frequently occur in the *BCR-ABL1*-negative MPNs including polycythemia vera (PV), primary myelofibrosis (PMF) and essential thrombocythemia (ET). These mutations lead to the activation of signalling pathways downstream of the erythropoietin receptor, thrombopoietin receptor, and granulocyte colony-stimulating factor receptor.[15] *JAK2* V617F mutation is present in the great majority of PV cases and is identified in around half of PMF and ET cases,[16–19] while *MPL* and *CALR* mutations are essentially limited to PMF and ET.[20–24]

Co-occurrence of spliceosome mutations and MPN-associated mutations is well recognised in certain disease entities. For example, *SF3B1* and *JAK2* are often both mutated in the rare WHO-defined entity MDS/MPN with ring sideroblasts and thrombocytosis (MDS/MPN-RS-T).[25 26] Both mutations contribute to the hallmark features of this rare entity including thrombocytosis and the presence of ring sideroblasts in association with anaemia. The routine application of target-panel

next-generation sequencing in suspected myeloid neoplasms leads to the identification of concurrent spliceosomal and MPN-associated mutations in many other entities defined by the WHO classification. However, the clinicopathologic features of these comutated neoplasms have not been fully described.

The goal of this study, therefore, was to describe the clinical and pathologic features of myeloid neoplasms with both spliceosomal and MPN-associated mutations. We also aim to examine the potential differences among neoplasms with different mutant splicing factors and to explore the composition of these overlapping neoplasms based on the current WHO classification that incorporates the clinical, pathologic, cytogenetic and molecular features into the diagnostic criteria.

MATERIALS AND METHODS

Case selection

Cases with mutations in both spliceosome genes (*SF3B1, SRSF2, U2AF1* or *ZRSR2*) and MPN-associated genes (*JAK2, MPL* or *CALR*) were retrospectively identified from the cases of suspected myeloid neoplasms that were submitted for target-panel next generation sequencing during the period 2016–2019. Review of the clinical records and pathology material was performed and was used to determine the disease classification based on WHO criteria.

Morphologic evaluation

Morphologic evaluation for the presence of increased blasts (>5%), dyserythropoiesis, dysgranulopoiesis, dysmegakaryopoiesis, ring sideroblasts and fibrosis was recorded. The evaluation for dysmegakaryopoiesis included assessment for megakaryocytes with MDS-like morphology (small size, hypo/monolobation and micromegakaryocytes) or megakaryocytes with more MPN-like morphology (larger size, hyperlobation and nuclear hyperchromasia). The morphologic evaluation was compromised by inadequate aspirate smears or an inadequate core biopsy specimen in a subset of cases.

Cytogenetic studies

Trypsin-Giemsa banded metaphase cells were obtained from 24 hours unstimulated cultures according to the standard protocol and analysed at a minimum 400-bands resolution. Results were reported according to standard ISCN nomenclature.[27]

Mutation analysis

DNA samples from the corresponding specimens were subjected to a polymerase chain reaction-based, amplicon target enrichment assay (Illumina TruSeq Amplicon Assay, San Diego, CA). Coding and non-coding regions of the selected genes were enriched and subsequently sequenced on an Illumina MiSeq instrument with paired end, 186 base pair reads. Following mapping of the read data to the human genome (GRCh37/hg19), single nucleotide variants, insertions and deletions with an allele frequency greater than 5% were identified. *FLT3* insertions greater than 15 base pairs were detected to a 0.5% allelic burden. The mutation hotspots of the following genes were interrogated by the test: *ABL1, ASXL1, BCOR, BCORL1, BRAF, CALR, CBL, CDKN2A, CSF3R, DNMT3A, ETV6, EZH2, FBXW7, FLT3, GATA2, HRAS, IDH1, IDH2, JAK2, KIT, KRAS, MPL, MYD88, NPM1, NRAS, PHF6, PTEN, PTPN11, RUNX1, SETBP1, SF3B1, SRSF2, TET2, TP53, U2AF1, WT1* and *ZRSR2*.

CEBPA mutation analysis was performed by fragment length analysis of the *CEBPA* gene for screening of the bZIP, TAD1 and TAD2 regions, followed by direct sequencing of positive amplicons utilising capillary electrophoresis with an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Statistical analysis

Continuous variables were analysed using Kruskal-Wallace rank sum test, and significant results were analysed using Wilcoxon tests with Benjamini-Hochberg correction for multiple comparisons. Categorical variables were analysed using the χ^2 test. Survival analysis was performed via the Kaplan-Meier method, and the log-rank test was used to compare survival curves. Multivariate analysis was performed using a Cox proportional hazards model. Statistical analysis was performed using R V.3.6.1.

RESULTS

Of 1128 cases submitted for myeloid NGS testing, 37 myeloid neoplasms containing both a spliceosome gene mutation and an MPN-associated mutation were identified. Of note, 12 myeloid neoplasms had mutations of *SF3B1*, 18 neoplasms had mutations of *SRSF2*, 8 neoplasms had mutations of *U2AF1* and 1 neoplasm had a mutation in *ZRSR2*. As only 1 *ZRSR2*-mutated case was identified, it was excluded from further statistical analysis. The remaining 36 cases were grouped into three categories based on the predominant spliceosome mutation: *SF3B1*-MPN, *SRSF2*-MPN and *U2AF1*-MPN; one case had mutations of both *SF3B1* and *SRSF2* and was classified as an *SF3B1*-MPN because the *SF3B1* mutation was present at a higher variant allele fraction (VAF) than the *SRSF2* mutation (*SF3B1* at 48% vs *SRSF2* at 5%), and another case had mutations of both *U2AF1* and *SRSF2* and was classified as a *U2AF1*-MPN because *U2AF1* mutation was present at a higher VAF than was that of *SRSF2* (*U2AF1* at 42% vs *SRSF2* at 21%). The clinicopathologic features of 12 *SF3B1*-MPNs, 16 *SRSF2*-MPNs and 8 *U2AF1*-MPNs were studied.

The clinical and demographic characteristics of the cases are presented in table 1. No significant differences in age were present among the three groups, with an overall median age of 74 years (range 46–90 years). The male:female ratio in *SF3B1*-MPN was 1.4:1; however, non-*SF3B1*-MPNs tended to be male predominant, and all eight cases of *U2AF1*-MPN were seen in males (p=0.04 vs *SF3B1*-MPN). The white blood cell (WBC) was significantly lower in *U2AF1*-MPN cases than in *SF3B1*-MPN and *SRSF2*-MPN, with median WBCs of 2.8 K/µL, 9.0 K/µL, and 13.0 K/µL, respectively (p=0.01 *U2AF1*-MPN vs *SF3B1*-MPN, p=0.02 *U2AF1*-MPN vs *SRSF2*-MPN). Haemoglobin concentrations were lower in the *U2AF1*-MPN group than in the *SRSF2*-MPN group (median 8.8 g/L vs 10.6 g/L, p=0.05), while the mean corpuscular volume was significantly higher in *SF3B1*-MPN than in *SRSF2*-MPN (median 99.4 fL vs 86.9 fL, p=0.008). The platelet count was higher in the *SF3B1*-MPN group than in the *U2AF1*-MPN group (median 474 K/µL vs 116 K/µL, p=0.03). The frequency of increased lactate dehydrogenase was higher in *SRSF2*-MPN patients than in *U2AF1*-MPN patients (83% vs 40%, p=0.05). No significant differences were seen among groups in the absolute monocyte count or in the presence of splenomegaly. Of 36 patients, three had deep venous thrombosis; no significant association between thrombosis and spliceosome group was present. No arterial thrombotic events were identified in our cohort.

Ring sideroblasts were significantly associated with *SF3B1*-MPNs, present in nine of 10 evaluable cases, while ring sideroblasts were less frequent in *SRSF2*-MPN (three of 14 cases) and *U2AF1*-MPN (two of seven cases) (p=0.003 *SF3B1*-MPN vs *SRSF2*-MPN and p=0.01 *SF3B1*-MPN vs *U2AF1*-MPN) (table 2). Morphologic dysplasia was generally common in

Table 1 Clinical and laboratory features of myeloid neoplasms with concurrent spliceosome and MPN-associated mutations

	SF3B1-MPN (n=12)	*SRSF2*-MPN (n=16)	*U2AF1*-MPN (n=8)	
Age (years)	74.5 (56–90)	72.5 (46–87)	75 (66–87)	
M:F	7:5	14:2	8:0	p=0.04 (U2AF1 vs SF3B1)
WBC (K/µL)	9.0 (3.1–22.4)	13 (2.3–68.6)	2.8 (0.9–19.8)	p=0.01 (U2AF1 vs SRSF2) p=0.02 (U2AF1 vs SF3B1)
Hgb (g/L)	9.7 (7.4–15.5)	10.6 (8–15.5)	8.8 (7.6–10.3)	p=0.05 (U2AF1 vs SRSF2)
Plt (K/µL)	474 (23–1437)	136 (26–1008)	116 (8–198)	p=0.03 (SF3B1 vs U2AF1)
MCV (fL)	99.4 (80.8–123)	86.9 (70.0–98.7)	95.8 (79–117)	p=0.008 (SF3B1 vs SRSF2)
Absolute monocyte count (K/µL)	0.57 (0.05–3.27)	0.77 (0.09–9.60)	0.16 (0.02–1.48)	
Lactate dehydrogenase (increased:normal)	5:2	10/2	2:5	p=0.05 (SRSF2 vs U2AF1)
Splenomegaly (Y:N)	3:9	6:9	1:7	

Values for quantitative parameters are presented as medians, with ranges in parentheses.
K, thousand; MCV, mean corpuscular volume; MPN, myeloproliferative neoplasms; N, no; WBC, white blood cell; Y, yes.

all groups, present in 88% of cases overall. Fibrosis was also common, present in the majority of cases in all groups, with no definite differences among groups. All groups contained some cases with increased blasts (defined as blasts>5% in the bone marrow), and there was no clear association between increased blasts as either a dichotomised or continuous variable and the spliceosome gene category.

All spliceosome-defined categories exhibited heterogeneity in the WHO-defined entities that were represented (table 3). As expected, cases morphologically consistent with MDS/MPN-RS-T were present exclusively in the *SF3B1*-MPN group and were not seen in the other two groups. However, MDS/MPN-RS-T represented only three of the 12 *SF3B1*-MPN cases, with other *SF3B1*-MPN cases exhibiting heterogeneous clinicopathologic features, including cases of AML, blast-phase MPN, MDS, PMF, CMML and MDS/MPN, unclassifiable. Similarly, *SRSF2*-MPN and *U2AF1*-MPN had highly heterogeneous clinicopathologic diagnoses, including cases of MDS, PMF, AML, CMML, blast-phase MPN and MDS/MPN, unclassifiable. Of note, three of the *SRSF2*-MPN cases had features that raised the differential diagnosis of PMF with monocytosis versus CMML (figure 1). Within the *SF3B1*-MPN group, one case with a consensus diagnosis of MDS/MPN, unclassifiable, demonstrated features that also caused consideration of PMF with ring sideroblasts and MDS/MPN-RS-T as diagnostic possibilities (figure 2).

The mutational profile of the cases is exhibited in figure 3. In all groups, the most common MPN-associated mutation by far was *JAK2* V617F, present in 32 of 36 total cases. Only low-frequency *MPL* (n=3) and *CALR* (n=2) mutations were identified (one case exhibited mutations of both *JAK2* and *MPL*). *SF3B1*-MPNs had fewer non-spliceosome/non-MPN-associated mutations than did non-*SF3B1*-MPN cases taken all together (median of 1.5 additional mutations in *SF3B1*-MPN cases vs median of three additional mutations in non-*SF3B1*-MPN cases, p=0.02). *ASXL1* mutations were enriched in *SRSF2*-MPNs, present in 14 of 16 cases (p=0.0005 *SRSF2*-MPN vs *SF3B1*-MPN and p=0.02 *SRSF2*-MPN vs *U2AF1*-MPN). *IDH2* mutations were also enriched in the *SRSF2*-MPN cases (p=0.03 *SRSF2*-MPN vs *SF3B1*-MPN and p=0.05 *SRSF2*-MPN vs *U2AF1*-MPN), and *IDH2* was specifically comutated with *ASXL1* (p=0.02). Abnormal karyotypes were enriched in *U2AF1*-mutated cases, present in five of six evaluable specimens (p=0.02, *U2AF1*-MPN vs both *SF3B1*-MPN and *SRSF2*-MPN.)

The codon-level distribution of *SF3B1* and *U2AF1* mutations in our cohort was atypical when compared with myeloid neoplasms overall. Only two of 12 *SF3B1*-MPN cases in our cohort had a K700Q mutation, rather most mutations in *SF3B1* occurred at the K666 residue or other nearby codons. In an internal comparison cohort of spliceosome-mutated myeloid neoplasms without MPN-associated mutations, Lys700Glu was the most common

Table 2 Morphologic and genetic features of myeloid neoplasms with concurrent spliceosome and MPN-associated mutations

	SF3B1-MPN (n=12)	*SRSF2*-MPN (n=16)	*U2AF1*-MPN (n=8)	
Increased blasts* (Y:N)	4:8	6:10	5:3	
Dysplasia of any lineage (Y:N)	9:1	13:2	6:1	
Erythroid dysplasia† (Y:N)	7:3	11:4	5:0	
Ring sideroblasts‡ (Y:N)	9:1	3:11	2:5	p=0.003 (SF3B1 vs SRSF2), p=0.01 (SF3B1 vs U2AF1)
Granulocytic dysplasia (Y:N)	2:8	6:9	2:5	
Megakaryocytic dysplasia (Y:N)	5:5	10:5	5:1	
Fibrosis (Y(MF-1–MF-3):N)	5:4	11:1	2:1	
Abnormal karyotype (Y:N)	2:8	4:12	5:1	p=0.02 (U2AF1vs both SF3B1 and SRSF2)
ASXL1 mutation (Y:N)	2:10	14:2	3:5	p=0.0005 (SRSF2 vs SF3B1), p=0.02 (SRSF2 vs U2AF1)
IDH2 mutation (Y:N)	0:12	6:10	0:8	p=0.03 (SF3B1 vs SRSF2), p=0.05 (SRSF2 vs U2AF1)

*Greater than 5% blasts in the bone marrow.
†Erythroid dysplasia, excluding ring sideroblasts.
‡The presence of any number of ring sideroblasts was considered to be positive for the purpose of this study.
MF-1–MF-3, myelofibrosis grade per WHO criteria; MPN, myeloproliferative neoplasms; N, no; Y, yes.

Table 3 WHO classification of cases with concurrent spliceosome and MPN-associated mutations

	SF3B1-MPN (N)	*SRSF2*-MPN (N)	*U2AF1*-MPN (N)
AML	1	2	3
BP-MPN	1	1	1
CMML	1	2	0
MDS	1	2	3
MDS/MPN-RS-T	3	0	0
MDS/MPN, U	2	2	1
MPN	3	3	0
PMF or CMML	0	3	0
SM-AHN-CMML	0	1	0

AML, acute myeloid leukaemia; BP-MPN, blast-phase myeloproliferative neoplasm; CMML, chronic myelomonocytic leukaemia; MDS, myelodysplastic syndrome; MDS/MPN-RS-T, myelodysplastic/myeloproliferative neoplasm with ring sideroblasts and thrombocytosis; MPN, myeloproliferative neoplasm; PMF, primary myelofibrosis; SM-AHN, systemic mastocytosis with associated haematologic neoplasm; MDS/MPN, U, myelodysplastic/myeloproliferative neoplasm, unclassifiable.

SF3B1 mutation (59%), a significantly different frequency than in our *SF3B1*-MPN cases (17%, p=0.02). Similarly, *U2AF1* S34 mutations represented 46% of all *U2AF1* mutations in cases without MPN-associated mutations, while only one *U2AF1* S34 mutation was identified in the *U2AF1*-MPN cohort (13%), where most mutations involved Q157 (p=0.11).

SRSF2 mutations tended to be present at a modestly higher VAF (median 47.5%) than were mutations in *SF3B1* or *U2AF1* (medians 43.1% and 37.1%, respectively, p=0.02 and 0.01). Spliceosome mutations were most frequently present at a higher VAF than were MPN-associated mutations (table 4); however, some differences in MPN gene VAFs were appreciated between WHO categories (table 5, overall p=0.05). As expected, neoplasms in categories that were more clinically myeloproliferative tended to have higher MPN-associated VAFs. The only categories where the spliceosome gene:MPN VAF ratio was uniformly less than 1 (ie, the MPN VAF was higher than the spliceosome gene VAF) were cases of blast-phase MPNs and cases with overlapping features between CMML and PMF. In contrast, cases of pathologically straightforward CMML had the highest average spliceosome gene:MPN VAF ratio in our cohort, with very low MPN VAFs.

In univariate analysis, the presence of *U2AF1* mutation was associated with poorer overall survival when compared with that of *SF3B1* mutation (figure 4, p=0.03 *SF3B1* vs *U2AF1*). However, in a multivariate model incorporating age, karyotype, number of mutations in addition to spliceosome/MPN-associated mutations, increase in blasts and spliceosome gene mutation; only age, number of mutations and the presence of increased blasts retained prognostic significance (figure 5).

DISCUSSION

Mutations in splicing machinery are found in a variety of haematopoietic malignancies, though spliceosome mutations are most common in MDS,[6–8 28–30] and these mutations are associated with morphologic features of myelodysplasia in other categories of myeloid neoplasia. RNA splicing is a highly regulated event involving orchestration of small nuclear ribonucleoprotein (snRNP) complexes (U1, U2 and either U4/5/6 or U11/12) as well as many other proteins.[6] *SF3B1*, *SRSF2*, *U2AF1* and *ZRSR2* are the most frequently mutated splicing factors in haematopoietic neoplasms. Mutated splicing factors lead to alternative splicing through different mechanisms including exon inclusion, exon skipping, intron retention and alternative 3′ and 5′ splice

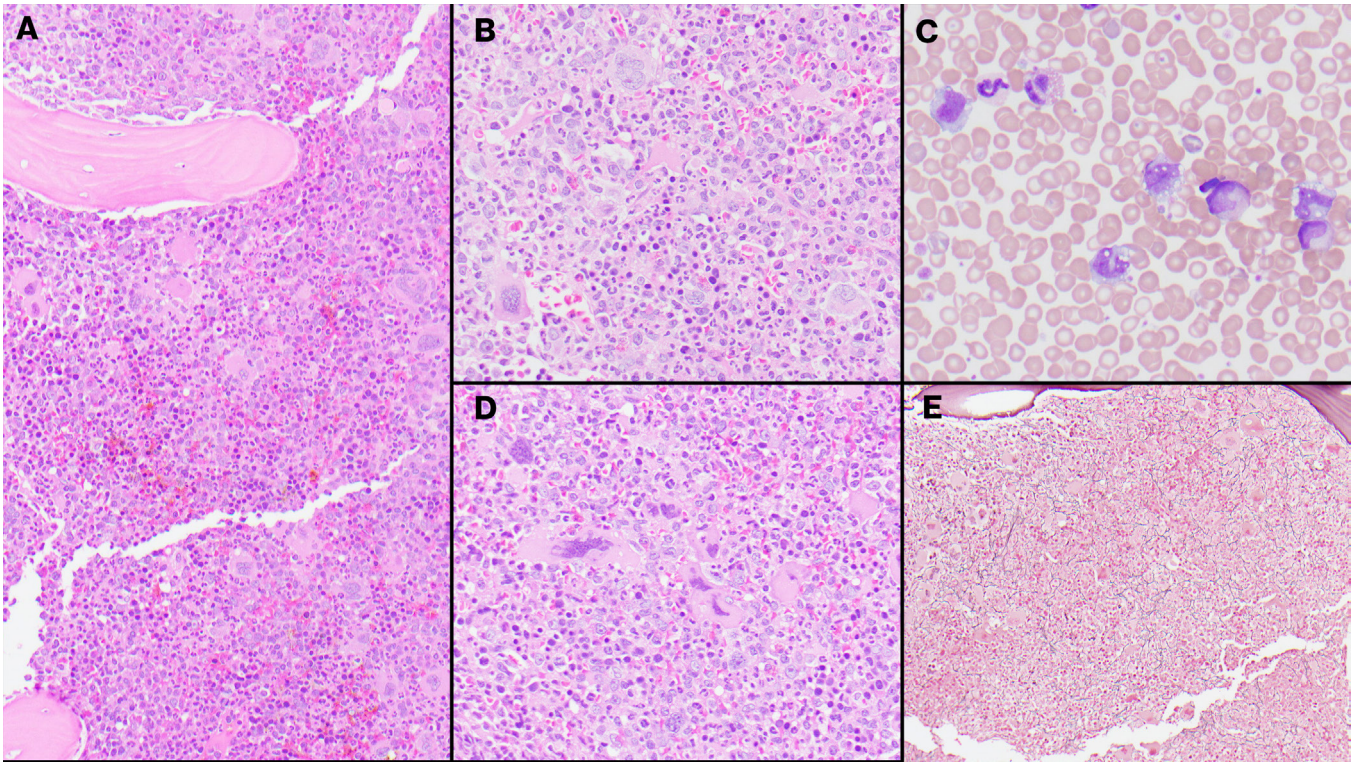

Figure 1 Example case with a differential diagnosis of chronic myelomonocytic leukaemia and primary myelofibrosis with monocytosis. (A–C) The biopsy section showed hypercellular marrow with increased megakaryocytes that appear variable in size but do not form large tight clusters (A) H&E 20X. (B & C) H&E 40X. (D) Peripheral blood showed leukocytosis with absolute monocytosis. (E) Reticulin stain showed MF-1 fibrosis.

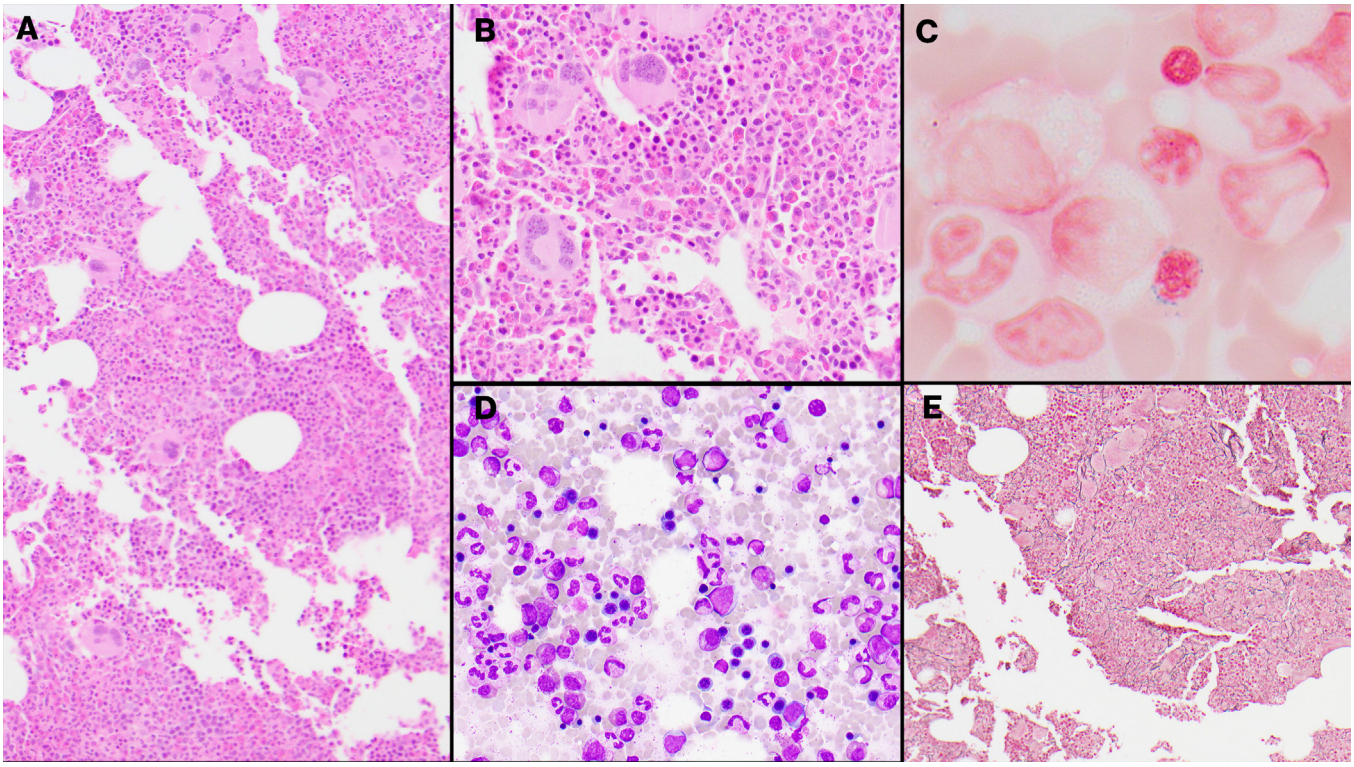

Figure 2 Case of myelodysplastic/myeloproliferative neoplasm, unclassifiable (MDS/MPN, U), with features of myelodysplastic/myeloproliferative neoplasm with ring sideroblasts and thrombocytosis (MDS/MPN-RS-T) versus primary myelofibrosis with ring sideroblasts. (A & B) The biopsy section showed a hypercellular marrow with increased number of megakaryocytes that appear largely scattered (A) H&E 20X. (B) H&E 40X. (C) Touch imprint of the biopsy section demonstrates megaloblastoid erythropoiesis and maturing granulopoiesis with a roughly normal M:E ratio. (D) Iron stain performed on the touch imprint of the biopsy section showed the presence of ring sideroblasts (<5%). (E) Reticulin stain showed MF-1 fibrosis.

sites and therefore impact the function of numerous genes.[5] Mutations in *JAK2*, *CALR* and *MPL* are characteristic of *BCR-ABL1*-negative MPNs. These mutations lead to the activation of signalling pathways downstream of cytokine receptors, including the erythropoietin receptor, thrombopoietin receptor (encoded by *MPL*) and granulocyte colony-stimulating factor receptor, to varying degrees.[15] Transgenic mouse models carrying mutated *JAK2* and *CALR* demonstrate myeloproliferative phenotypes, confirming that these mutations are essential to the pathogenesis of MPN.[31–37]

Mutations in splicing factor genes tend to occur at certain hotspot regions, with the exception of *ZRSR2*. Intriguingly, in our cohort composed of myeloid neoplasms with concurrent spliceosome mutations and mutations associated with MPNs, *SF3B1* and *U2AF1* demonstrated mutation distribution patterns deviating from that identified in general haematologic malignancies, as the common *SF3B1* K700 and *U2AF1* S34 hotspots were underrepresented. *U2AF1* mutations cause altered 3′ splice site recognition, and U2AF1 Q175 mutants preferentially recognise different splice site sequences than do U2AF1 S34 mutants, subsequently affecting splicing of different target genes.[38] A higher incidence of *U2AF1* Q157 mutations compared with S34 mutations has been previously reported in PMF,[39] and it is tempting to speculate that the differences in the mutation distribution of the spliceosome genes seen in our cohort and prior MPN cohorts suggest that there are specific target genes essential for the coordination between MPN-associated mutations and spliceosome mutations. In contrast to *U2AF1*, to our knowledge, no clear functional differences have been identified among *SF3B1* hotspot mutations, but the paucity of *SF3B1* K700 mutations in our cohort is striking in the context of myeloid neoplasms. The fact that different *SF3B1* codons are preferentially mutated in uveal melanoma versus in myeloid neoplasms suggests the possibility of *SF3B1*-mutation-specific biologic differences.[40 41] Additional studies of neoplasms with both spliceosome and MPN-associated mutations would be needed to confirm our findings of increased *U2AF1* Q157 and decreased *SF3B1* K700 mutations in these diseases.

In our study, myeloid neoplasms with spliceosome mutations and concurrent MPN-associated mutations had a variety of clinical and pathologic features. Some of our findings were expected and reflect known phenotypes associated with specific splicing factor mutations, such as the high percentage of ring sideroblasts in *SF3B1*-mutated myeloid neoplasms. In our cohort, neoplasms with concurrent *U2AF1* mutation and MPN-associated mutations were more likely to demonstrate features of MDS/AML with lower leucocyte and platelet counts, while neoplasms with *SRSF2* and *SF3B1* mutations were more likely to demonstrate features suggestive of MPN or MDS/MPN and were less likely to have abnormal karyotypes. In addition, neoplasms with concurrent *SRSF2* mutation and MPN-associated mutations were more likely to show *ASXL1* and *IDH2* mutations, two adverse prognostic markers in multiple PMF prognostic scoring models.[42 43] The finding is not entirely surprising in light of the recently discovered coordination between SRSF2 and IDH2 mutant proteins in leukemogenesis.[44] Our findings suggest that coordination among the spliceosome mutations, MPN-associated mutations and other comutations may contribute to the specific biology and clinical presentation of these neoplasms. It will be of interest to validate these clinicopathologic associations with specific genotypes in larger scale studies.

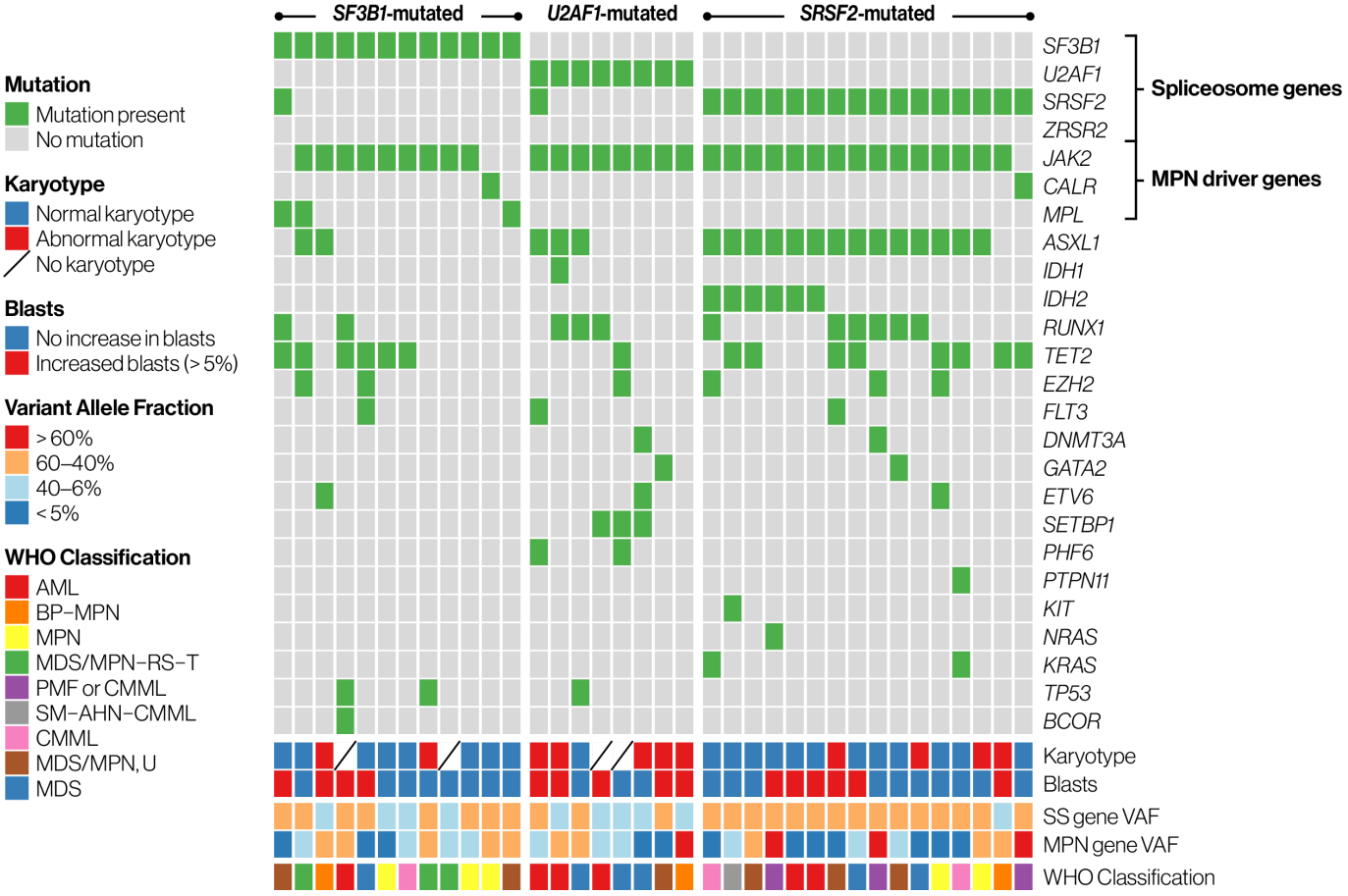

Figure 3 Mutational and pathologic features of myeloid neoplasms with both spliceosome and myeloproliferative-neoplasm-associated mutations. AML, acute myeloid leukaemia; BP-MPN, blast-phase myeloproliferative neoplasm; CMML, chronic myelomonocytic leukaemia; MDS, myelodysplastic syndrome; MDS/MPN-RS-T, myelodysplastic/myeloproliferative neoplasm with ring sideroblasts and thrombocytosis; MDS/MPN, U, myelodysplastic/myeloproliferative neoplasm, unclassifiable; MPN, myeloproliferative neoplasm; PMF, primary myelofibrosis; SM-AHN, systemic mastocytosis with associated haematologic neoplasm; VAF, variant allele fraction.

Although there were some clinical and genetic features associated with specific combinations of spliceosome mutations and MPN-associated mutations, we found generally heterogeneous clinical phenotypes among myeloid neoplasms with similar genotypes, as cases encompassed entities in various MDS, AML, MDS/MPN and MPN WHO-defined categories. Although cases fulfilling the diagnostic criteria of MDS/MPN-RS-T were only identified in the group with concurrent *SF3B1* mutation and MPN-associated mutations, they represented only one fourth of the cases with that genotypic combination; therefore, identification of comutated *SF3B1* and MPN-associated genes should not lead to disease reclassification.

Classifying myeloid neoplasms with a mixture of both MDS-like and MPN-like features by WHO criteria may be challenging, and our findings suggest that incorporation of genetic information may be contributory in select circumstances. In our study, three cases demonstrated clinicopathologic features that could lead to consideration of both CMML and PMF with monocytosis as diagnostic possibilities. Interestingly, all such cases in this series were *SRSF2* mutated. A prior history of MPN, clinical features and *JAK2* mutation VAF have previously been suggested to facilitate diagnosis between PMF and MDS/MPN.[45 46] Our series supports the suggestion that a high MPN-associated mutation VAF may be a useful diagnostic adjunct, as the only categories in which the VAF ratio of spliceosome mutation/MPN-associated mutation was uniformly less than one were these cases with overlapping features between CMML and PMF with monocytosis and cases of blast-phase MPN. The very high MPN VAF in our cases with overlapping features indicated loss of heterozygosity of the mutated MPN gene, a common finding in MPNs,[17 47 48] and this is in stark contrast to our cases of

Table 4 Spliceosome and MPN variant allele fractions by mutation category

	SF3B1-MPN	*SRSF2*-MPN	*U2AF1*-MPN	
Spliceosome VAF (%)	43.1 (11.1–47.4)	47.5 (28.9–52.7)	37.1 (24.0–47.4)	p=0.02 SRSF2 vs SF3B1, p=0.01 SRSF2 vs U2AF1
MPN VAF (%)	18.9 (1.0–50.8)	15.4 (0.53–78.6)	11.4 (2.2–70.4)	
SS/MPN VAF ratio	1.96 (0.9–12.6)	3.19 (0.55–87)	3.48 (0.53–16.2)	

The values presented are medians with the range in parentheses.
MPN, myeloproliferative neoplasm; VAF, variant allele fraction.

Table 5 Spliceosome and MPN variant allele fractions by WHO category

	N	Spliceosome VAF (%)	MPN VAF (%)	SS/MPN VAF ratio
AML	6	43.0 (35.1–46.7)	11.4 (0.53–50.7)	3.48 (0.92–87)
BP-MPN	3	37.0 (28.9–37.0)	52.2 (38–70.4)	0.55 (0.53–0.97)
CMML	3	42.4 (34.9–47.0)	3.0 (0.85–7.08)	15.7 (4.93–49.9)
MDS	6	40.0 (24.0–48.0)	5.6 (2.23–40)	7.07 (0.98–12.6)
MDS/MPN-RS-T	4	42.4 (25.5–45.0)	11.4 (9.03–43.8)	2.24 (0.97–5.0)
MDS/MPN,U	4	45.1 (41.0–48.4)	5.4 (1.38–50.8)	11.9 (0.9–29.9)
MPN	6	46.9 (11.1–52.0)	37.3 (1.04–52)	1.36 (0.9–46.8)
PMF vs CMML	3	49.7 (45.7–52.7)	73.5 (67.5–78.6)	0.67 (0.62–0.74)
SM-AHN-CMML	1	49.8	16.9	2.95
			p=0.05	p=0.03

The values presented are medians with the range in parentheses, as applicable.
AML, acute myeloid leukaemia; BP-MPN, blast-phase myeloproliferative neoplasm; CMML, chronic myelomonocytic leukaemia; MDS, myelodysplastic syndrome; MDS/MPN-RS-T, myelodysplastic/myeloproliferative neoplasm with ring sideroblasts and thrombocytosis; MPN, myeloproliferative neoplasm; PMF, primary myelofibrosis; SM-AHN, systemic mastocytosis with associated haematologic neoplasm; MDS/MPN, U, myelodysplastic/myeloproliferative neoplasm, unclassifiable; VAF, variant allele fraction.

straightforward CMML that had very low MPN VAFs, suggesting that the MPN mutation was present in a minor subclone.

Although MPN-associated mutations lead to myeloproliferative phenotypes, these mutations are not the initial somatic mutation in all MPN cases. A study of MPNs with concurrent *TET2* and *JAK2* mutations showed that the order of mutation acquisition impacts the phenotype of MPN.[49] Cases that acquired *TET2* mutation first tended to be older at diagnosis and showed lower risk of thrombocytosis, while cases that acquired *JAK2* mutation first tended to show expanded megakaryocyte–erythroid progenitors and bigger subclones carrying homozygous *JAK2* mutation, both features associated with PV. It is likely that the order of other mutations, such as spliceosome mutations, may also lead to different clinical manifestations of disease. Prior studies of MPNs containing spliceosome mutations have documented heterogeneity in the stability of both spliceosome and MPN-associated mutations during patients' disease course. In some patients, both mutations are relatively stable during therapy, while in other patients either the spliceosome or MPN-associated mutation persists, with loss of the other.[50–52] In at least some cases, this change in mutational burden has correlated with the clinical features of relapse (eg, a more MDS-like presentation with persistence of a spliceosome mutation and loss of MPN-associated mutation).[50] Our two cases with mutational follow-up exhibited similar heterogeneity. One of our PMF cases developed a newly emergent *SRSF2* mutation during disease, along with persistent *SF3B1* and *CALR* mutations. Another of our PMF cases demonstrated persistent spliceosome mutations but subsequently lost *JAK2* and *ETV6* mutations, without receiving JAK inhibitors. This temporal mutational variation highlights the complexity of disease progression in MPN and underscores the intricate clonal hierarchy and complicated association between genotypes and phenotypes in myeloid neoplasms.

In univariate analysis, *U2AF1*-mutated patients had lower overall survival than did patients with *SF3B1* mutations. However, cases of MDS and AML were somewhat enriched in

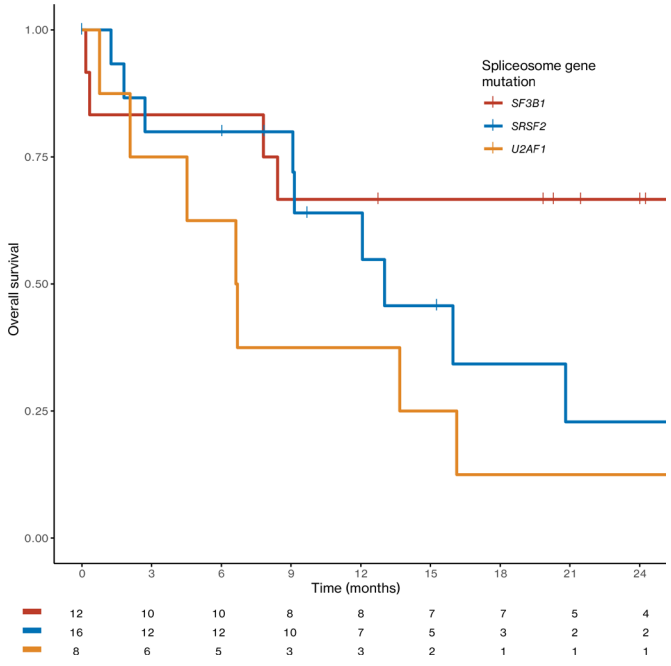

Figure 4 Impact of spliceosome mutation on survival in myeloid neoplasms with both spliceosome and myeloproliferative-neoplasm-associated mutations. In univariate analysis, *U2AF1* mutations were associated with significantly shorter overall survival than were *SF3B1* mutations (p=0.03).

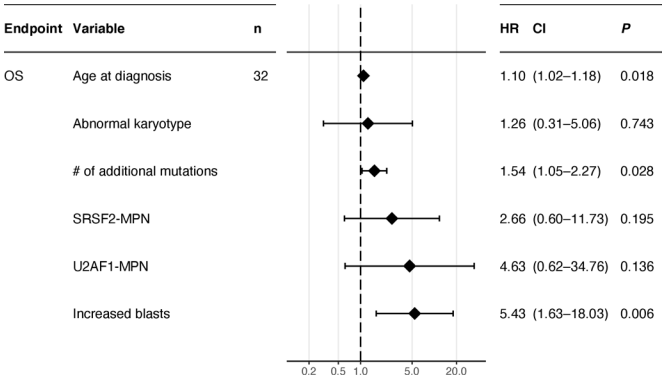

Figure 5 Forest plot of multivariate analysis showing factors associated with decreased overall survival in myeloid neoplasms with both spliceosome and myeloproliferative-neoplasm-associated mutations. Age and number of additional non-spliceosome/non-MPN-associated mutations were associated with decreased survival as continuous variables, and increased bone marrow blast percentage (>5%) was associated with decreased survival as a dichotomous variable. Spliceosome gene class (with SF3B1-MPN as the reference group) and karyotypic abnormalities did not reach significance in the model. MPN, myeloproliferative neoplasms; OS, overall survival.

the group with *U2AF1* mutations, and in a multivariate model, only age; the number of additional non-spliceosome, non-MPN-associated mutations and blast increase were associated with worse overall survival. Patients in our retrospective study were treated heterogeneously for their myeloid neoplasms, making our study less than optimal for survival assessment. Larger cohorts of patients would be needed to draw definitive conclusions regarding the relationship between specific spliceosome mutations and survival in myeloid neoplasms with both spliceosome and MPN-associated mutations.

In summary, we showed myeloid neoplasms with concurrent spliceosome mutations and MPN-associated mutations tend to demonstrate spliceosome mutations deviating from the most common *SF3B1* K700 and *U2AF1* S34 hotspots present in other myeloid neoplasms. Myeloid neoplasms with concurrent spliceosome and MPN-associated mutations appear to demonstrate somewhat different clinical and pathologic features depending on their specific spliceosome mutations, such as enrichment of MDS-MPN-RS-T in *SF3B1*-mutated cases, neoplasms with overlapping PMF/CMML features in *SRSF2*-mutated cases and more MDS/AML-like features in *U2AF1*-mutated cases. However, numerous categories of myeloid neoplasms were represented in all spliceosome groups, and genotype-phenotype correlation is not straightforward due to the complicated clonal hierarchy and other factors in these overlapping neoplasms. Although *U2AF1*-mutated patients tended to do poorly in our cohort, mutational categories were not clearly prognostically significant when taking into account conventional myeloid neoplasm risk factors such as age and the presence of increased blasts. Some myeloid neoplasms with overlapping genetic features of MDS and MPN also demonstrate overlapping clinical and pathologic features of MDS and MPN, which could potentially pose diagnostic challenges. Although these cases should primarily be classified on the basis of their clinical, morphologic and immunophenotypic features, incorporation of molecular data informs diagnostic classification in some cases.

Take home messages

- Myeloid neoplasms with concurrent spliceosome and myeloproliferative neoplasms (MPN) associated mutations comprise many different WHO defined clinicopathologic entities.
- Different spliceosome mutations are associated with different comutations and different laboratory and morphologic features.
- In multivariate analysis, conventional prognostic factors are associated with survival in neoplasms with both spliceosome and MPN associated mutations.
- Incorporation of mutational information may impact WHO category assignment in certain situations.

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