Diagnostic challenges and proposed classification of myeloid neoplasms with overlapping features of thrombocytosis, ring sideroblasts and concurrent del(5q) and *SF3B1* mutations

Myelodysplastic syndromes/neoplasms (MDS) are a spectrum of clonal bone marrow failure disorders at risk of progression to acute myeloid leukemia (AML). The classification of MDS relies on peripheral blood findings, morphology, cytogenetics, and molecular data. However, a subset of cases may be challenging to differentiate given overlapping features of MDS and myeloproliferative neoplasms (MPN). For example, MDS with low blasts and isolated deletion 5q (MDSdel(5q)) is characterized by cytopenia, usually anemia, and deletion 5q which may co-occur with another cytogenetic abnormality except monosomy 7 or deletion 7q. This entity is recognized by both the World Health Organization (WHO) and International Consensus Classification (ICC) systems under slightly different terminology.[1-3] The presence of ring sideroblasts (RS) or *SF3B1* mutations does not exclude the diagnosis of MDS-del(5q) if other diagnostic criteria are met.[1-3]

Up to a third of patients with MDS-del(5q) have sustained thrombocytosis, and a subset harbor additional *JAK2* or *MPL* mutations.[1-5] In some of these cases, the mutation and del(5q) are found in different clones.[6] These features therefore overlap with MDS/MPN with *SF3B1* mutation and thrombocytosis (MDS/MPN-*SF3B1*-T), another entity recognized by both the WHO and ICC classification systems.[1-3] Notably, cases that fulfil the criteria for MDS-del(5q) are excluded from MDS/MPN-*SF3B1*-T.[1,3] Recent classification updates also include a new MDS entity with low blasts and *SF3B1* mutation (MDS-*SF3B1*), which may further complicate the differential diagnosis.[1,7,8] According to the WHO, these cases are classified as MDS-*SF3B1* in the absence of del(5q), monosomy 7/7q deletion, or a complex karyotype.[3] The ICC has similar criteria and also specifies that the variant allele frequency of *SF3B1* must be >10% without multi-hit *TP53* or *RUNX1* alterations.[1] In addition, cases of MDS-*SF3B1* that later develop thrombocytosis should not be reclassified as MDS/MPN-*SF3B1*-T.[1] While most myeloid neoplasms in this differential diagnosis can be categorized with existing classification schema, some cases may show features that overlap between entities, presenting a diagnostic challenge. The clinical features and how to best classify these overlap cases remain unclear.

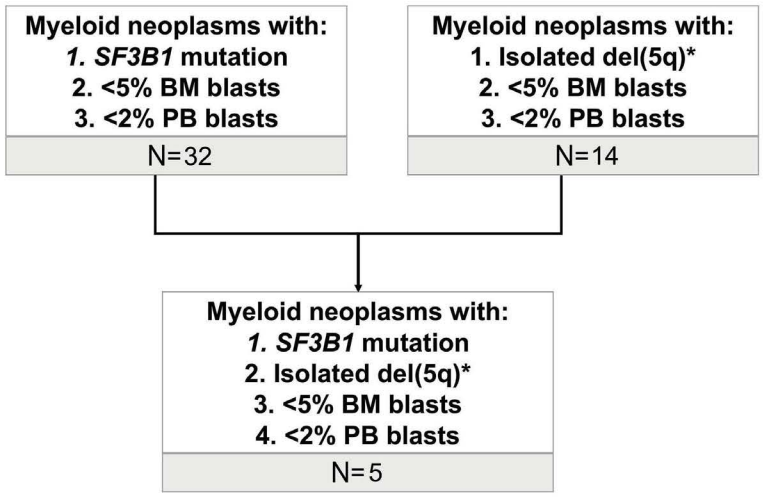
We sought to systematically characterize myeloid neoplasms with overlapping features of del(5q) and *SF3B1* mutations to assess their frequency, clinicopathological features, and their cell of origin. We searched the pathology archives at Memorial Sloan Kettering (MSK) Cancer Center for in-house bone marrow biopsy specimens from 2014 through 2018 for myeloid neoplasms with <5% blasts in the bone marrow, <2% circulating blasts in peripheral blood, and either del(5q) by cytogenetic studies or *SF3B1* variants by mutational testing (Figure 1A). Bone marrow cytogenetic testing included both conventional karyotyping and fluorescence *in situ* hybridization (FISH) for MDS-related abnormalities. Molecular data were collected from targeted next-generation sequencing-based mutational analysis performed on either a bone marrow or peripheral blood specimen using MSK laboratory-developed microdroplet amplicon-based 30 or 49 gene panels[9] or a hybridization-capture-based 400 gene panel (MSK-IMPACT), as previously described (*Online Supplementary Table S1*).[10] These assays report variants in key oncogenes and tumor suppressor genes implicated in hematolymphoid malignancies. This study was approved by the Institutional Review Board of the MSK Cancer Center.

In total, 41 unique patients were identified: nine patients met WHO 5th edition criteria for MDS-del(5q), 27 patients had myeloid neoplasms with *SF3B1* mutation (MN-*SF3B1*), and five patients had overlapping features (Figure 1B). Of the 27 patients in the MN-*SF3B1* category, five met the criteria for MDS/MPN-*SF3B1*-T, although the variant allele frequency of *SF3B1* in one of the five patients could not be confirmed. The remaining 22 patients fell under the category of MDS-*SF3B1,* although the variant allele frequency could not be evaluated in six of these patients. The summary of clinicopathological features of all patients in the study is shown in *Online Supplementary Table S2*. Twenty-six of 37 patients (70%) with reviewable iron-stained aspirate smears demonstrated RS: five had <5% RS, one had 5-15% RS, and 20 had >15% RS as a proportion of total erythroid precursors; 25 of these patients harbored *SF3B1* mutations. Twelve of 41 (29.3%) patients had a history of thrombocytosis (platelet count >450x10^9/L). Nineteen cases (46.3%) demonstrated morphological evidence of megakaryocytic dysplasia, which included forms with small, non-lobated and/or hypolobated nuclei, widely spaced nuclei, and hyperlobated nuclei. Twelve (29.3%) had significant reticulin fibrosis, with a grading of at least 2 on a scale from 0 to 3.

We identified five overlap cases that simultaneously met criteria for MDS-del(5q) while also harboring an *SF3B1* mutation. Three of these patients also had a history of sustained thrombocytosis, thus demonstrating overlapping diagnostic features between MDS-del(5q), MDS-*SF3B1*, and MDS/MPN-*SF3B1*-T. Based on the current classification, all five cases

were assigned to the diagnostic group of MDS-del(5q). The five overlap patients had a median age of 69 years and comprised three females and two males. None had received prior cytotoxic therapy. The median hemoglobin was 8.6 g/dL, platelet count 528x10^9/L, and absolute neutrophil count 1.4 x10^9/L in these overlap cases. No circulating blasts were

A



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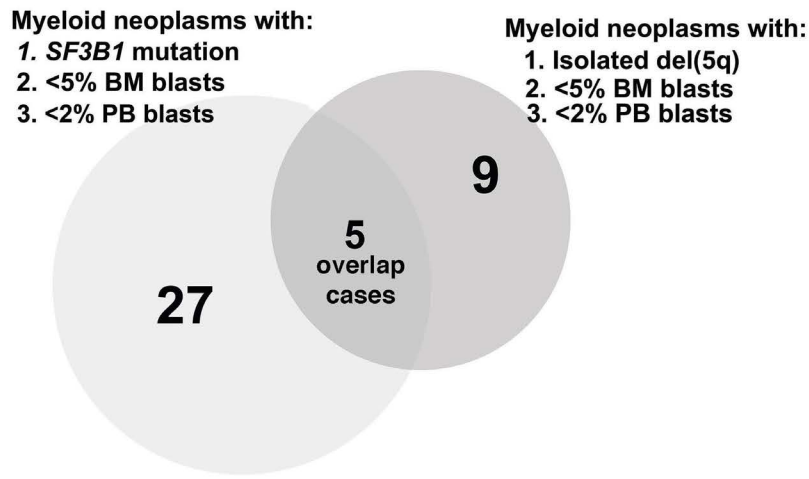
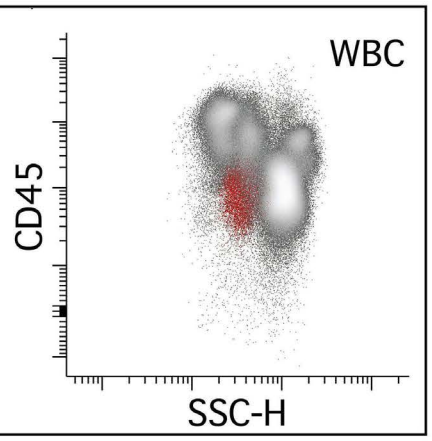
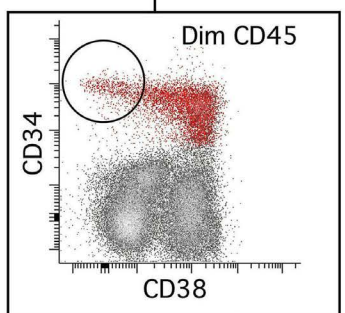


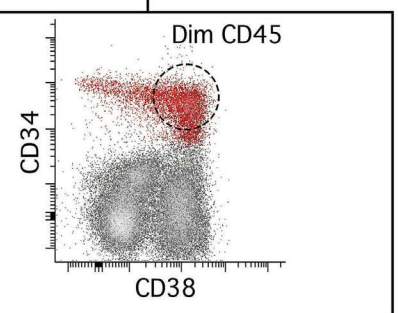
C

Bulk marrow	Pt A	Pt B
del(5q)	+	+
SF3B1 mut	+	+
MPL mut	+	NA

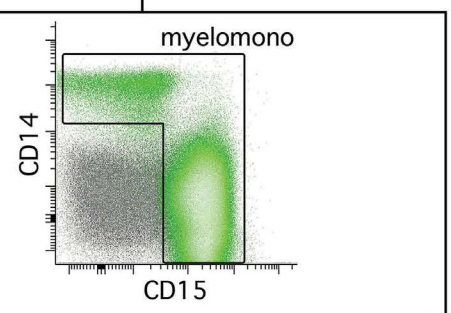




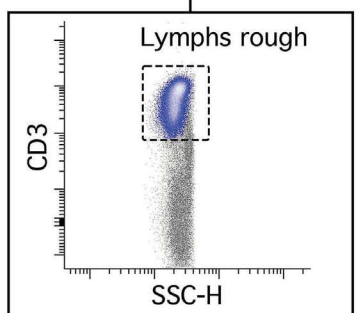
$CD38^{dim}$ blasts	Pt A
del(5q)	+
SF3B1 mut	50.5%
MPL mut	49.8%



$CD38^{bright}$ blasts	Pt A	Pt B
del(5q)	+	+
SF3B1 mut	46.8%	48.2%
MPL mut	51.3%	NA



Mature myelomonocytic	Pt A	Pt B
del(5q)	+	+
SF3B1 mut	52.3%	42.1%
MPL mut	52.4%	NA



T-cells	Pt A	Pt B
del(5q)	-	-
SF3B1 mut	0%	0%
MPL mut	0%	NA

Figure 1. Patient and specimen selection criteria, case distribution by category, and flow cytometric sorting data. (A) Flow chart demonstrating criteria for selecting cases of myeloid neoplasms with <5% blasts in the bone marrow, <2% circulating blasts in peripheral blood, and either del(5q) by cytogenetic studies or *SF3B1* variants by mutational testing. (B) Venn diagram illustrating the number of cases within each category. Of the 27 patients in the category of MN-SF3B1 (myeloid neoplasms with *SF3B1* mutation), five meet the criteria for MDS/MPN-*SF3B1*-T (myelodysplastic syndrome/myeloproliferative neoplasm with *SF3B1* mutation and thrombocytosis), although the variant allele frequency (VAF) of *SF3B1* in one of the five patients could not be confirmed. The remaining 22 patients fall under the category of MDS-*SF3B1*, of which the VAF could not be evaluated in six patients. (C) For patients A and B, cells were sorted by flow cytometry into four populations: hematopoietic stem cells, progenitor cells, mature myelomonocytic cells, and T cells as a negative control. Fluorescence *in situ* hybridization testing using probes for del(5q) and digital droplet polymerase chain reaction analyses were performed in each cell population. The presence or absence of del(5q) is indicated with + or –, respectively. The VAF of a mutation in *SF3B1* or *MPL*, if present, is listed as a percent. BM: bone marrow; PB: peripheral blood; NA: not applicable; WBC: white blood cells.

seen in the peripheral blood. The overlap patients showed significantly increased maximum platelet counts compared to MDS-del(5q) patients (*P*=0.029). Histologically, all five (100%) showed dysplastic megakaryocytes, and four out of five (80%) also showed RS: two cases had >15% RS on aspirate smear, and two had <5% RS, although one of these patients had absent iron stores on an iron-stained aspirate and evidence of a concurrent iron-deficiency anemia. The overlap patients had a median of 3% blasts on bone marrow aspirate differential (range, 0-4%), and two of five (40%) showed evidence of at least grade 2 reticulin fibrosis. The five overlap cases are described in detail in Table 1. During a median follow-up of 26 months, two of the five (40%) patients progressed to AML and ultimately died. In contrast, one of nine (11.1%) patients with MDS-del(5q) and three of 27 (11.1%) with MN-*SF3B1* evolved to AML.

Evaluating bone marrow morphology, the prevalence of megakaryocytic dysplasia was higher in the overlap cases (*P*=0.0099) and the isolated del(5q) cases (*P*=0.001) than in the MN-SF3B1 cases. The megakaryocytes were more heterogenous and ranged in size from small to large in the overlap cases. RS were more common in MN-*SF3B1* (*P*=0.0003) and overlap cases (*P*=0.032) when compared to MDS-del(5q) cases. While there were no significant differences in complete blood count findings between the overlap patients and the remaining patients, patients with MN-*SF3B1* showed a significantly lower median hemoglobin concentration compared to MDS-del(5q) patients (*P*=0.011), and a significantly higher median absolute neutrophil count (*P*=0.0062) when assessed using non-parametric Mann-Whitney tests.

An oncoplot was generated to evaluate the mutational landscape of the entire cohort (Figure 2).[11,12] An *MPL* variant was seen in two of the five patients (40%) in the overlap group compared to none in the MDS-del(5q) (*P*>0.05) and MN-*SF3B1* (*P*=0.02) groups. Mono-allelic *TP53* variants were more common in the overlap (2/5, 40%) and MDS-del(5q) groups (4/9, 44.4%) than in the MN-*SF3B1* cohort (2/27, 7.4%), which was a statistically significant difference when comparing MDS-del(5q) and MN-*SF3B1* (*P*=0.025). Cases of MN-*SF3B1* also showed greater frequencies of mutations in *TET2* and *ASXL1* genes compared to MDS-del(5q) and overlap groups, but these were not statistically significant differences.

To infer the cell of origin of genetic events, we used fluorescence assisted cell sorting (FACS) in two overlap patients who had cryopreserved cells from bone marrow aspirates. Cell populations were sorted using a panel of six antibodies (CD34, CD38, CD45, CD15, CD14, CD3) and scatter characteristics to separate four populations: $CD38^{dim}/CD34^{bright}$ blasts (enriched for hematopoietic stem cells), $CD38^{bright}/CD34^{positive}$ blasts (enriched for progenitor cells), mature myelomonocytic cells based on scatter characteristics and CD45 expression, and $CD3^{positive}$ T-cells as a negative control (Figure 1C). The data were analyzed using custom software ("Woodlist," a gift from B.L. Wood, Children's Hospital of Los Angeles, Los Angeles, CA, USA). In all sorted cell populations, FISH testing using probes for del(5q) and digital droplet polymerase chain reaction (ddPCR) were performed. For one case we were unable to sort enough viable cells for ddPCR testing on the $CD38^{dim}$ stem cell population but acquired enough cells for ddPCR testing in the other three cell populations. A commercially available QX100 Droplet Digital PCR System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used following the manufacturer's protocols and with custom PCR primers targeting the specific variants identified in the corresponding next-generation sequencing studies.

We found that the major genetic alterations that occurred within these cases, including del(5q), *SF3B1* mutations, and MPN-related mutations, were present in all sorted myeloid cell populations and not identified in the T-cell component (Figure 1C). Given that there was no evidence of loss of heterozygosity and the gene alterations had similar variant allele frequencies as compared to the proportion of cells harboring del(5q) by FISH (Table 1), our findings support that all genetic alterations occur within the same clone of early myeloid stem cells. Interestingly, these results contrast with those of some previously published studies, although the data are limited.[4-7] For example, MDS cases with isolated del(5q) harboring concomitant *JAK2* V617F were shown to occur in different clones.[6] Another study reported that *JAK2* alterations were a subclonal event present in the same clone, although not all del(5q) cells in that cohort harbored the *JAK2* variant.[4] Our data suggest the possibility that these alterations occur within the same clone, although the timeline of these mutational events is unclear. Future studies should seek to determine the impact of clonal architecture and the chronology of mutation acquisition on pathogenesis, as this may clarify classification of these challenging cases.

This is the first study to summarize this rare type of overlap myeloid neoplasm with thrombocytosis, ring sideroblasts, *SF3B1* and *MPL* mutations, which may create a diagnostic challenge. Currently, WHO and ICC recommend classifying these cases as MDS-del(5q). However, two of five of these cases had *MPL* mutations that showed moderate to severe reticulin fibrosis, a feature uncommon for MDS-del(5q). Of note, two of the patients within the overlap cohort on lenalidomide therapy progressed to AML. Typically, patients with MDS-del(5q) and MDS/MPN-*SF3B1*-T have a better prognosis than patients with other myeloid neoplasms.[13-15]

Moreover, the updated prognostic schema, the Molecular International Prognostic Scoring System (IPSS-M), demonstrated that cases of *SF3B1* mutation in the presence of isolated del(5q) are assigned to higher risk categories compared to cases of del(5q) or *SF3B1* alterations occurring in isolation.[16] Based on this observation along with the results our study, it may be prudent to classify these patients as a subcategory under MDS/MPN-*SF3B1*-T. Features suggesting subcategorization under MDS/MPN-*SF3B1*-T include a more

Table 1. Clinicopathological findings in patients with myeloid neoplasias with features overlapping between myelodysplastic syndrome with low blasts and isolated del(5q), myelodysplastic syndrome with low blasts and *SF3B1* mutation, and myelodysplastic/myeloproliferative neoplasm with thrombocytosis and *SF3B1* mutation.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Age in years	62	77	78	51	68
Sex	Female	Male	Male	Female	Female
Other cancer history	None	None	None	None	None
Blood counts					
Hemoglobin, g/dL	10.1	8.6	8.6	10.5	7.8
Max. platelet count x10^9/L	528	600	562	299	415
ANC x10^9/L	2.8	1.4	6.9	1.3	1.4
AMC x10^9/L	0.2	0.0	0.3	0.3	0.2
Blasts, %	0	0	0	0	0
Bone marrow findings					
Megakaryocyte dysplasia	Present	Present	Present	Present	Present
Megakaryocyte morphology	Hypolobated nuclei	Monolobated nuclei, separated nuclear lobes	Hypolobated nuclei, separated nuclei, clustering	Hypolobated nuclei, clustering	Variably sized hypolobated and hyperchromatic nuclei
Ring sideroblasts, %	>15	Present, <5	>15	Absent	Present, <5
Blasts, %	4	0	0	3	4
Fibrosis, 2+ out of 3 or higher	Absent	Present	Present	Absent	Absent
Pathological diagnosis	MDS-del(5q)	MDS-del(5q)	MDS-del(5q)	MDS-del(5q)	MDS-del(5q)
Genetic findings					
Deletion 5q	Present	Present	Present	Present	Present
ISCN karyotype	46,XX,del(5)(q13q31)[16] / 46,XX[4]	46,XY,del(5)(q22q35)[8] / 46,idem,del(20)(q11.2)[1] / 46,XY[1]	46,XY,del(5)(q31q31)[9] / 46,XY[11]	46,XX,del(12)(p11.2p13)[1] / 46,idem,del(5)(q13q31)[4] / 46,XX[15]	46,XX,del(5)(q13q33)[19] / 46,XX[1]
FISH proportion, %	66	81.7	83	42	33.3
Other abnormalities	None	del(20q12)	None	del(12p13)	None
SF3B1 mutation (VAF %)	K666E (38)	K666N (42)	K666Q (46)	K700E (23.4)	K700E (13.4)
JAK2, CALR, MPL mutations (VAF %)	None	*MPL* W515S (44)	*MPL* W515S (39)	None	None
Other gene mutations (VAF %)	None	*IDH1* R132C (8.7)	None	*TP53* R248W (30)	*TP53* R175H (16.3)
Clinical data					
IPSS-R	1 (very low)	2 (low)	2 (low)	1 (very low)	2.5 (low)
IPSS-M	-1 (low)	-0.29 (moderate low)	-0.63 (low)	-1.10 (low)	-0.43 (moderate low)
Initial therapy	Darbopoietin	Lenalidomide	Darbopoietin	Lenalidomide	5-azacytidine
Transformation to acute leukemia	No	Yes	No	Yes	No
Time to transformation in months	NA	7	NA	28	NA
Overall survival in months	26.1	8.9	22.0	44.5	45.7
Alive/died, Cause of death	Died, NSCLC	Died, AML	Alive	Died, AML	Died, unknown cause

Max: maximum; ANC: absolute neutrophil count; AMC: absolute monocyte count; MDS-del(5q): myelodysplastic syndrome with low blasts and isolated del(5q); ISCN: International System for Human Cytogenetic Nomenclature; FISH: fluorescence *in situ* hybridization; VAF: variant allele frequency; IPSS-R: Revised International Prognostic Scoring System; IPSS-M: Molecular International Prognostic Scoring System; NA: not applicable; NSCLC: non-small cell lung carcinoma; AML: acute myeloid leukemia.

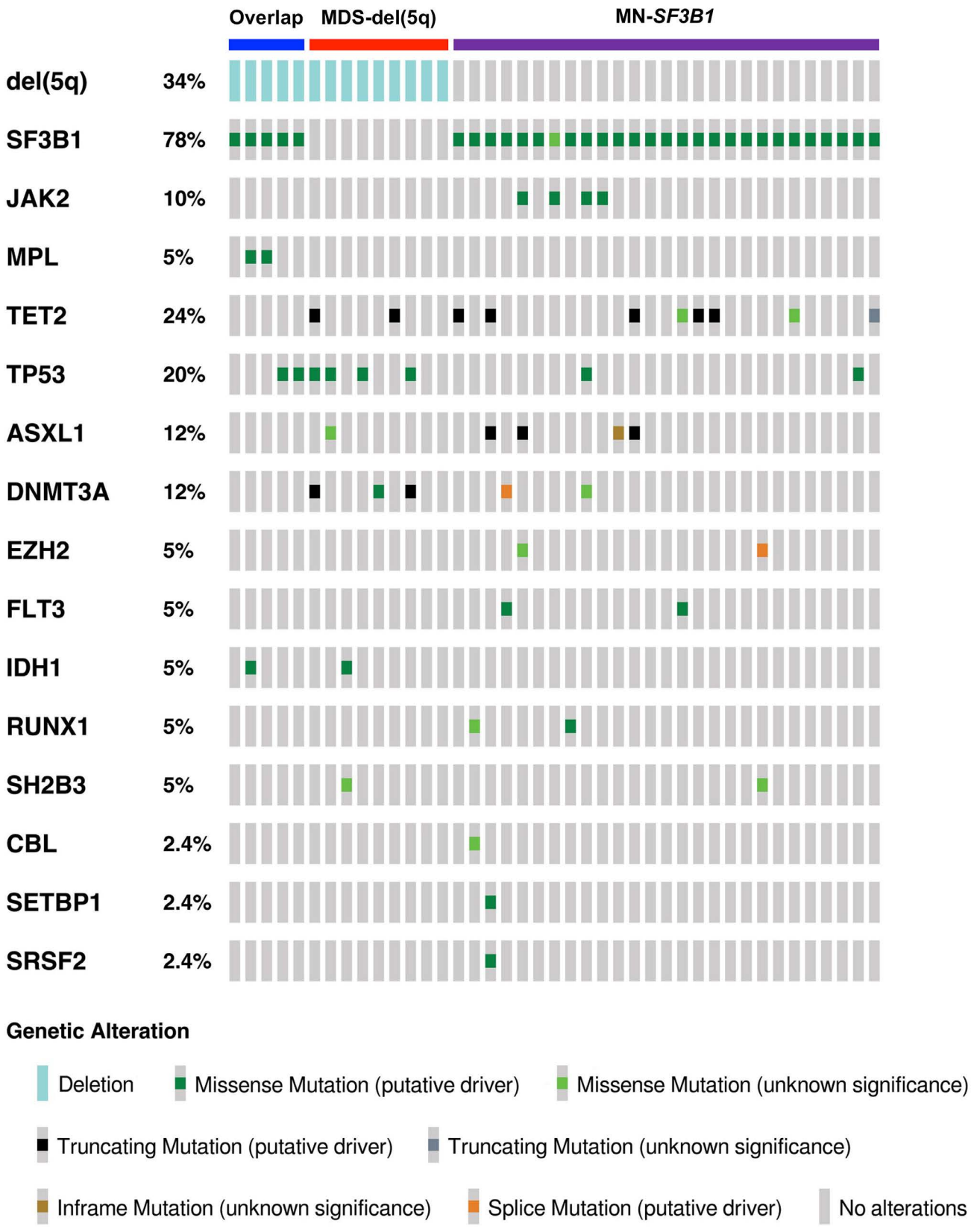


Figure 2. Oncoplot of the most frequent genetic aberrations by category. Overlap cases harbored both del(5q) and *SF3B1* mutation. MDS-del(5q): myelodysplastic syndrome with low blasts and del(5q); MN-*SF3B1*: myeloid neoplasm with *SF3B1* mutation.

heterogeneous megakaryocytic appearance in the overlap cases than in MDS-del(5q) cases. Although our cohort of overlap cases showed frequent hypolobated megakaryocytes, there were also forms with widely separated nuclei and others with hyperlobated nuclei and some clustering. Furthermore, MDS-del(5q) rarely transforms into AML, but in our cohort, we identified two patients on lenalidomide therapy without response who ultimately progressed to AML. There have been limited studies exploring del(5q) as an exclusion criterion from MDS/MPN-*SF3B1*-T, as currently defined in the updated classifications. Thus, additional studies utilizing single-cell sequencing techniques are warranted, as they may provide insight regarding the acquisition of a particular genetic alteration and its influence on morphology within dysplastic cells.

Although limited by sample availability, results from our sorted subpopulations suggest that in at least some overlap cases, driver genetic alterations occur at similar stages in early myeloid stem cells. Future studies are needed to study the oncogenic cooperation between these genetic events, determine the impact of clonal architecture and chronology of mutation acquisition, and explore the relationship between genotype and phenotype. Together, this additional information may help inform the pathogenesis of these unique myeloid neoplasms.

Authors

Jyoti Kumar,[1,2] Natasha E. Lewis,[1*] Sarina Sherpa,[3] Dory Londono,[3]

Xiaotian Sun,[1] Qi Gao,[1] Maria E. Arcila,[1,2] Mikhail Roshal,[1] Yanming Zhang,[3] Wenbin Xiao[1#] and Alexander Chan[1#]

[1]Hematopathology Service; [2]Diagnostic Molecular Pathology Service and [3]Cytogenetics Laboratory, Department of Pathology and Laboratory Medicine, Memorial Sloan Kettering Cancer Center, New York, NY, USA

[°]Current address: Division of Hematopathology, Department of Laboratory Medicine and Pathology, Mayo Clinic Arizona, Phoenix, AZ, USA
[#]WX and AC contributed equally as senior authors.

Correspondence:
A. CHAN - chana7@mskcc.org
W. XIAO - xiaow@mskcc.org

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Disclosures

No conflicts of interest to disclose.

Contributions

JK, NEL, WX, and AC compiled and annotated the cohort of patients. JK and MEA annotated the mutations. DL and YZ helped with cytogenetic analysis. XS, QG, MR, and AC designed/performed flow sorting analyses. WX and AC designed/validated the digital droplet polymerase chain reaction assay primers. WX designed and supervised the entire study. JK AC, and WX prepared the manuscript. All authors edited or reviewed the manuscript.

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Data-sharing statement

The original data that support the findings of this study will be made available upon request.

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