High frequencies of *SF3B1* and *JAK2* mutations in refractory anemia with ring sideroblasts associated with marked thrombocytosis strengthen the assignment to the category of myelodysplastic/myeloproliferative neoplasms

Mutations of spliceosome genes were shown to occur frequently in different entities.[1] Remarkably, mutations in *SF3B1* (splicing factor 3b, subunit 1) were associated with the morphological feature of ring sideroblasts[1-3] and were also found in refractory anemia with ring sideroblasts and marked thrombocytosis (RARS-T).[2,4-6] This malignancy has been assigned as a provisional entity in the chapter "Myelodyplastic/myeloproliferative neoplasms, unclassifiable" of the WHO classification.[7] Patients have anemia, clinical and morphological features of myelodysplastic syndromes (MDS), but also show marked thrombocytosis associated with abnormal megakaryocytes resembling *BCR-ABL1* negative myeloproliferative neoplasms (MPN).[7] In line with the myeloproliferative character of this disease, RARS-T patients often show *JAK2*V617F mutations and, much less common, *MPL*W515 mutations (*MPL*W515mut).[7,8] To further characterize this entity, we analyzed 47 RARS-T patients for the occurrence of mutations in *SF3B1* (*SF3B1*mut) and its association with *JAK2*V617F and *MPL*W515mut. To our knowledge, this is the largest cohort studied so far. All patients strictly met the criteria for RARS-T according to the WHO classification 2008.[7] Twenty-seven of 47 (57.4%) were female and 20 of 47 (42.6%) were male. The median age was 76 years (range 44-89 years), white blood cell count (WBC) 7.9x10^9/L (range 2.9-60.0x10^9/L), hemoglobin level (Hb) 9.7 g/dL (range 6.9-13.1 g/dL), platelet count 691x10^9/L (range 466-1,500x10^9/L), and percentage of ring sideroblasts 60% (range 18-97%). Most patients had a normal karyotype (39 of 47, 83.0%) and 8 of 47 (17.0%) had various chromosomal aberrations consistent with MDS/MPN. Screening for *SF3B1*mut was performed by direct Sanger sequencing of exons 11-16. Using cDNA, a 1kb amplicon was generated with primers 5`-TGACCAGCCATCTGGAAATC-3` (forward, exon 10) and 5`-CACCATCTGTCCCACAACAC-3` (reverse, exon 17). Sequencing was performed with previous primers and 5`-GCTTGCCAGGACTTCTTGCT-3` (reverse, exon 14), 5`-AGCTTTTGCTGTTGTAGCCTCTG-3` (forward, exon 14). Mutation load was determined according to the ratio of mutated/wild-type in the electropherograms of forward and reverse reaction. Cases being *SF3B1* unmutated by Sanger sequencing were reanalyzed with a more sensitive 454 deep-sequencing approach (NGS) on genomic DNA. The lowest detectable mutation load with NGS was about 3% (1,013 reads) and, for Sanger sequencing, 5-10% as confirmed by NGS. *JAK2*V617F, *JAK2*exon12 and *MPL*W515 mutations were analyzed as published previously.[9-11] Sensitivities of the assays were approximately 1%, 10%, and 5%, respectively. The frequency of *SF3B1*mut was 41 of 47 (87.2%). The 6 patients without *SF3B1*mut were reanalyzed by NGS but, despite higher sensitivity, no *SF3B1*mut was found. In the 41 *SF3B1*mut patients, 43 mutations with median mutation load of 40% (range 15-70%) were detected. The most frequent mutation was Lys700Glu (22 of 43, 51.2%), followed by Lys666Arg/Asn/Thr (7 of 43, 16.3%), Glu622Asp (4 of 43, 9.3%), His662Gln (4 of 43, 9.3%), Arg625Lys/Cys (3 of 43, 7.0%), Thr627Pro, Met784_Lys785delinsIle and a synonymous Leu575Leu (1 of 43, 2.3% each). The two double mutated cases had: i) Arg625Cys (load 45%) and Thr627Pro (load 45%); or ii) Lys700Glu (load 30%) and the synonymous Leu575Leu (load 70%) mutation. *JAK2*V617F was also very frequent (36 of 47, 76.6%). Twenty-five of 36 (69.4%) revealed a mutation load of 10-50%, 6 of 36

Table 1. Characteristics of patients according to *SF3B1* and *JAK2*V617F mutations.

	Total cohort	*SF3B1* wildtype	*SF3B1* mutated	*P*	*JAK2*V617F wildtype	*JAK2*V617F mutated	*P*	All others	*SF3B1* mutated/ *JAK2*V617F	*P*
All patients, n. (%)	47	6 (12.8)	41 (87.2)	–	11 (23.4)	36 (76.6)	–	17 (36.2)	30 (63.8)	–
Female, n. (%)	27 (57.4)	1 (3.7)	26 (96.3)	0.070	7 (25.9)	20 (74.1)	0.737	8 (29.6)	19 (70.4)	0.362
Male, n. (%)	20 (42.6)	5 (25.0)	15 (75.0)		4 (20.0)	16 (80.0)		9 (45.0)	11 (55.0)	
Age (years, mean, range*)	74 (44-89)	71 (55-86)	74 (44-89)	0.452	72 (44-87)	74 (55-89)	0.398	71 (44-87)	75 (58-89)	0.203
WBC (x10^9/L, mean, range*)	10.2 (2.9-60.0)	9.7 (4.2-14.8)	10.3 (2.9-60.0)	0.880	6.8 (4.6-10.4)	11.3 (2.9-60.0)	0.160	7.8 (4.2-14.8)	11.6 (2.9-60.0)	0.180
Hb (g/dL, mean, range*)	9.7 (6.9-13.1)	9.5 (7.7-12.0)	9.8 (6.9-13.1)	0.689	9.7 (7.9-12.7)	9.8 (6.9-13.1)	0.819	9.6 (7.7-12.7)	9.8 (6.9-13.1)	0.631
Platelet count (x10^9/L, mean, range*)	762 (466-1,500)	776 (473-1,100)	759 (466-1,500)	0.885	639 (497-892)	799 (466-1,500)	0.013	687 (473-1,100)	804 (466-1,500)	0.132
Ring sideroblasts (%, mean, range*)	61 (18-97)	42 (18-85)	63 (20-97)	0.016	55 (25-88)	62 (18-97)	0.345	50 (18-88)	66 (20-97)	0.011

*in brackets.

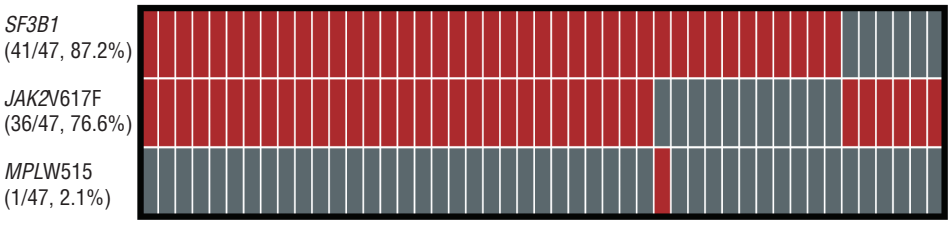

Figure 1. Frequencies and distribution of *SF3B1*, *JAK2*V617F and *MPL*W515 mutations. Each analyzed case is represented by a box. Mutated cases are colored in red, wild-type cases in gray.

(16.7%) had low (≤10%) and 5 of 36 (13.9%) high (>50%) mutation load. *SF3B1*mut and *JAK2*V617F were found concomitantly in 63.8% (30 of 47) patients. Remarkably, each of the 47 patients had at least either *SF3B1*mut or *JAK2*V617F (Figure 1). All *JAK2*V617F negative patients were additionally analyzed for *JAK2*exon12 but no mutation was detected. Only one patient (1 of 47, 2.1%) carried an *MPL*W515L in addition to *SF3B1*Lys700Glu. Age, Hb, WBC, platelet count, and percentage of ring sideroblasts were analyzed with t-test according to *SF3B1*mut and *JAK2*V617F. Platelet counts were significantly lower in *JAK2*wt compared to *JAK2*V617F patients (639 *vs.* 799x10^9/L, *P*=0.013). In contrast, *SF3B1*mut were associated with higher percentages of ring sideroblasts (*SF3B1*mut *vs.* *SF3B1*wt: 63% *vs.* 42%, *P*=0.016). *SF3B1*mut and *JAK2*V617F double mutated cases had significantly higher percentage of ring sideroblasts than all other cases (*SF3B1*mut/*JAK2*V617F *vs.* all others: 66 *vs.* 50%, *P*=0.011) (Table 1). However, there was no correlation between percentage of ring sideroblasts with *SF3B1* mutation load (*P*=0.632). In accordance with the high *SF3B1*mut frequency in our cohort (87.2%), previous publications have also reported high frequencies of 66.7% (12 of 18),[2] 72.2% (13 of 18),[4] 83.3% (5 of 6)[5] and 90.1% (10 of 11).[6] Furthermore, we and others showed *SF3B1*mut to occur in combination with *MPL*W515mut and mainly *JAK2*V617F.[2,4] Overlap of *SF3B1*mut and *JAK2*V617F were present in 63.8% patients, which is much more frequent than in previous studies with 11.1% (2 of 18)[2] and 22.2% (4 of 18).[4] This is mainly related to the high frequency of *JAK2*V617F (76.6%) in our RARS-T cohort, rarely seen in other studies (31-100%) in 3 to 23 analyzed patients.[8] A very large recent multicenter study with 175 RARS-T patients analyzed for *JAK2*V617F reported a frequency of 42.9%.[12] The only study of hematologic parameters in dependence of *SF3B1*mut in RARS-T patients detected no difference between *SF3B1*mut and *SF3B1*wt.[2] Our results confirmed this finding except that *SF3B1*mut cases had a significantly higher percentage of ring sideroblasts. This is comparable to results seen in MDS patients.[2] There is still ongoing discussion as to whether RARS-T is a distinct entity, or a subset of essential thrombocythemia or a progression of RARS.[7,8] In contrast to MDS, almost no *SF3B1*mut were found in MPN patients,[1,13] whereas *JAK2*V617F is characteristic for MPN and rare in MDS.[12,14] Thus, the combination of high *SF3B1*mut and *JAK2*V617F frequencies supports the idea that RARS-T actually represents an overlap of MDS and MPN. Single RARS-T cases have been reported that developed from a pre-existing RARS through increasing *JAK2*V617F mutation load or the acquisition of *JAK2*V617F.[12,15] As we could detect both RARS-T patients with high *SF3B1*mut and low *JAK2*V617F load and vice versa, our data support the idea that RARS-T might develop from two directions. In this process, MDS patients with *SF3B1*mut or MPN patients with *JAK2*V617F might each acquire the other mutation.

In summary, our results show that RARS-T is characterized by high frequencies of *SF3B1*mut associated with percentages of ring sideroblasts and *JAK2*V617F correlated with platelet counts. Our data clearly demonstrate that the assignment of RARS-T into the WHO category "Myelodyplastic/myeloproliferative neoplasms" is not only justified based on cytomorphology, but is also reflected by molecular mutations. We strongly recommend screening for *JAK2*V617F and *SF3B1*mut in all confirmed or suspected RARS-T patients to facilitate diagnosis and distinguish RARS-T from other disease entities or reactive conditions.

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