Refractory anemia with ring sideroblasts and marked thrombocytosis cases harbor mutations in *SF3B1* or other spliceosome genes accompanied by *JAK2*V617F and *ASXL1* mutations

Refractory anemia with ring sideroblasts and marked thrombocytosis (RARS-T) is a rare entity with characteristics of both myelodysplastic syndromes (MDS) and myeloproliferative neoplasms (MPN).[1] Patients have been shown to be frequently *JAK2*V617F and *SF3B1*, and less commonly *MPL*W515, mutated (mut).[1-4] Gene mutations that have been occasionally analyzed in limited cohorts include *TET2, ASXL1* and *DNMT3A*.[2,5,6] Recently, also *CALR*mut have been reported with varying frequencies of 0%-25%.[7-10] However, a comprehensive mutational analysis of both MPN and MDS related markers has still not been made. To further characterize the genetic landscape *of RARS-T,* we analyzed 17 genes *(ASXL1, CALR, CBL, DNMT3A, ETV6, EZH2, IDH1, IDH2, JAK2, MPL, NPM1, RUNX1, SF3B1, SRSF2, TET2, U2AF1, ZRSR2*) in a large cohort of 92 RARS-T patients, and we were able to create a comprehensive mutational landscape in 75 patients.

Patients were diagnosed with RARS-T if they strictly fulfilled the criteria according to the WHO classification 2008.[1] Part of the data has been published previously in Flach *et al*. (18 of 92),[5] Jeromin *et al*. (47 of 92),[3] and Broseus *et al*. (54 of 92)[3] and (54 of 95),[9] respectively, but these have been included in our study with the purpose of analyzing features not examined in the aforementioned articles. Of 92 patients, data for 36 (39.1%) have not been published before. Patients gave their informed consent to laboratory analyses and scientific studies. The study design adhered to the tenets of the Declaration of Helsinki and was approved by our institutional review board.

Male:female ratio was 1:1.4 and median age was 75 years (range: 44-89 years). Median white blood cell count (WBC) was 7.5 x10^9/L (2.9-60.0 x10^9/L), hemoglobin level 9.6 g/dL (6.9-13.2 g/dL), platelet count 659x10^9/L (454-1500 x10^9/L), and percentage of ring sideroblasts (RS) 61% (18%-97%). Eighty-six patients were cytogenetically analyzed. Seventy-one patients (82.6%) had normal karyotype and 15 chromosomal aberrations (*Online Supplementary Table S1*). Screening for mutations in *ASXL1, CALR, SF3B1*, and *SRSF2* was performed by direct Sanger sequencing. *JAK2*V617F, *JAK2*exon12 and *MPLW*515 were analyzed by melting curve analysis. All other genes were analyzed by a

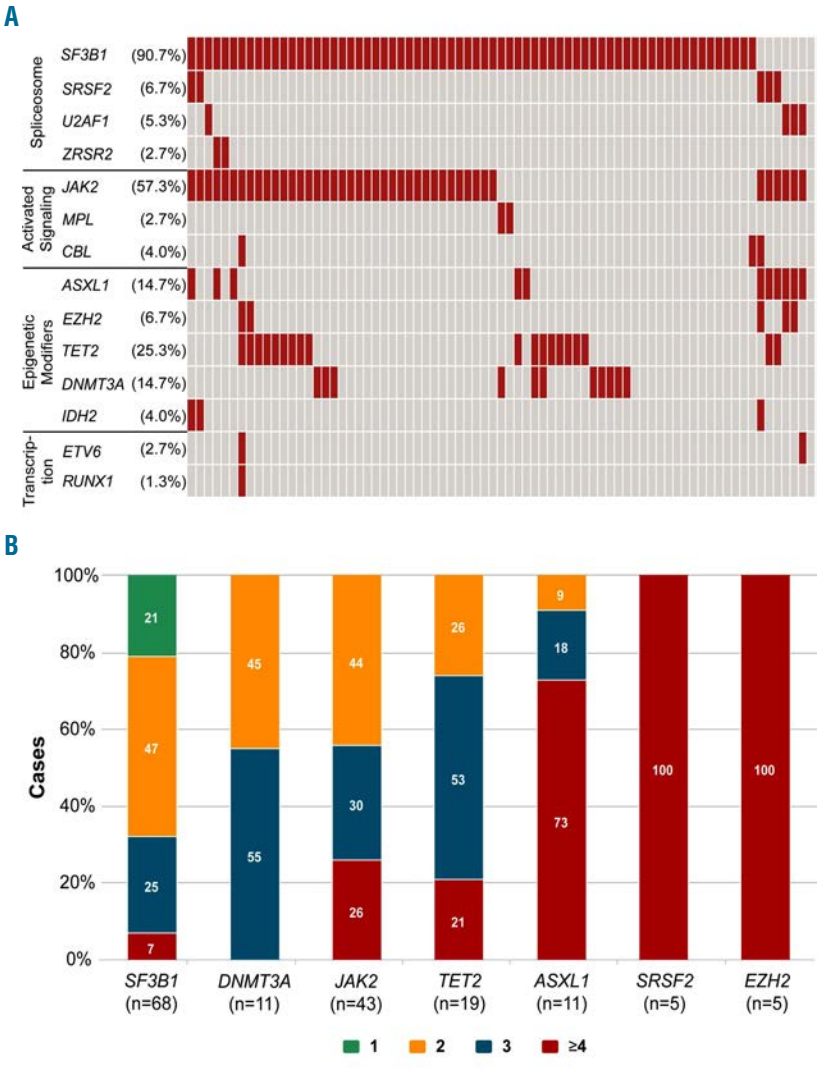

Figure 1. Distribution of mutations in 14 genes in 75 RARS-T patients. (A) Rows correspond to the depicted genes and columns represent individual patients. Cases presented with a mutation are colored in red and wild-type cases in gray. (B) Mutations occurring as sole alteration (1 mutation) (1 mutation) or concomitantly with other mutations (2, 3, ≥4 mutations) for the single gene mutations are shown. The percentage is depicted in the columns.

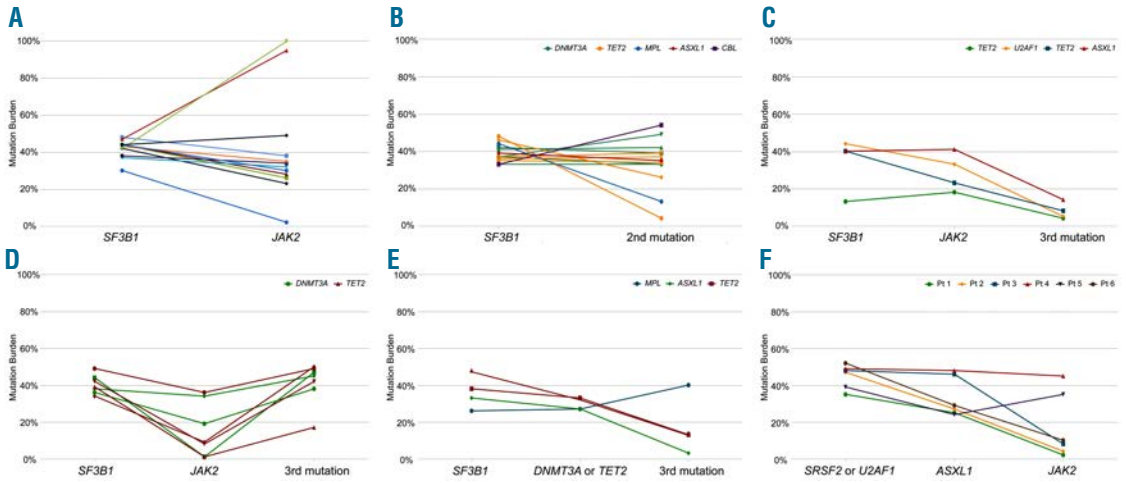

Figure 2. Mutation burdens of different co-occurring mutations. Each line defines an individual patient. (A) *SF3B1*mut/*JAK2*V617F double-mutated cases. (B) Cases with *SF3B1*mut and a second mutation other than *JAK2*V617F. Patients with *SF3B1*mut, *JAK2*V617F and a third mutation with low (C) and high (D) mutation burdens. (E) Cases with *SF3B1*mut and two additional mutations. Second mutation was in *TET2* in the case of *ASXL1*mut and in *DNMT3A* in the remaining patients. (F) Mutation burdens of *SRSF2*mut, *U2AF1*mut, *ASXL1*mut and *JAK2*V617F in 6 patients (Pt) with *SF3B1*wt.

454 deep-sequencing approach (NGS). For analysis of mutation burdens, cases were re-analyzed by NGS or a real-time quantitative approach for *JAK2*V617F (*Online Supplementary Appendix*).

Ninety-two patients were analyzed for mutations in the above-mentioned 17 different genes. As material was limited in some cases, 7.7% of all intended analyses could not be performed (*Online Supplementary Table S2*). The five most frequently mutated genes were: *SF3B1* (90.2%), *JAK2*V617F (58.7%), *TET2* (23.3%), *DNMT3A* (16.7%), and *ASXL1* (14.3%). *JAK2*exon12, *CALR*, *IDH1* and *NPM1* showed no mutation. The remaining gene mutations occurred in less than 10% of cases (*Online Supplementary Table S2*). Altogether, in 98.9% of the patients at least one mutation was found. Only one patient carried no mutation and had normal karyotype. We further analyzed this case for mutations in *PRPF8* that have recently been shown to occur in myeloid malignancies and to associate with ring sideroblasts.[11] In addition, a pan-myeloid NGS gene panel was applied providing higher sensitivity and data on 11 additional genes (*Online Supplementary Appendix*). However, also with these approaches no mutation was detectable.

For the final comprehensive analysis we focused on 75 patients that had available mutation status for all 14 genes mutated in at least one of the cases (Figure 1A). *JAK2*V617F presented in combination with *SF3B1*mut in 49.3% (37 of 75). Interestingly, nearly all *SF3B1* wild-type (wt) cases carried a *JAK2*V617F (85.7%; n.s.) and an *ASXL1*mut (85.7% *vs.* 7.4%; $P<0.001$). Furthermore, mutations in *SRSF2* (2.9% *vs.* 42.9%; $P=0.005$) and *U2AF1* (1.5% *vs.* 42.9%; $P=0.002$) were rare in *SF3B1*mut cases, but were associated with *ASXL1*mut (*SRSF2*mut: 36.4% *vs.* 1.6%; $P=0.001$; *U2AF1*mut: 27.3% *vs.* 1.6%; $P=0.009$). Of note, all 13 *DNMT3A*mut occurred concomitantly with *SF3B1*mut (n.s.) and were negatively associated with *JAK2*V617F (7.0% *vs.* 25.0%; $P=0.046$) (*Online Supplementary Figure S1*).

The number of gene mutations per patient ranged from 0 to 7 (mean: 2.4; median: 2.0). *SF3B1*mut were the only mutations that occurred as sole alteration (14 of 68, 20.6%) (Figure 1B) and were significantly associated with a low number of mutations (mean: 2.3 *vs.* 3.7; $P=0.002$) (*Online Supplementary Tables S3 and S4*). In contrast, 6 of 7 *SF3B1*wt patients carried a *JAK2*V617F and *ASXL1*mut accompanied by either an *SRSF2*mut (n=3) or *U2AF1*mut (n=3) (Figure 1A). Accordingly, *JAK2*V617F (3.0 *vs.* 1.6; $P<0.001$) and *ASXL1*mut (3.9 *vs.* 2.1; $P<0.001$) were correlated with high numbers of accompanied gene mutations (*Online Supplementary Table S4*).

We also analyzed the mutation burden of accompanying gene mutations in 50 cases with quantitative data. These analyses were carried out on unsorted cells, but aimed to analyze the relation of allelic frequencies and not absolute counts. In cases with *SF3B1*mut, this mutation usually had the highest burden (Figure 2A-E and *Online Supplementary Figure S2A-D*). Cases with *SF3B1*mut/*JAK2*V617F showed slightly lower mutation burdens for *JAK2*V617F in most patients. Furthermore, in one case a small clone and in 2 patients a homozygous state of *JAK2*V617F were detected, respectively (Figure 2A). In a further 13 patients, *SF3B1*mut were accompanied by a second mutation with similar mutation burden in 8 cases. In 3 cases, the additional mutation presented with a clearly lower and in 2 with a higher burden (Figure 2B). In 11 patients with *SF3B1*mut/*JAK2*V617F and a third mutation, two groups could be distinguished: either the third mutation (n=4) (Figure 2C) or *JAK2*V617F (n=8) (Figure 2D) showed the lowest burden. In 4 patients with three alterations not including *JAK2*V617F, the only mutation with a clearly higher burden than *SF3B1*mut was *MPL*W515. For the remaining cases, the third mutation showed a burden of 15% or less (Figure 2E). In 4 patients with 4 to 7 different gene mutations, including *SF3B1*mut, no definite pattern could be detected (*Online Supplementary Figure S2A-D*). Analysis of 6 *SF3B1*wt cases revealed that *SRSF2*mut or *U2AF1*mut had the highest mutation burdens, whereas *ASXL1*mut had either similar (n=2) or lower (n=4) burdens. The *JAK2*V617F presented as a subclonal mutation in 4 cases (Figure 2F and *Online Supplementary Figure S3*). The broad spectrum of *JAK2*V617F mutation burdens suggests

diverse sequences of acquirement. Altogether, these data implicate that, in most RARS-T cases, spliceosome mutations either in *SF3B1*, *U2AF1* or *SRSF2* are the founder mutations. *ASXL1*mut and *JAK2*V617F seem to be acquired at different time points of the evolution of RARS-T and are in most cases subclonal mutations.

Analyzing the clinical characteristics, we detected associations of *SF3B1*mut with higher percentages of RS (mean: 61% *vs.* 41%; *P*=0.006) and of *JAK2*V617F with higher WBC (10.7 *vs.* 6.9x10^9/L; *P*=0.004) and platelet counts (807 *vs.* 599x10^9/L; *P*<0.001). *ASXL1*mut cases had lower percentages of RS, even though approximately half of these patients had concomitant *SF3B1*mut (43% *vs.* 62%; *P*=0.004). In contrast, *DNMT3A*mut/*SF3B1*mut patients had higher percentages of RS than *DNMT3A*wt/*SF3B1*mut cases (69% *vs.* 58%; *P*=0.017). Interestingly, the cases with sole *SF3B1*mut had lower platelet counts (546 *vs.* 749x10^9/L; *P*<0.001) and were younger (68.4 *vs.* 74.2 years; *P*=0.040). Survival analyses were not applicable since available data were limited (n=52) and because of the mild course of the disease (events=5). However, part of our cohort has been integrated into a large multicenter study showing that *SF3B1*mut, *JAK2*V617F and younger age at diagnosis were independent factors for a good prognosis in RARS-T.[3]

Taken together, we showed that *SF3B1*mut have a unique relevance in the development of RARS-T and seem to be the founding mutation in most cases. In *SF3B1*wt cases, however, *U2AF1* and *SRSF2* seem to be the founder mutations. Hence, 98.7% (74 of 75) RARS-T cases carried a spliceosome mutation. Furthermore, the high prevalence of *ASXL1*/*JAK2*V617F double mutations (6 of 7; 85.7%) in *SF3B1*wt cases is unique for RARS-T. It might be presumed that in contrast to *SF3B1*mut patients, *SRSF2*mut and *U2AF1*mut cases require mutations in *ASXL1* and *JAK2*V617F for evolution of RARS-T.

There has been discussion as to whether RARS-T is a distinct entity, or a subset of essential thrombocythemia or a progression of RARS.[1] Besides *JAK2*V617F and *MPL*W515 mutations that are characteristic for MPN, approximately half of the patients in our cohort show a more MDS-characteristic pattern of mutations. *SF3B1*mut, *TET2*mut, *ASXL1*mut and *EZH2*mut show similar frequencies as those found in MDS and exceed those seen in MPN.[12-14] Of note, we found a high occurrence of *DNMT3A*mut in RARS-T, as previously described by others.[2] However, in our cohort, *DNMT3A*mut was always accompanied by an *SF3B1*mut, resembling data from RARS patients.[15] Interestingly, *CALR*mut were not detected in any of our patients, whereas they can be found frequently in essential thrombocythemia and primary myelofibrosis.[7,8] In other studies, varying frequencies of *CALR*mut of 0-25% have been reported in RARS-T.[7-10] However, the frequency of *CALR*mut is most likely very low. High frequencies have been detected only in small cohorts: 12.5% (n=24)[7] and 25.0% (n=12),[10] whereas a large multicenter study reported a frequency of only 1.1% (n=95).[9]

In summary, RARS-T patients carry more mutations than previously reported and are specified by frequent occurrence of *SF3B1*mut (90.2%). Nearly all *SF3B1*wt patients show *SRSF2*mut or *U2AF1*mut in addition to *ASXL1*mut and *JAK2*V617F. Therefore, 99% of RARS-T cases can be characterized by mutations in these five genes. The alterations that mediate myeloproliferative character in those cases with a more MDS-characteristic mutation pattern are still unknown. Moreover, the patient with no detectable mutation in our cohort highlights the fact that some gene alterations in RARS-T have still not been discovered.

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