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ORIGINAL ARTICLE

Age, *JAK2*^{V617F} and *SF3B1* mutations are the main predicting factors for survival in refractory anaemia with ring sideroblasts and marked thrombocytosis

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Refractory anaemia with ring sideroblasts (RARS) and marked thrombocytosis (RARS-T) is a provisional entity in the World Health Organisation 2008 classification and has previously been shown to have a high proportion of *JAK2*^{V617F} (*Janus Kinase 2*) and *SF3B1* (*Splicing Factor 3B subunit 1*) mutations. The purpose of the present study was to analyse the frequency of *SF3B1* mutations in a large cohort of 111 patients with RARS-T and 33 patients with RARS and to explore the prognostic impact of *SF3B1* mutational status on RARS-T. The frequency of *SF3B1* mutations in RARS-T (96/111, 86.5%) and RARS (28/33, 84.8%) was similar. In RARS-T, median survival was better in *SF3B1*-mutated patients than in *SF3B1*-non-mutated patients (6.9 and 3.3 years, respectively, $P = 0.003$). RARS can be differentiated from RARS-T by the frequency of *JAK2*^{V617F} (0% vs 48.6%). In RARS-T patients, *SF3B1* ($P = 0.021$) and *JAK2* mutations ($P = 0.016$) were independent factors for a better prognosis. Altogether, our results confirm that RARS-T is an independent entity that should be recognised by the next World Health Organisation classification. The assessment of *SF3B1* mutations is of prognostic interest in RARS-T patients. Younger age, *JAK2*^{V617F} and *SF3B1* mutations are the main predicting factors for survival in RARS-T.

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Keywords: refractory anaemia with ring sideroblasts and marked thrombocytosis; *SF3B1*; prognostic impact; survival

INTRODUCTION

Refractory anaemia with ring sideroblasts (RARS) and marked thrombocytosis (RARS-T) has been proposed in the World Health Organisation 2001 classification of tumours of haematopoietic and lymphoid tissues and retained as a provisional entity in the classification of 2008.[1] RARS-T is classified in the myelodysplastic/myeloproliferative (MDS/MPN) disorders group, because it presents with the dysplastic features of RARS[2] and the myeloproliferative features of essential thrombocythemia (ET).[3] RARS-T is characterised by a high rate of *JAK2*^{V617F} (*Janus Kinase 2*) mutations[4–15] and/or the presence of the mutation *MPL*$^{W515L/R}$ (*MyeloProliferative Leukemia*).[16,17] The classification of RARS-T as an entity that is independent from RARS or ET is currently a matter of debate. Several specialists favour the hypothesis that RARS-T is a form of ET with $\geq$15% of ring sideroblasts in the bone marrow[18] while others think that RARS-T develops from RARS with secondary thrombocytosis accompanying the acquisition of the *JAK2*^{V617F} mutation.[19]

Recent publication from our group in a European retrospective multicentre collaborative study demonstrated that RARS-T was independent from RARS and ET from a clinical and biological as well as prognostic point of view.[20]

Our results have recently been strengthened by the discovery of the association between myelodysplastic syndromes and mutations involving components of the RNA splicing machinery, including *U2AF35* (*U2 small nuclear RNA Auxiliary Factor 35*), *ZRSR2* (*Zinc finger CCCH type*, *RNA-binding motif and Serine/aRginine rich 2*), *SRSF2* (*Serine/aRginine-rich Splicing Factor 2*) and *SF3B1* (*Splicing Factor 3B subunit 1*). *SF3B1* mutations (*SF3B1*^{mut}) are found in about 20% of total MDS and correlate strongly with the presence of $\geq$15% of ring sideroblasts (MDS-RS; 64–82.6% in RARS, 57–76% in refractory cytopenia with multilineage dysplasia and ring sideroblasts (RCMD-RS) and 66.7–72% in RARS-T).[21–27] On the other hand, mutations of *SF3B1* are found at a lower frequency in MDS with $<$15% ring sideroblasts, which confirms the specificity of *SF3B1*^{mut} in MDS-RS. *SF3B1* mutations are rare in myeloproliferative neoplasms and particularly in ET (0–3%).[22,28] The high frequency of *SF3B1* mutations suggests that these mutations have a pathophysiological role in these diseases, probably through perturbations of RNA splicing. The link between *SF3B1*-mutated status and ring sideroblasts has been confirmed in a recent experimental study on murine models.[29] About one quarter of MDS-RS are *SF3B1*^{wt} and somatic mutations of *SRSF2* or *ZRSR2* have been described in about 7% of MDS-RS,[21]

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which suggests that other mutant genes may have a role in the appearance of ring sideroblasts. Furthermore, a recent study showed that RARS-T presented with a particular genetic pattern with a high frequency of *JAK2*^{V617F} and *SF3B1* mutations, confirming the classification of RARS-T in the category of myelodysplastic/myeloproliferative neoplasms.[30]

Finally, as precedent studies have been performed on little RARS-T cohorts, the prognostic impact of *SF3B1*^{mut} status remains controversial,[22,24,25,27,31] and there is a need for a study on a larger cohort. Our purpose was to analyse the frequency of *SF3B1* mutations in a large cohort of 111 RARS-T and to explore the prognostic impact of *SF3B1* mutations in this disorder.

PATIENTS AND METHODS

Patient selection

According to the World Health Organisation 2008 classification, patients were diagnosed with RARS-T if they fulfilled the following criteria: (i) anaemia (haemoglobin level <125 g/l for females and <135 g/l for males) with erythroid dysplasia and $\geq 15\%$ ring sideroblasts; (ii) thrombocytosis of $\geq 450 \times 10^9$ platelets/l; (iii) $<5\%$ blast cells in the bone marrow; (iv) the presence of large atypical megakaryocytes similar to those observed in *BCR-ABL1*-negative myeloproliferative neoplasms; (v) no secondary cause of ring sideroblasts; and (vi) no karyotype abnormalities, such as del(5q), t(3;3)(q21;q26) or inv(3)(q21q26).[1]

To obtain a comprehensive data set of this rare entity, samples from seven European centres in three European countries were collected. The total cohort comprised 111 cases of RARS-T and 33 cases of RARS.

Data record

For each patient, demographic (gender, age at diagnosis, date of death or last follow-up) and biological data (blood cell count, bone marrow exploration, ring sideroblasts, karyotype, molecular explorations) were collected.

The *SF3B1* mutations were analysed with a sensitive next-generation amplicon deep-sequencing assay (454 Life Sciences, Branford, CT, USA) with a median coverage of 500 reads. This approach was able to detect mutations with a sensitivity $<1\%$.

The *JAK2*^{V617F} mutation was analysed by allele-specific real-time PCR to estimate allele burden according to methods published by Lippert *et al.*[32] and Schnittger *et al.*[33] with a sensitivity of 1%. *JAK2*^{exon12} analysis was performed according to the method of Schnittger *et al.*,[34] and the *MPL*^{W515} mutations were analysed by high-resolution melting curve analyses followed by Sanger sequencing if positive, as previously published by Schnittger *et al.*[35]

Statistical analyses

Standardised overall survival was estimated by the Kaplan–Meier method and based on the time from diagnosis to death or last contact. Survival curves for the different groups were compared using the log rank test. Multivariate analysis was performed using Cox's proportional hazards model.

Approval for the study was obtained from the ethics committee of each institution, and the procedures were carried out in accordance with the Helsinki Declaration of 1975, as revised in 2000.

RESULTS

Demographic and biological features

A total of 144 cases (111 RARS-T and 33 RARS including 72 males and 72 females) were recorded in the study. Median age at diagnosis was 73.9 years (range: 44.4–96.1 years). The median follow-up was 37.5 and 55.2 months for the RARS-T and RARS cohort, respectively (Table 1). Survival data were available in 130 (97 RARS-T and 33 RARS) of the 144 patients.

Frequencies and characterisation of mutations

A karyotype was available in 128 cases. One hundred and ten (85.9%) patients carried a normal karyotype, whereas 18 (14.1%) patients showed aberrant karyotypes, which was equally distributed between RARS-T and RARS patients. Even if the IPSS (International Prognostic Scoring System) score can only be applied to MDS *de novo*, we calculated it to check if we had a homogeneous group of patients. Most of the patients of the total cohort (133 out of 144) had an IPSS score of 0.

A *SF3B1*^{mut} was noted in 124 out of the 144 patients (86.1%). A total of 127 *SF3B1* mutations were detected in these 124 patients (28 RARS and 96 RARS-T). Three RARS-T cases carried two

Table 1. Demographic and biological characteristics of RARS-T and RARS patients

	RARS-T patients	*RARS patients*
n	111	33
Male (%)	46.8	60.6
Age at diagnosis (years)		
Median	74.3	71.1
Range	44.4–92.1	48.4–96.1
20–50 years, *n* (%)	4 (3.6)	2 (6.1)
50–70 years, *n* (%)	36 (32.5)	9 (27.2)
>70 years, *n* (%)	71 (63.9)	22 (66.7)
Available survival data (*n*)	97	33
Median follow-up (years)	3.1	4.6
WBC ($\times 10^9$/l)		
Median	7.6	5.2
Range	2.1–60.0	1.6–17.3
Hb (g/l)		
Median	96.5	91.0
Range	51.0–131.0	69.0–128.0
Platelets ($\times 10^9$/l)		
Median	646	314
Range	452–1500	61–444
450–600, *n* (%)	54 (48.6)	
>600, *n* (%)	57 (51.4)	
Ring sideroblasts (%)		
Median	52	40
Range	16–97	19–85
SF3B1 mutations (%)	86.5	84.8
Tested (*n*)	111	33
Mutated (*n*)	96	28
p.Lys700Glu	51	16
p.Lys666Glu/Thr/Asp/Asn	16	2
p.His662Asp/Gln	11	2
p.Glu622Asp	7	2
p.Arg625Gys/Leu/Gly	5	3
p.Thr663Pro	2	2
p.Met784_Lys785delinslle	1	0
p.Asp781Gly	0	1
Two different mutations	3	0
JAK2^{V617F} mutations (%)	48.6	0
Tested (*n*)	111	33
MPL mutations (%)	1	0
Tested (*n*)	102	27
IPSS		
0	104	29
0.5	5	3
1	2	1

Abbreviations: Hb, haemoglobin; IPSS, International Prognostic Scoring System; *JAK2*, Janus Kinase 2; *MPL*, MyeloProliferative Leukaemia; RARS, refractory anaemia with ring sideroblasts; RARS-T, refractory anaemia with ring sideroblasts and marked thrombocytosis; *SF3B1*, Splicing Factor 3B subunit 1; WBC, white blood cells.

different mutations. With the exception of one p.Arg549Cys in exon 12 and two in exon 16, all mutations were located in exons 14 and 15. All but one del/ins mutations (p.Met784_Lys785del/inslle) were missense mutations. In detail, the most frequent mutation was p.Lys700Glu (68/127 53.5%), followed by p.Lys666-Glu/Thr/Asp/Asn mutations ($n=18$, 14.2%), p.His662Asp/Gln ($n=13$, 10.2%), p.Arg625Cys/Leu ($n=10$, 7.9%), p.Glu622Asp ($n=9$, 7.1%) and p.Thr663Pro ($n=4$, 3.1%). Five further mutations were detected in single cases only. Frequencies and positions of mutations are illustrated in Table 1 and Figure 1. Median mutation/wildtype load was 40% (range: 15–78%). Small subclones with *SF3B1*^{mut} were not detected.

Frequency of mutations in RARS and RARS-T

The frequency of *SF3B1* mutations in RARS-T (96/111, 86.5%) was similar to that in RARS (28/33, 84.8%). By contrast, both entities differed by the presence of the *JAK2*^{V617F} mutation, which was detected in 54/144 (37.5%) in the total cohort but in 54/111 (48.6%) in RARS-T compared with none (0/33) in RARS ($P<0.001$). Among the RARS-T *SF3B1*^{mut}, 46/96 (47.9%) harboured a *JAK2*^{V617F} mutation. *JAK2*^{V617F} allele burden was very heterogeneous with a median of 49% (range: 1–100%). No *JAK2*^{exon12} mutation (111 tested) was observed, whereas only one case with the *MPL*^{W515L} mutation was noted in a RARS-T (102 tested; Table 1 and Figure 2).

Biological association

The presence of *SF3B1*^{mut} was analysed with respect to age, sex, white blood cell count, haemoglobin levels, platelet counts, blast counts, percentage of ring sideroblasts, karyotype and *JAK2*^{V617F} allele burden. In RARS-T, *SF3B1* mutations were more frequent in females (56/59, 94.9%) than in males (40/52, 76.9%) ($P=0.010$), and mean ring sideroblast counts were higher in *SF3B1*^{mut} than in *SF3B1*^{wt} (55% vs 38%) ($P=0.007$). No further correlations were detected for these parameters.

Impact of mutations on outcome

The difference in survival between RARS-T and RARS was not statistically significant (median survival 10.7 vs 6.2 years, respectively, $P>0.05$). On the other hand, in the total cohort, patients with *SF3B1*^{mut} had longer survival than those with

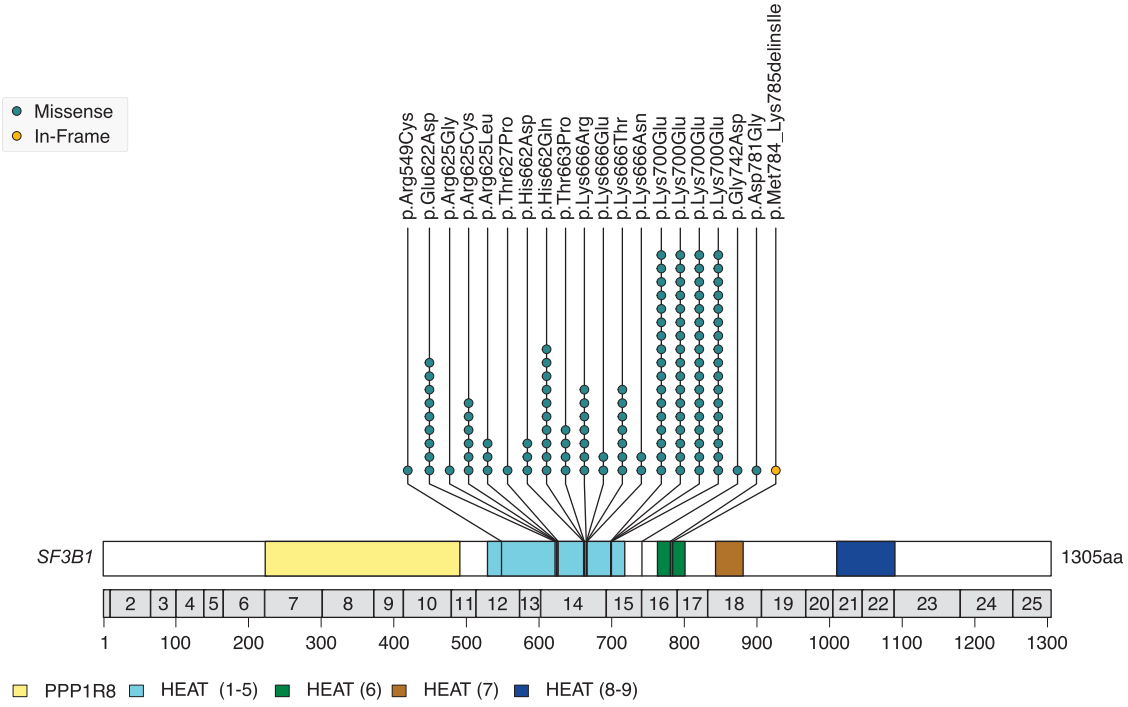


Figure 1. Positions and characterisation of mutations in the HEAT domains 1–6 (exons 12–16) of the *SF3B1* gene. Missense mutations are indicated in green and the rare ins/del mutation in yellow. HEAT, Huntingtin, elongation factor 3, protein phosphatase 2A, Tor1; PPP1R8, protein phosphatase 1, regulatory subunit 8; aa, amino acid.

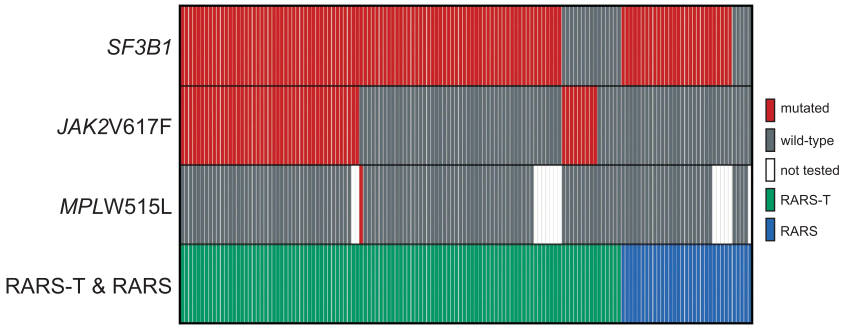


Figure 2. Distribution of *SF3B1*, *JAK2*^{V617F} and *MPL*^{W515L} mutations in RARS-T and RARS.

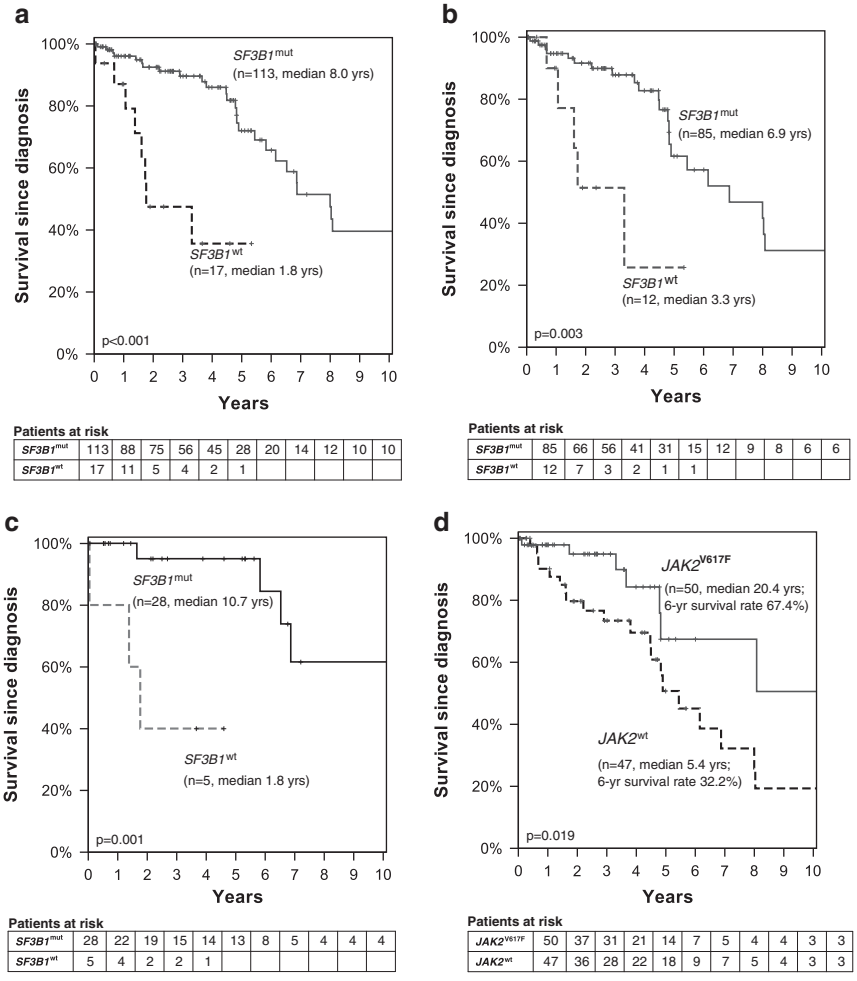


Figure 3. Kaplan–Meier analysis for survival since diagnosis according to *SF3B1* and *JAK2*^{V617F} mutation status. *SF3B1* in (**a**) total cohort, (**b**) RARS-T, (**c**) RARS and (**d**) *JAK2*^{V617F} in RARS-T.

SF3B1^{wt} (8.0 vs 1.8 years, respectively, $P<0.001$; Figure 3a). When restricted to RARS-T (85 *SF3B1*^{mut} and 12 *SF3B1*^{wt}), median overall survival was 6.9 years in *SF3B1*^{mut} vs 3.3 years in *SF3B1*^{wt} ($P=0.003$; Figure 3b). In RARS, survival was 10.7 years in the *SF3B1*^{mut} ($n=28$) and 1.8 years in *SF3B1*^{wt} patients ($n=5$; $P=0.001$; Figure 3c). In RARS-T patients, the survival rates at 2, 4 and 6 years within the *JAK2*^{V617F} sub-cohort were 94.9, 84.3 and 67.4%, respectively, while within the *JAK2*^{wt} sub-cohort, they were 79.7, 69.6 and 32.2%, respectively. *JAK2*^{V617F} ($n=50$) was then associated with a more favourable outcome compared with *JAK2*^{wt} ($n=47$; $P=0.019$; Figure 3d).

Cox regression analysis

In the total cohort including RARS-T and RARS cases in univariate analysis, age ($P=0.020$), ring sideroblast count ($P=0.008$) and *SF3B1* mutational status ($P<0.001$) were prognostically significant, but *SF3B1* mutational status was the only independent prognostic factor ($P=0.001$) in multivariable analysis.

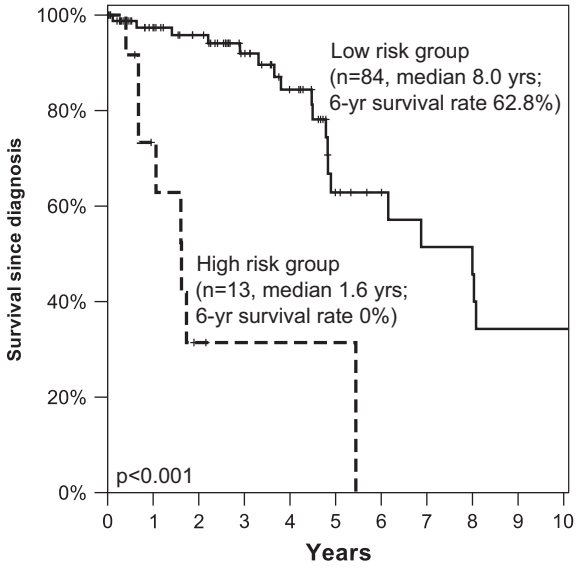
In the RARS-T cohort in univariate analysis, age ($P=0.038$), ring sideroblast count ($P=0.014$), *JAK2*^{V617F} ($P=0.024$) and *SF3B1*^{mut} ($P=0.006$) were prognostically significant but only age ($P=0.044$), *JAK2*^{V617F} ($P=0.016$) and *SF3B1*^{mut} ($P=0.021$) were independent prognostic parameters in multivariable analysis. Survival was better in patients with age ≤ 80 years, *JAK2*^{V617F} and *SF3B1* mutations. Taking into account these three prognostic factors, a model for survival in RARS-T patients was constructed in which each risk factor (that is, age >80 years, *SF3B1*^{wt} and *JAK2*^{wt}) was worth 1 point and defined two groups of risk: high risk for patients with a score of 2 or 3 ($n=13$) and low risk when the score is 0 or 1 ($n=84$). Median survival in the high-risk group was 1.6 vs 8.0 years in the low-risk group ($P<0.001$; corresponding survival rates at 2, 4 and 6 years are 31.4, 31.4 and 0% for high-risk patients vs 95.8, 84.4 and 62.8% for low-risk patients; Figure 4).

DISCUSSION

Up to now, *SF3B1* mutations have been studied in small series of RARS-T patients. In this study, we provide data on *SF3B1* mutations in a large cohort of RARS-T patients, which is, to the best of our knowledge, the largest published to date.

SF3B1 mutations were observed with a high frequency in both RARS-T and RARS patients (86.5% and 84.8%, respectively). These proportions are slightly higher than those already published, as *SF3B1* mutations have been found in 64–82.6% of RARS[21–24] and 66.7–72% in RARS-T.[23,24] The slightly higher frequency of *SF3B1* mutations in the current study may be due to the larger cohort of RARS-T patients than in other studies. There may also be differences in methodology, for example, direct sequencing vs non-sequencing-based screening strategies.

However, RARS differs from RARS-T in that there are no *JAK2*^{V617F} mutations in RARS,[4,14] whereas there is a high frequency in RARS-T.[4–13,15,19] We recently showed that RARS-T differed from RARS and ET from a clinical, biological and



Patients at risk

Lowrisk	84	66	57	42	31	15	12	9	8	6	6
High risk	13	7	2	1	1	1					

Figure 4. Kaplan–Meier analysis for survival according to two risk groups. High risk comprising patients with at least two of the following risk factors: age >80 years, *SF3B1*wt, *JAK2*wt; and low risk: one or less of the risk factors.

prognostic point of view, suggesting that RARS-T could be considered as a unique entity.[20] The results of our current study showing the presence of both *SF3B1* and *JAK2*^{V617F} mutations in a high proportion of RARS-T confirm that RARS-T is a unique entity. Indeed, RARS-T was associated with high rates of *SF3B1* mutations (86.5%) and *JAK2*^{V617F} mutations while ET patients have a low frequency of *SF3B1* mutations, and in RARS, no *JAK2*^{V617F} mutations were detected.

A minority of RARS-T patients (13.5%) presented without *SF3B1* mutations. This could be due to another mutation affecting components of the RNA splicing machinery as mutations of *SRSF2*, *U2AF35* and *ZRSR2* have already been described in 12.4, 7.3 and 3.1% of MDS, respectively,[36] or due to mutations of proteins associated with SF3B1 (SF3B4 and SF3B14).

These results are in line with our hypothesis that RARS-T is an independent entity characterised by a particular molecular combination associating mutations that give a myeloproliferative advantage (*JAK2*^{V617F}, *MPL*$^{W515R/L}$ or other unknown mutations) and mutations of components of the splicing machinery responsible for myelodysplastic features (*SF3B1* in most cases, and possibly *SRSF2*, *U2AF35* or *ZRSF2* in the remaining cases).

Conflicting results on the prognostic impact of *SF3B1* mutations in MDS have been reported as several studies noted a good prognostic impact in MDS, whereas others hypothesised that *SF3B1* mutations were associated with good-prognosis MDS subgroups but lost their prognostic impact in RARS and in RCMD-RS.[27]

In our large cohort of RARS-T patients, *SF3B1* mutations were associated with female sex, higher ring sideroblast counts and a longer overall survival than in *SF3B1*^{wt} patients. In a multivariable analysis, age >80 years at diagnosis, *SF3B1*^{wt} as well as *JAK2*^{wt} were independent factors of a worse prognosis. Based on these three independent parameters, a prognostic score for RARS-T patients was created to define two risk groups: high risk when there were two or three risk factors, low when there was only one or no risk factor. Median survival was 1.6 vs 8.0 years in the high- and low-risk group, respectively, underlying the relevance of such score in RARS-T patients.

Exploring *SF3B1* mutations in MDS associated with ring sideroblasts is of interest from a prognostic point of view, particularly as specific treatment will be available.[37,38] Also, allele-specific PCR have been designed, and these could be useful for monitoring minimal residual disease in MDS.[39]

In summary, this study confirms that RARS-T should be considered an independent entity. In RARS-T patients, age <80 years at diagnosis, *SF3B1* and *JAK2* mutations are independent factors for better survival and may be used to stratify patients.

CONFLICT OF INTEREST

SS and TH declare part ownership of the MLL Munich Leukemia Laboratory. TA, SJ and VG are employed by the MLL Munich Leukemia Laboratory. All the other authors declare no conflict of interest.

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