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Low rate of calreticulin mutations in refractory anaemia with ring sideroblasts and marked thrombocytosis

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Refractory anaemia with ring sideroblasts (RARS) and marked thrombocytosis (RARS-T) was proposed in the World Health Organisation 2001 classification of tumours of haematopoietic and lymphoid tissues and retained as a provisional entity in the 2008 version.[1] RARS-T is characterised by a high rate of *JAK2*^{V617F} mutations[2–4] or the presence of mutations in exon 10 of *MPL* (myeloproliferative leukaemia).[5,6] The existence of RARS-T as an entity independent from RARS and essential thrombocythemia (ET) was a matter of debate, as certain specialists favoured the hypothesis that RARS-T was a form of ET with $>15\%$ of ring sideroblasts in the bone marrow, whereas others thought that RARS-T is derived from RARS with secondary thrombocytosis developing through the acquisition of the *JAK2*^{V617F} mutation.

We recently demonstrated that RARS-T differed from RARS and ET from a clinical, biological and prognostic point of view.[4] The presence of high rates of splicing factor 3B subunit 1 (*SF3B1*) mutations in RARS-T and the absence of these mutations in ET strengthened the hypothesis that RARS-T was a distinct entity.[7–9]

However, the myeloproliferative features of RARS-T are not totally explained by *JAK2*^{V617F} and *MPL*^{W515L} mutations, as these account for only 50% and 1% of RARS-T, respectively. The question remains about other causative mutations responsible for the myeloproliferative part of RARS-T. The challenge is the same in myeloproliferative neoplasms (MPN), as 50–60% of ET and primary myelofibrosis (PMF) do not present with any *JAK2* or *MPL* mutations.

The recent discovery of a high rate of calreticulin (*CALR*) mutations in *JAK2*-non-mutated MPNs was a great step towards better understanding of the molecular pattern of these diseases. Mutations in exon 9 of *CALR* have been reported as mutually exclusive of *JAK2* and *MPL* mutations, and found in 67–71% of ET and 56–88% of PMF with wild-type *JAK2* or *MPL*.[10,11] These mutations are insertions or deletions leading to a frameshift responsible for modifying the C-terminal part of the protein. Following these modifications, the C-terminal part of the protein becomes positively charged and the reticulum targeting KDEL sequence is abrogated, which disturbs its cellular localisation.

Up to now, *CALR* mutations have only been explored in small RARS-T cohorts, with a mutation frequency ranging from 0 to 12.5%.[10,11] The purpose of our study was to analyse the frequency of *CALR* mutations in a large RARS-T cohort in order to determine whether *CALR* mutations may also be responsible for thrombocytosis in this disease. We therefore analysed a large cohort of 95 RARS-T patients, with 29 RARS as the control.

According to the WHO 2008 classification, patients are diagnosed with RARS-T if they fulfil the following criteria: i) anaemia (haemoglobin level <125 g/l for females and <135 g/l for males) with erythroid dysplasia and $>15\%$ ring sideroblasts; ii) thrombocytosis with $>450\times10^9$ platelets/l; iii) $<5\%$ blast cells in the bone marrow; iv) presence of large atypical megakaryocytes similar to those observed in *BCR-ABL1*-negative MPN; 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] One hundred and twenty-four samples including 95 RARS-T and 29 RARS from seven European centres in three European countries were collected and tested. This cohort has been published previously.[4,9]

For each patient, demographic (gender and age at diagnosis) and biological data (blood cell count, bone marrow exploration, ring sideroblasts, karyotype and 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 below 1%.[9]

The *JAK2*^{V617F} mutation was analysed by allele-specific real-time PCR to estimate the allele burden according to the method published by Lippert *et al.*[12] with a sensitivity of 1%. *JAK2* exon 12 analysis was performed according to the method by Schnittger *et al.*[13] 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.*[14]

The *CALR* exon 9 mutations were screened by fragment analysis and Sanger sequencing according to the method by Klampfl *et al.*[10]

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.

A total of 124 cases with 95 RARS-T and 29 RARS (including 62 males and 62 females) were recorded in the study, which is, to our best knowledge, the largest series of myelodysplastic syndromes with ring sideroblasts (MDS-RS) studied for *CALR* mutations. The median age at diagnosis was 74 years and 73 years for the RARS-T and RARS cohort, respectively (Table 1). A karyotype was available in 112 cases (87 RARS-T and 25 RARS). Seventy-five (86.2%) RARS-T

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

	RARS-T patients	*RARS patients*
n	95	29
Male (%)	45.2	65.5
Age at diagnosis (years)		
Median	74.0	73.0
Range	44.4–88.1	48.4–93.0
20–50 (*n*)	4	2
50–70 (*n*)	30	8
>70 (*n*)	61	19
WBC ($\times 10^9$/l)		
Median	7.6	5.5
Range	2.1–60	1.9–17.3
Hb (g/l)		
Median	9.6	9.1
Range	5.1–12.9	6.9–12.8
Platelets ($\times 10^9$/l)		
Median	655	274
Range	455–1500	61–444
450–600 (*n*)	45	
>600 (*n*)	50	
Ring sideroblasts (%)		
Median	53.5	46.5
Range	16–97	19–85
Karyotype		
Tested (*n*)	87	25
Abnormal (*n*)	12	6
SF3B1 *mutations (%)*	88.3	82.7
Tested (*n*)	94	29
JAK2^{V617F} *mutations (%)*	49.4	0.0
Tested (*n*)	95	29
MPL mutations (%)	1.1	0.0
Tested (*n*)	88	23
CALR mutations (%)	1	3.4
Tested (*n*)	95	29

Abbreviations: CALR, calreticulin; JAK2, Janus kinase 2; MPL, myeloproliferative leukaemia; SF3B1, splicing factor 3B subunit 1; RARS, refractory anaemia with ring sideroblasts; RARS-T, refractory anaemia with ring sideroblasts and marked thrombocytosis; WBC, white blood cells.

patients carried a normal karyotype, whereas 12 (13.8%) patients showed aberrant karyotypes. The rates in RARS-T and RARS patients were comparable.

An *SF3B1* mutation was noted in 24 (82.7%) RARS and 83 (88.3%) RARS-T confirming that this mutation is highly represented in MDS-RS.

A *JAK2*^{V617F} mutation was noted in 47 of the 95 RARS-T patients (49.4%) but in no RARS patients. The *JAK2*^{V617F} allele burden was very heterogeneous with a median of 33.3% (range: 1–92%). No *JAK2* exon 12 mutation (19 tested) was observed, whereas only one *MPL*^{W515L} mutation was noted in a patient with RARS-T (88 tested; Table 1 and Figure 1).

A *CALR* mutation was noted in only one (1%) RARS-T and one (3.4%) RARS patient. The *CALR* mutation found in the RARS-T patient was a 10-bp (1129–1138) deletion located in exon 9. Surprisingly, this patient also presented a *JAK2*^{V617F} mutation with a low allele burden (4%) and an *SF3B1* mutation. The mutational status of this patient was confirmed by two independent laboratories on two different DNA samples. This patient is a 73-year-old women diagnosed in 2010. At diagnosis, her blood cell count revealed anaemia (haemoglobin 106 g/l) and a marked thrombocytosis (992×10^9 platelets/l). She is currently under cytoreductive and antiplatelet treatment and her last blood count revealed a mild anaemia (haemoglobin 104 g/l) and a platelet count lowered to 600×10^9/l.

Among the 29 RARS, we also identified a single case with a *CALR* mutation. This unique *CALR*-positive RARS case was a 70-year-old man who had thrombocytopenia and carried an *SF3B1* mutation.

In the present study on *JAK2* and *MPL* mutations, our results are in accordance with the usually reported prevalence of *JAK2*^{V617F} in half of RARS-T and the low frequency of *MPL* mutations.[2–6]

Recent publications have reported a variable frequency of *CALR* mutations in MDS-RS: 0/6 to 3/24 in RARS-T, 3/27 in RARS and 0/3 in refractory cytopenia with multilineage dysplasia and ring sideroblasts.[10,11] However, these results were obtained in small series of patients, thus leading to potential biases. Of note, a high proportion (12.5%) of *CALR* mutations in RARS-T in one series of 24 patients contrasts with our results where only one positive case in 95 patients was noted. In particular, one of the three previously reported *CALR*-positive RARS-T cases did not harbour the *SF3B1* mutation, a frequent hallmark of RARS-T. Moreover, in this series, the frequency of the *SF3B1* mutation was much lower than in ours (66% vs 88%) suggesting that this *CALR*-positive *SF3B1*-negative RARS-T case could be MPN rather than RARS-T, owing to the difficulty of asserting the diagnosis in some cases. Finally, the low rate of *CALR* mutations in the present RARS-T series is similar (0–3%) to that reported in chronic myelomonocytic leukaemia, another MDS/MPN.[10,11]

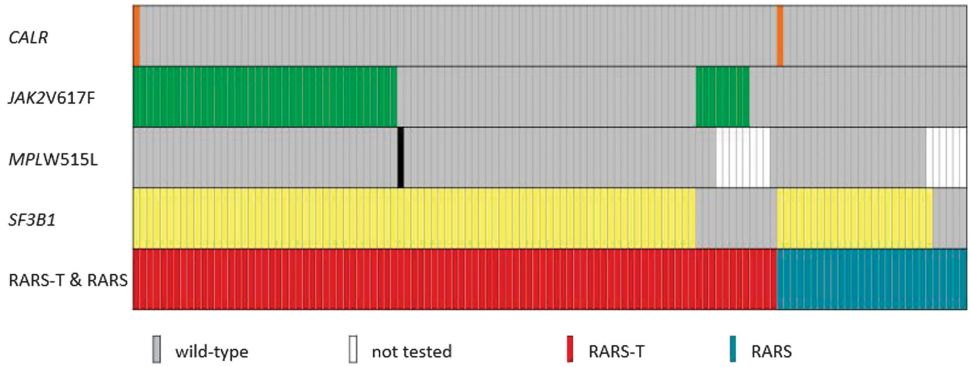

Figure 1. Distribution of mutations in *SF3B1*, *JAK2*^{V617F}, *MPL*^{W515L} and *CALR* mutations in 95 RARS-T and 29 RARS. In RARS-T patients, *SF3B1*, *JAK2*^{V617F}, *MPL* and *CALR* mutations were observed in 88.3%, 49.4%, 1.1% and 1%, respectively. In RARS patients, *SF3B1*, *JAK2*^{V617F}, *MPL* and *CALR* mutations were observed in 82.7%, 0%, 0% and 3.4%, respectively.

Among the 29 RARS, we also identified a single case with a *CALR* mutation, which is consistent with a previous report.[11]

Surprisingly, the *CALR*-positive RARS-T patient also carried the *JAK2*^{V617F} mutation, which is to date, the first double-mutant *JAK2*/*CALR* ever described in RARS-T. Indeed, the *CALR* and *JAK2*^{V617F} mutations were described as mutually exclusive in the two recent studies of >2300 haematologic cancers.[10,11] However, another group recently described a case of a *JAK2*/*CALR* double mutation in PMF,[15] which is in keeping with our results and proves that these mutations coexist in rare cases. One can hypothesise that this RARS-T patient with a *JAK2*/*CALR* mutation had two different clones. However, owing to absence of frozen progenitor cells, no clonogenic assay was feasible.

The two major types of CALR mutations are type 1 mutations (52 bp deletion; c.1192_1143del) and type 2 mutations (5 bp insertion; c.1154_1155insTTGTC).[10] The *CARL* mutation described in our RARS-T is a 10-bp deletion (1129–1138del), which is a rare type of *CALR* exon 9 mutation that shifts the reading frame in a similar way to all reported *CALR* mutations.

Finally, our study showed that *CALR* mutations are rare in MDS-RS and thus do not explain the myeloproliferative part of RARS-T. Approximately 40–50% of RARS-T are triple-negative for *JAK2*, *MPL* and *CALR* mutations, indicating that further research is required to explain the myeloproliferation in this rare MDS/MPN.

CONFLICT OF INTEREST

SS and TH declare part ownership of the MLL Munich Leukemia Laboratory. SJ is employed by the MLL Munich Leukemia Laboratory. The remaining authors declare no conflict of interest.

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