Acute myeloid leukemias with ring sideroblasts show a unique molecular signature straddling secondary acute myeloid leukemia and *de novo* acute myeloid leukemia

Ring sideroblasts (RS) are a distinct morphological feature present in myelodysplastic syndromes (MDS), myelodysplastic/myeloproliferative neoplasms (MDS/MPN) and acute myeloid leukemia (AML). The International Working Group on Morphology of Myelodysplastic Syndrome (IWGM-MDS) defines them as erythroblasts with a minimum of 5 siderotic granules covering at least one third of the circumference of the nucleus. Their presence ≥15% has been associated with mutations in the splicing factor 3B subunit 1 (SF3B1) in 64-83% of patients with refractory anemia with ring sideroblasts (RARS), 57-76% of patients with refractory cytopenia with multilineage dysplasia and ringed sideroblasts (RCMD-RS) and in 90% of RARS with thrombocytosis (RARS-T).[1-5] Recently, the 2016 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemias has recognized the biological importance of *SF3B1*mut and the correlation with RS, classifying all MDS without excess blasts or 5q deletion into a category of their own.[6] Mutations in *SF3B1* are so frequently associated with RS[7] that the threshold at which MDS may be classified as bearing RS has been lowered from the classical 15% to 5% if *SF3B1* mutations are demonstrated.[6] Although the impact of *SF3B1* mutations was initially associated with better outcomes in MDS,[1,7] this can be explained through a higher incidence in low-grade MDS and due to the lack of prognostic significance carried by the mutation itself.[4] Recent studies have shown the incidence of *SF3B1* in *de novo* AML is low.[7,8] However the significance of RS and *SF3B1*mut in AML had not been assessed in a larger cohort. In an attempt to analyze whether RS could differentiate a subgroup of AML with different biological characteristics, we herein present the biological and clinical associations of RS and *SF3B1* mutations in patients with AML (n=1857).

From a total of 1857 AML patients (excluding those with cytogenetically defined entities according to WHO), bone marrow assessment revealed 473 (25%) with RS ≥1%, of which 290 (16% of all 1857 patients) had RS ≥5%, and 183 (10% of all 1857 patients) had ≥15% RS, indicating a lower incidence of RS in comparison to cohorts of MDS patients described in the literature (57% of cases showing RS ≥1%).[9] This incidence was, however, significantly higher to that recently reported in AML patients (5%).[7] It must be acknowledged, however, that especially in those cases with lower RS counts, other causes of RS, like alcohol consumption or drug toxicity, cannot be excluded.

Next-generation sequencing (NGS) for a panmyeloid panel, gene scan and quantitative polymerase chain reaction (PCR) were performed in a subcohort of 340/473 patients (due to sample availability) for the detection of

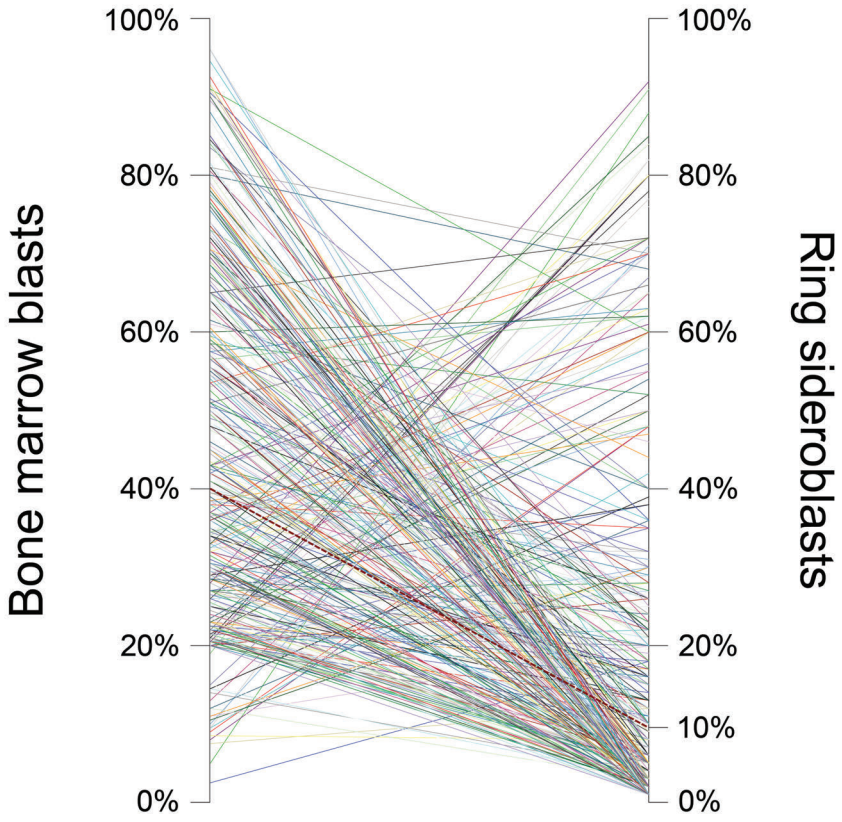

Figure 1. Inverse relation between percentage of blasts and ring sideroblasts. The percentages of bone marrow blasts are given on the left side in relation to the percentages of ring sideroblasts on the right side. The percentage of bone marrow blasts correlated inversely with the percentage of RS present (r=0.213, *P*<0.001).

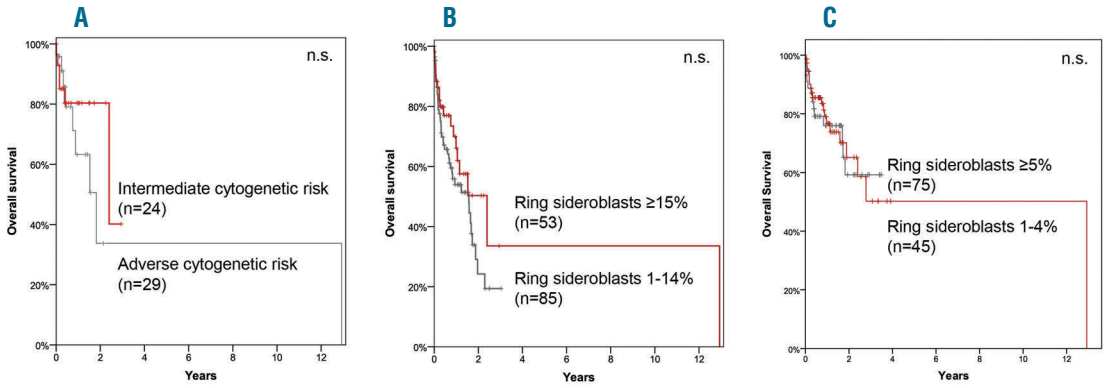

Figure 2. Percentage of ring sideroblasts has no impact on OS. Survival plots on patients with ≥15% ring sideroblasts stratified in A: by cytogenetic risk groups according to MRC criteria;[13] B: stratified by the threshold of ≥15% ring sideroblasts, and C: stratified by the threshold of ≥5% ring sideroblasts. The case numbers as well as the median OS is given in months. n.s: not significant.

mutations in: *SF3B1, ASXL1, DNMT3A, FLT3-TKD, IDH1R132, IDH2R140, IDH2R172, KRAS, NPM1, NRAS, RUNX1, TET2* and *TP53*. Either complete genes or hotspots were first amplified by a microdroplet-based assay (Raindance, Lexington, MA, USA) and then sequenced with a MiSeq instrument (Ilumina, San Diego, CA, USA). *RUNX1* was sequenced on a JS junior system (Roche 454, Branford, CT, USA). The median coverage per amplicon was 2215 reads (range 100-24716). The lowest limit of detection was set at a cutoff of 3%. *FLT3*-ITD was analyzed by gene scan and *MLL*-PTD by quantitative PCR as described elsewhere.[10,11] These 340 cases were subject to the study.

Out of this subcohort of 340 patients, 225 (66%) had RS ≥5% (141 (41%) of them ≥15%) and the remaining 115/340 (33%) had RS ≥1 to 4%. The cohort consisted of 303 (89%) *de novo* AML, which showed a normal distribution of French-American-British (FAB) subtypes (FAB: M0 n=18, M1 n=67, M2 n=165, M4 n=30, M5 n=3, M6 n=20) and 37 (11%) therapy-related AML (t-AML). Secondary AML (s-AML) stemming from a previously known MDS were excluded. The male to female ratio was 192:148 with a median age of 74 years, range: 20-93 years. Chromosome banding analysis (assisted by fluorescence *in situ* hybridization (FISH) if needed) was performed in all 340 cases.

The percentage of bone marrow blasts correlated inversely with the percentage of RS present (r=0.213, P<0.001) (Figure 1). This is most likely linked to the higher prevalence of subtypes M2 and M4, which are the most frequent subtypes occurring after MDS, in cases with higher RS. This correlates well with two of the most frequently mutated genes in our cohort, (*ASXL1* and *SF3B1*), which are among those frequently found to be specifically mutated in s-AML. We could then argue in favor of a previous clinically unnoticed dysplastic phase in these cases.[12]

A normal karyotype was found in 136 (40%) patients. 204 patients (60%) showed chromosome aberrations in metaphase cytogenetics. For prognostication, intermediate-risk cytogenetics according to the Medical Research Council (MRC) criteria[13] were found in 193 (57%), and adverse-risk in 147 (43%). Patients with RS ≥15% had adverse cytogenetics more frequently in comparison to those with RS between 1-14% (54% *vs.* 36%, P=0.001). However, this does not seem to translate into a negative impact on the prognosis of patients with RS ≥15% (Figure 2A,B). Unknown, or thus far poorly understood molecular mechanisms could be playing a role in the outcome of these patients. Studies with wider gene panels could help further elucidate the biology of these conditions in a similar way to that which has been described in the literature.[8,12]

The frequencies of gene mutations in the 340 patients with available samples for sequencing were as follows: *TP53* 103/331 (31%), *RUNX1* 84/315 (26%), *DNMT3A* 86/337 (25%), *TET2* 68/330 (20%), *ASXL1* 58/334 (17%), *IDH2*R140 53/338 (15%), *NPM1* 43/340 (12%), *SF3B1* 34/334 (10%), *FLT3*-ITD 33/340 (10%), *NRAS* 29/340 (8%), *IDH1*R132 21/339 (6%), *MLL*-PTD 22/337 (6%), *FLT3*-TKD 18/333 (5%), *KRAS* codon 12 13/299 (4%), *IDH2*R172 13/338 (3%) and *KRAS* codon 61 3/299 (1%) (Figure 3A).

If we stratify the mutational analysis in patients with RS ≥1% to <15% and those with RS ≥15% (standard MDS definition according to morphology), we discover that the latter have more frequent *TP53* mutations (22% *vs.* 44%, P<0.001) and less frequent *IDH2*R140 mutations (19% *vs.* 11%, P=0.094) and *MLL*-PTD (6% *vs.* 2%, P=0.006) (Figure 3A). If we drop the threshold to ≥5% RS, patients still had more frequent statistically significant *TP53*mut in comparison to patients with RS ≥1 to 4% (39% *vs.* 14%, P<0.001).

Accordingly, patients with *TP53* mutations had higher percentages of RS as compared to those without (28% *vs.* 16%, P<0.001), and patients with *IDH2*R140 mutations and *MLL*-PTD had lower percentages of RS as compared to those without (15% *vs.* 21%, P=0.043 and 11% *vs.* 21%, P=0.025, respectively). Furthermore, patients with mutations in the following genes had fewer RS than patients with the respective wild-types: *ASXL1* (15% vs. 21%, P=0.040), *FLT3*-ITD (14% *vs.* 21%, P=0.049), *IDH2*R140 (15% *vs.* 21%, P=0.043), *MLL*-PTD (11% *vs.* 21%, P=0.025), *NPM1* (13% *vs.* 21%, P=0.018) and

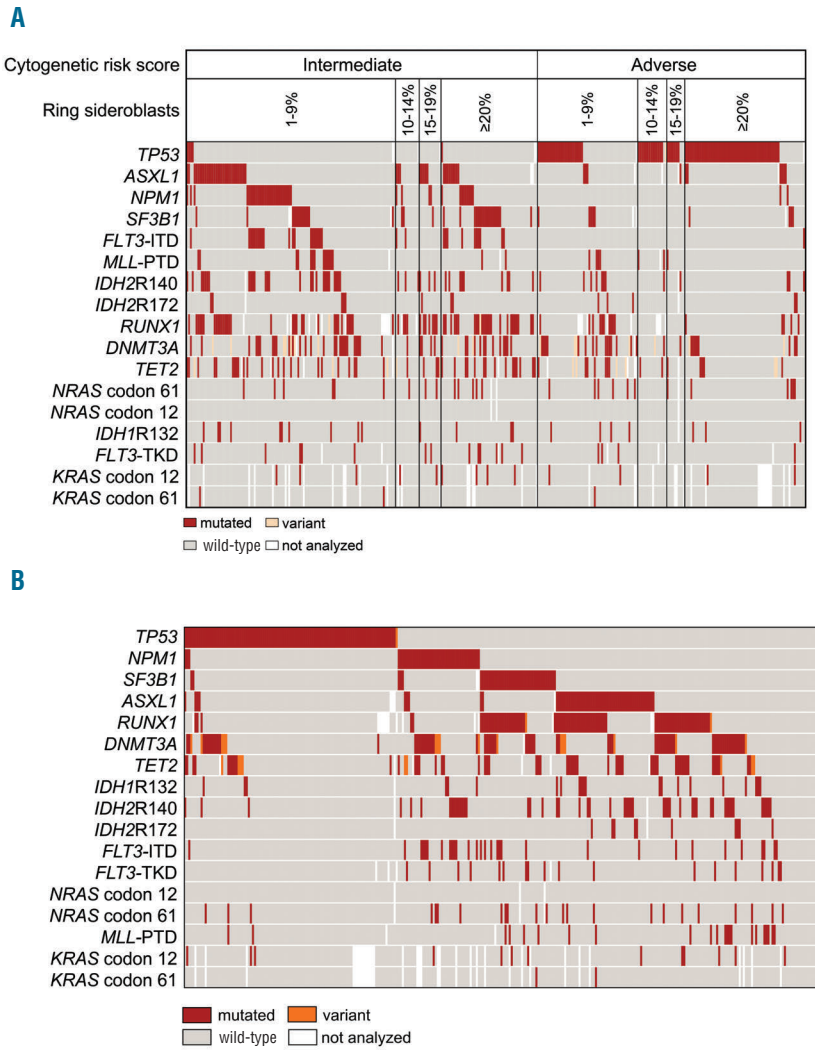

Figure 3. Molecular signature of the cohort pf patients with AML with RS. A: molecular genetic mutation pattern of the 340 patients classified by the cytogenetic risk group according to the MRC criteria[13] and the percentage of ring sideroblasts. All analyzed genes are illustrated, each column represents one patient. B: molecular signature of the 340 patients.

KRAS codon 61 (3% *vs.* 20%, $P<0.001$). Conversely, patients with mutated *SF3B1* had more RS than patients with wild-type (27% *vs.* 19%, respectively, $P=0.054$) as reported in the literature[14] (Figure 3). Also, contrary to what has been described in the literature up to now, the percentage of RS did not translate into an increase in the mutational burden of *SF3B1*[7] ($r=0.63$, $P<0.001$).

The thresholds (≥15% and ≥5%) did not have an impact on the overall survival (OS) (Figure 2A-C) and event-free survival (EFS) of patients. The molecular signature (Figure 3B) described in our cohort would correlate with that described in s-AML by Lindsley *et al.* (*ASXL1*mut and *SF3B1*mut), suggesting this cohort of *de novo* AML with ring sideroblasts likely had a MDS background (although clinically unnoticed).[12] As with Lindsley's cohort, the presence of *TP53*mut (defining a unique group of AML patients with different ontogeny) excluded mutations in *SF3B1* ($P<0.001$), *ASXL1* ($P<0.001$) and *NPM1* ($P<0.001$). Strikingly, mutations in *NPM1*, specifically associated to *de novo* AML did not exclude mutations in *ASXL1* ($P=0.09$) or *SF3B1* ($P=0.1$), which are, in turn, specifically associated to s-AML in our cohort. In addition, the fact that we also found other mutations with less specificity for both s-AML or *de novo* AML (*RUNX1, NRAS, TET2, KRAS, IDH1, IDH2, FLT3* and *DNMT3A*) suggests a more complex ontogeny in AML cases with RS.

To summarize, in contrast to low-grade MDS where the presence of RS identifies patients with less adverse cytogenetics and less somatic mutations, AML with RS more frequently harbors adverse cytogenetics and mutations in genes generally associated with poorer outcomes (i.e., *TP53*). This, however, does not translate into a decreased overall survival (Figure 2). Moreover, patients with *TP53* or *SF3B1* mutations have higher RS.

Lindsley *et al.* recently confirmed that from a genetic point of view, the clinical distinction of AML ontogeny in 3 groups (s-AML, t-AML and *de novo* AML) based on different molecular signatures is correct.[12] As described in the aforementioned study, we detected in our cohort of patients with AML and no clinical background of dysplasia, mutations linked to s-AML, indicating that these patients might have evolved through a phase of unnoticed dysplasia. We believe this is the most plausible explanation for this finding given the fact that these patients in our cohort all had ring sideroblasts, a distinct feature of dysplasia.

Most strikingly, however, and in contrast to what Lindsley *et al.* described, mutations like *NMP1* (significantly underrepresented in s-AML) did not significantly exclude mutations in *ASXL1* or *SF3B1* in our cohort (mutations significantly associated to s-AML and that in turn excluded mutations in *NMP1*). This highlights the complex ontogeny of AML with RS, a group of

AML patients that seem to straddle between *de novo* and s-AML.[8,12]

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