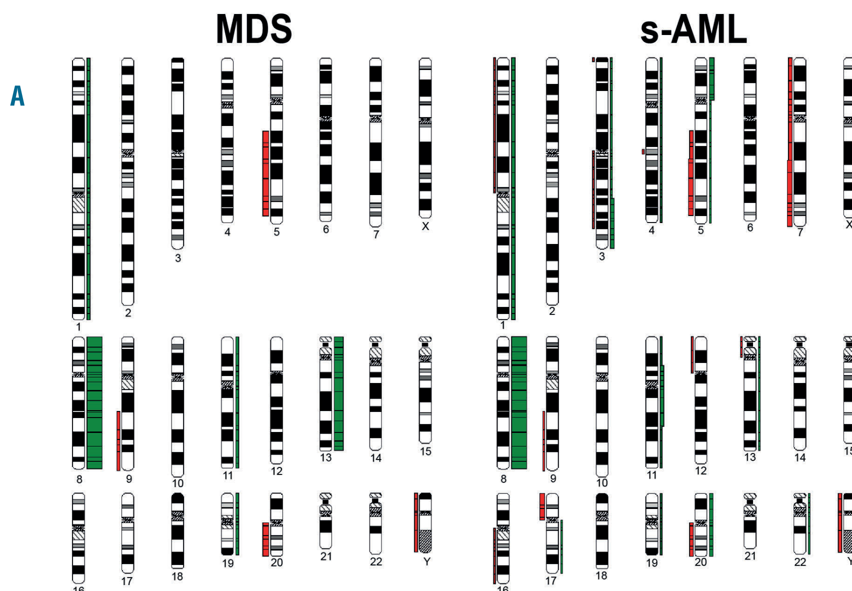
Karyotype evolution and acquisition of *FLT3* or RAS pathway alterations drive progression of myelodysplastic syndrome to acute myeloid leukemia

Myelodysplastic syndromes (MDS) are a heterogeneous group of myeloid neoplasms associated with aberrant myeloid differentiation and ineffective hematopoiesis leading to cytopenias. In more than 20% of affected patients the MDS transforms into secondary acute myeloid leukemia (s-AML). To date, information on the molecular and cytogenetic bases of leukemic transformation into AML are rare and there is a compelling need to identify the specific molecular events potentially driving this process. We, therefore, analyzed a cohort of 38 patients with paired samples from when they had MDS and s-AML and applied cytogenetics and a 33-gene panmyeloid panel for comparison of mutation frequencies and patterns within these two groups of samples. We further compared these findings with those in a large cohort of 494 MDS patients, showing no transformation to s-AML. We identified that mutations in genes of cellular signal transduction drive leukemic transformation, and found that several MDS typical mutations predispose to s-AML transformation. In addition, karyotype evolution was detected in more than one third of patients underlining its important impact on s-AML transformation.

We identified 38 patients (11 female, 27 male) who were analyzed in our laboratory by cytomorphology and cytogenetics both at diagnosis of MDS and later at progression to s-AML. The median age at diagnosis of MDS was 71 years (range, 59-86 years). The median time to progression was 18 months (range, 2-72 months). The diagnosis was made according to World Health Organization (WHO) criteria.[1] Chromosome banding analysis was performed for all patients according to standard procedures in combination with fluorescence *in situ* hybridization, if needed. All 76 samples were analyzed by next-generation sequencing or polymerase chain reaction with a 33-gene panel targeting *ASXL1, BCOR, BRAF, CBL, DNMT3A, ETV6, EZH2, FLT3* (*FLT3*-ITD and *FLT3*-TDK*), GATA1, GATA2, IDH1, IDH2, JAK2, KIT, KRAS, MLL*-PTD*, MPL, NPM1, NRAS, PHF6, RAD21, RUNX1, SETBP1, SF3B1, SMC1A, SMC3, SRSF2, STAG2, TET2, TP53, U2AF1, WT1*, and *ZRSR2*. Further details on the characterization of the patients and the study methodology are provided in the *Online Supplementary Material*.

Cytogenetically, a total of 14/38 (37%) MDS and 22/38 (58%) s-AML samples had an aberrant karyotype and 13 patients (34%) gained chromosome abnormalities during progression to s-AML. One of these patients showed in parallel clonal evolution in one cytogenetic clone but lost another cytogenetic clone completely. These findings again confirm that cytogenetic evolution in MDS has been associated with progression to AML.[2,3] The most

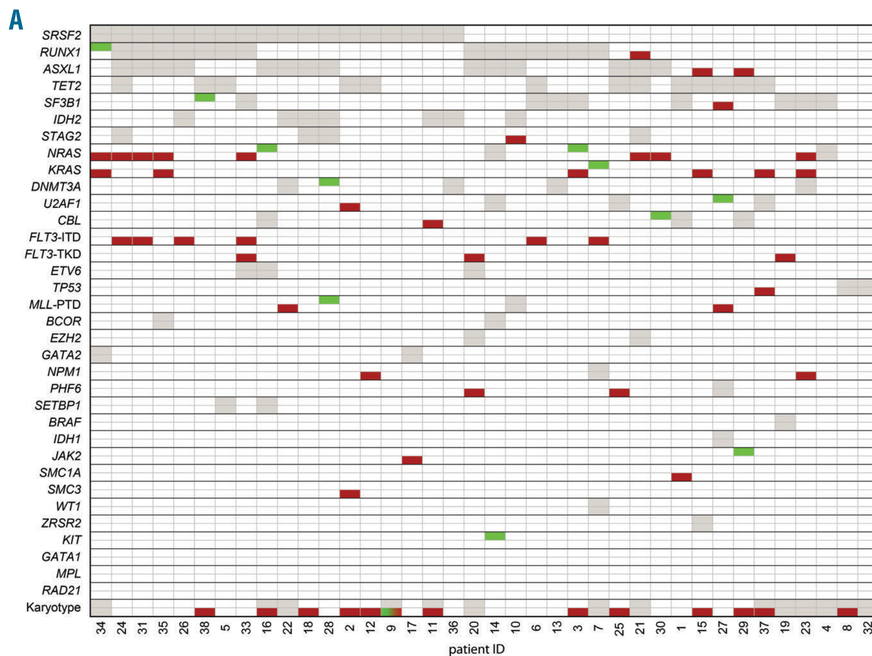
A

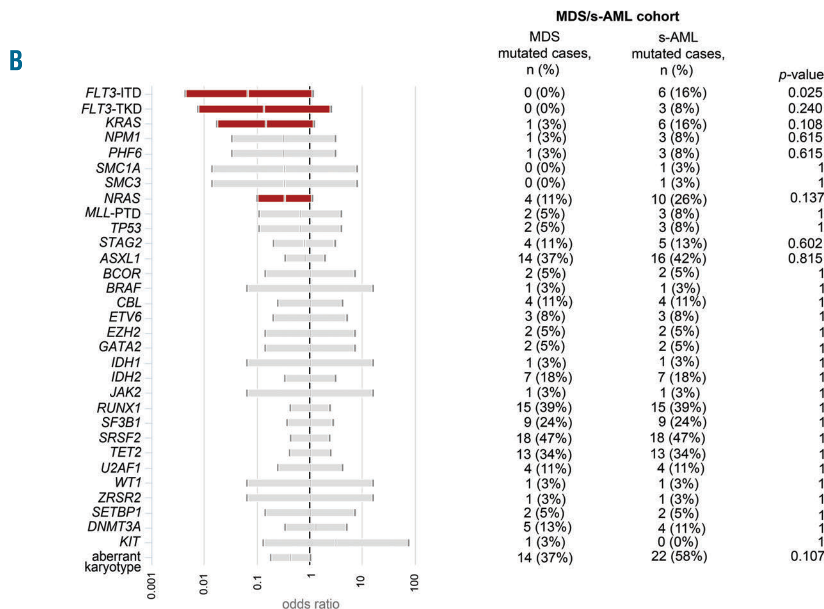


B

Karyotype	MDS case number, n (%)	s-AML case number, n (%)	*p*
aberrant	14 (37%)	22 (58%)	0.107
+8	5 (13%)	9 (24%)	0.375
del(5q)	2 (5%)	3 (8%)	1
del(20q)	2 (5%)	2 (5%)	1

Figure 1. Cytogenetic characterization of paired MDS and s-AML samples. (A) Illustration of the cytogenetic patterns with the support of CYDAS (*http://www.cydas.org/OnlineAnalysis/*). Chromosomal gains are marked in green to the right, losses in red to the left. The thickness of the bars represents the number of cases showing the respective chromosomal gain or loss. Illustrations of the cytogenetic alterations in the MDS state (left panel) and in the s-AML state (right panel) are shown. (B) Table giving the case numbers of recurrent cytogenetic abnormalities in both disease states. None of the differences was statistically significant. *P*-values are given.



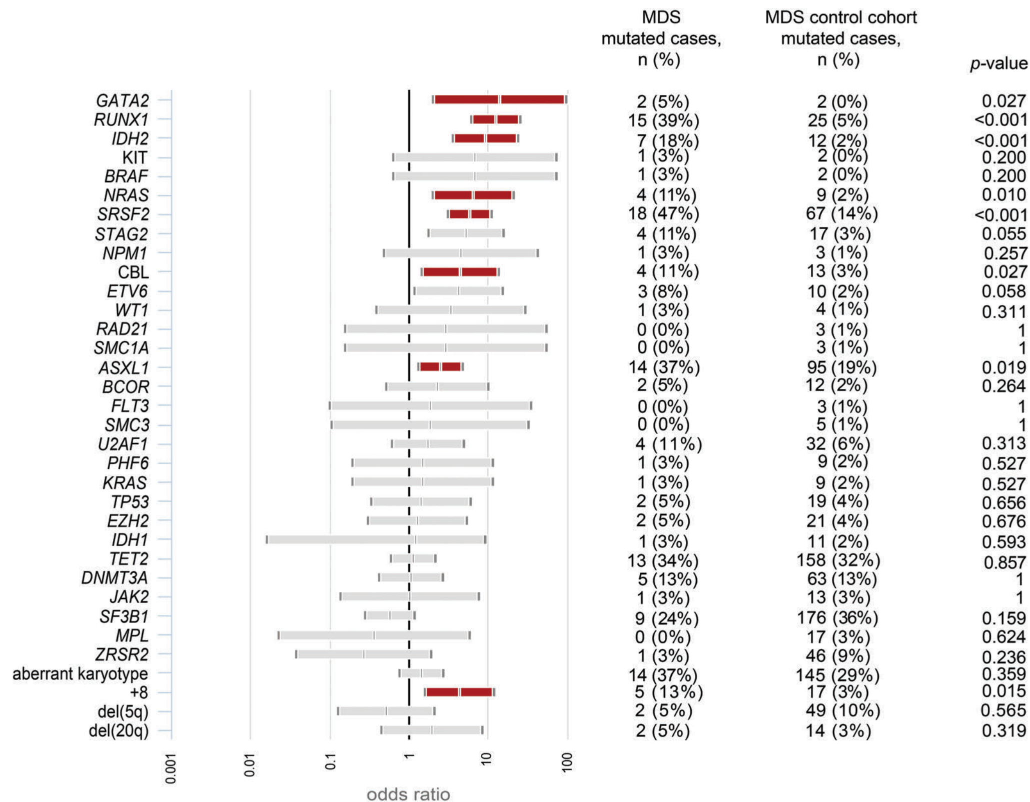


	MDS/s-AML cohort MDS mutated cases, n (%)	s-AML mutated cases, n (%)	*p*-value
FLT3-ITD	0 (0%)	6 (16%)	0.025
FLT3-TKD	0 (0%)	3 (8%)	0.240
KRAS	1 (3%)	6 (16%)	0.108
NPM1	1 (3%)	3 (8%)	0.615
PHF6	1 (3%)	3 (8%)	0.615
SMC1A	0 (0%)	1 (3%)	1
SMC3	0 (0%)	1 (3%)	1
NRAS	4 (11%)	10 (26%)	0.137
MLL-PTD	2 (5%)	3 (8%)	1
TP53	2 (5%)	3 (8%)	1
STAG2	4 (11%)	5 (13%)	0.602
ASXL1	14 (37%)	16 (42%)	0.815
BCOR	2 (5%)	2 (5%)	1
BRAF	1 (3%)	1 (3%)	1
CBL	4 (11%)	4 (11%)	1
ETV6	3 (8%)	3 (8%)	1
EZH2	2 (5%)	2 (5%)	1
GATA2	2 (5%)	2 (5%)	1
IDH1	1 (3%)	1 (3%)	1
IDH2	7 (18%)	7 (18%)	1
JAK2	1 (3%)	1 (3%)	1
RUNX1	15 (39%)	15 (39%)	1
SF3B1	9 (24%)	9 (24%)	1
SRSF2	18 (47%)	18 (47%)	1
TET2	13 (34%)	13 (34%)	1
U2AF1	4 (11%)	4 (11%)	1
WT1	1 (3%)	1 (3%)	1
ZRSR2	1 (3%)	1 (3%)	1
SETBP1	2 (5%)	2 (5%)	1
DNMT3A	5 (13%)	4 (11%)	1
KIT	1 (3%)	0 (0%)	1
aberrant karyotype	14 (37%)	22 (58%)	0.107

Figure 2. Molecular characterization of paired MDS and s-AML samples. (A) Illustration of all 38 samples in both disease states, MDS and s-AML. Each column represents one patient. White: no mutation/normal karyotype; gray: mutation/aberrant karyotype; red: aberration acquired at s-AML state; green: aberration only during MDS. The upper lane shows data from MDS and the lower lane shows data from s-AML for each of the genes analyzed. (B) Associations of aberrations with MDS or s-AML are depicted by the odds ratio of MDS/s-AML, the 95% confidence intervals are given as width of the bars. An odds ratio <1 indicates that mutations are less frequent in MDS and more common in the s-AML state. Red bars indicate differences between MDS and s-AML states. Numbers of cases with respective mutations and karyotype aberrations as well as *P*-values are given in the table beside.

frequently occurring recurrent abnormality during the stage of MDS was trisomy 8 (+8; n=5), followed by del(5q) and del(20q) both present in two patients each. While a +8 occurred in four additional patients with s-AML, thus being present in nine cases in total, del(5q) and del(20q) were rarely gained in s-AML [one case with del(5q)]. Although a normal karyotype was more often found at the MDS stage than in s-AML transformed samples, this difference was not statistically significant. The cytogenetic patterns during both MDS and s-AML states are illustrated in Figure 1 and detailed in *Online Supplementary Table S1*.

We next analyzed the distribution of molecular mutations in patients during the stages of MDS and s-AML. All samples had at least one mutation in both MDS and s-AML disease states. The most frequently mutated genes largely matched in MDS and s-AML and showed the following mutation frequencies: *SRSF2* (47% MDS; 47% s-AML), *RUNX1* (37%; 40%), *ASXL1* (37%; 42%), *TET2* (34%; 34%), and *SF3B1* (24%; 24%). In contrast, mutations in *GATA1, MPL* and *RAD21* genes were not found in either MDS or s-AML stages. One *KIT* mutation was detected in a patient only in the MDS stage and was lost with transformation to s-AML, while *FLT3*-ITD and *FLT3*-TKD mutations were identified in s-AML samples only (16% and 8%, respectively). A total of 20 (53%) patients gained mutations in at least one of the 33 analyzed genes during progression to s-AML, four (11%) patients lost at least one mutation and six (16%) patients had simultaneous losses and gains of mutations (Figure 2). There were, however, no recurrently lost gene mutations at transformation. Looking at the distribution of molecular mutations revealed that *FLT3*-ITD was significantly associated with s-AML compared to the corresponding MDS cases (16% *versus* 0%, *P*=0.025). Although the differences were not statistically significant,



	MDS mutated cases, n (%)	MDS control cohort mutated cases, n (%)	*p*-value
GATA2	2 (5%)	2 (0%)	0.027
RUNX1	15 (39%)	25 (5%)	<0.001
IDH2	7 (18%)	12 (2%)	<0.001
KIT	1 (3%)	2 (0%)	0.200
BRAF	1 (3%)	2 (0%)	0.200
NRAS	4 (11%)	9 (2%)	0.010
SRSF2	18 (47%)	67 (14%)	<0.001
STAG2	4 (11%)	17 (3%)	0.055
NPM1	1 (3%)	3 (1%)	0.257
CBL	4 (11%)	13 (3%)	0.027
ETV6	3 (8%)	10 (2%)	0.058
WT1	1 (3%)	4 (1%)	0.311
RAD21	0 (0%)	3 (1%)	1
SMC1A	0 (0%)	3 (1%)	1
ASXL1	14 (37%)	95 (19%)	0.019
BCOR	2 (5%)	12 (2%)	0.264
FLT3	0 (0%)	3 (1%)	1
SMC3	0 (0%)	5 (1%)	1
U2AF1	4 (11%)	32 (6%)	0.313
PHF6	1 (3%)	9 (2%)	0.527
KRAS	1 (3%)	9 (2%)	0.527
TP53	2 (5%)	19 (4%)	0.656
EZH2	2 (5%)	21 (4%)	0.676
IDH1	1 (3%)	11 (2%)	0.593
TET2	13 (34%)	158 (32%)	0.857
DNMT3A	5 (13%)	63 (13%)	1
JAK2	1 (3%)	13 (3%)	1
SF3B1	9 (24%)	176 (36%)	0.159
MPL	0 (0%)	17 (3%)	0.624
ZRSR2	1 (3%)	46 (9%)	0.236
aberrant karyotype	14 (37%)	145 (29%)	0.359
+8	5 (13%)	17 (3%)	0.015
del(5q)	2 (5%)	49 (10%)	0.565
del(20q)	2 (5%)	14 (3%)	0.319

Figure 3. **Associations of aberrations to MDS progressing to s-AML and to the MDS control cohort (not including patients with s-AML transformation) are depicted by the odds ratios of MDS/MDS without s-AML transformation; the 95% confidence intervals are given. Odds ratios >1 indicate that mutations are more frequent in MDS progressing to AML and less frequent in the MDS control cohort. Red bars indicate statistically significant differences between MDS progressing to s-AML and the MDS control cohort. Numbers of cases with respective mutations and karyotype aberrations as well as *P*-values are given in the table beside.**

FLT3-TKD (8% *versus* 0%), *NRAS* (26% *versus* 11%), and *KRAS* (16% *versus* 3%) were more often mutated in s-AML than in MDS cases, while mutations in all other genes were equally distributed between both disease states (Figure 2). Summing the cases with at least one of these four gene mutations resulted in a significant association of mutations in these genes with s-AML compared to MDS (47% *versus* 13%, *P*=0.002). In total, 15/20 patients (75%) who acquired new mutations showed mutations in the latter mentioned signal transduction proteins (*FLT3* or RAS pathway), indicating that these might be mutations driving s-AML transformation. *FLT3* and *NRAS* mutations are thought to be important genetic events contributing to the pathogenesis of AML[4-6] and the expected increase in the frequencies of mutations in s-AML cases was observed, confirming previously reported data.[7,8] In an very elegant study, recently published by Lindsley *et al*., the three different AML subtypes – secondary, therapy-related and *de novo* – were genetically compared and also in a small group of 17 MDS/s-AML matched samples the authors showed that 78% of the patients gained mutations in transcription factors as well as signal transduction proteins (*FLT3* and RAS pathway) during s-AML transformation.[9] These results are in line with our data showing predominantly acquired mutations in signal transduction. Although *FLT3* and *RAS* mutations are rare in MDS, the assessment of these mutations during the disease course is potentially useful as an indicator of progression to AML.[10]

Next-generation sequencing allows further investigation of variant allele frequencies. However, the major changes between MDS and s-AML stages were gains or losses of mutations. The allele frequencies of persisting mutations remained quite stable with only single exceptions (see *Online Supplementary Table S2*).

Comparing the frequencies of mutational acquisition in cases with and without karyotype alterations at leukemic transformation revealed that only eight of the 26 cases that acquired mutations also showed gains of cytogenetic aberrations, while 18 cases remained cytogenetically stable. Interestingly, none of the patients who acquired a mutation in *NRAS* or *FLT3* (n=12) presented with karyotype transformation, and only three patients acquiring a *KRAS* mutation also showed cytogenetic transformation (Figure 2).

We further compared the MDS data set of the presented study to our results of an independent previously published MDS cohort.[11] WHO diagnosis categories were matched between cohorts, and cases showing transformation to s-AML were excluded. Cases with a follow-up of less than 18 months (the median time to transformation of the MDS/s-AML cohort) were also excluded, resulting in a final cohort of 494 patients. The frequencies of patients with aberrant karyotypes during their MDS stage (37% *versus* 29% in the published control cohort, respectively) were similar. However, trisomy 8 appeared

more frequently in MDS patients with progression to s-AML than in the control MDS cohort (13% *versus* 3%; *P*=0.015) (Figure 3). We further compared the mutation frequencies between these two cohorts, and found that in MDS cases transforming to s-AML the following mutations were more frequent than in the MDS control cohort: *ASXL1* (37% *versus* 19%; *P*=0.019), *CBL* (11% *versus* 3%; *P*=0.027), *GATA2* (5% *versus* 0; *P*=0.027), *IDH2* (18% *versus* 2%; *P*<0.001), *NRAS* (11% *versus* 2%; *P*=0.010), *RUNX1* (39% *versus* 5%; *P*<0.001), and *SRSF2* (47% *versus* 14%; *P*<0.001). Mutations in these genes might, therefore, predispose to progression to s-AML (Figure 3). To further validate our findings we then focused on the patients previously excluded from the control cohort who also developed s-AML (n=78) and analyzed the differences in cytogenetics and mutation frequencies to the MDS cases of the present MDS/s-AML cohort. Both MDS to s-AML cohorts showed comparable frequencies of aberrations and mutations, again supporting that the previous findings are specific for MDS cases transforming to s-AML (*Online Supplementary Figure S3*). Previous studies on MDS patients showed that mutations in *ASXL1, ETV6, EZH2, IDH2, RUNX1*, and *TP53* are predictors of poor overall survival in MDS patients independently of other established risk factors.[11-13] Furthermore the splicing machinery is one of the most frequently affected pathways in MDS,[14] and mutations in *SRSF2* have been shown to be independently associated with a negative prognosis for overall survival and AML transformation.[15] Recently, several of these gene mutations were identified as specific for s-AML in comparison to *de novo* AML underlining their function in dysplastic differentiation since they occur already at the stage of MDS.[9] However, these gene mutations must be differentiated clearly from the mutations exclusively found during s-AML.

In conclusion, our data suggest that different underlying molecular mechanisms drive the progression from MDS to s-AML. On the one hand, karyotype evolution has an important impact on s-AML transformation. On the other hand, several mutations including those in *ASXL1, ETV6, GATA2, IDH2, NRAS, RUNX1*, and *SRSF2* predispose to transformation to s-AML. However, mutations in signal transduction genes (*FLT3, KRAS*, and *NRAS*) seem to drive the progression from MDS to s-AML more quickly if mutated at a specific time-point and should, therefore, be considered as prognostically informative during the disease course.

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