MYELOID NEOPLASIA

Isolated trisomy 13 defines a homogeneous AML subgroup with high frequency of mutations in spliceosome genes and poor prognosis

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Key Points

- AML patients with isolated trisomy 13 have a very poor clinical outcome
- Isolated trisomy 13 in AML is associated with a high frequency of mutations in *SRSF2* (81%) and *RUNX1* (75%)

In acute myeloid leukemia (AML), isolated trisomy 13 (AML+13) is a rare chromosomal abnormality whose prognostic relevance is poorly characterized. We analyzed the clinical course of 34 AML+13 patients enrolled in the German AMLCG-1999 and SAL trials and performed exome sequencing, targeted candidate gene sequencing and gene expression profiling. Relapse-free (RFS) and overall survival (OS) of AML+13 patients were inferior compared to other ELN Intermediate-II patients (n=855) (median RFS, 7.8 vs 14.1 months, *P* = .006; median OS 9.3 vs. 14.8 months, *P* = .004). Besides the known high frequency of *RUNX1* mutations (75%), we identified mutations in spliceosome components in 88%, including *SRSF2* codon 95 mutations in 81%. Recurring mutations were detected in *ASXL1* (44%) and *BCOR* (25%). Two patients carried mutations in *CEBPZ*, suggesting that *CEBPZ* is a novel recurrently mutated gene in AML. Gene expression analysis revealed a homogeneous expression profile including upregulation of *FOXO1* and *FLT3* and downregulation of *SPRY2*. This is the most comprehensive clinical and biological characterization of AML+13 to date, and reveals a striking clustering of lesions in a few genes, defining AML+13 as a genetically homogeneous subgroup with alterations in a few critical cellular pathways. Clinicaltrials.gov identifiers: AMLCG-1999: NCT00266136; AML96: NCT00180115; AML2003: NCT00180102; and AML60+: NCT00893373 (*Blood*. 2014;124(8):1304-1311)

Introduction

Acquired isolated trisomy 13 (+13) is a rare cytogenetic alteration in acute myeloid leukemia (AML). In a retrospective study of 22 856 AML patients from the Mayo Clinic, its incidence was 0.7%.[1] So far, the prognostic relevance of AML+13 has not been extensively studied, but assumed to be unfavorable based on small or heterogeneous patient cohorts.[2-4] However, according to the European LeukemiaNet (ELN) classification, AML+13 is currently classified in the Intermediate-II genetic group.[5] AML+13 is frequently associated with FAB M0 morphology and shows a high frequency (80% to 100%) of *RUNX1* mutations.[6,7] Overexpression of *FLT3* (located in band q12 on chromosome 13) due to a gene dosage effect was proposed as a potential mechanism of leukemogenesis in AML+13.[6,7] The possibility that AML+13 might be a marker for treatment response to lenalidomide has recently been raised.[8]

Constitutional aneuploidy is linked to increased cancer risk.[9] For example, Down syndrome (trisomy 21) predisposes to megakaryoblastic leukemia with a high frequency of acquired *GATA1* mutations.[10] Trisomy 13 (Patau syndrome) is a severe congenital disorder with cerebral, cardiac, and renal malformations.[11] An association of Patau syndrome and solid neoplasms including neuroblastoma and nephroblastoma was reported.[12] In the literature, we found a single case report of Patau syndrome with congenital myeloid leukemia.[13]

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Table 1. Patient characteristics

Variable	AML+13*	Control Group*	*P*
No. of patients	34	850	
Median age, years *(range)*	64 (43-80)	59 (17-84)	**.004**
Male sex, no. *(%)*	24 (70)	465 (55)	.08
WBC count, G/l, median *(range)*	10 (1-318)	11 (0.1-365)	.64
Hemoglobin, g/dl, median *(range)*	8.9 (4.6-12.8)	9.2 (2.9-17.2)	.2
Platelet count, G/l, median *(range)*	77 (1-399)	54 (1-1760)	.23
LDH (U/l), median *(range)*	269 (155-1011)	414 (115-11140)	**.009**
BM blasts, %, median *(range)*	80 (11-100)	68 (11-100)	**.02**
BM blasts at day 16, %, median *(range)*	5 (0-85)	9 (0-100)	.78
Performance status (ECOG) ≥ 2 *(%)*	8 (26)	263 (34)	.44
de novo AML *(%)*	26 (76)	646 (76)	1.0
Allogeneic transplantation, no. *(%)*	6 (18)	180 (21)	.83
CR, no. *(%)*	21 (62)	471 (55)	.49
Relapse, no. *(%)*	18 (86)	327 (69)	.14
Deceased, no. *(%)*	31 (91)	644 (76)	**.04**

Significant *P* values are indicated in bold.

*All patients were enrolled in the AMLCG-99 or SAL trials and received intensive induction treatment. All patients are classified as ELN Intermediate-II; AML+13: patients with isolated tri- or tetrasomy 13, additional aberrations of the sex chromosomes are allowed.

Considering that the vast majority of infants with Patau syndrome die before 1 year of age,[11] it remains unclear whether constitutional trisomy 13 predisposes to myeloid neoplasia.

We set out to characterize the clinical course of AML+13 patients and to elucidate the underlying spectrum of molecular genetic changes by exome sequencing, targeted sequencing, and gene expression profiling.

Materials and methods

Patients

In this analysis, a subgroup of patients enrolled in the German AML Cooperative Group (AMLCG) (NCT00266136) multicenter AMLCG-1999 trial, and the AML96, AML2003, and AML60+ trials of the Study Alliance Leukemia (SAL) was studied (for details, see supplemental Figure 1A-B on the *Blood* Web site).[14-17] All patients received intensive induction chemotherapy as described elsewhere.[14-17] The AMLCG and SAL clinical trials were approved by the local institutional review boards of all participating centers and informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

Exome sequencing

To perform exome sequencing, genomic DNA of available paired diagnostic and remission samples was extracted from archived bone marrow (BM) samples and fragmented for library preparation as described previously.[18,19] Protein-coding regions were enriched using the SureSelect Human All Exon V4 Kit (Agilent), followed by multiplexed 80 bp paired-end sequencing on an Illumina Genome Analyzer IIx. In total, at least 3.2 Gb of raw sequence data were generated per sample (mean 3.5 Gb; quality metrics are summarized in supplemental Table 1). Raw sequence reads were filtered by Illumina's chastity filter and mapped to the NCBI human hg19 RefSeq reference genome using BWA mapper with default parameters.[20] Insufficiently mapped sequence reads (cutoff Q13, according to 95% confidence of correct mapping) and polymerase chain reaction (PCR) duplicate reads were removed using SAMtools[21]; realignment of mapped reads was performed using the Genome Analysis Toolkit to reduce false-positive single nucleotide variant calls.[22] Candidates for somatically acquired mutations were detected using VarScan with the following parameters: coverage ≥ 10×, variant allele frequency ≥ 20%, variant base calling quality ≥ Q13, and variant reads ≥ 3.[23] Positions with evidence for a variant in the corresponding remission sample or annotated polymorphism (as listed in dbSNP v135) were excluded.

Targeted amplicon sequencing

A selection of genes identified by exome sequencing (n = 9) and a panel of genes recurringly mutated in AML (n = 42) were studied by targeted amplicon sequencing (Haloplex; Agilent) in all AMLCG AML+13 patients with available material (16 of 23). The resulting libraries were sequenced in a single run on a MiSeq instrument. Sequence data were aligned to the human reference genome (version hg19) using BWA.[20] Single nucleotide variants and short insertions or deletions were called using VarScan 2 and Pindel, respectively.[24,25]

In addition, Sanger sequencing of genomic DNA was performed for additional validation of selected mutations. Primer sequences and PCR conditions (for *SRSF2*) are shown in supplemental Tables 2 and 3). PCR products were purified using NucleoFast 96 PCR Clean-up Kit (Macherey Nagel, Düren, Germany) and bi-directional sequencing was performed on an ABI 3500xL Genetic Analyzer using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Sequences were aligned and compared with the reference sequences (NCBI accession numbers: NC_000002.11 [CEBPZ], NG_027868.1 [ASXL1], and NG_032905.1 [SRSF2]) using the Sequencher software (Gene Codes Corporation, Ann Arbor, MI)

Gene expression analysis

To further characterize the AML+13 subgroup, we compared gene expression profiles of 9 patients with AML+13 to 509 AML patients with various genetic abnormalities (except for numerical alterations affecting chromosome 13). The gene expression data set was published previously and is publicly available through the Gene Expression Omnibus Web site (GSE37642).[26] Eight of 9 patients were also included in the genetic analysis. Details of sample preparation, hybridization, and image acquisition were described previously.[26] For probe-to-probe set summarization, we used custom chip definition files based on GeneAnnot version 2.0 (available at http://www.xlab.unimo.it/GA_CDF/) as reported before.[18] Only the 17 389 probe sets present on both the Affymetrix HG-U133A and B chips, and the HG-U133 plus 2.0 chips were included in the analysis. To eliminate the batch effect resulting from the use of different chip designs, we applied an empirical Bayesian method as described previously.[27]

Gene set enrichment analysis (GSEA) was performed with GSEA software (MIT) using the "c5_all" collection consisting of 1454 gene sets derived from the controlled vocabulary of the Gene Ontology project.[28]

The Linear Models for Microarray Data package was used to compute differentially regulated probe sets. Differential regional gene expression on chromosome 13 was analyzed using MACAT (MicroArray Chromosome Analysis Tool) as described previously.[29,30]

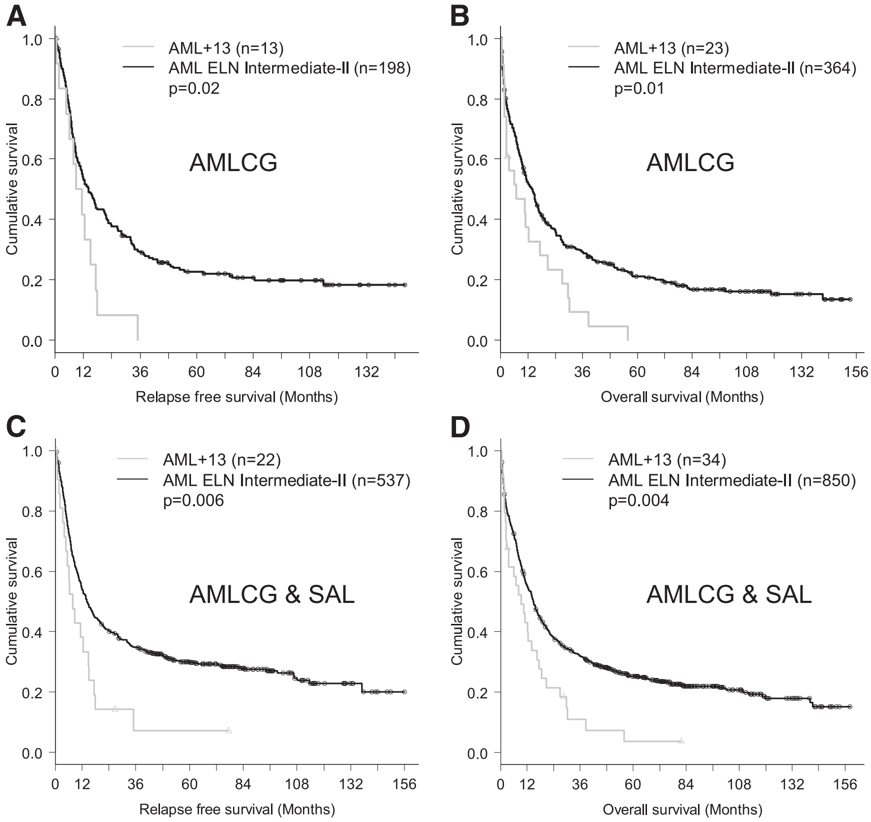


Figure 1. RFS and OS in AML patients. (A-B) AMLCG cohort. (C-D) Combined AMLCG and SAL cohort. Kaplan–Meier estimates of RFS and OS are significantly reduced for the AML+13 subgroup within the ELN Intermediate-II genetic group.

Statistical analyses

All statistical analyses were performed using the R 2.12.2 and 3.0.1 software[31] and routines from the biostatistics software repository Bioconductor, and SPSS version 21.0 (SPSS Inc., Chicago, IL). Two-sided Fisher's exact test was used to compare categorical variables, while Wilcoxon Mann-Whitney *U* test was applied for continuous variables. Adjustment for multiple hypothesis testing was performed using the Benjamini-Hochberg procedure.[32] Complete remission (CR) was defined as hematologic recovery with at least 1000 neutrophils per μL and at least 100 000 platelets per μL, and < 5% BM blasts in at least one measurement.[33] Relapse-free survival (RFS) was defined as time from the date of CR until relapse, or death. Overall survival (OS) was defined as time from study entry until death from any cause. Patients alive without an event were censored at the time of their last follow-up. The prognostic impact of AML+13 was evaluated according to the Kaplan-Meier method and the log-rank test. To adjust for other potential prognostic variables, we derived multivariate Cox models for RFS and OS. The following variables were included in the models, based on their role as potential confounders and availability of data: age (as a continuous parameter), sex, BM blasts at initial diagnosis and on day 16, Eastern Cooperative Oncology Group (ECOG) performance status, white blood cell (WBC) count, platelet count, hemoglobin, serum lactate dehydrogenase (LDH) level, de novo vs secondary AML, and presence of AML+13. No variable selection technique was applied, and all variables were retained in the final models. $P \leq .05$ was considered significant.

Results

Isolated trisomy 13 is associated with poor prognosis

We evaluated the cytogenetic reports of 6836 AML patients with available follow up data treated within the multicenter AMLCG-1999 and SAL trials for aneuploidy of chromosome 13. A total of 264 patients (3.9%) lacked sufficient cytogenetic data. Additional copies of chromosome 13 were reported in 99 of 6572 patients (incidence, 1.5%). Our analyses focused on patients with isolated trisomy (n = 33) or tetrasomy 13 (n = 1) (incidence, 0.5%). Patients with additional

Table 2. Multivariate analysis

	RFS*		OS†	
Variable‡	HR (95% CI)	*P*	HR (95% CI)	*P*
Age *(10 y increase)*	1.33 (1.21-1.46)	**<.001**	1.38 (1.27-1.5)	**<.001**
BM blasts on day 16 (*10% increase*)	1.04 (0.97-1.09)	.08	1.02 (1.02-1.09)	**.002**
WBC (*10 G/l increase*)	1.02 (0.99-1.05)	.15	1.02 (1-1.05)	**.04**
de novo vs secondary AML	1.02 (0.75-1.4)	.89	1.26 (1-1.59)	**.05**
AML+13	1.47 (0.82-2.62)	.2	1.65 (1.03-2.63)	**.04**

Significant P values are indicated in bold.

*n = 378, number of events = 275 (114 patients excluded due to missing covariables).

†n = 549, number of events = 410 (335 patients excluded due to missing covariables).

‡Only variables with $P \leq .05$ in either model are shown. The following variables were included in both models: sex, age (continuous variable), BM blasts at initial diagnosis and day 16, ECOG performance status, WBC count, platelet count, hemoglobin, serum LDH level, de novo vs secondary AML, and AML+13 status.

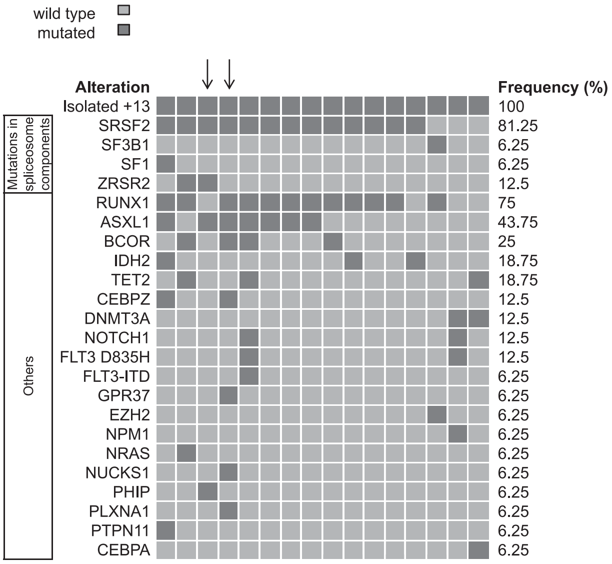


Figure 2. Frequency distribution of recurrently mutated genes in AML+13. Distribution of mutated genes in 16 patients with AML+13. Patients show a high frequency of mutations in spliceosome components and in *RUNX1*, *ASXL1*, and *BCOR*. Arrows highlight the 2 patients who were exome-sequenced.

numerical alterations of the sex chromosomes (n = 2) were included. These 34 patients (AML+13) were categorized into the Intermediate-II genetic category according to the ELN recommendations.[5] The remaining 65 patients had heterogeneous additional cytogenetic aberrations (aAML+13), frequently in the context of a complex karyotype, and were mostly classified as "adverse" according to ELN criteria (Favorable, n = 1; Intermediate-II, n = 20; Adverse, n = 44). AML+13 patients (n = 34 [AMLCG, n = 23; SAL, n = 11]) were compared with 850 ELN Intermediate-II genetic group patients without +13 enrolled in the same clinical trials. Detailed patient characteristics are given in Table 1 (and separated for the AMLCG and SAL subgroups in supplemental Table 4A-B). The study design is summarized in supplemental Figure 1A-B. In the combined data set, AML+13 patients were significantly older (P = .004) and had higher initial BM blast counts (P = .02), but significantly lower LDH levels (P = .009) than other patients in the ELN Intermediate-II genetic group. AML+13 and aAML+13 patients had similar baseline characteristics, except for significantly lower LDH levels and a higher CR rate in AML+13 and lower platelet counts than aAML+13 (supplemental Table 4C).

Twenty-one AML+13 patients (62%, 95% confidence interval [CI]: 44% to 77%) reached CR, compared with 471 (55%, 95% CI: 52% to 59%) of ELN Intermediate-II patients without +13 (P = .49). However, 18 of these 21 patients (86%, 95% CI: 63% to 96%) relapsed.

In the AMLCG trial, AML+13 was associated with inferior RFS and OS (median RFS = 8.7 vs 14.1 months, P = .02; median OS = 7 vs 13.9 months, P = .01; Figure 1A-B), whereas in the SAL cohort, the differences between AML+13 and other ELN Intermediate-II patients did not reach significance (RFS, P = .12; OS, P = .29; supplemental Figure 2A), possibly due to the small number of AML+13 cases (n = 11). RFS and OS in the combined SAL and AMLCG cohort were inferior for the AML+13 group compared with other ELN Intermediate-II patients (median RFS = 7.8 vs 14.1 months, P = .006; median OS = 9.3 vs 14.8 months, P = .004; Figure 1C-D).

In a multivariate analysis in the combined AMLCG and SAL cohorts that adjusted for other known prognostic markers, AML+13 remained a significant variable within the ELN Intermediate-II genetic group for OS, but not for RFS (Table 2).

There was no significant difference in RFS (P = .74) or OS (P = .82) between the AML+13 and aAML+13 subgroups, despite the high frequency of adverse cytogenetic alterations in the aAML+13 group (supplemental Figure 2B). We also compared the AMLCG AML+13 group (n = 23) to 463 patients treated on the AMLCG-1999 trial who had adverse cytogenetics. Baseline characteristics for these cohorts are shown in supplemental Table 4D. There was no significant difference regarding RFS (P = .78) or OS (P = .98) between both groups (supplemental Figure 2C).

High frequency of mutations affecting *SRSF2*, *RUNX1*, *ASXL1*, and *BCOR* in AML+13

To systematically identify somatic mutations associated with AML+13, we performed exome sequencing of paired diagnostic and remission samples from 2 patients with AML+13 (patients no. 8 and 11). We identified nonsynonymous leukemia-specific mutations affecting 36 genes, including *RUNX1*, *ASXL1*, *BCOR*, *ZRSR2*, *NUP188*, and *CEBPZ*. No recurring mutations were observed between the 2 patients. Nonsynonymous mutations in protein-coding transcripts are summarized in supplemental Table 5.

Targeted amplicon sequencing was performed on 16 AML+13 patient samples. Consistent with previous reports,[6,7] we found a high frequency of *RUNX1* mutations (n = 12, 75%). In addition, we detected mutations in spliceosome components in 14 AML+13 patients (88%), including *SRSF2* codon 95 mutations in 13 patients (81%) and an *SF3B1* mutation in 1 patient. The association of spliceosome component mutations (*SRSF2*, *SF3B1*, *SF1*, and *ZRSR2*) with *RUNX1* mutations was significant (P = .05). Additional recurring mutations affected *ASXL1* (n = 7, 44%) and *BCOR* (n = 4, 25%), and occurred with *RUNX1* and *SRSF2* mutations but these associations did not reach statistical significance (*ASXL1-SRSF2*, P = .21; *ASXL1-RUNX1*, P = .34; *BCOR-SRSF2*, P = .53; and *BCOR-RUNX1*, P = .53). The 2 patients without mutations in the splicing machinery had *DNMT3A* mutations, which were also mutually exclusive with mutations in *RUNX1* or *ASXL1*. Two patients carried mutations in *CEBPZ*, thus establishing *CEBPZ* as a novel recurrently mutated gene in AML. Details of all detected nonsynonymous variants are shown in Figure 2 and supplemental Table 6.

The mutations in *SRSF2* and *CEBPZ* were confirmed by Sanger sequencing (results summarized in supplemental Table 6). The correlation of the results from Sanger sequencing and targeted high throughput sequencing was 100% (for details, see supplemental Figure 3). In one of the patients with a *CEBPZ* mutation and an available remission sample, we could confirm the somatic nature of the mutation (supplemental Figure 3).

Both patients characterized by exome sequencing carried *SRSF2* mutations at codon 95, as identified by amplicon sequencing. However, these mutations were not detected by exome sequencing due to low coverage of this region in both samples. These results show that our targeted sequencing approach detects mutations in AML candidate genes with high sensitivity and specificity, including mutations in regions not covered by exome sequencing.

To further explore the association between *RUNX1* and *SRSF2* mutations, we analyzed the *SRSF2* gene in a cohort of 14 patients with a known *RUNX1* mutation and normal karyotype AML (CN-AML).[34] We found mutations in *SRSF2* in 3 of the 14 patients (21%).

Distinct gene expression pattern of AML+13

We identified 678 probe sets as significantly ($P \leq .05$ after adjustment for multiple testing) deregulated (upregulated, 492; downregulated, 186) in AML+13 patients (n = 9), when compared

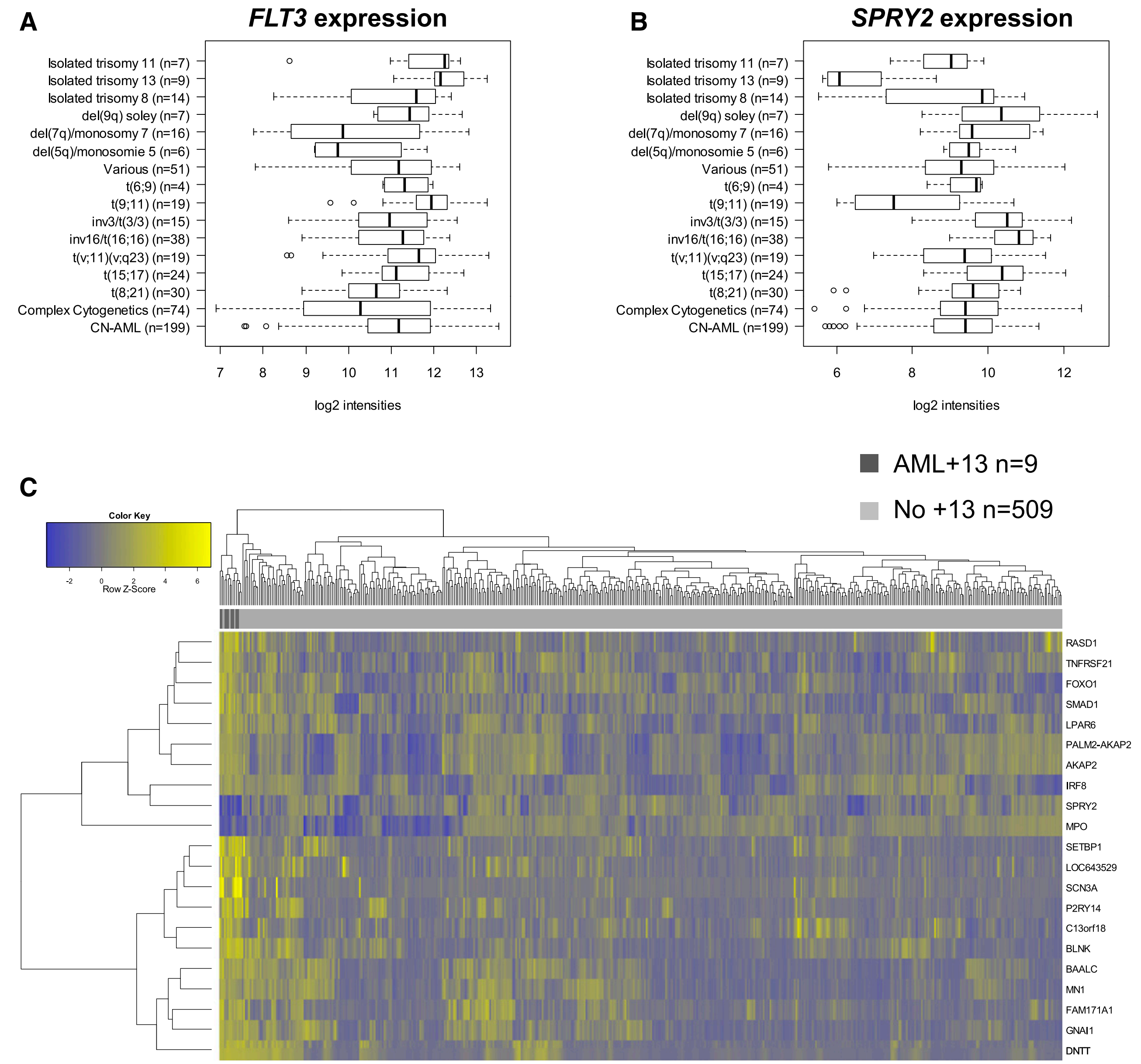
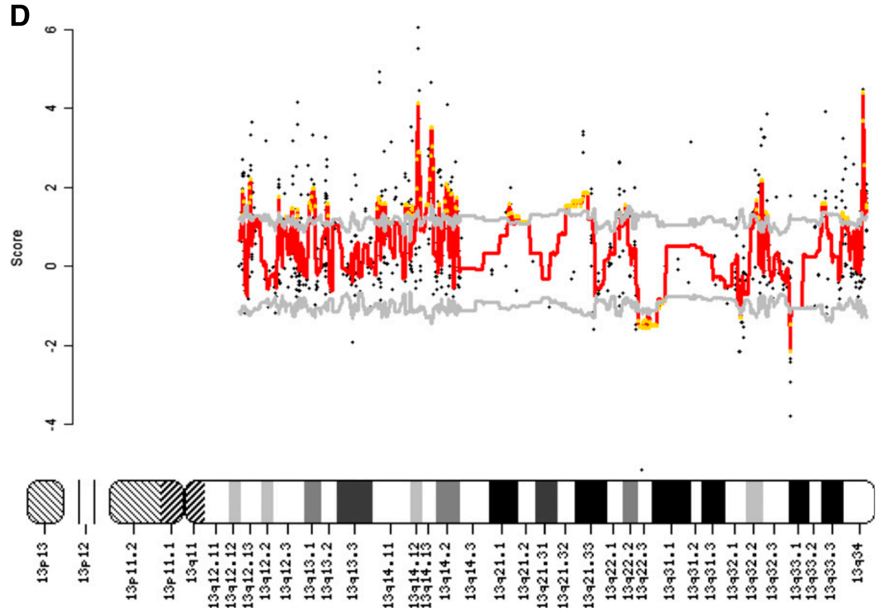


Figure 3. Gene expression profile of AML+13. (A-B) *FLT3* and *SPRY2* expression in AML subgroups. Boxplot showing *FLT3* (A) and *SPRY2* (B) expression levels in various cytogenetic AML subgroups. The boxes indicate the upper and lower quartiles. The band within the boxes represents the median. Outliers are plotted as individual points. *FLT3* expression is significantly higher in AML+13 compared with all other samples (P = .04). However, in several individual samples of various cytogenetic subgroups, *FLT3* was expressed at higher levels compared with AML+13. *SPRY2* expression is significantly lower in AML+13 ($P < .001$). (C) Clustering of AML+13 using 21 probe sets. Heatmap visualizing hierarchical clustering of AML+13 samples according to the 21 most differentially expressed probe sets (log-fold change ≥ 2 or ≤ −2 and adjusted P-value < .001) compared with AML with various other cytogenetic aberrations except for +13. All AML+13 samples cluster closely together, indicating a highly homogenous expression profile of this subgroup. (D) Regional gene expression on chromosome 13 in AML+13. Expression levels of probe sets located on chromosome 13 displayed by MACAT analysis in AML+13 patients (n = 9) compared with AML with various other cytogenetic abnormalities (except +13, n = 519). Scores for probe sets are shown as black dots. The sliding average of the 0.025 and 0.975 quantiles of the permuted scores are visualized as gray lines. The sliding average permuted scores (red line), and highlighted regions (yellow-dotted), where the score exceeds the quantiles, are plotted along chromosome 13. Despite the majority of probe sets showing elevated expression levels as expected, some regions were characterized by significantly lower expression levels.

to AML patients with various other cytogenetic abnormalities (n = 509). Detailed patient characteristics are given in supplemental Table 7. Only 59 (8.7%) of these probe sets were localized on chromosome 13, but of those, 55 were upregulated and only 4 were downregulated. Upregulated probe sets on chromosome 13 included *FOXO1*, *FLT3*, (Figure 3A) and *RB1*. The strongest downregulated probe set on chromosome 13 belonged to the tumor suppressor gene *SPRY2* (Figure 3B), which is a negative regulator of receptor tyrosine kinases. As described before, *FLT3* is significantly upregulated in AML+13, compared with all other AML samples in our gene expression data set (P = .04). However, as shown in Figure 3A, *FLT3* expression in AML shows a complex pattern with a wide range of expression levels, and AML+13 is not the only entity associated with high *FLT3* levels.

A total of 21 probe sets showed highly significant deregulation (log-fold change ≥ 2 or ≤ −2 and adjusted P-value < .001) and were therefore used for clustering (supplemental Table 8). The result of the clustering is shown in Figure 3C. Consistent with the results from our

Figure 3. Continued



genetic analysis, AML+13 shows a homogenous gene expression profile that is distinct from other AML subsets.

Surprisingly, some genes located on chromosome 13 showed significantly lower expression in AML+13 compared with patients with two copies of chromosome 13. The differential regional gene expression of AML+13 patient samples across chromosome 13 is visualized in Figure 3D (for details, see supplemental Table 9A-B). Despite the additional copy of chromosome 13, we identified several regions on chromosome 13 with significantly reduced gene expression levels compared with patients with two copies of chromosome 13.

By using GSEA, we see a potential deregulation of gene sets associated with cytoplasmatic and nuclear transport and the regulation of transcription. Details are given in supplemental Table 10. We could also observe that the expression levels of the transcription factor *FOXO1* correlated with higher expression levels of a predefined gene set consisting of target genes of this transcription factor (nominal *P*-value: .02; false discovery rate: .23). In summary, our gene expression studies reveal a complex picture of deregulated genes in AML+13 patients with a potential role in leukemogenesis. Some of these genes, such as *SPRY2* (Figure 3B) are downregulated despite their location on chromosome 13.

Finally, we compared the results of our gene expression analysis with data derived from the comparison of *RUNX1*-mutated and wild type AML with CN-AML.[34] This 85 gene *RUNX1* signature showed an overlap of 28 genes (33%) with differentially expressed genes in AML+13 (supplemental Table 11).

Discussion

Our study is the first to show that AML+13 patients have a significantly inferior RFS and OS compared with patients with other intermediate-risk cytogenetic abnormalities in a homogeneously treated cohort. Based on these findings, AML+13 should be considered as a subgroup associated with an extremely poor outcome. Furthermore, we provide evidence that AML+13 leukemia is genetically homogenous, not only on the cytogenetic but also on the molecular level. AML+13 is not only associated with a high frequency of *RUNX1* mutations, but also with mutations in *SRSF2*, *ASXL1*, and *BCOR*. To our knowledge, the incidence of mutations in *SRSF2* in AML+13 is the highest of any AML or myelodysplastic syndrome (MDS) subgroup reported so far.[35,36] An association between *SRSF2* and *RUNX1* mutations was already reported in patients with MDS.[35] We provide first evidence that an association between these mutations could also be observed in AML with *RUNX1* mutations. However, larger studies are necessary to verify this observation.

It is intriguing to speculate about functional interactions between mutations in these two genes and trisomy 13. It remains unclear whether mutations targeting *SRSF2* and *RUNX1*, and trisomy 13, affect a common pathway or different but complementary pathways on the way to leukemia. Although one of these lesions likely represents a near compulsory additional hit required by the initial event, the order of these events remains elusive. In light of the high prevalence of acquired *GATA1* mutations in AML of Down syndrome patients,[10] it is very likely that the chromosomal aneuploidy is the first event and determines the subsequent acquisition of mutations in precisely defined genes.

There is some, but limited overlap of recurrently mutated genes in AML and MDS. However, the high incidence of spliceosome gene mutations in both MDS and AML+13 is striking. A case report of 2 AML+13 patients who achieved sustained complete morphologic and cytogenetic remission while treated with high-dose, single-agent lenalidomide suggests a potential role of spliceosome gene mutations in the response to lenalidomide, which is also used in MDS therapy.[8] Otrock et al recently reported an association of lenalidome response with distinct mutation patterns.[37]

Of note, only one *SRSF2* mutation was found in 200 AML patients studied by whole exome or whole genome sequencing.[38] This *SRSF2*-mutated patient also had a *RUNX1* mutation. The study included a total of 19 *RUNX1*-mutated patients.[38] As is obvious from our study, it is likely that some *SRSF2* mutations in this study might have gone undetected, since exome sequencing may miss these mutations due to inefficient target enrichment.

It was proposed that overexpression of *FLT3,* which localizes to chromosome 13, could play a crucial role in AML+13.[6,7] Our study confirms an elevated expression level of *FLT3* in the AML+13 subgroup. However, the levels are similar to other cytogenetic AML subgroups without additional chromosome 13, showing that high *FLT3* expression levels are not a defining feature of AML+13. Nevertheless, these findings do not rule out that high *FLT3* expression levels are an important leukemic driver in AML+13. High *FLT3* expression levels might be achieved by other mechanisms than an additional copy of chromosome 13 in other leukemias. Our gene expression analysis suggests several possible alternative or additional consequences of trisomy 13. *FOXO1* is overexpressed in AML+13, and GSEA revealed upregulated sets of *FOXO1* target genes. Recurrent mutations in *FOXO1* associated with poor survival were recently discovered in diffuse large B-cell lymphoma.[39] Furthermore, activation of *FOXO1* was observed in ~40% of AML patients.[40] Inhibition of *FOXO1* leads to reduced leukemic cell growth.[40] The tumor suppressor gene *SPRY2*, a negative regulator of receptor tyrosine kinases, had strikingly low expression levels even though it is located on chromosome 13 (Figure 3B). Downregulation of *SPRY2* was previously reported in a variety of solid tumors.[41-44] It is challenging to explain the underlying mechanism for this apparently contradictory result (ie, the downregulation despite an additional gene copy). Potential mechanisms for low *SPRY2* expression include epigenetic inactivation, submicroscopic deletions of *SPRY2*, or mutations in upstream regulators of *SPRY2*. These results again demonstrate the complexity of gene regulation and indicate that the concept of gene dosage is inadequate to explain all effects of an additional chromosome 13. Our gene expression data show a distinct gene expression profile of AML+13 partially overlapping with *RUNX1*- mutated CN-AML.

The striking association of mutations affecting only a few distinct genes in AML+13 suggests a strong synergism of these lesions during leukemogenesis. The fact that mutations in *RUNX1*, *ASXL1*, and upregulation of *FLT3* were previously reported as markers of poor prognosis in AML clearly suggests that the combination of these lesions is responsible for the extremely poor outcome of AML+13.

In summary, we discovered the highest incidence of *SRSF2* mutations in a specific AML subgroup reported so far. This rare, but genetically extremely homogenous group of AML+13 leukemia is characterized by concurrent mutations of *SRSF2* and *RUNX1*, as well as a specific gene expression profile. Consistent with other studies, our findings suggest a connection between mutations of *RUNX1* and *SRSF2* in myeloid leukemogenesis. AML+13 is associated with inferior survival despite intensive treatment. Therefore, new treatment strategies are highly warranted.

The discovery of rare, genetically homogenous AML subgroups indicates that the genetic complexity of AML is extremely high but mutations do not occur randomly. Despite the increasing number of comprehensively characterized AML cases, the understanding of oncogenic collaboration poses a challenge ahead.

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Authorship

Contribution: T.H., K.H.M., and. P.A.G. conceived and designed the experiments; T.H., K.H.M., L.H., E.Z., B.K., and S.K. performed experiments; T.H., K.H.M., S.V., M.H., and V.J. analyzed data; S.V. and A.G. provided bioinformatics support; H.B. managed the Genome Analyzer IIx platform; B.K., A.D., E.Z., Z.P., P.M.K., S.S., S.K.B., and K.S. characterized patient samples; M.C.S., W.E.B., T.B., B.J.W., and W.H. coordinated the AMLCG clinical trial; P.A.G., U.M., K.S., and S.K.B. supervised the project; T.H., K.H.M., S.K.B., and P.A.G. wrote the manuscript; and C.R., F.S., M.B., and G.E. coordinated the SAL clinical trials, selected, contributed, and analyzed SAL data.

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References

1. Mesa RA, Hanson CA, Ketterling RP, Schwager S, Knudson RA, Tefferi A. Trisomy 13: prevalence and clinicopathologic correlates of another potentially lenalidomide-sensitive cytogenetic abnormality. *Blood.* 2009;113(5):1200-1201.
2. Grimwade D, Hills RK, Moorman AV, et al; National Cancer Research Institute Adult Leukaemia Working Group. Refinement of cytogenetic classification in acute myeloid leukemia: determination of prognostic significance of rare recurring chromosomal abnormalities among 5876 younger adult patients treated in the United Kingdom Medical Research Council trials. *Blood.* 2010;116(3):354-365.
3. Baer MR, Bloomfield CD. Trisomy 13 in acute leukemia. *Leuk Lymphoma.* 1992;7(1-2):1-6.
4. Döhner H, Arthur DC, Ball ED, et al. Trisomy 13: a new recurring chromosome abnormality in acute leukemia. *Blood.* 1990;76(8):1614-1621.
5. Döhner H, Estey EH, Amadori S, et al; European LeukemiaNet. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood.* 2010;115(3):453-474.
6. Silva FP, Lind A, Brouwer-Mandema G, Valk PJ, Giphart-Gassler M. Trisomy 13 correlates with RUNX1 mutation and increased FLT3 expression in AML-M0 patients. *Haematologica.* 2007;92(8):1123-1126.
7. Dicker F, Haferlach C, Kern W, Haferlach T, Schnittger S. Trisomy 13 is strongly associated with AML1/RUNX1 mutations and increased FLT3 expression in acute myeloid leukemia. *Blood.* 2007;110(4):1308-1316.
8. Fehniger TA, Byrd JC, Marcucci G, et al. Single-agent lenalidomide induces complete remission of acute myeloid leukemia in patients with isolated trisomy 13. *Blood.* 2009;113(5):1002-1005.
9. Ganmore I, Smooha G, Izraeli S. Constitutional aneuploidy and cancer predisposition. *Hum Mol Genet.* 2009;18(R1):R84-R93.
10. Wechsler J, Greene M, McDevitt MA, et al. Acquired mutations in GATA1 in the megakaryoblastic leukemia of Down syndrome. *Nat Genet.* 2002;32(1):148-152.
11. Baty BJ, Blackburn BL, Carey JC. Natural history of trisomy 18 and trisomy 13: I. Growth, physical assessment, medical histories, survival, and recurrence risk. *Am J Med Genet.* 1994;49(2):175-188.
12. Satge D, Van Den Berghe H. Aspects of the neoplasms observed in patients with constitutional autosomal trisomy. *Cancer Genet Cytogenet.* 1996;87(1):63-70.

13. Schade H, Schoeller L, Schultze KW. [D-trisomy (Paetau syndrome) with congenital myeloid leukemia]. *Med Welt.* 1962;50:2690-2692.

14. Schaich M, Parmentier S, Kramer M, et al. High-dose cytarabine consolidation with or without additional amsacrine and mitoxantrone in acute myeloid leukemia: results of the prospective randomized AML2003 trial. *J Clin Oncol.* 2013; 31(17):2094-2102.

15. Schaich M, Röllig C, Soucek S, et al. Cytarabine dose of 36 g/m compared with 12 g/m within first consolidation in acute myeloid leukemia: results of patients enrolled onto the prospective randomized AML96 study. *J Clin Oncol.* 2011;29(19): 2696-2702.

16. Rollig C, Kramer M, Hanel M, et al. Induction treatment in elderly patients with acute myeloid leukemia (AML): randomized comparison of intermediate-dose cytarabine plus mitoxantrone (IMA) versus standard-dose cytarabine plus daunorubicin (DA) in 492 AML patients >60 years - Results from the SAL 60plus trial [abstract]. *Blood (ASH Annual Meeting Abstracts).* 2010;116(21). Abstract 334.

17. Büchner T, Berdel WE, Schoch C, et al. Double induction containing either two courses or one course of high-dose cytarabine plus mitoxantrone and postremission therapy by either autologous stem-cell transplantation or by prolonged maintenance for acute myeloid leukemia. *J Clin Oncol.* 2006;24(16):2480-2489.

18. Opatz S, Polzer H, Herold T, et al. Exome sequencing identifies recurring FLT3 N676K mutations in core-binding factor leukemia. *Blood.* 2013;122(10):1761-1769.

19. Greif PA, Dufour A, Konstandin NP, et al. GATA2 zinc finger 1 mutations associated with biallelic CEBPA mutations define a unique genetic entity of acute myeloid leukemia. *Blood.* 2012;120(2): 395-403.

20. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics.* 2009;25(14):1754-1760.

21. Li H, Handsaker B, Wysoker A, et al; 1000 Genome Project Data Processing Subgroup. The Sequence Alignment/Map format and SAMtools. *Bioinformatics.* 2009;25(16):2078-2079.

22. McKenna A, Hanna M, Banks E, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res.* 2010;20(9): 1297-1303.

23. Koboldt DC, Chen K, Wylie T, et al. VarScan: variant detection in massively parallel sequencing of individual and pooled samples. *Bioinformatics.* 2009;25(17):2283-2285.

24. Ye K, Schulz MH, Long Q, Apweiler R, Ning Z. Pindel: a pattern growth approach to detect break points of large deletions and medium sized insertions from paired-end short reads. *Bioinformatics.* 2009;25(21):2865-2871.

25. Koboldt DC, Zhang Q, Larson DE, et al. VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. *Genome Res.* 2012;22(3):568-576.

26. Li Z, Herold T, He C, et al. Identification of a 24-gene prognostic signature that improves the European LeukemiaNet risk classification of acute myeloid leukemia: an international collaborative study. *J Clin Oncol.* 2013;31(9):1172-1181.

27. Herold T, Jurinovic V, Metzeler KH, et al. An eight-gene expression signature for the prediction of survival and time to treatment in chronic lymphocytic leukemia. *Leukemia.* 2011;25(10): 1639-1645.

28. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA.* 2005;102(43):15545-15550.

29. Herold T, Jurinovic V, Mulaw M, et al. Expression analysis of genes located in the minimally deleted regions of 13q14 and 11q22-23 in chronic lymphocytic leukemia-unexpected expression pattern of the RHO GTPase activator ARHGAP20. *Genes Chromosomes Cancer.* 2011;50(7):546-558.

30. Toedling J, Schmeier S, Heinig M, Georgi B, Roepcke S. MACAT—microarray chromosome analysis tool. *Bioinformatics.* 2005;21(9): 2112-2113.

31. R Development Core Team (2008). R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, www.R-project.org.

32. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc, B.* 1995;57(1): 289-300.

33. Cheson BD, Bennett JM, Kopecky KJ, et al; International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia. Revised recommendations of the International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia [published correction appears in J Clin Oncol. 2004;22(3):576]. *J Clin Oncol.* 2003;21(24):4642-4649.

34. Greif PA, Konstandin NP, Metzeler KH, et al. RUNX1 mutations in cytogenetically normal acute myeloid leukemia are associated with a poor prognosis and up-regulation of lymphoid genes. *Haematologica.* 2012;97(12):1909-1915.

35. Thol F, Kade S, Schlarmann C, et al. Frequency and prognostic impact of mutations in SRSF2, U2AF1, and ZRSR2 in patients with myelodysplastic syndromes. *Blood.* 2012; 119(15):3578-3584.

36. Yoshida K, Sanada M, Shiraishi Y, et al. Frequent pathway mutations of splicing machinery in myelodysplasia. *Nature.* 2011;478(7367):64-69.

37. Otrock ZK, Przychodzen BP, Husseinzadeh HD, et al. Molecular predictors of response to lenalidomide in myeloid malignancies. *Blood.* 2013;122(21):Abstract 2807.

38. Cancer Genome Atlas Research Network. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *N Engl J Med.* 2013;368(22):2059-2074.

39. Trinh DL, Scott DW, Morin RD, et al. Analysis of FOXO1 mutations in diffuse large B-cell lymphoma. *Blood.* 2013;121(18):3666-3674.

40. Sykes SM, Lane SW, Bullinger L, et al. AKT/FOXO signaling enforces reversible differentiation blockade in myeloid leukemias [published correction appears in *Cell.* 2011;147(1):247]. *Cell.* 2011;146(5):697-708.

41. Kwak HJ, Kim YJ, Chun KR, et al. Downregulation of Spry2 by miR-21 triggers malignancy in human gliomas. *Oncogene.* 2011;30(21):2433-2442.

42. Fritzsche S, Kenzelmann M, Hoffmann MJ, et al. Concomitant down-regulation of SPRY1 and SPRY2 in prostate carcinoma. *Endocr Relat Cancer.* 2006;13(3):839-849.

43. Fong CW, Chua MS, McKie AB, et al. Sprouty 2, an inhibitor of mitogen-activated protein kinase signaling, is down-regulated in hepatocellular carcinoma. *Cancer Res.* 2006;66(4):2048-2058.

44. Lo TL, Yusoff P, Fong CW, et al. The ras/mitogen-activated protein kinase pathway inhibitor and likely tumor suppressor proteins, sprouty 1 and sprouty 2 are deregulated in breast cancer. *Cancer Res.* 2004;64(17):6127-6136.