# Recurrent deletions of IKZF1 in pediatric acute myeloid leukemia

**Jasmijn D.E. de Rooij,[1*] Eva Beuling,[1*] Marry M. van den Heuvel-Eibrink,[1,2] Askar Obulkasim,[1] André Baruchel,[3] Jan Trka,[4] Dirk Reinhardt,[5] Edwin Sonneveld,[6] Brenda E.S. Gibson,[7] Rob Pieters,[2] Martin Zimmermann,[4] C. Michel Zwaan,[1†] and Maarten Fornerod[1†]**

[1]Pediatric Oncology, Erasmus MC-Sophia Children's Hospital Rotterdam, the Netherlands; [2]Princess Máxima Center for Pediatric Oncology, Utrecht, the Netherlands; [3]Hematology, Hopital Saint- Louis, Paris, France; [4]Pediatric Hematology/Oncology, 2nd Medical School, Charles University, Prague, Czech Republic; [5]AML-BFM Study Group, Pediatric Hematology/Oncology, Medical School Hannover, Germany; [6]Dutch Childhood Oncology Group (DCOG), The Hague, the Netherlands; and [7]Royal Hospital for Sick Children, Glasgow, UK

**JDEdR and EB contributed equally to this work as 1st authors*

*†CMZ and MF contributed equally to this work*

## ABSTRACT

IKAROS family zinc finger 1/IKZF1 is a transcription factor important in lymphoid differentiation, and a known tumor suppressor in acute lymphoid leukemia. Recent studies suggest that IKZF1 is also involved in myeloid differentiation. To investigate whether *IKZF1* deletions also play a role in pediatric acute myeloid leukemia, we screened a panel of pediatric acute myeloid leukemia samples for deletions of the *IKZF1* locus using multiplex ligation-dependent probe amplification and for mutations using direct sequencing. Three patients were identified with a single amino acid variant without change of IKZF1 length. No frame-shift mutations were found. Out of 11 patients with an *IKZF1* deletion, 8 samples revealed a complete loss of chromosome 7, and 3 cases a focal deletion of 0.1-0.9Mb. These deletions included the complete *IKZF1* gene (n=2) or exons 1-4 (n=1), all leading to a loss of IKZF1 function. Interestingly, differentially expressed genes in monosomy 7 cases (n=8) when compared to non-deleted samples (n=247) significantly correlated with gene expression changes in focal *IKZF1*-deleted cases (n=3). Genes with increased expression included genes involved in myeloid cell self-renewal and cell cycle, and a significant portion of GATA target genes and *GATA* factors. Together, these results suggest that loss of *IKZF1* is recurrent in pediatric acute myeloid leukemia and might be a determinant of oncogenesis in acute myeloid leukemia with monosomy 7.

## Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease characterized by dysregulated hematopoiesis initiated by recurrent non-random genetic aberrations.[1,2] Despite major progress in improving treatment over the past decades, AML remains a life-threatening malignancy in children. The 5-year event-free survival (EFS) rates of pediatric AML are currently approximately 50%-60% and the overall survival (OS) rate approximately 70%.[3,4] Treatment stratification is mainly based on early response to treatment and prognostic relevant genetic abnormalities. Although many driving recurrent genetic aberrations causing AML have been defined, in approximately 20% of the pediatric AML patients the driving genetic events remain unidentified.[3]

The DNA-binding protein IKAROS family zinc finger 1 (IKZF1) is a zinc finger containing transcription factor that is encoded in 8 exons on chromosome 7 (7p12.2). IKZF1 functions upon homo-dimerization and acts as a chromatin remodeling protein involved in several chromatin remodeling complexes, such as nucleosome-remodeling and histone deacetylation (NuRD).[5] Alternative splicing of *IKZF1* pre-mRNA gives rise to several isoforms.[6] The functional differences among the various isoforms of IKZF1 are mostly due to alterations of zinc finger structures required for DNA binding (4 zinc fingers in the N-terminal domain) or protein-protein interactions (2 zinc fingers in the C-terminal domain).[6,7] Some of these splice variants lack the ability to properly bind DNA and act as dominant-negative repressors when dimerized with functional isoforms. Loss of Ikzf1 function in mice homozygous for an *Ikzf1* null allele or homozygous for a dominant-negative isoform of Ikzf1 results in an early arrest of lymphoid differentiation with lack of T and B lymphocytes and NK cells, as well as their earliest identifiable progenitors.[8,9] In heterozygous *Ikzf1* mutant mice, lymphocytes appear normal immediately after birth, but proliferation increases and differentiation arrest occurs rapidly, leading to the development of leukemia and lymphoma within three months after birth.[10] In humans, heterozygous loss of *IKZF1* is found in 15% of B-cell precursor acute lymphoblastic leukemia (BCP-ALL) patients; in 70%-80% of *BCR/ABL1* (t(9;22)(q34;q11)) positive BCP-ALL patients and in 33% of *BCR/ABL1* negative BCP-ALL patients.[11-15] In *BCR/ABL1* ALL patients, heterozygous loss of *IKZF1* associates with a poor clinical outcome. In T-ALL heterozygous loss of *IKZF1* is found in only 4%, but it remains unclear if this has any prognostic value.[11,12]

While the role of IKZF1 in lymphoid differentiation and ALL is firmly established, the role of IKZF1 in myeloid differentiation is less clear. However, there are some strong indications that IKZF1 is also involved in myeloid differentiation. In early myeloid progenitors, loss of IKZF1 function prolongs cell survival.[16] During erythropoiesis, IKZF1 supports survival of the erythroid lineage, and promotes erythrocyte differentiation at the expense of granulocyte and monocyte differentiation.[17-19] In early megakaryopoiesis, IKZF1 represses differentiation by inhibiting genes associated with the NOTCH pathway and later on in differentiation towards megakaryocytes

 doi:10.3324/haematol.2015.124321
The online version of this article has a Supplementary Appendix.
Manuscript received on January 23, 2015. Manuscript accepted on June 5, 2015.
Correspondence: m.fornerod@erasmusmc.nl

by controlling transcriptional regulators, such as GATA1 and RUNX1.[18,20] Although IKZF1 is not required for the initial differentiation towards granulocytes and monocytes, IKZF1 represses differentiation of the basophilic granulocyte lineage and promotes early maturation and survival of the neutrophil granulocyte lineage.[17,21,22]

In pediatric AML, monosomy 7 is found in only 4%-5% of patients. The 5-year OS and EFS of pediatric AML patients with monosomy 7 with or without additional cytogenetic or chromosomal aberrations are poor.[23] In adult AML, monosomy 7 is the most frequent single monosomy and has a similar poor 4-year OS as compared to other single autosomal monosomies.[24] Deletions of the short arm of chromosome 7, 7p (del7p) were recurrently found in adult *de novo* AML and secondary AML developed from myelodysplastic syndrome (MDS) or myeloproliferative neoplasms (MPN).[25-27] Furthermore, 7p deletions were mapped to the *IKZF1* locus and it was shown that loss of *IKZF1* is acquired during the progression of MPN to secondairy AML, indicating that loss of *IKZF1* contributes to the transformation from MPN to AML.[25]

Together, these studies demonstrate that IKZF1 may play a role in myeloid differentiation and that loss of *IKZF1* may contribute to myeloid oncogenesis. We, therefore, hypothesized that, in pediatric AML patients with monosomy 7, IKZF1 may be an important player. In this study, we analyzed the frequency of *IKZF1* deletions in a pediatric AML cohort, and studied the difference in gene expression of cases with focal *IKZF1* deletions, and cases with monosomy 7.

## Methods

### Patients' samples

Samples were provided by the Dutch Childhood Oncology Group (DCOG, the Netherlands), the AML–Berliner-Frankfurt-Münster Study Group (Germany and Czech Republic), the Saint-Louis Hospital (France), and the Royal Hospital for Sick Children (UK). Isolation of genomic DNA and total cellular RNA was performed using Trizol reagent. Each study group provided morphological and cytogenetic classification and clinical data. Institutional review board approval for these studies had been obtained in the participating centers.

### Detection of deletions and mutations of IKZF1

Multiplex ligation-dependent probe amplification (MLPA) was performed using the p335-B1;ALL-IKZF1 kit (MRC Holland, Amsterdam, the Netherlands; data available at *http://www.mlpa.com*). The data were analyzed with GeneMarker v. 1.85 (SoftGenetics, State College, Pennsylvania, USA). Data were normalized to reference probes and control samples. A deletion was defined as a peak ratio below 0.75; an amplification was defined as a peak ratio above 1.25.

Array comparative genomic hybridization (Array-CGH) was performed using the human genome CGH Microarray 105K (Agilent Technologies, Santa Clara, California, USA) according to the manufacturer's protocol; data were analyzed with Genomic Workbench v. 5.0.14 (Agilent Technologies, Santa Clara, California, USA).

Direct sequencing was used to analyze mutations or frameshifts in exons 4, 5 and 8, which are found to be hotspot areas of mutations in pediatric BCP-ALL (primers are listed in *Online Supplementary Table S1*).[13,28]

### Cytogenetic and molecular characterization

Reverse-transcription-PCR was performed on hotspot areas as previously described by Balgobind *et al.*,[29] and to analyze the frequency of the *BCR/ABL1* translocation (primers are listed in *Online Supplementary Table S1*).

### Gene expression profiling

Gene expression profiling (GEP) data were available from earlier studies.[30] Quantitative reverse-transcription-PCR (qRT-PCR) analysis was performed on a subset of patients to analyze the correlation between GEP data and qRT-PCR on relevant genes in patients of different cytogenetic subgroups and a variety of expression measured with GEP. The mRNA expression was determined using the average cycle threshold (Ct) in comparison to expression levels of *GAPDH*, using the comparative cycle threshold method.

### Statistical analysis

Statistical analyses for disease outcome and correlation were performed with IBM SPSS 21 (IBM, Armonk, New York, USA). To assess clinical outcome, complete remission (CR; defined as less than 5% blasts in the bone marrow, with regeneration of tri-lineage hematopoiesis, plus absence of leukemic cells), probability of event-free survival (pEFS; defined as the time between diagnosis and first event including non-responders calculated as an event on day 0), and probability of overall survival (pOS; defined as the time between diagnosis and death) were analyzed. Both pEFS and pOS were estimated by the Kaplan-Meier method and differences compared using log rank tests. The cumulative incidence of non-response or relapse (pCIR; defined as time between diagnosis and relapse, and with non-responders included as an event on day 0) was analyzed by the Kalbfleisch and Prentice method, and compared with the Gray's test. $P<0.05$ was considered significant.

Differential expression analyses between groups were conducted using the R-package ShirnkBayes[31] at the probe level. In contrast to commonly used two-sample tests, this test is proven to be powerful for small sample sizes. Bayesian false discovery rate (BFDR)<0.05 was considered statistically significant.

## Results

### Identification of IKZF1 deletions in pediatric AML

The cohort of newly diagnosed pediatric AML patients with available peripheral blood or bone marrow samples taken at initial diagnosis included in this study has been previously described by Hollink *et al.* (n=293, age 0-18 years).[30] Of 293 well-characterized pediatric AML cases, 258 with available high quality DNA were analyzed with MLPA using 250ng of DNA. Cytogenetic aberrations in this group were representative for the frequencies seen in pediatric AML.[3,32] In addition, 14 healthy control samples as negative controls, and one pediatric ALL sample known to harbor an *IKZF1* deletion as positive control were analyzed. A subsequent MLPA was considered if all reference probes had a peak ratio between 0.75 and 1.25, except where a deletion or amplification of a reference probe could be explained by an aberration visible in the karyotype. The P335-B1;ALL-IKZF1 MLPA kit contains a probe in each of the 8 exons of *IKZF1*. All probes on chromosome 7 were analyzed individually, including reference probe 15370-L13762 located on 7q11.23 in *P450 cytochrome oxidorectase (POR)*. Additional high-resolution array-CGH was performed on patients with an *IKZF1* deletion detected by MLPA, but without knowledge of the existence of monosomy 7, and one additional case of monosomy 7

**Table 1.** Patients' characteristics of *IKZF1* deleted pediatric AML cases (n=11).

| ID | IKZF1 status MLPA | IKZF1 status array-CGH | Karyotype provided | Age (y) | Sex | WBC x10$^9$/L | FAB | Aberrations* | Therapy protocol | CR | Relapse | Death | SCT | COD |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | del ex1-8 | NA | 45, XX, -7, t(8;16)(p11;p13) [21] | 7.4 | F | 58.1 | M5 | *KAT6A/CREBBP*, *FLT3/ITD* | MRC12 | yes | no | no | no | – |
| 2 | del ex1-8 | del 7p12.2 | 47,XY,del(12)(p12p13).ish t(7;12)(q3;p13),+19 [20] | 1.5 | M | 226.8 | M3 | *MNX1/ETV6* | DCOG94 | yes | yes | yes | yes | leukemia |
| 3 | del ex1-8 | del 7p12.2 | NA | 11.3 | F | NA | M5 | *WT1* | MRC12 | yes | yes | no | no | – |
| 4 | del ex1-8 | del 7 | NA | 4.9 | F | 31.4 | M6 | None | BFM04 | yes | yes | no | yes | – |
| 5 | del ex1-8 | NA | 45, XX, inv(2)(p24q14), -7 | 10.9 | F | 54.9 | M4 | None | DCOG94 | yes | yes | yes | no | infection |
| 6 | del ex1-8 | del 7 | 46,XY | 0.7 | M | 8.2 | M5 | *PTPN11* | BFM87 | yes | yes | yes | yes | leukemia |
| 7 | del ex1-8 | NA | 45,XY,-7 | 9.6 | M | 66.0 | M2 | *CEBPA*dm, *NRAS*, *IDH2* | BFM98 | yes | no | no | no | – |
| 8 | del ex1-8 | NA | 45,XX,-7[21] | 14.1 | F | 4.4 | M4 | *NRAS* | ELAM 2001 | yes | yes | yes | yes | toxicity |
| 9 | del ex1-8 | del 7 | 46, XY | 10.6 | M | 22.0 | M2 | *RUNX1/RUNX1T*, *CKIT* | BFM98 | yes | no | no | no | – |
| 10 | del ex1-4 | del 7 p12.2 | 46, XY, t(6;11)(q27;q23)[15] | 2.3 | M | 29.0 | M4 | None | BFM04 | yes | no | no | yes | – |
| 11 | NA | del 7 | NA | 9.1 | F | 4.4 | M5 | *PTPN11* | BFM98 | yes | yes | yes | yes | |

*del: deletion; WBC: white blood cell count; FAB: French-American-British classification; CR: complete remission; SCT: stem-cell transplantation; COD: cause of death; NA: not available. *Aberrations screened are AML associated fusions and hotspot areas in NRAS, KRAS, KIT, WT1, CEBPA, FLT3, and PTPN11.*

from which no material was available for MLPA analysis. Mutational screening of exons 4, 5 and 8 of *IKZF1* was performed on the same cohort of 258 patients. This revealed no patient harboring a frameshift mutation, which has been described as damaging in BCP-ALL. We did identify 3 patients with a heterozygous point mutation, p.V382M, which was predicted as tolerated by SIFT analysis and as benign by PoyPhen analysis, and p.G158S and p.L188P, which were predicted as deleterious by SIFT analysis and probably damaging by PolyPhen analysis, but not resulting in a truncated protein. Seventy-seven of 258 patients presented with synonymous SNP rs61731355, 17/258 with synonymous SNP rs61731356, and 1/258 with non-synonymous SNP rs376657964 (p.T333A), which is in line with the presence of these SNPs in the normal population (21%, 4% and 1%, respectively).[33]

In total, 10 patients carried a heterozygous *IKZF1* deletion as identified by MLPA, and one additional case as identified by array-CGH (Table 1). As determined by MLPA, of 258 patients, 9 cases showed an *IKZF1* deletion in all 8 exons, and one case showed a deletion of exon 1-4 with a normal peak ratio in the remaining exons (Figure 1). Four of these 10 cases showed a monosomy 7 with conventional karyotyping (Table 1). Using array CGH on 7 patients, we identified 2 cases with monosomy 7 in patients with an unknown karyotype, 2 patients with monosomy 7 of whom the karyotype was (wrongly) defined as cytogenetically normal, and 3 cases with a focal 0.1-0.9Mb deletion of 7p12.2, where the *IKZF1* gene is located (Table 1 and Figure 2).

Combining the data gathered from MLPA, karyotype and array CGH, we defined 11 patients with an *IKZF1* deletion, 8 of which showed complete loss of one copy of chromosome 7 (monosomy 7), and 3 of which showed a focal *IKZF1* deletion. As shown in Figure 2, patient #10 demonstrated a focal deletion (~0.1Mb) including exon 1-4 of *IKZF1*. The focal deletion in patient #2 included the *IKZF1* gene and part of the C7orf72 gene (~0.3Mb), whereas patient #3 showed a focal deletion (~0.9Mb) including the *IKZF1* gene and the genes for *VWC2, ZPBP, C7orf72* and *DDC*. Together, these results map the deletions in these patients to the *IKZF1* gene locus as the common deleted region.

### *Characteristics of* IKZF1 *deleted cases in pediatric AML*

*IKZF1* deleted cases did not differ significantly from the other pediatric AML cases in terms of age at diagnosis [median age 9.1 years (range 0.7-14.1) *vs.* 9.5 years (range 0.1-18.5) respectively, *P*=0.41]; nor in terms of sex (55% female *vs.* 42% female; *P*=0.41), and white blood cell count at diagnosis (median 30.2x10$^9$/L *vs.* 47.5 x10$^9$/L; *P*=0.24) (*Online Supplementary Table S2*). All AML patients were treated with intensive collaborative group cytarabine-anthracycline-based pediatric AML treatment protocols. There was no significance difference in clinical outcomes between *IKZF1* focal deleted, monosomy 7 and other AML cases. The 3-year pOS in *IKZF1* deleted cases (n=11) was 70%±14% versus 63%±3% (*P*=0.82) in cases with 2 *IKZF1* alleles (n=231); the 3-year pEFS was 36%±15% *versus* 46%±3% (*P*=0.87), and 3-year pCIR was 64%±16% versus 36±3% (*P*=0.09), respectively (*Online Supplementary Figure S1*).

No specific morphology subtypes based on the French–American–British (FAB) classification were related to *IKZF1* deletions. *IKZF1*-deleted samples showed none or various additional somatic mutations, most frequently

activating the RAS pathway with mutations in *NRAS* or *PTPN11* (n=4) (Table 1). As in BCP-ALL, *IKZF1* deletions are associated with *BCR-ABL1* fusions. Therefore, in addition we screened the *IKZF1*-deleted cases for the *BCR/ABL1* fusion and all were negative.[12]

### IKZF1 *haploinsufficient AML cells display an AML-specific gene expression signature*

Original gene expression data are available in the Gene Expression Omnibus repository (*http://www.ncbi.nlm.nih.gov/geo;accession GSE17855*). Focal IKZF1 deletions are also found in mixed phenotype acute leukemia.[34] To ensure the included cases were AML patients and not patients with a mixed ALL and AML phenotype, we used a previously defined AML *versus* ALL classifier.[35] Patients with focal *IKZF1* deletions (dark green triangles) and patients with monosomy 7 (light green squares) clustered amongst the other AML patients (red circles) and away from the ALL patients (blue circles), based on the expression signatures of the AML-specific genes (Figure 3A), the ALL-specific genes (Figure 3B), and the combined AML/ALL- specific genes (Figure 3C). This indicates that the *IKZF1* haploinsufficient AML cells of included patients indeed displayed pure AML cell gene expression profiles. Only of one of the 3 patients with a focal *IKZF1* deletion immunophenotypical data was available (patient #2); this patient was classified as *PML/RARA* negative AML FAB M3V ($TdT^-$/$MPO^+$/cyCD79a$^-$/$CD34^+$/$HLADR^+$/$CD19^-$/$CD10^-$/$CD7^+$/$CD13^+$/$CD33^+$).

### *Similarity between gene expression profiles of monosomy 7 and focal* IKZF1*-deleted cases*

Loss of IKZF1 function, a well-known tumor-inducing event in ALL, is conceivably one of the reasons for the recurrent heterozygous loss of chromosome 7, as this would include losing the *IKZF1* locus on 7p12.2. We hypothesized that, if loss of *IKZF1* were to be one of the driving leukemic events in monosomy 7, the gene expression signature of monosomy 7 AML cells may resemble those that only have a focal *IKZF1* deletion. However, we anticipated that this similarity might be quite weak because of the heterogeneity across the samples in our cohort, both derived from somatic and germ-line genetic variation and the small sample size of monosomy 7 and *IKZF1*-deleted cases. Therefore, we performed genome-wide differential gene expression analyses using a robust Bayesian method implemented in the R-package ShrinkBayes.[31] Probe sets were limited to those targeting unique genes or having a selectivity score of more than 0.8 (*www.geneannot.com*). Further statistical analyses were performed in R v. 3.0.0. (R scripts are available upon request.) We first compared gene expression profiles of monosomy 7 samples (n=8) to those of *IKZF1*-non-deleted samples (n=247). We detected 244 probe sets representing 198 genes differently expressed (BFDR<0.05) between these two groups (Figure 4A). As might be expected, a large proportion (44%) of these genes are located on chromosome 7, and are down-regulated (Figure 4B and *Online Supplementary Table S3*).

Next, we compared the mean differences in expression of these 198 genes in monosomy 7 samples (n=8) to those in samples with *IKZF1* focal deletions (n=3) (Figure 4C). For non-chromosome 7 genes, highly significant positive correlation was observed in gene expression differences

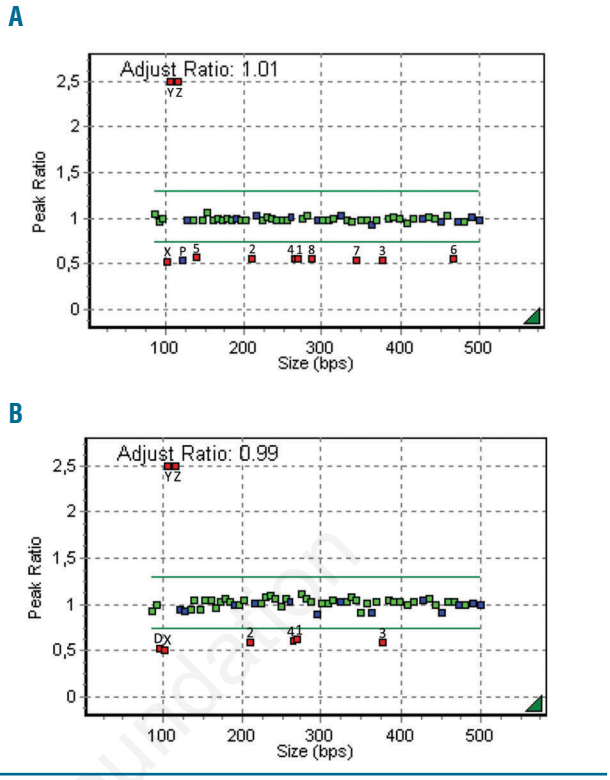


**Figure 1. Heterozygous IKZF1 deletions in pediatric AML. (A) Multiplex ligation-dependent probe amplification results of a patient with heterozygous deletion of exons 1-8 of *IKZF1* including the reference probe in *POR* located on 7q11.23. (B) MLPA results of a patient with heterozygous deletion of exons 1 through 4 of *IKZF1*. 1-8 indicates *IKZF1* exons 1-8; D: reference D-fragment 96, a control probe for denaturation during the procedure; P: POR (7q11.23); X: X-chromosome specific fragment, a reference probe for gender; Y, Y-chromosome specific fragment, a reference probe for gender; Z: *ZFY* (Yp11.31), a reference probe for gender.**

(r=0.50, *P*=3e-10). A less significant positive correlation (r=0.33, *P*=5e-4) was observed for chromosome 7 genes (Figure 4B). To further test that the correlation of gene expression changes between monosomy 7 and focal *IKZF1* deletions is not based on chance, we performed a bootstrap analyses taking 3 random patients and comparing them to the 8 patients with monosomy 7. None of 100,000 comparisons displays a correlation approaching the value of 0.5 found for the patients with focal *IKZF1* deletions (Figure 4D).

We next examined which non-chromosome 7 genes were responsible for the similarity between monosomy 7 and focal *IKZF1* deletion gene expression profiles. Genes that substantially contribute to the correlation between monosomy 7 and *IKZF1*-only deleted gene expression differences are up-regulated in both groups (Figure 4C). Interestingly, the gene most clearly up-regulated in both monosomy 7 and *IKZF1*-focal deleted samples was Hemogen (*HEMGN*), the overexpression of which has been described to induce expansion of myeloid progenitor cells in a murine model.[36] Other genes with known pro-leukemic functions in the top-correlating list are Four and a half LIM domains 2 (*FHL2*), Frizzled 6 (*FZD6*) and SET binding protein 1 (*SETBP1*) (Figure 4E). Very strong correlations (r=0.9117-0.9562, *P*<0.0001) were found between the GEP data and qRT-PCR analyses for *HEMGN* and *FHL2*, and strong correlations (r=0.7913-0.7962, *P*<0.0001)

were found for SETBP1 and FZD6 (n=30) (*Online Supplementary Figure S2*).

As upregulation of *HEMGN* in *IKZF1* hemizygous cells is relative to a fairly heterogeneous group, we stratified the control group in various more homogenous cytogenetic AML subgroups (Figure 5A). Median expression of *HEMGN* in each of those subgroups is clearly lower than in the *IKZF1*-deleted group. The same is true for *FHL2, FZD6* and *SETBP1* (Figure 5B-D), although it should be noted that *FZD6* and *SETBP1* are also highly expressed in patients with *MNX1-ETV6* (t(7;12)). When excluding the patient harboring both an *IKZF1* deletion and *MNX1-ETV6* (t(7;12)), *FZD6* and *SETBP1* remain significantly more highly expressed in the *IKZF1*-deleted cases compared to the other pediatric AML subgroups (Figure 5E and F). These data indicate that upregulation of these genes in *IKZF1*-deleted samples is not confounded by specific cytogenetic aberrations in pediatric AML.

### *Gene expression profiles of the* IKZF1*-deleted samples demonstrate enrichment in up-regulated GATA targets*

*HEMGN*, the gene most clearly up-regulated in both monosomy 7 and *IKFZ1*-focal deleted samples, is transcriptionally activated by GATA1 in the myeloid cell line K562.[37] We, therefore, investigated whether other genes that demonstrated a correlation between monosomy 7

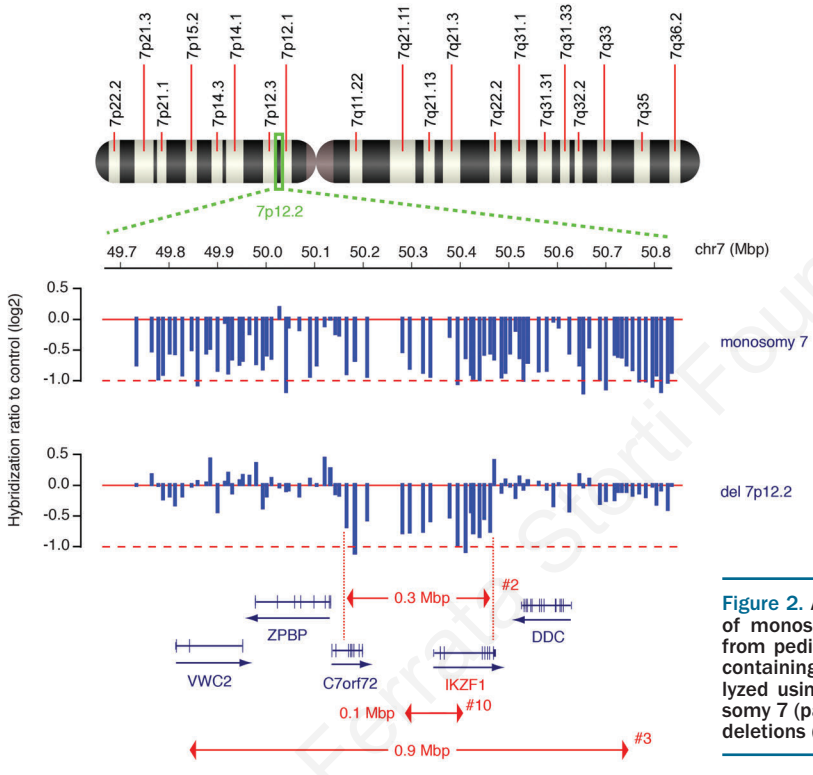


**Figure 2.** Array comparative genomic hybridization analysis of monosomy 7 and focal *IKZF1* deletions. DNA isolated from pediatric AML samples taken at initial diagnosis and containing *IKZF1* deletion based on MLPA results were analyzed using array CGH. Representative samples for monosomy 7 (patient #4, upper panel) and IKZF1 focal del7p12.2 deletions (patient #2, #3 and #10, lower panel) are shown.

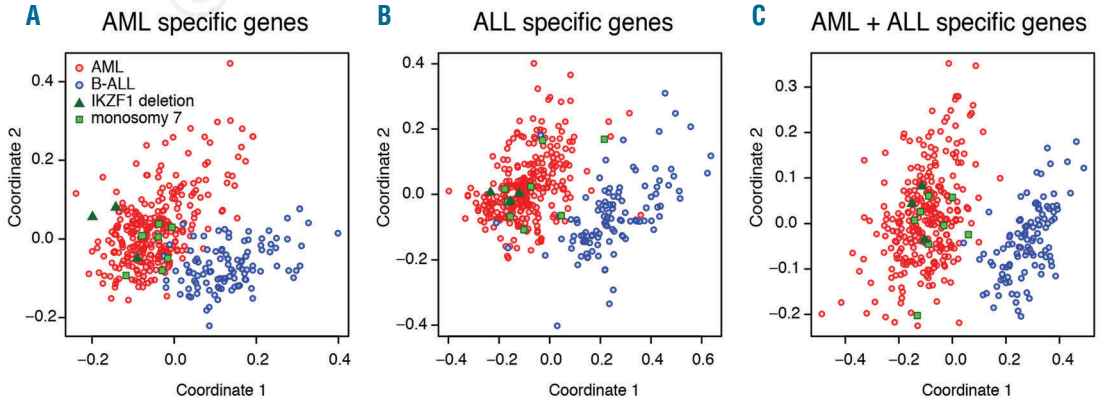


**Figure 3.** *IKZF1* haploinsufficient acute myeloid leukemia (AML) cells display an AML-specific gene expression signature. AML (n=297, red) and B-cell acute lymphoblastic leukemia (B-ALL) (n=107, blue) patients were projected onto the first two principal coordinates, obtained via multidimensional scaling of expression values of AML specific genes (A), ALL specific (B) genes and the combination of these (C) (*see* Thomas *et al.*[35]). Focally deleted *IKZF1* (dark green triangles) and monosomy 7 (light green squares) AMLs were located among other AMLs and away from B-ALLs.

and focally deleted *IKFZ1* samples are also GATA1 targets in K562. For this purpose, we used a GATA1 ChIP seq dataset in K562 cells.[38] From the 23 genes significantly up-regulated in the *IKZF1*-deleted samples, 11 genes had a GATA1 peak ratio larger than 25, indicating the gene being a GATA1 target (Figure 6A). Further analysis showed that whereas 13% of genes on our expression platform is a GATA1 target, 55% of genes that are up-regulated in IKZF1-deleted cases are GATA1 targets (*P*=3.5 e-7) (Figure 6B). While GATA transcription factors exhibit diverse functions in cellular proliferation, differentiation and gene regulation, only GATA1, GATA2, and GATA3 are expressed in developing blood cells and are critical for hematopoiesis.[39,40] GATA1 (*P*=0.07) and GATA3 (*P*=0.006) were up-regulated in the *IKZF1*-deleted samples (n=11) as compared to the rest (n=247) (Figure 6C). Direct interaction of IKZF1 with GATA proteins has been previously demonstrated.[41] Together, these data emphasize the similarity in gene expression between monosomy 7 and focal *IKZF1*-deleted pediatric AML patients.

## Discussion

In the last ten years, *IKZF1* has been widely studied in the context of B-cell differentiation and acute lymphoblastic leukemia, both in adults and children. There is growing evidence to indicate that IKZF1 also plays a role in various stages of myeloid differentiation. So far, the best indication that loss of *IKZF1* may contribute to myeloid leukemogenesis are deletions of the short arm of chromosome 7 associated with MPN-preceded secondary AML in adults, where the commonly deleted region is mapped to the *IKZF1* locus.[25] Our finding of recurrent focal deletions that map to the *IKZF1* gene locus in pediatric AML patients cells now provides additional support for a role for IKZF1 in myeloid leukemia.

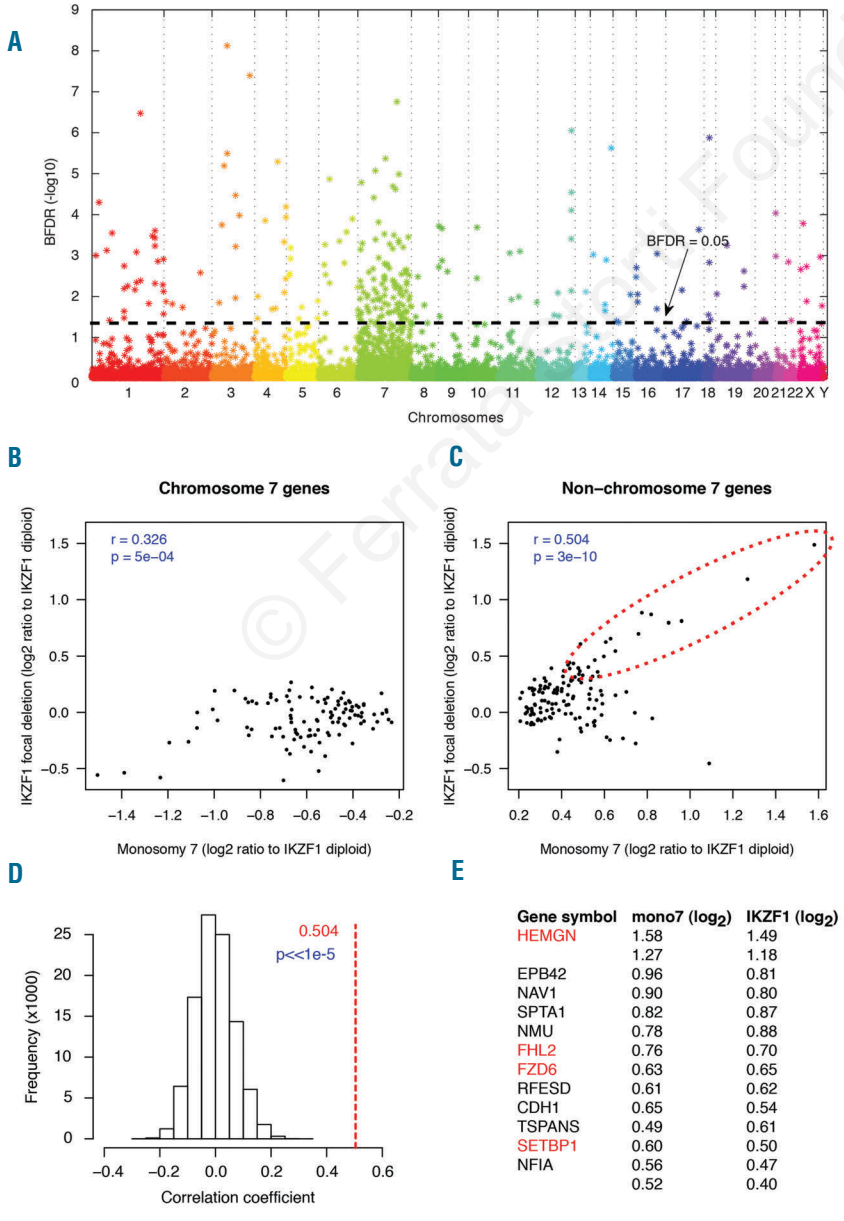


**Figure 4.** Monosomy 7 and focal *IKZF1* deletion associate with gene expression profiles with significant similarity. (A) Analysis of differences in gene expression between monosomy 7 samples (n=8) and *IKZF1*-non-deleted samples (n=247). Genes are ranked on Bayesian false discovery rate (BFDR) (y-axis) and binned in their chromosome of origin (x-axis). Using a BFDR of 5% (dashed line), a total of 198 genes are detected differentially expressed, of which 111 are not encoded on chromosome 7. (B and C) Mean differences per gene between monosomy 7 and non-*IKZF1*-deleted samples versus those of *IKZF1* focal deletions and non-*IKZF1*-deleted samples for chromosome 7 encoded genes (B) and non-chromosome 7 encoded genes (C). Pearson's correlation coefficients (r) are indicated in the plot, together with accompanying *P* values. (D) Bootstrap analyses of the correlation between monosomy 7 cases (n=8) and 3 randomly picked *IKZF1*-non-deleted samples (n=247). The red dashed line indicates correlation coefficient from the analyses displayed in Figure 4C. (E) Top correlating genes [dashed elipse in (C)] between monosomy 7 and focal *IKZF1* deletions. Highlighted in red are those reported to be positively acting on leukemic cell self-renewal or cell cycle.

Reduced *IKZF1* gene function is a well-known recurrent event in BCP-ALL. In pediatric BCP-ALL, the overall frequency of focal *IKZF1* deletions is approximately 15%, but is greatly enriched in *BCR/ABL1* positive BCP-ALL at 70%-80%.[15,42] Here we report that focal *IKZF1* deletions are also a recurrent event in pediatric AML, although with much lower frequency. In a cohort of 258 patients, representing all cytogenetic AML subgroups, we found 3 cases of a focal inactivating deletion of this gene (1.2%). In addition, 3 patients were identified with a single nucleotide variation leading to amino acid changes. In contrast to mutations found in BCP-ALL, no frame-shift mutations were detected, and the functional status of these SNV containing alleles remain to be determined.

In adult and pediatric BCP-ALL, in addition to focal *IKZF1* deletions, monosomy 7 is a recurrent chromosomal aberration. In *BCR/ABL1* positive BCP-ALLs, 16% (adult) and 13% (pediatric) of the cases of *IKZF1* deletion can be attributed to monosomy 7.[14,42] In pediatric AML, monosomy 7 is a recurrent chromosome loss, in particular in myeloproliferative diseases, such as juvenile myelomonocytic leukemia that have progressed towards AML (40% of cases), but also in primary AML (4%-5% of cases).[23,43] In our pediatric *de novo* AML cohort, we found 8 cases (3.1%) of monosomy 7, consistent with earlier estimates. Across these monosomy 7 AML cases, we find various additional somatic mutations, most prominently *PTPN11* (n=2) and *NRAS* mutations (n=2), adding up to half of the cases presenting with an activated RAS pathway.

We hypothesized that, in pediatric AML, as presumed in BCP-ALL, an important biological determinant for leukemogenesis in monosomy 7 patients is loss of the *IKZF1* gene.[44] Evidence for this hypothesis was obtained by comparing gene expression profiles of monosomy 7 and focal *IKZF1* deletions. We found a statistically significant correlation on non-chromosome 7 genes. We attribute the changes in chromosome 7 genes to bystander effects of deletion of the entire chromosome 7, leading to a widespread reduction in chromosome 7 encoded genes, many of which can be assumed to no impact on the leukemogenic process. In contrast, the effects on non-chromosome 7 genes can be expected to be more enriched for leukemia-related changes in the genetic program due to loss of one or more chromosome 7 encoded driving oncogenic mutations. Therefore, the similarity of gene expression signatures between AML cases with monosomy 7 and those with a focal *IKZF1* deletion suggests that *IKZF1* is one of the driving oncogenic events in monosomy 7 pediatric AML. Further support for this hypothesis is the observation that pediatric AML patients harboring monosomy 7 have a worse disease outcome compared to those harboring 7q deletions.[23] Our findings may also explain the prevalence of monosomy 7 and focal *IKZF1* deletions in mixed phenotype acute leukemia, as loss of *IKZF1* now appears to be able to contribute to both acute lymphoblastic and myeloid leukemogenesis.[34]

Several genes up-regulated in both monosomy 7 and focal *IKZF1*-deleted AML cases are previously implicated in leukemogenesis. In a large cohort of adult BCP-ALL, comparison between focal *IKZF1* deletions and *IKZF1*

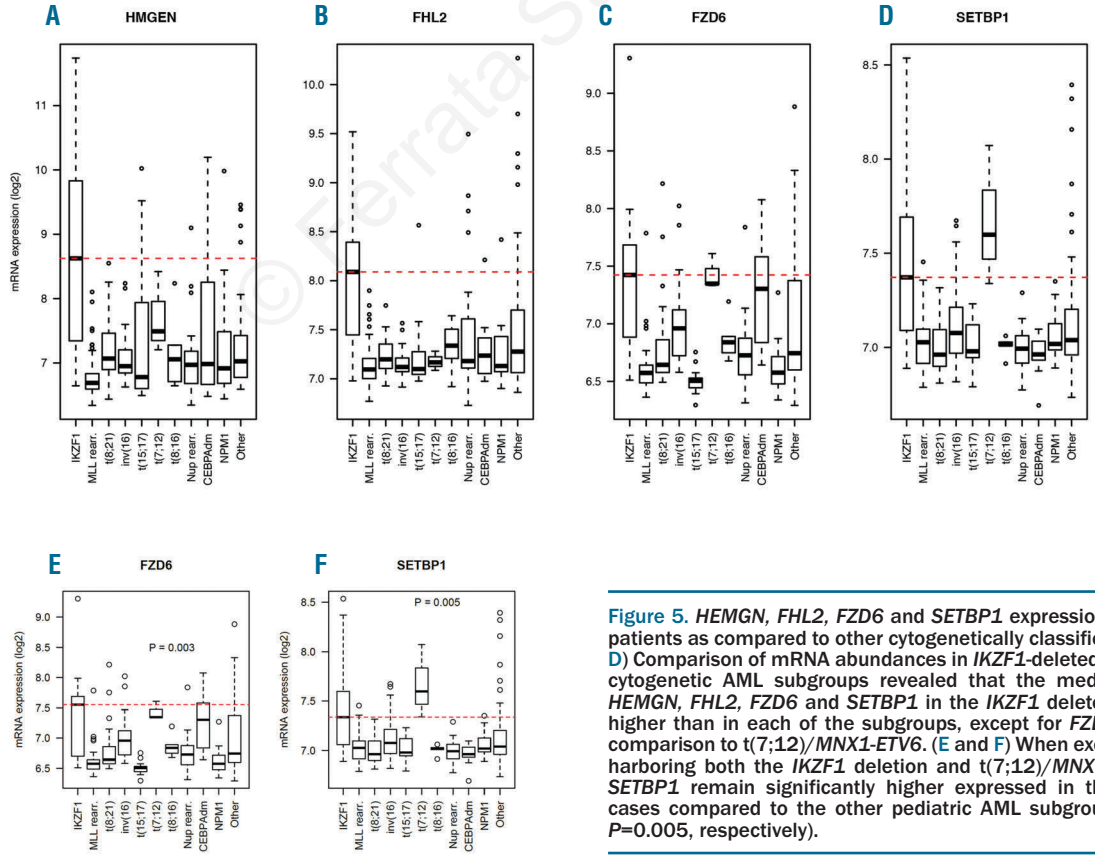


**Figure 5.** ***HEMGN, FHL2, FZD6*** **and** ***SETBP1*** **expression in** ***IKZF1*****-deleted patients as compared to other cytogenetically classified AML groups. (A-D) Comparison of mRNA abundances in** ***IKZF1*****-deleted samples to major cytogenetic AML subgroups revealed that the median expression of** ***HEMGN, FHL2, FZD6*** **and** ***SETBP1*** **in the** ***IKZF1*** **deleted group is clearly higher than in each of the subgroups, except for** ***FZD6*** **and** ***SETBP1*** **in comparison to t(7;12)/*MNX1-ETV6*. (E and F) When excluding the patient harboring both the** ***IKZF1*** **deletion and t(7;12)/*MNX1-ETV6*, *FZD6* and *SETBP1* remain significantly higher expressed in the *IKZF1* -deleted cases compared to the other pediatric AML subgroups (*P*=0.003 and *P*=0.005, respectively).**

**A**

| Gene symbol | GATA1 peak value |
|---|---|
| HEMGN | 83 |
| EPB42 | 40 |
| NAV1 | ≤25 |
| SPTA1 | ≤25 |
| NMU | ≤25 |
| FHL2 | 39 |
| FZD6 | ≤25 |
| RFESD | 48 |
| CDH1 | 36 |
| TSPAN5 | ≤25 |
| SETBP1 | ≤25 |
| NFIA | 34 |
| SLC2A1 | 113 |
| HBBP1 | ≤25 |
| KANK2 | ≤25 |
| OPTN | ≤25 |
| MOSPD1 | ≤25 |
| RHCE | 28 |
| C6orf192 | 33 |
| ANK1 | 75 |
| MKI67 | ≤25 |
| YPEL4 | 75 |
| TSPO2 | ≤25 |

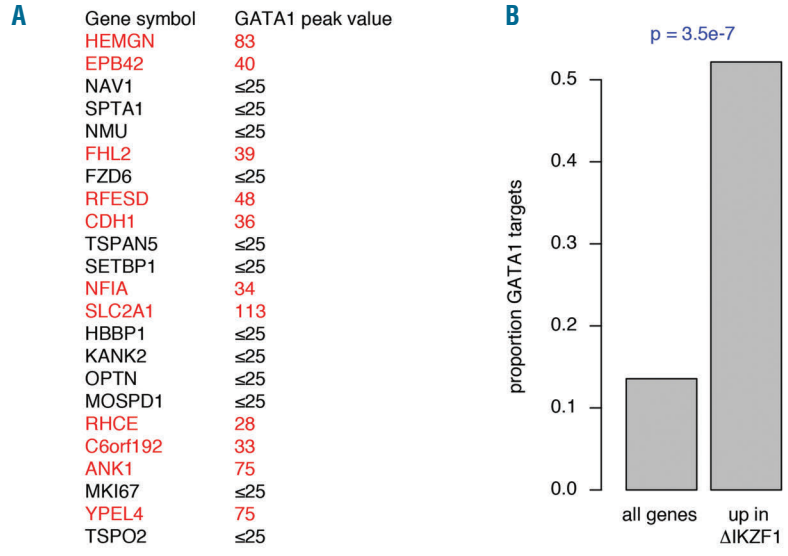


**Figure 6.** Genes commonly up-regulated in *IKZF1* focally deleted and monosomy 7 cases are enriched in myeloid GATA1 targets. (A) GATA1 chromatin interaction values (*GATA1* peak value) in K562 cells of commonly up-regulated genes (n=23) in monosomy 7 and focal *IKZF1* deletion (Δ*IKZF1*). (B) Proportion of all matching genes (n=22564) or Δ*IKZF1* up-regulated (n=23) displaying a GATA1 chromatin interaction in K562 cells. *P* value from Fisher's exact test. (C) mRNA expression of GATA1, GATA2 and GATA3 in IKZF1 deleted samples (red) *versus IKZF1* WT samples (gray). *P* values according to Mann-Whitney tests.



wild-type cases revealed upregulation of genes involved in the cell cycle, JAK-STAT signaling and stem cell self-renewal.[45] We find upregulation of *HEMGN*, (a transcription factor implicated in myeloid self-renewal[36]), *FHL2* (enhanced expression of which leads to enhanced cell cycle entry), *FZD6* (shown to be involved in the pathologically reactivation of the Wnt/Fzd-mediated self-renewal signals that are enlisted during B-cell development and may be pathologically reactivated in the neoplastic transformation of mature B cells), and *SETBP1* (overexpression of which promotes the self-renewal of myeloid progenitors[46-48]). It is interesting that similar cellular processes are affected in the *IKZF1* deleted cases, while none of the genes deregulated in *IKZF1* deleted adult BCP-ALL are found deregulated in this pediatric *IKZF1* deleted AML cohort.[45]

It is worthy of note that we find a strong enrichment of myeloid GATA1 targets among genes commonly up-regulated in focal *IKZF1* deleted AML cases and monosomy 7 AML cases. We interpret this as a confirmation that the genes commonly up-regulated in *IKZF1* focal deletions and monosomy 7 did not present themselves by chance. However, it is tempting to speculate that *IKZF1* haploinsufficiency in pediatric AML is influencing GATA transcription factor function. Supporting this idea, *IKZF1* overexpression in transgenic mice represses GATA1 expression, implying that loss of IKZF1 function may increase GATA1 function.[21]

Taken together, we find evidence for *IKZF1* as a tumor suppressor gene in pediatric acute myeloid leukemia and suggest that *IKZF1* deletion might be one of the driving events of monosomy 7 in various myeloid diseases.

### *Funding*

*JdR was funded by Kinder Oncologisch Centrum Rotterdam (KOCR). AO was funded by KIKA project #109. MF was funded by KWF project EMCR 2012-5546. The authors declare no conflict of interest.*

### *Authorship and Disclosures*

*Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.*

## References


1. Pui CH, Carroll WL, Meshinchi S, Arceci RJ. Biology, risk stratification, and therapy of pediatric acute leukemias: an update. J Clin Oncol. 2011;29(5):551-565.
2. Shih AH, Abdel-Wahab O, Patel JP, Levine RL. The role of mutations in epigenetic regulators in myeloid malignancies. Nat Rev Cancer. 2012;12(9):599-612.
3. Creutzig U, van den Heuvel-Eibrink MM, Gibson B, et al. Diagnosis and management of acute myeloid leukemia in children and adolescents: recommendations from an international expert panel. Blood. 2012;120(16):3187-3205.
4. Kaspers GJ, Zwaan CM. Pediatric acute myeloid leukemia: towards high-quality cure of all patients. Haematologica. 2007;92(11):1519-1532.
5. Oestreich KJ, Weinmann AS. Ikaros changes the face of NuRD remodeling. Nat Immunol. 2012;13(1):16-18.
6. Molnar A, Georgopoulos K. The Ikaros gene encodes a family of functionally diverse zinc finger DNA-binding proteins. Mol Cell Biol. 1994;14(12):8292-8303.
7. Sun L, Liu A, Georgopoulos K. Zinc finger-mediated protein interactions modulate Ikaros activity, a molecular control of lymphocyte development. EMBO J. 1996;15(19):5358-5369.
8. Georgopoulos K, Bigby M, Wang JH, et al. The Ikaros gene is required for the development of all lymphoid lineages. Cell. 1994;79(1):143-156.
9. Wang JH, Nichogiannopoulou A, Wu L, et al. Selective defects in the development of the fetal and adult lymphoid system in mice with an Ikaros null mutation. Immunity. 1996;5(6):537-549.
10. Winandy S, Wu P, Georgopoulos K. A dominant mutation in the Ikaros gene leads to rapid development of leukemia and lymphoma. Cell. 1995;83(2):289-299.
11. Mullighan CG, Goorha S, Radtke I, et al. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. Nature. 2007;446(7137):758-764.
12. Mullighan CG, Miller CB, Radtke I, et al. BCR-ABL1 lymphoblastic leukaemia is characterized by the deletion of Ikaros. Nature. 2008;453(7191):110-114.
13. Mullighan CG, Su X, Zhang J, et al. Deletion of IKZF1 and prognosis in acute lymphoblastic leukemia. N Engl J Med. 2009;360(5):470-480.
14. Martinelli G, Iacobucci I, Storlazzi CT, et al. IKZF1 (Ikaros) deletions in BCR-ABL1-positive acute lymphoblastic leukemia are associated with short disease-free survival and high rate of cumulative incidence of relapse: a GIMEMA AL WP report. J Clin Oncol. 2009;27(31):5202-5207.
15. van der Veer A, Zaliova M, Mottadelli F, et al. IKZF1 status as a prognostic feature in BCR-ABL1-positive childhood ALL. Blood. 2014;123(11):1691-1698.
16. Ruiz A, Williams O, Brady HJ. The Ikaros splice isoform, Ikaros 6, immortalizes murine haematopoietic progenitor cells. Int J Cancer. 2008;123(6):1240-1245.
17. Dijon M, Bardin F, Murati A, Batoz M, Chabannon C, Tonnelle C. The role of Ikaros in human erythroid differentiation. Blood. 2008;111(3):1138-1146.
18. Lopez RA, Schoetz S, DeAngelis K, O'Neill D, Bank A. Multiple hematopoietic defects and delayed globin switching in Ikaros null mice. Proc Natl Acad Sci USA. 2002;99(2):602-607.
19. Papathanasiou P, Perkins AC, Cobb BS, et al. Widespread failure of hematolymphoid differentiation caused by a recessive niche-filling allele of the Ikaros transcription factor. Immunity. 2003;19(1):131-144.
20. Malinge S, Thiollier C, Chlon TM, et al. Ikaros inhibits megakaryopoiesis through functional interaction with GATA-1 and NOTCH signaling. Blood. 2013;121(13):2440-2451.
21. Rao KN, Smuda C, Gregory GD, Min B, Brown MA. Ikaros limits basophil development by suppressing C/EBP-alpha expression. Blood. 2013;122(15):2572-2581.
22. Dumortier A, Kirstetter P, Kastner P, Chan S. Ikaros regulates neutrophil differentiation. Blood. 2003;101(6):2219-2226.
23. Hasle H, Alonzo TA, Auvrignon A, et al. Monosomy 7 and deletion 7q in children and adolescents with acute myeloid leukemia: an international retrospective study. Blood. 2007;109(11):4641-4647.
24. Breems DA, Van Putten WL, De Greef GE, et al. Monosomal karyotype in acute myeloid leukemia: a better indicator of poor prognosis than a complex karyotype. J Clin Oncol. 2008;26(29):4791-4797.
25. Jager R, Gisslinger H, Passamonti F, et al. Deletions of the transcription factor Ikaros in myeloproliferative neoplasms. Leukemia. 2010;24(7):1290-1298.
26. Klampfl T, Harutyunyan A, Berg T, et al. Genome integrity of myeloproliferative neoplasms in chronic phase and during disease progression. Blood. 2011;118(1):167-176.
27. Milosevic JD, Puda A, Malcovati L, et al. Clinical significance of genetic aberrations in secondary acute myeloid leukemia. Am J Hematol. 2012;87(11):1010-1016.
28. Lana T, de Lorenzo P, Bresolin S, et al. Refinement of IKZF1 status in pediatric philadelphia positive acute lymphoblastic leukemia. Leukemia. 2015;Mar 17.[Epub ahead of print].
29. Balgobind BV, Hollink IH, Arentsen-Peters ST, et al. Integrative analysis of type-I and type-II aberrations underscores the genetic heterogeneity of pediatric acute myeloid leukemia. Haematologica. 2011;96(10):1478-1487.
30. Hollink IH, van den Heuvel-Eibrink MM, Arentsen-Peters ST, et al. NUP98/NSD1 characterizes a novel poor prognostic group in acute myeloid leukemia with a distinct HOX gene expression pattern. Blood. 2011;118(13):3645-3656.
31. Van De Wiel MA, Leday GG, Pardo L, Rue H, Van Der Vaart AW, Van Wieringen WN. Bayesian analysis of RNA sequencing data by estimating multiple shrinkage priors. Biostatistics. 2013;14(1):113-128.
32. Pui CH, Schrappe M, Ribeiro RC, Niemeyer CM. Childhood and adolescent lymphoid and myeloid leukemia. Hematology Am Soc Hematol Educ Program. 2004;118-145.
33. Flicek P, Amode MR, Barrell D, et al. Ensembl 2014. Nucleic Acids Res. 2014;42(Database issue):D749-755.
34. Yan L, Ping N, Zhu M, et al. Clinical, immunophenotypic, cytogenetic, and molecular genetic features in 117 adult patients with mixed-phenotype acute leukemia defined by WHO-2008 classification. Haematologica. 2012;97(11):1708-1712.
35. Thomas JG, Olson JM, Tapscott SJ, Zhao LP. An efficient and robust statistical modeling approach to discover differentially expressed genes using genomic expression profiles. Genome Res. 2001;11(7):1227-1236.
36. Jiang J, Yu H, Shou Y, et al. Hemgn is a direct transcriptional target of HOXB4 and induces expansion of murine myeloid progenitor cells. Blood. 2010;116(5):711-719.
37. Yang LV, Wan J, Ge Y, et al. The GATA site-dependent hemogen promoter is transcriptionally regulated by GATA1 in hematopoietic and leukemia cells. Leukemia. 2006;20(3):417-425.
38. Fujiwara T, O'Geen H, Keles S, et al. Discovering hematopoietic mechanisms through genome-wide analysis of GATA factor chromatin occupancy. Mol Cell. 2009;36(4):667-681.
39. Patient RK, McGhee JD. The GATA family (vertebrates and invertebrates). Curr Opin Genet Dev. 2002;12(4):416-422.
40. Orkin SH. Embryonic stem cells and transgenic mice in the study of hematopoiesis. Int J Dev Biol. 1998;42(7):927-934.
41. Bottardi S, Mavoungou L, Bourgoin V, Mashtalir N, Affar el B, Milot E. Direct protein interactions are responsible for Ikaros-GATA and Ikaros-Cdk9 cooperativeness in hematopoietic cells. Mol Cell Biol. 2013;33(16):3064-3076.
42. Iacobucci I, Storlazzi CT, Cilloni D, et al. Identification and molecular characterization of recurrent genomic deletions on 7p12 in the IKZF1 gene in a large cohort of BCR-ABL1-positive acute lymphoblastic leukemia patients: on behalf of Gruppo Italiano Malattie Ematologiche dell'Adulto Acute Leukemia Working Party (GIMEMA AL WP). Blood. 2009;114(10):2159-2167.
43. Hasle H, Arico M, Basso G, et al. Myelodysplastic syndrome, juvenile myelomonocytic leukemia, and acute myeloid leukemia associated with complete or partial monosomy 7. European Working Group on MDS in Childhood (EWOG-MDS). Leukemia. 1999;13(3):376-385.
44. Tirado CA, Valdez F, Klesse L, et al. Acute myeloid leukemia with inv(16) with CBFB-MYH11, 3'CBFB deletion, variant t(9;22) with BCR-ABL1, and del(7)(q22q32) in a pediatric patient: case report and literature review. Cancer Genet Cytogenet. 2010;200(1):54-59.
45. Iacobucci I, Iraci N, Messina M, et al. IKAROS deletions dictate a unique gene expression signature in patients with adult B-cell acute lymphoblastic leukemia. PLoS One. 2012;7(7):e40934.
46. Qian Z, Mao L, Fernald AA, et al. Enhanced expression of FHL2 leads to abnormal myelopoiesis in vivo. Leukemia. 2009;23(9):1650-1657.
47. Wu QL, Zierold C, Ranheim EA. Dysregulation of Frizzled 6 is a critical component of B-cell leukemogenesis in a mouse model of chronic lymphocytic leukemia. Blood. 2009;113(13):3031-3039.
48. Oakley K, Han Y, Vishwakarma BA, et al. Setbp1 promotes the self-renewal of murine myeloid progenitors via activation of Hoxa9 and Hoxa10. Blood. 2012;119(25):6099-6108.