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Absence of Genomic *Ikaros/IKZF1* Deletions in Pediatric B-Precursor Acute Lymphoblastic Leukemia

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Abstract

Here we report the results of gene expression analyses using multiple probesets aimed at determining the incidence of *Ikaros/IKZF1* deletions in pediatric B-precursor acute lymphoblastic leukemia (BPL). Primary leukemia cells from 122 Philadelphia chromosome (Ph)⁺ BPL patients and 237 Ph⁻ BPL patients as well as normal hematopoietic cells from 74 normal non-leukemic bone marrow specimens were organized according to expression levels of *IKZF1* transcripts utilizing two-way hierarchical clustering technique to identify specimens with low *IKZF1* expression for the 10 probesets interrogating Exons 1 through 4 and Exon 8. Our analysis demonstrated no changes in expression that would be expected from homozygous or heterozygous deletions of *IKZF1* in primary leukemic cells. Similar results were obtained in gene expression analysis of primary leukemic cells from 20 Ph⁺ positive and 155 Ph⁻ BPL patients in a validation dataset. Taken together, our gene expression analyses in 534 pediatric BPL cases, including 142 cases with Ph⁺ BPL, contradict previous reports that were based on SNP array data and suggested that Ph⁺ pediatric BPL is characterized by a high frequency of homozygous or heterozygous *IKZF1* deletions. Further, exon-specific genomic PCR analysis of primary leukemia cells from 21 high-risk pediatric BPL patients and 11 standard-risk pediatric BPL patients, and 8 patients with infant BPL did not show any evidence for homozygous *IKZF1* locus deletions. Nor was there any evidence for homozygous or heterozygous intragenic *IKZF1* deletions.

Introduction

Ikaros (IK) is a zinc finger (ZF)-containing sequence-specific DNA-binding protein encoded by the *IKZF1* gene. It plays a pivotal role in immune homeostasis through transcriptional regulation of the earliest stages of lymphocyte ontogeny and differentiation by both (a) gene transcriptional activation via efficient transcription initiation and elongation as well as (b) repression.^{1,2} In recent years, there have been a series of contradictory reports based on

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Author Contributions

F.M.U conceived and supervised this study and wrote the final manuscript. H.M and S.Q have equally contributed to this study. All authors contributed to the design of the experiments and performed research. All authors reviewed and revised the paper.

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studies that employed single-nucleotide polymorphism (SNP) arrays regarding the incidence and prognostic significance of *IKZF1* deletions in primary leukemic cells from pediatric patients with high-risk B-cell precursor ALL (BPL).³⁻⁹ Some of the initial reports have proposed that genomic *IKZF1* deletions (not alternative splicing) are the cause of expression of dominant-negative IK isoforms.^{3,4} Mullighan et al. reported deletions of *IKZF1* in 84% of Ph+ BPL, including 76% of pediatric and 91% of adult Ph+ BPL cases.³ The same authors also reported a >25% frequency of *IKZF1* deletions in Ph- high-risk BPL patients.⁴ In both studies, *IKZF1* deletions included homozygous/biallelic as well as heterozygous/monoallelic deletion of the entire gene locus as well as intragenic deletions.^{3,4} Subsequently, Volejnikova et al reported discordant results in 206 children with Ph⁻ ALL.⁷ Of 24 patients with overexpression of dominant-negative IK isoforms other than IK6, only one patient had a deletion within the *IKZF1* locus and only half of the IK6⁺ cases were found to have monoallelic *IKZF1* deletions.⁷ The overall incidence of *IKZF1* deletions was only 7% and no patient had homozygous *IKZF1* deletions and no patient had evidence of decreased IK protein expression even in the presence of monoallelic *IKZF1* deletions.⁷ Although Mullighan et al. reported *IKZF1* deletions as a significant predictor of poor outcome for high-risk BPL patients on Children's Oncology Group (COG) Study P9906⁴, a subsequent study by Chen et al. could not confirm the independent prognostic significance of *IKZF1* deletions for 499 high-risk BPL patients.⁵ In their most recent paper, Palmi et al. reported an elegant study, which raises further questions about the clinical significance of *IKZF1* deletions in pediatric BPL.⁹ They documented no homozygous *IKZF1* deletions and heterozygous *IKZF1* deletions were detected in only ~13% of their Ph- BPL patient population. In approximately half of the cases with deletions (7.1%), the deletion involved the entire *IKZF1* locus and in the other half a portion of the *IKZF1* gene.⁹ Most importantly, *IKZF1* deletions were not an independent prognostic factor of the hazard of relapse.⁹

The purpose of the present study was to gain further insights into the incidence of *IKZF1* deletions by using gene expression analysis using multiple probesets and exon-specific genomic PCR. Our analysis revealed no evidence for homozygous or heterozygous *IKZF1* deletions in Ph⁺ or Ph⁻ BPL and contradicts previous reports of *IKZF1* deletions in BPL that were based on SNP array data.

Materials and Methods

Cells

Cryopreserved leukemia cells from 21 children with newly diagnosed high risk B-precursor ALL, 11 children with newly diagnosed standard risk B-precursor ALL, 9 children with B-precursor ALL in first bone marrow relapse occurring within 12 months of the completion of primary therapy, and 8 infants (age <1 year) with newly diagnosed B-precursor ALL were examined for IK expression using multiple assay platforms. We also used ALL cells isolated from spleen specimens of xenografted NOD/SCID mice in the described experiments. The xenografts were established using primary cells from 7 pediatric B-precursor ALL patients. The IRB (CCI) at Children's Hospital Los Angeles (CHLA) (Human Subject Assurance Number: FWA0001914) determined that the use of leukemic cells in our research did not meet the definition of human subject research per 45 CFR 46.102 (d and f) since it does not include identifiable private information. The research was approved by the CHLA CCI. The IRB approved project numbers were CCI-09-00304 (CCI Review Date 12/21/2009, Approval Date: 12/29/09) for cryopreserved cells and CCI-10-00141 (CCI Review Date 7/27/2010, Approval Date 7/27/2010) for freshly obtained primary leukemia cells.

Genomic PCR Analysis of the *Ikaros/IKZF1* Gene in Leukemia Cells

DNA sequencing was carried out using a “primer-walking” strategy and the BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA), as previously reported.¹⁰ Total genomic DNA was extracted from patients’ leukemia cells using the Qiagen DNeasy Blood & Tissue kit (Catalog No. 6950) according to the manufacturer’s specifications. PCR products encompassing the *IKZF1* exons 4, 5, 6, 7 and their exon-intron junctions were amplified using previously reported genomic PCR primer sets.^{10,11} PCR products were separated on 1% agarose gels and sized using the 1-Kb Plus DNA ladder from Invitrogen (Cat. No. 10787-018). The PCR products were cleaned using the Qiagen QIAquick PCR Purification Kit (Cat No. 28104) and submitted to the DNA Sequencing Facility of Genewiz Inc (CA) using the corresponding forward primer and the Applied Biosystems’ dye-based (BigDye V3.1™) DNA sequencing method. DNA sequencing was performed on an ABI 3730 DNA Analyzer using a long read protocol. Sequence obtained from each genomic PCR product was analyzed and aligned using SeqMan II contiguous alignment software in the LaserGene suite from DNASTAR Inc. and the MegAlign multisequence alignment software in comparison with the wild-type *IKZF1* sequence (NCBI Reference Sequence: NCBI Reference Sequence: NT_007819.17 Homo sapiens chromosome 7, Genome Reference Consortium Human Build 37 (GRCh37.p9) primary reference assembly, www.ncbi.nih.gov).¹¹

RT-PCR Analysis of *IKZF1* Exons 5-7 for Detection of *IK6* Transcripts

Reverse transcription (RT) and polymerase chain reaction (PCR) were used according to the Qiagen OneStep RT-PCR (Qiagen, Cat# 210210) guidelines to amplify specific regions of the *IKZF1* transcript. Total cellular RNA was extracted from primary ALL cells or ALL cell lines using the Qiagen RNeasy Mini Kit (Cat No 74104) (Qiagen, Santa Clarita, CA). Total RNA (~ 100 ng/sample) was reversely transcribed and directly amplified in the same tube using 2 specific primer sets (viz. P1 and P2). The P1 primer set (Forward: *CCAATGTGCTCATGGTTCAC*, Reverse: *CTCTTACGGTTTGGCGACGTT*) was used to amplify a 442-bp region of the *IKZF1* mRNA spanning Exons E5-E7. The P2 primer set (Forward: *CCAATGTGCTCATGGTTCAC*, Reverse: *TAGCTTCGGCCACAATATCC*) was used to amplify a 244-bp region (c.2180–c.2361) of the *IKZF1* mRNA extending from E5 to E6. Both PCR products included ~1/8th of E4 and E4/E5 junction as well. The cycling conditions were: 30 min reverse-transcription reaction at 50°C immediately followed by 15 min initial PCR activation step, and 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 58°C, and 1 min extension at 72°C. PCR products were separated on a 1.2% agarose gel and visualized after ethidium bromide staining using a UVP Epi Chemi II Darkroom Transilluminator.

Bioinformatics and Statistical Analysis of Gene Expression Profiles

BLAT analysis on *IKZF1* target sequences deposited in Affymetrix NetAffx™ Analysis Center (<http://www.affymetrix.com/analysis/index.affx>) mapped these probesets onto specific *IKZF1* exons visualized using the UCSC genome browser (<http://genome.ucsc.edu/cgi-bin/hgBlat?command=start>). This analysis is designed to locate sequences of 95% and greater similarity of length 25 bases or more in the entire genome.¹³ The exon designation by comparing the BLAT analysis to 3 reference sequences (UCSC genes, Ensembl gene predictions, Human mRNA Genbank) were as follows: 1565817_at: Exon 1, chr7:50,348,436-50,348,502; 1565816_at: Exon 1-4 reverse strand, chr7:50,348,485-50,444,456; 1565818_s_at: Exon 4, chr7:50,444,230-50,444,480; 220704_at: Exon 3, chr7:50,368,140-50,368,398; 1557632_at: Exon 3, chr7:50,370,493-50,370,631; 216901_s_at: Exon 8, chr7:50,467,793-50,468,294; 205039_s_at: Exon 8, chr7:50,469,625-50,470,188; 205038_at: Exon 8,

chr7:50,471,237-50,471,785; 227346_at: Exon 8, chr7:50,471,780-50,472,114; 227344_at: Exon 8, chr7:50,472,198-50,472,761.

We compiled the archived “The Microarray Innovations in Leukemia” (MILE) study gene expression profiling (GEP) data on primary leukemic cells (GSE13159) from 122 pediatric BCR-ABL⁺ BPL patients with t(9;22) translocation (Ph⁺), 237 pediatric BCR-ABL⁻ BPL patients without t(9;22) translocation (Ph⁻) and 74 normal bone marrow specimens.¹³ Signal values for 10 *IKZF1* probesets (Human Genome U133 Plus 2.0 Array: 1565816_at, 1565817_at, 1565818_s_at, 205038_at, 205039_s_at, 216901_s_at, 220704_at, 227344_at, 227346_at, 1557632_at) obtained from hybridization onto the Affymetrix Human Genome U133 Plus 2.0 Arrays were calculated using non-central trimmed mean of differences between perfect match and mismatch intensities with quantile normalization (DQN3, signal normalized with quantiles of the beta distribution with parameters $p=1.2$ and $q=3$).¹⁴ Mean expression levels of the 50 least abundant probesets were used as a comparison for absence of signal in the *IKZF1* probesets.

As a validation set, we compiled the archived gene expression data on in primary leukemic cells from 155 pediatric Ph⁻ BPL patients and 20 Ph⁺ BPL patients on the Mullighan study (GSE12995).⁴ Transcript signal values were obtained from hybridization onto the Affymetrix Human Genome U133A genechip arrays. Trimmed mean target intensity of each array was globally scaled to 500 (MAS5 values) as the normalization method and log₁₀ transformed to homogenize within group variances. Mean expression levels of the 25 least abundant probesets were used as a comparison for absence of signal in the *IKZF1* probesets.

We performed a two-way hierarchical clustering technique to organize expression patterns (MAS5 values or DQN3 values) such that sample and *IKZF1* probesets having the similar expression profiles were grouped together using the average distance metric. The *IKZF1* probesets were arranged on the x-axis and samples were arranged along the y-axis such that similar expression patterns were placed adjacent to each other ordered by the cluster algorithm. The heat map represented the color coded expression values of probesets ranging from low levels (blue) to high levels (red). Dendrograms were formed using the clustering algorithm that finds similar expression pattern of a pair of expression values and joins them together, then clusters of expression values are joined together to form larger and larger clusters until all of the responses were joined into one giant cluster. The joining of the clusters was depicted by the branch structure that connects individual expression patterns with larger groups and color coded for major cluster groupings. Outlier boxplots of the expression levels for all the subgroups were utilized to compare differences in distribution of expression for each probeset across all sub-groups of samples (depicting median, 75th and 25th quartiles, outlier whiskers defined as 1.5 times outside the interquartile range and a red brackets defining the modal concentration of 50% of the expression values). This non-parametric method identifies specimen samples with low expression across all *IKZF1* probesets covering exons 1-8 and the absence of a probeset was determined by comparing the expression distribution of individual *IKZF1* probesets with the upper outlier whisker of the least abundantly expressed probesets. A non-parametric medians test was performed to determine significant differences in the median rank scores between Ph⁺ and Ph⁻ BPL groups in the Mullighan study⁴ and between Ph⁺ BPL, Ph⁻ BPL and normal bone marrow groups in the MILE study.¹³ The median rank scores are either 1 or 0, depending on whether a rank is above or below the median rank and the Chi-Square statistic calculated the P-values (<0.05 deemed significant). We expect significant reductions in the rank scores across all *IKZF1* probesets in samples with *IKZF1* locus deletions. Mixed Model Analysis of Variance analysis for standardized expression scores for combined MILE (GSE13159) and Mullighan (GSE12995) datasets (N= 534 samples (392 Ph⁻, 142 Ph⁺) with three fixed factors (“Diagnosis” Ph⁻, Ph⁺), “Probeset” (4 *IKZF1* probesets common in both MILE and

Mullighan studies), an interaction term for diagnosis x probeset and a random factor, “case” for sample identification) was utilized for the analysis of differential *IKZF1* probeset expression levels. Planned Linear contrasts were performed using the fitted parameters from the interaction term to compare Ph⁺ versus Ph⁻ BPL for each of the 4 probesets (205038_at, 205039_s_at, 216901_s_at and 220704_at). All calculations were performed using JMP statistical package (JMP v10, SAS, Cary, NC).

Results

Exon-Specific Genomic PCR Analysis of Primary BPL Cells Shows No Evidence for Homozygous *IKZF1* Deletions

In an effort to identify homozygous *IKZF1* deletions, we performed exon-specific genomic PCR with DNA sequencing on purified genomic DNA samples from 21 patients with high risk BPL, including 3 patients with BCR-ABL⁺ ALL. As shown in **Figure 1A-D**, no homozygous deletions involving E4-E7 were found in any of the 21 cases. Further, no mutations were detected in E4-E7 or adjacent intronic segments. This absence of genomic deletions in the E4-E7 segment encoding the DNA binding domain of IK prompted us to further validate our findings using RT-PCR analyses designed to detect IK6 transcripts with high accuracy. Specifically, primary leukemia cells from 6 high-risk B-precursor ALL patients, including 3 patients with BCR-ABL⁺ ALL, were subjected to RT-PCR analyses designed to amplify a 442-bp region of the *IKZF1* cDNA extending from distal portion of E4 to E7 as well as a 244-bp region of the *IKZF1* cDNA extending from distal portion of E4 to E6. A single PCR product of the appropriate size was detected in these PCR assays without any evidence of a homozygous or heterozygous deletion of E5, E6, or E7 either alone or in combination, which would have resulted in substantially smaller transcripts alone or in combination with the regular size transcripts (**Figure 1E**). Likewise, no exonic deletions or mutations involving E4-E7 were detected in genomic DNA samples from 11 patients with standard risk pediatric BPL (**Figure 2A**) or 8 patients with infant BPL (**Figure 2B**). Taken together, these findings collectively demonstrate that homozygous *IKZF1* deletions are not consistent abnormalities in pediatric high-risk BPL and they are not signature characteristics of pediatric BCR-ABL⁺ BPL.

Gene Expression Analysis Using Multiple Probesets Contradicts Previous Reports Suggesting Philadelphia-Chromosome (Ph) Positive Pediatric ALL is Characterized by Homozygous or Heterozygous *IKZF1* Deletions

In order to gain further insights into the incidence and biological significance of *IKZF1* deletions, we examined the expression levels of *IKZF1* transcripts in primary leukemic cells from Ph⁻ BPL patients in side by side comparison with pediatric Ph⁺ BPL cases and normal bone marrow specimens in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database GSE 13159.¹³ BLAT analysis on *IKZF1* target sequences mapped the 10 probesets from the Affymetrix Human Genome U133 Plus 2.0 Arrays used in the analysis onto specific *IKZF1* exons visualized using the UCSC genome browser (<http://genome.ucsc.edu/cgi-bin/hgBlat?command=start>). The *IKZF1* probesets were mapped onto common *IKZF1* deletion regions to identify which probesets would be expected to exhibit reduced gene expression in samples with the *IKZF1* deletions.¹⁵ Specifically, the examination of the Affymetrix probeset coverage in relation to most common *IKZF1* micro deletions and complete *IKZF1* locus deletions reported to occur in Ph⁺ BPL cases revealed that each of these deletions can be detected by gene expression profiling using multiple *IKZF1* probesets. The most common microdeletion occurs between exons 4-7 (30%)¹⁶ and can be detected by 1565816_at and 1565818_s_at followed by deletion of exons 2-7 (15%) that can be detected by 1565816_at, 1565818_s_at, 220704_at, 1557632_at) and large chromosome deletions (15%) that can be detected by 9 of 10 *IKZF1*

probesets.¹⁶ Primary leukemia cells from 122 Ph⁺ BPL patients and 237 Ph⁻ BPL patients as well as normal hematopoietic cells from 74 normal non-leukemic bone marrow specimens from the MILE study (GSE 13159)¹³ were organized according to DQN3 expression levels of 10 *IKZF1* transcripts utilizing two-way hierarchical clustering technique (Average distance metric) to identify specimens with low *IKZF1* expression for the 10 probesets interrogating Exons 1 through 4 and Exon 8. Our analysis demonstrated no changes in expression that would be expected from homozygous or heterozygous deletions of *IKZF1* in primary leukemic cells. In particular, the probesets 1565816_at and 1565818_s_at specific for Exon 4 did not detect any reduced expression levels in Ph⁺ or Ph⁻ BPL vs. normal bone marrow specimens, to suggest a heterozygous intragenic deletion between Exons 4-7 or Exons 2-7. Box plots depicted in **Figure 3** illustrate the distribution of expression levels for each probeset color coded with respect to the sample clustering order. Four probesets for exon 8 (205038_at, 205039_s_at, 227344_at, 227346_at) were highly correlated across 433 samples. In comparison to the negative control expression values (mean of 50 lowest abundant probesets), all 433 samples for 7 probesets (1565818_s_at, 205038_at, 205039_s_at, 220704_at, 227344_at, 227346_at, 1557632_at) were greater than the upper outlier range for negative controls. Only one Ph⁺ BPL case exhibited lower expression across all 10 probesets (**Figure 3A**). For 8 out of the 10 probesets, the proportions of above median expression values were greater for Ph⁺ vs. Ph⁻ BPL cells and expression values for only one probeset, 1565818_s_at, exhibited borderline significance (P=0.063) for reduced proportion above median expression in Ph⁺ BPL cells compared to either Ph⁻ BPL cells or normal hematopoietic cells (**Figure 3B**). Likewise, gene expression profiles of primary leukemic cells from 20 Ph⁺ positive and 155 Ph⁻ BPL patients from the original Mullighan study (GSE12995)⁴ were organized according to log₁₀ transformed MAS5 expression levels of 4 *IKZF1* probesets utilizing a two-way hierarchical clustering technique to identify specimens with low *IKZF1* expression for the 4 probesets interrogating Exons 3 and 8. As with the MILE study dataset, we found no changes in *IKZF1* expression that would be expected from homozygous or heterozygous deletions of *IKZF1* in primary leukemic cells. Box plots depicted in **Figure 4** illustrate the distribution of expression levels for each probeset and color coded with respect to the sample clustering order. Two probesets for *IKZF1* expression were highly correlated across 175 samples (205038_at and 205039_s_at). In comparison to the mean expression of 25 lowest abundant probesets, all samples for probesets 205038_at and 205039_s_at were greater than the upper outlier whisker of these negative controls. Only one Ph⁺ BPL case, that was a member of the cluster of expression values below the outlier range for probeset 216901_s_at, exhibited low expression across all 4 probesets (**Figure 4A**). No statistically significant differences were found between Ph⁺ vs. Ph⁻ BPL cases relative to the proportions of cases with above median expression values for the 4 probesets (**Figure 4B**).

We next employed a mixed ANOVA model for the combined datasets comparing Ph⁺ and Ph⁻ BPL cases using standardized expression values. The model explained 56% of the total variation in the probeset expression data across all 4 *IKZF1* transcripts accounting for both fixed and random factors. Relative variance components of the total variation explained for the fixed and random factors for individual cases were 54.6% and 45.4% respectively. Significant effects of Probeset ($F_{3,1596} = 2.668$, $P=0.046$), a strong trend in the Diagnosis factor exhibiting an increase in expression of 4 probesets in Ph⁺ BPL cases ($F_{1,532} = 3.188$, $P = 0.075$) and a non-significant Interaction term ($F_{3,1596} = 0.869$, $P=0.456$) were observed for these fixed factors. Planned Linear Contrasts of Ph⁺ versus Ph⁻ BPL cases for each of the probesets exhibited a borderline increase in exon 3 probeset (220704_at) expression for Ph⁺ BPL cases (Effect size = 0.21 standard deviation units, T-ratio = 2.011, $P = 0.0445$) as well as a borderline increase for the 3 exon 8 probesets (**Figure 5**; Effect sizes for 205038_at, 205039_s_at and 216901_s_at showed increases of 0.046 ($P = 0.66$), 0.135 ($P = 0.20$) and 0.183 ($P = 0.081$) standard deviation units in Ph⁺ BPL cases respectively). These

findings from the parametric ANOVA model are the opposite of what would be expected from a increased incidence of homozygous or heterozygous deletions of *IKZF1* in primary leukemia cells from Ph⁺ BPL patients.

Discussion

In the present study we examined the expression of *IKZF1* in pediatric BPL. The analysis demonstrated no changes in expression that would be expected from homozygous or heterozygous deletions of *IKZF1* in primary leukemic cells from Ph⁺ or Ph⁻ BPL patients. In particular, the probesets 1565816_at (specific for Exons 1-4) and 1565818_s_at (specific for Exon 4 only) did not detect any significantly reduced expression levels in Ph⁺ or Ph⁻ BPL vs. normal bone marrow specimens controlling for repeated measures taken from individual cases, that would have suggested heterozygous intragenic deletions between exons 4-7 or exons 2-7.¹⁵ Our results demonstrate that *IKZF1* deletions are not common characteristics of high-risk BPL or its BCR-ABL⁺ subset in children. In particular, our *IKZF1* gene expression analysis using multiple probesets contradicts previous reports suggesting Ph⁺ BPL in children is characterized by homozygous as well as heterozygous *IKZF1* Deletions. Furthermore, our genotyping of primary leukemia cells from pediatric patients with high-risk ALL using exon-specific genomic PCR and sequencing revealed no homozygous deletions involving E4-E7 and no mutations in E4-E7 or their adjacent intronic segments. Based on the reported detection of *IKZF1* deletions by SNP arrays, our findings indicate that *IKZF1* deletions either occur in a minority of leukemic cells in a oligoclonal heterogeneous population of leukemic B-cell precursors or *IKZF1* expression is characterized by “allelic imbalance” or “allelic exclusion” and deletions occur in “inactive” alleles.¹⁵

While the earlier contradictory reports regarding the true incidence of *IKZF1* deletions may be related to the different techniques used in the research laboratories and have little biologic significance since primary leukemia cells from high-risk B-precursor ALL patients are not IK-deficient, they need to be reconciled if *IKZF1* deletions are to be used for clinical risk assignment and subsequent treatment decisions. Data quality of SNP arrays plays a key role in the accuracy and precision of downstream data analyses.^{17,18} An analysis of possibly contaminated data from SNP arrays or genotyping experiments can yield false-positive discoveries.^{17,18} Therefore, for each SNP, simultaneous comparisons between matched leukemic DNA from diagnostic bone marrow specimens and normal DNA from skin or buccal biopsies is considered critically important.¹⁷ Hence, it is of paramount importance that IK deletions be used as a diagnostic tool for clinical assignment of patients to a high-risk group only when the tests utilized are subject to additional validation of the results with inclusion of normal DNA controls from skin biopsies or buccal swabs.¹⁷

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Figure 1. [A-D] Genomic PCR Analysis of the Human *Ikzf1/IKZF1* Gene Exons E4-E7 in High Risk B-precursor ALL

We performed exon-specific PCR with DNA sequencing on purified genomic DNA samples from 21 patients with high risk B-precursor ALL, including 3 patients with BCR-ABL⁺ ALL. Exons E4-E7 and their intron-exon junctions were PCR amplified, as described in Materials and Methods using the PCR primers in Table S3 (Data Supplement). Normal size PCR products were obtained in each of the 21 patients, providing strong evidence against the existence of homozygous deletions of the entire *IKZF1* locus or within *IKZF1* exons E4-E7. [E] RT-PCR analysis was performed to amplify a 442-bp region of the human (h) *IKZF1* cDNA covering exons E5 to E7 as well as a 244-bp region of the *IKZF1* cDNA covering E5 to E6. Both PCR products included ~1/8th of E4 and E4/E5 junction as well. A single PCR product of the appropriate size was detected in both PCR assays without any evidence of a homozygous or heterozygous deletion of E5, E6, or E7 either alone or in combination, which would have resulted in substantially smaller transcripts alone or in combination with the regular size transcripts.

Figure 2. Genomic PCR Analysis of the Human *IKZF1/Ikaros* Gene Exons E4-E7

Exons 4-7 and their intron-exon junctions were PCR amplified, as described in Materials and Methods using the PCR primers in Table S3. [A1-D1] PCR products from primary leukemic cells from 11 newly diagnosed pediatric patients with standard risk B-lineage ALL. [A.2-D.2] PCR products from primary leukemic cells from 8 newly diagnosed infant ALL patients. Normal size PCR products were obtained in each of the 19 patients, providing strong evidence against the existence of homozygous deletions of the entire *IKZF1* locus or within human *IKZF1* exons E4-E7.

Figure 3. Analysis of Primary Ph⁺ vs. Ph⁻ BPL Cells for *IKZF1* Deletions by Gene Expression Analysis Using 10 Probesets for Exons 1-4 and 8
IKZF1 transcript levels of primary leukemic cells from 122 Ph⁺ positive and 237 Ph⁻ negative BPL patients along with normal lymphohematopoietic cells from 74 normal bone marrow specimens were organized according to DQN3 expression levels of 10 *IKZF1* transcripts utilizing a 2-way hierarchical clustering technique (Average distance metric) to identify specimens with low *IKZF1* expression for the 10 probesets interrogating Exons 1 through 4 and Exon 8. The depicted box plots illustrate the distribution of expression levels for each probeset color coded with respect to the sample clustering order. Four probesets for Exon 8 (205038_at, 205039_s_at, 227344_at, 227346_at) were highly correlated across 433 samples. In comparison to the negative control expression values (mean of 50 lowest abundant probesets), the expression levels of all 433 samples for 7 probesets (1565818_s_at, 205038_at, 205039_s_at, 220704_at, 227344_at, 227346_at, 1557632_at) were higher than

the upper outlier range for negative controls. Only one Ph⁺ BPL case exhibited lower expression across all 10 probesets (panel A). For 8 out of the 10 probesets, the proportion above median expression values were greater for primary leukemia cells from Ph⁺ vs. Ph⁻ BPL cases and expression values for a single probeset (viz.:1565818_s_at) exhibited borderline significance ($P = 0.063$) for reduced proportion above median expression in primary leukemia cells from Ph⁺ BPL vs. Ph⁻ BPL patients or normal bone marrow cells (panel B).

Figure 4. Analysis of Primary Ph⁺ vs. Ph⁻ BPL Cells for *IKZF1* Deletions by Gene Expression Analysis Using Exon 3 and Exon 8 Probesets
IKZF1 transcript levels of 20 Ph⁺ positive and 155 Ph⁻ negative BPL cases were organized according to log₁₀ transformed MAS5 expression levels of 4 *IKZF1* probesets utilizing a 2-way hierarchical clustering technique (Average distance metric) to identify specimens with low *IKZF1* expression for the 4 probesets interrogating Exons 3 and 8. The depicted box plots illustrate the distribution of expression levels for each probeset and color coded with respect to the sample clustering order. Only one PH positive specimen, that was a member of the purple cluster of expression values below the outlier range for probeset 216901_s_at,

exhibited low expression across all 4 probesets (panel A). The proportion above median values comparing Ph^+ vs. Ph^- BPL cases were not significantly different (panel B)

Figure 5. Analysis of Primary Ph⁺ vs. Ph⁻ BPL Cells for *IKZF1* Deletions by Gene Expression Analysis Using Exon 3 and Exon 8 Probesets

Standardized expression scores for combined MILE and Mullighan datasets (N= 534 BPL samples; 392 Ph⁻ BPL and 142 Ph⁺ BPL) were utilized to compare Ph⁺ versus Ph⁻ BPL samples using a mixed ANOVA model. Mean standardized scores ($\pm$ SEM) for Ph⁺ and Ph⁻ BPL groups are depicted for each of the 4 probesets (205038_at, 205039_s_at, 216901_s_at and 220704_at) common in both studies.