JAK mutations in high-risk childhood acute lymphoblastic leukemia

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Pediatric acute lymphoblastic leukemia (ALL) is a heterogeneous disease consisting of distinct clinical and biological subtypes that are characterized by specific chromosomal abnormalities or gene mutations. Mutation of genes encoding tyrosine kinases is uncommon in ALL, with the exception of Philadelphia chromosome-positive ALL, where the t(9,22)(q34;q11) translocation encodes the constitutively active BCR-ABL1 tyrosine kinase. We recently identified a poor prognostic subgroup of pediatric *BCR-ABL1*-negative ALL patients characterized by deletion of *IKZF1* (encoding the lymphoid transcription factor IKAROS) and a gene expression signature similar to *BCR-ABL1*-positive ALL, raising the possibility of activated tyrosine kinase signaling within this leukemia subtype. Here, we report activating mutations in the Janus kinases *JAK1* ($n = 3$), *JAK2* ($n = 16$), and *JAK3* ($n = 1$) in 20 (10.7%) of 187 *BCR-ABL1*-negative, high-risk pediatric ALL cases. The JAK1 and JAK2 mutations involved highly conserved residues in the kinase and pseudokinase domains and resulted in constitutive JAK-STAT activation and growth factor independence of Ba/F3-EpoR cells. The presence of JAK mutations was significantly associated with alteration of *IKZF1* (70% of all JAK-mutated cases and 87.5% of cases with *JAK2* mutations; $P = 0.001$) and deletion of *CDKN2A/B* (70% of all *JAK*-mutated cases and 68.9% of *JAK2*-mutated cases). The JAK-mutated cases had a gene expression signature similar to *BCR-ABL1* pediatric ALL, and they had a poor outcome. These results suggest that inhibition of JAK signaling is a logical target for therapeutic intervention in JAK mutated ALL.

IKAROS | kinase | mutation

Acute lymphoblastic leukemia (ALL) is the most common pediatric cancer, and despite high overall cure rates (1), ALL remains the second leading cause of cancer death in children. To improve outcome, it is necessary to identify high-risk patients at the time of diagnosis and then tailor therapy toward the genetic lesions driving their leukemia.

Recent genome-wide analyses have identified common genetic alterations in childhood ALL that contribute to leukemogenesis (2, 3). To identify genetic lesions predictive of poor outcome in childhood ALL, we recently performed genome-wide analysis of DNA copy number alterations, transcriptional profiling, and gene resequencing in a cohort of 221 children with B progenitor ALL predicted to be at high risk for relapse based on age and presentation leukocyte count (4). These patients were treated on the Children's Oncology Group P9906 trial by using an augmented reinduction/reconsolidation strategy ("Berlin–Frankfurt–Münster" regimen) (5, 6). This cohort excluded patients with known good (*ETV6-RUNX1* or trisomies 4 and 10) or very poor (hypodiploid, *BCR-ABL1*) risk sentinel genetic lesions, and it represents ≈12% of noninfant B precursor ALL cases (Table S1). Alteration of the lymphoid transcription factor *IKZF1* (IKAROS) was associated with poor outcome and a leukemic cell gene expression signature highly similar to that of *BCR-ABL1* pediatric ALL (4). Furthermore, hierarchical clustering of gene expression profiling data identified a subset of 24 cases with poor outcome (4-year incidence of relapse, death, or second malignancy: 79.1%; 95% C.I., 58.6–99.6%) and expression of outlier genes similar to those seen in *BCR-ABL1* ALL. Together, these observations suggested that the poor-outcome, *IKZF1*-deleted, *BCR-ABL1*-negative cases might harbor activating tyrosine kinase mutations. The JAK-STAT pathway may mediate *BCR-ABL1* signaling and transformation (7, 8), and *JAK1* and *JAK2* are mutated in myeloproliferative diseases (9), Down syndrome-associated ALL (DS-ALL), and T lineage ALL (10–12). Here, we have performed genomic resequencing of *JAK1*, *JAK2*, *JAK3*, and *TYK2* in 187 diagnostic samples from this high risk B-progenitor ALL cohort that had available DNA and gene expression profiling data. This identified mutations in *JAK1*, *JAK2*, and *JAK3* in 20 patients (10.7%). The *JAK*-mutated cases had a high frequency of concomitant deletion of *IKZF1* (IKAROS) and *CDKN2A/B*, a gene expression profile similar to *BCR-ABL1* ALL, and extremely poor outcome.

Results

***JAK1*, *JAK2*, and *JAK3* Mutations in High-Risk Pediatric ALL.** Genomic resequencing of *JAK1*, *JAK2*, *JAK3*, and *TYK* was performed for

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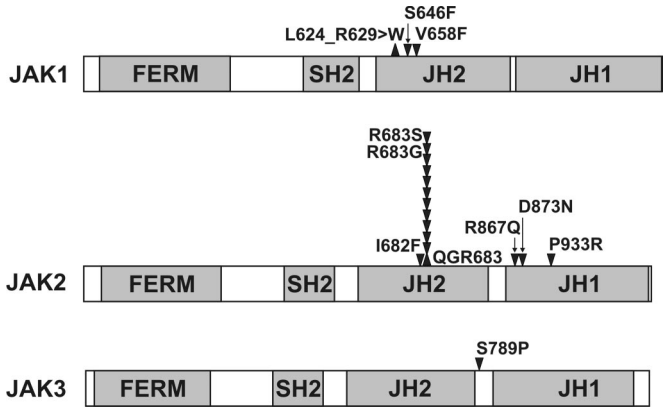

Fig. 1. Primary structure of JAK1, JAK2, and JAK3 showing the location of missense (▼) and insertion/deletion (▲) mutations. FERM, band 4.1 ezrin, radixin, and moesin domain; SH2, src-homology domain; JH2, pseudokinase domain; and JH1, kinase domain.

187 cases in the P9906 cohort that had available DNA, single-nucleotide polymorphism array, and gene expression profiling data. This identified 20 pediatric ALL patients (10.7%) with 20 heterozygous, somatic mutations of *JAK1*, *JAK2*, and *JAK3* (Fig. 1, Tables S2 and S3, and Fig. S1). All patients with JAK mutations lacked known common chromosomal translocations.

A total of 16 cases had *JAK2* mutations, with 13 located in the pseudokinase domain (R683G, $n = 10$; R683S, $n = 1$; I682F, $n = 1$; and QGinsR683, $n = 1$) and 3 within the kinase domain (R867Q, D873N, and P933R). Three previously undescribed missense or in-frame deletion mutations were also identified in the pseudokinase domain of *JAK1* (L624_R629>W, S646F, and V658F), as well as a single *JAK3* mutation, S789P. A total of 2 of the 9 DS-ALL cases in our cohort harbored *JAK2* mutations (QGinsR683 and R683G), with the remaining 18 *JAK* mutations occurring in non-DS-ALL patients (Tables S2 and S3). With the exception of JAK3 S789P, each mutation was located in highly conserved residues in either the pseudokinase or kinase JAK domains (Fig. S2). Mutation of JAK2 R683 and JAK1 V658F (which is homologous to the JAK2 V617F mutation common in myeloproliferative disease) (13–16) results in cytokine-independent in vitro growth of Ba/F3-Epo-R or Ba/F3 cells (10, 11, 17).

Concomitant Genomic Abnormalities in JAK-Mutated ALL. The presence of JAK mutations in this cohort was significantly associated with alterations of *IKZF1* and *CDKN2A/CDKN2B* (Table S2). *IKZF1* deletions or mutations were present in 14 (70%) *JAK*-mutated cases (and in 14 of 16 cases with *JAK2* mutations) but in only 25.7% of cases that lacked a *JAK* mutation ($P = 0.0001$). *JAK* mutations were also associated with *CDKN2A/B* deletion (70% vs. 47%, $P = 0.06$). An increased frequency of copy number alterations at or flanking the *IL3RA/CSF2RA/CRLF2* locus at the pseudoautosomal region of Xp22.3/Yp11.3 was also observed in *JAK*-mutated cases (45.0% vs. 4.2%; $P < 0.0001$). A trend to a significantly higher presenting leukocyte count in JAK-mutated cases was observed (158×10^9/L vs. 101×10^9/L; $P = 0.06$), but there was no difference in age of presentation.

Structural Modeling of JAK Mutations. The JAK pseudokinase domain is thought to negatively regulate activity of the kinase domain (18) and may mediate protein–protein interactions (19–21). JAK2 I682 and R683 map to the junction between the N and C lobes of the pseudokinase domain (Fig. S3*A*). All 4 pseudokinase domain mutations identified affect these residues and are predicted to influence the structure and dynamics of the loops that pack together at the interlobe interface, and this may result in a loss of the inhibitory activity of the pseudokinase domain. Accordingly, the R683G and R683S mutations result in the activation of the tyrosine kinase activity of JAK2 (10, 11). R867Q and D873N map to the $\beta 2$-$\beta 3$ loop of the kinase domain and are predicted to alter surface electrostatic properties of this region (Fig. S3*B*). The P933 residue lies in the JAK2 kinase hinge region, adjacent to the ATP-binding site (22), and is thought to impart rigidity to this hinge that may be important for catalytic activity. These data suggest that the kinase mutations may lead to enhanced kinase activity.

In Vitro Analysis of JAK Mutations. To examine the functional consequences of the JAK variants, we transduced murine pro-B Ba/F3 cells expressing the erythropoietin receptor (Ba/F3-EpoR cells) with retroviral constructs expressing wild-type or mutant murine Jak1 or Jak2 alleles. Each Jak mutation examined conferred growth factor independence to Ba/F3-EpoR cells (Fig. 2 *A* and *B*) and resulted in constitutive Jak-Stat activation, as assessed by western blotting (Fig. 2*E*), and by phosphoflow cytometry analysis of Jak2 and Stat5 phosphorylation after serum and cytokine starvation and subsequent erythropoietin or pervanadate stimulation (Fig. 3*A* and *C*). Interestingly, expression of the Jak2 pseudokinase domain mutants resulted in higher growth rates and Jak-Stat phosphorylation than that observed for the Jak2 kinase domain mutants (Figs. 2 *A* and *E* and 3 *A* and *C*).

This transformation was abrogated by the pan-Jak-specific inhibitor Jak inhibitor I (Fig. 2 *C*, *D*, and *F*). The Jak2 inhibitor XL019 abrogated ligand-induced Jak-Stat activation induced by all tested Jak2 mutants (Fig. 3*B*). Treatment of the cells with the tyrosine phosphatase inhibitor pervanadate led to greater levels of Jak2 and Stat5 phosphorylation (Fig. 3*C*), which was more completely inhibited for mutations involving the pseudokinase domain than the kinase domain (Fig. 3*D*). The basis of this variable inhibition is unknown but raises the possibility of differences in the mechanism of transformation induced by each JAK mutation.

Similarity of the Gene Expression Profiles of *JAK*-Mutated and *BCR-ABL1*-Positive ALL. The similarity in gene expression signatures between *IKZF1*-deleted *BCR-ABL1*-positive and *BCR-ABL1*-negative ALL suggested the possibility of activated tyrosine kinase signaling in the *BCR-ABL1*-negative cases (4). As expected, the JAK-mutated cases exhibited a *BCR-ABL1*-like gene expression signature (Fig. 4 *A* and *B*). Notably, additional *IKZF1*-mutated cases that lacked JAK mutations also showed enrichment of the *BCR-ABL1*-like signature (Fig. 4*B*), suggesting that these cases may harbor additional tyrosine kinase or JAK-STAT-activating mutations.

JAK Mutations and *IKZF1* Alteration Are Associated with Poor Outcome in Pediatric ALL. We observed highly significant associations between *IKZF1* and JAK lesions and outcome. The 4-year cumulative incidence of events (relapse, death, or second malignancy) was 78.2% for patients with both a JAK mutation and *IKZF1* alteration, compared with 54.4% for *IKZF1* alteration only, 33.3% for JAK mutation only, and 24.3% for neither lesion ($P = 0.0002$; Fig. 4*C*). This was primarily attributable to differences in the risk of relapse. The 4-year cumulative incidence of relapse was 76.6% for patients with both a JAK mutation and *IKZF1* alteration, compared with 53.6% for *IKZF1* alteration only, 33.3% for JAK mutation only, and 23.2% for neither lesion ($P = 0.0004$; Fig. 4*D*). In multivariable analyses incorporating clinical and laboratory variables, there was a trend toward an association between JAK mutations and increased risk of events or relapse (Table S4). However, no independent association was observed after incorporation of *IKZF1* status in the model (Table S5). This is in part due to the highly significant correlation

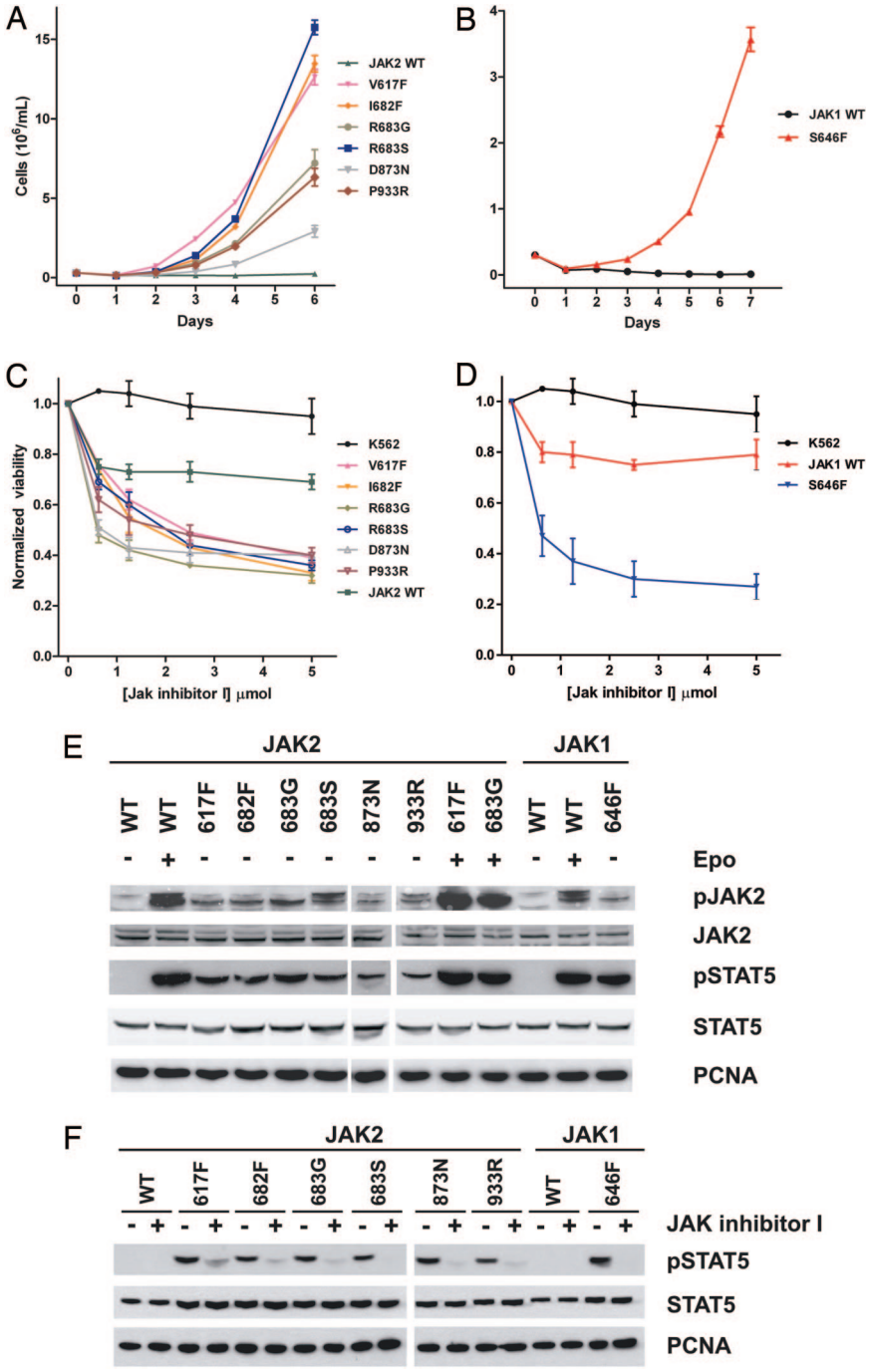

Fig. 2. Functional effects of JAK mutations. (*A*) Ba/F3-EpoR cells were transduced with retroviruses expressing wild-type or mutant Jak2 alleles and cultured in the absence of cytokine. Each Jak2 mutant examined resulted in cytokine-independent growth. Untransduced cells and cells transduced with wild-type mJak2 remained cytokine-dependent. Mean ± SDs of triplicates are shown. (*B*) Transduction of Ba/F3-EpoR cells with retrovirus expressing mJak1 S646F resulted in factor independence. (*C*) Ba/F3-EpoR cells transduced with wild-type or mutant Jak2 were cultured without cytokine in the presence of increasing concentrations of Jak inhibitor I. Each mutation was sensitive to Jak inhibition. The *BCR-ABL1*-positive cell line K562 is shown as a control. (*D*) Growth of Ba/F3 cells transduced with S646F was inhibited by Jak inhibitor I. (*E*) Western blots showing activation of JAK-STAT signaling in Ba/F3-EpoR cells transduced with each mutant Jak allele. Cells were cultured without erythropoietin for 15 h and then harvested for blotting before and after 15 min of erythropoietin at 5 units/mL. Each mutation resulted in constitutive Jak2 and Stat5 phosphorylation that was augmented by pulsed erythropoietin (shown for Jak2 617F and 683G). The Jak2 kinase domain mutations showed less constitutive Jak-Stat activation than the pseudokinase domain mutations. Epo, erythropoietin; WT, wild type. (*F*) Western blots demonstrating abrogation of Jak-Stat activation by Jak inhibitor I. Ba/F3-EpoR cells transduced with each Jak allele were grown in the absence of cytokine, then harvested after 5 h of exposure to 5 mM Jak inhibitor I or vehicle (DMSO).

of JAK mutations and *IKZF1* alterations. Moreover, additional *IKZF1*-mutated, JAK wild-type cases also have a "*BCR-ABL1*-like" signature and poor outcome, suggesting additional unidentified kinase-activating lesions in these cases.

Discussion

These results demonstrate that JAK kinase mutations are not limited to patients with DS-ALL, but also occur in about 10% of high-risk pediatric B-progenitor ALL patients. Notably, the

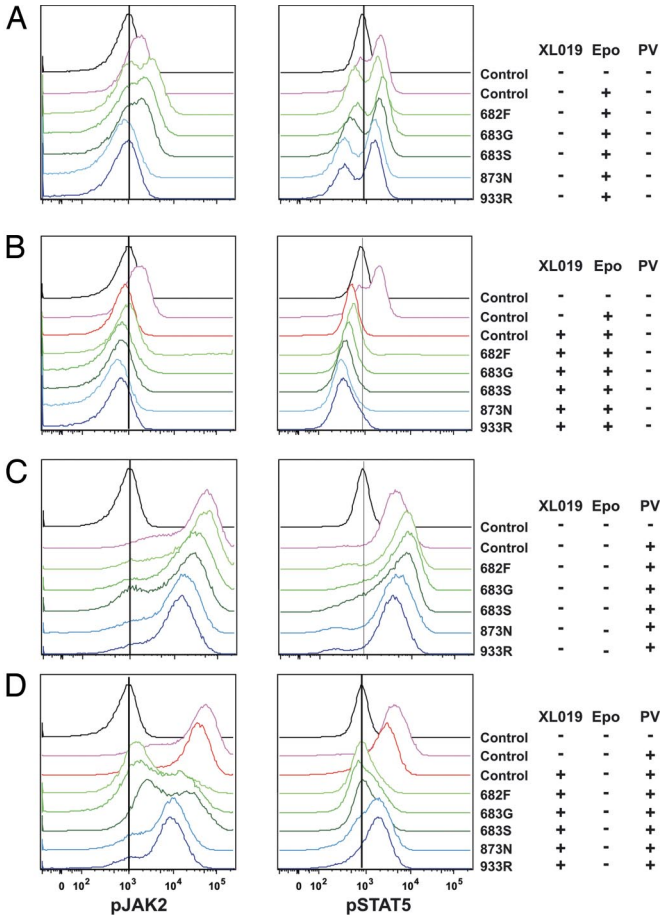

Fig. 3. Phosphoflow cytometry analysis of Jak-Stat activation in Ba/F3-EpoR cells transduced with Jak2 retroviral constructs. Transduced cells were serum-starved and cytokine-starved and then stimulated either with erythropoietin (Epo; *A* and *B*) or pervanadate (PV; *C* and *D*), either without pharmacologic Jak inhibition (*A* and *C*) or after administration of the Jak2 inhibitor XL019 (*B* and *D*). (*A*) Activation of Jak-Stat phosphorylation with erythropoietin stimulation. Notably, Jak2 phosphorylation was evident for Jak2 pseudokinase mutant alleles but not the kinase domain mutants. (*B*) Signaling was abrogated in control and mutants treated with XL019 with subsequent erythropoietin stimulation. (*C*) Marked Jak2 and Stat5 phosphorylation was observed for each mutant after pervanadate stimulation. (*D*) Jak2 and Stat5 signaling was preferentially abrogated in mutants treated with XL019 with subsequent pervanadate stimulation.

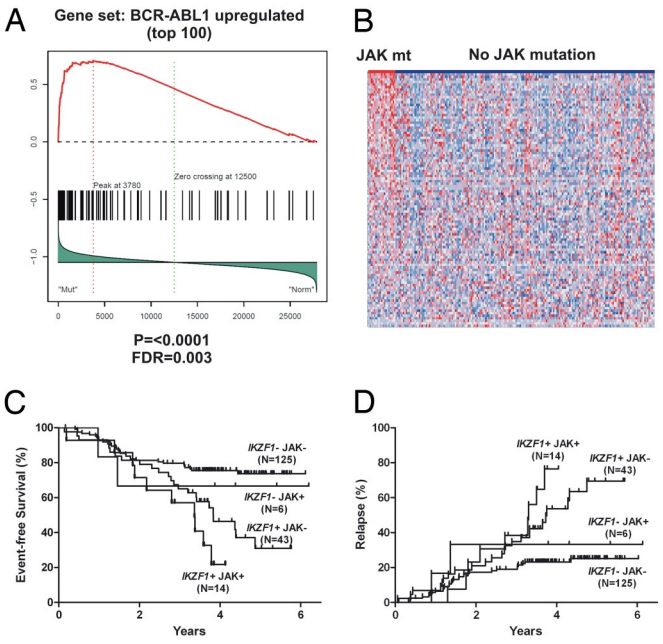

Fig. 4. Gene expression profile and outcome of JAK-mutated B-progenitor ALL. (*A*) Gene set enrichment analysis demonstrates significant enrichment of the BCR-ABL1 gene expression signature in JAK-mutated ALL. (*B*) Heatmap of the enriched *BCR-ABL1* up-regulated gene set in the P9906 cohort, showing overexpression of *BCR-ABL1* up-regulated genes in JAK-mutated ALL. Notably, several cases lacking JAK mutations also have a *BCR-ABL1* signature, suggesting the presence of additional kinase mutations in these cases. (*C* and *D*) JAK mutation and *IKZF1* alteration are associated with a high incidence of events (*C*) and relapse (*D*).

P9906 cohort studied here is not an unselected series of childhood ALL cases, but comprises patients with high white blood cell counts and/or older age that were predicted to have a poor outcome. Patients with high hyperdiploidy, hypodiploidy, *ETV6-RUNX1*, or *BCR-ABL1* were not included; however, the cohort did include *TCF3-PBX1* ($n = 22$) and *MLL*-rearranged ($n = 18$) B-progenitor cases, none of whom had JAK mutations. These differences in cohort composition provide a potential explanation as to why JAK mutations have not been detected more frequently in non-DS-ALL in other studies (10, 11). Future studies including larger numbers of patients with recurring cytogenetic alterations will be of interest to determine whether JAK mutations occur predominantly among ALL patients lacking known translocations and aneuploidy.

JAK mutations were associated with concomitant *IKZF1* and *CDKN2A/B* alterations, suggesting that genetic lesions targeting multiple cellular pathways, including lymphoid development (*IKZF1*), tumor suppression (*CDKN2A/B*), and activation of tyrosine kinases (*BCR-ABL1*, JAK, or other kinase mutations) cooperate to induce aggressive lymphoid leukemia in both DS-ALL and non-DS-ALL that is resistant to conventional ALL therapy. The majority of the identified JAK mutations occur in the pseudokinase domain of JAK2 in a region (R683) distinct from the predominant mutation (V617F) seen in polycythemia vera and related myeloproliferative diseases (9). It has been hypothesized that the nature of the JAK mutation plays a direct role in establishing the disease phenotype (9, 23, 24), and mutations at R683 have been identified almost exclusively in DS-ALL-related ALL (10, 11). Experiments testing the in vivo-transforming activity of the JAK mutations and the cooperative effect of concomitant genetic lesions, such as alteration of *IKZF1*, should provide valuable insights into how these lesions contribute to leukemogenesis and treatment resistance. Notably, both DS-ALL cases with JAK mutations in this study had concomitant alterations of *IKZF1* and *CDKN2A/B*, suggesting that the cooccurrence of these lesions is important in the pathogenesis of DS and non-DS high-risk ALL. The identification of JAK mutations in a subset of the *IKZF1*-mutated, poor-outcome group raises the possibility that inhibition of JAK activity will be a logical therapeutic approach in these patients. Indeed, our data demonstrate impressive inhibition of the Jak-Stat activation induced by Jak pseudokinase domain mutations by the JAK2 inhibitor XL019, a drug currently in early-phase trials for myeloproliferative disorders. Finally, the absence of JAK mutations in additional cases with a *BCR-ABL1*-like expression signature suggests that efforts to identify the causes of activated kinase signaling in these cases should identify additional therapeutic targets in high-risk pediatric ALL.

Materials and Methods

Patients and Treatment. Patients were enrolled in the Children's Oncology Group P9906 trial and treated with an augmented reinduction/reconsolida-

tion strategy (5). All patients were high-risk based on the presence of central nervous system or testicular disease, *MLL* rearrangement, or based on age, sex, and presentation leukocyte count (25). *BCR-ABL1* and hypodiploid ALL, as well as cases of primary induction failure were excluded. The cohort is described further in the *SI Methods*.

Genomic Resequencing and Structural Modeling of JAK2 Mutations. Resequencing of the coding exons of *JAK1*, *JAK2*, *JAK3*, and *TYK2* was performed by Agencourt Biosciences. Sequencing, sequence analysis, structural modeling, and homology alignment of JAK mutations are described in the *SI Methods*.

Functional Assays of JAK Mutants. The *JAK1* S646F and *JAK2* V617F, I682F, R683G, R683S, D873N, and P933R mutations were introduced into the bicistronic MSCV-IRES-GFP retroviral vector encoding either murine Jak1 or Jak2 containing the C-terminal HA tag (26) by site-directed mutagenesis (QuikChange XL II; Stratagene). Retroviral supernatants were produced by using ecotropic Phoenix packaging cells (G.P. Nolan; www.stanford.edu/group/nolan/). Murine pro-B Ba/F3 cells were transduced with MSCV-EpoR-IRES-puro, and after puromycin selection they were transduced with wild-type or mutant Jak retroviral supernatants. Transduced cells were purified by flow sorting for GFP and were maintained in RPMI-1640 with 10% FCS (HyClone) penicillin-streptomycin, L-glutamine, and 5 units/mL erythropoietin. To assess growth factor independence, cells were washed 3 times and were plated at 500,000 cells per milliliter in media without cytokine, with or without JAK inhibitor I (Calbiochem), and growth was monitored daily by using a ViCell cell counter (Beckman Coulter).

For Western blotting, Jak-transduced Ba/F3-EpoR cells were cultured for 15 h without erythropoietin, followed by 15 min of treatment with erythropoietin at 5 units/mL or vehicle (DMSO). Whole-cell lysates were blotted and probed with anti-Jak2, anti-phospho-Jak2 (Tyr 1007–1008), anti-Stat5, and anti-phospho-Stat5 (Cell Signaling Technology), and with anti-PCNA (Santa Cruz Biotechnology).

Cytokine stimulation and intracellular phosphoprotein analysis using flow cytometry was performed as described previously (27). Ba/F3-EpoR cells were serum- and cytokine-starved for 30 min, then incubated with the JAK2 inhibitor XL019 (Exelixis) at a concentration of 5 μM for 30 min. Control and XL019-treated cells were subsequently stimulated with 5 ng/mL murine IL-3, 2 units/mL human erythropoietin, or 125 μM pervanadate for 15 min. Cells were fixed, permeabilized, rehydrated overnight, and then stained with anti-phospho-Stat5-Alexa 647 (Tyr-694; BD Biosciences), anti-phospho-Jak2 (Tyr 1007–1008), and phycoerythrin-conjugated donkey anti-rabbit IgG secondary antibody (Jackson ImmunoResearch). Samples were analyzed on an LSRII flow cytometer (BD Biosciences), and data were collected and analyzed by using DIVA (BD Biosciences) and FlowJo (Tree Star).

Gene Set Enrichment Analysis (GSEA). GSEA (28) was performed as described previously (2, 4) by using the collection of publicly available gene sets (www.broad.mit.edu/gsea/msigdb/) and gene sets derived from the top up- and down-regulated genes of *BCR-ABL1* de novo pediatric ALL (29, 30).

Statistical Analysis. Associations between clinical, laboratory, and genetic variables and outcome (event-free survival and relapse) were performed as described previously (4). Cumulative incidence of relapse according to *IKZF1* and JAK status was analyzed by using Gray's test (31). Associations with event-free survival were examined by using the methods of Kaplan and Meier and the Mantel–Haenszel test (32). Multivariable analyses of event-free survival were performed by using the EFS-PHREG procedure in SAS version 9.1.3 (SAS Institute); multivariable analyses of relapse were performed by using the Fine and Gray method (33) in S-Plus version 7.0.6 (Insightful).

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