ARTICLE

RNA sequencing unravels the genetics of refractory/relapsed T-cell acute lymphoblastic leukemia. Prognostic and therapeutic implications

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ABSTRACT

Despite therapeutic improvements, a sizable number of patients with T-cell acute lymphoblastic leukemia still have a poor outcome. To unravel the genomic background associated with refractoriness, we evaluated the transcriptome of 19 cases of refractory/early relapsed T-cell acute lymphoblastic leukemia (discovery cohort) by performing RNA-sequencing on diagnostic material. The incidence and prognostic impact of the most frequently mutated pathways were validated by Sanger sequencing on genomic DNA from diagnostic samples of an independent cohort of 49 cases (validation cohort), including refractory, relapsed and responsive cases. Combined gene expression and fusion transcript analyses in the discovery cohort revealed the presence of known oncogenes and identified novel rearrangements inducing overexpression, as well as inactivation of tumor suppressor genes. Mutation analysis identified JAK/STAT and RAS/PTEN as the most commonly disrupted pathways in patients with chemorefractory disease or early relapse, frequently in association with NOTCH1/FBXW7 mutations. The analysis on the validation cohort documented a significantly higher risk of relapse, inferior overall survival, disease-free survival and event-free survival in patients with JAK/STAT or RAS/PTEN alterations. Conversely, a significantly better survival was observed in patients harboring only NOTCH1/FBXW7 mutations: this favorable prognostic effect was abrogated by the presence of concomitant mutations. Preliminary *in vitro* assays on primary cells demonstrated sensitivity to specific inhibitors. These data document the negative prognostic impact of JAK/STAT and RAS/PTEN mutations in T-cell acute lymphoblastic leukemia and suggest the potential clinical application of JAK and PI3K/mTOR inhibitors in patients harboring mutations in these pathways.

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Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is a genetically heterogeneous disease caused by the accumulation of molecular lesions acting in a multistep pathogenic process.[1,2] While more than 80% of children can expect to be cured nowadays, among adults younger than 60 years managed with conventional treatment the survival rates are in the range of 40-50% and older patients have a much worse outcome.[3-6] Although the use of intensified strategies results in a survival advantage, many patients still relapse and eventually experience refractory leukemia associated with a poor likelihood of cure.[4,5,7,8]

Over the last years much effort has been put into understanding the molecular background of relapsed and chemotherapy-resistant ALL.[9-12] In the pediatric setting, Tzoneva *et al.* identified mutations affecting the *NT5C2* gene[13], which appeared to be acquired at relapse and, overall, to be more frequent in T-ALL than in B-ALL; similar results were recently reported in T-ALL also by Kunz *et al.*[14]

A comprehensive description of the genetic alterations in patients with chemorefractory T-ALL has thus far been lacking. We previously reported that whole transcriptome sequencing (RNAseq) is a powerful approach for the detection of translocations, single nucleotide variants, small insertions/deletions (INDEL) and gene expression deregulation in T-ALL.[15]

In this study, we applied RNAseq to 19 diagnostic T-ALL samples from patients who were refractory to treatment or experienced an early relapse. Our aim was to identify key oncogenic pathways that may predict treatment failure and that could be targets for molecularly tailored therapies. The recurrence and the prognostic value of the lesions identified were validated in an additional set of 49 newly diagnosed T-ALL samples.

Methods

Discovery cohort

This study was carried out on T-ALL samples collected at diagnosis from 19 patients who proved refractory to first-line treatment (n=11), experienced an early relapse after first complete remission (n=7: median time to relapse 4 months; range, 1-9), and a single patient who relapsed after 16 months. Fourteen were male and five were female and they had a median age of 36 years (range, 11-55). The diagnosis of T-ALL was based on the World Health Organization (WHO) classification.[16] Cytogenetic and molecular analyses were performed as reported elsewhere.[17-19] Molecular screening included searches for recurrent fusion genes, e.g. *BCR/ABL1*, *MLL* and *MLLT10* fusions, *STIL/TAL1*, *NUP98/RAP1GDS1*, *SET/NUP214*, *NUP214/ABL1*, *ETV6/RUNX1* and *E2A/PBX1*. *SET-NUP214* was identified in three cases and *STIL-TAL1* in one, while the remaining 15 patients were negative for recurrent fusion genes (*Online Supplementary Table S1*).

For all 19 patients, RNA from peripheral blood or bone marrow samples was analyzed by RNAseq on the initial diagnostic material collected before starting treatment at the Hematology Center, 'Sapienza' University of Rome. In the three relapsed cases with the longest duration of first complete remission, RNAseq was also performed on the matched relapse samples.

All patients or guardians gave their informed consent to blood/marrow collection and biological analyses, in agreement with the Declaration of Helsinki. The study was approved by the Institutional Review Board.

RNA sequencing analysis on the discovery cohort

RNAseq and bioinformatic analyses were performed at the Laboratory for the Molecular Biology of Leukemia, VIB-KU Leuven, Belgium. Paired-end sequencing was performed on an Illumina HiSeq2000 instrument. Sequence reads were processed to identify fusion transcripts, single nucleotide variants, INDEL and gene expression levels, as reported previously (*Online Supplementary Figure S1*).[15]

Validation of RNA sequencing data on the discovery cohort

Candidate fusion transcripts were validated by reverse-transcription polymerase-chain reaction (RT-PCR) and Sanger sequencing (*Online Supplementary Table S2*). Fluorescence *in situ* hybridization (FISH) was applied to confirm the presence of fusion transcripts in four samples (*Online Supplementary Methods*). Candidate variants (single nucleotide variants and INDEL) were confirmed by PCR amplification and Sanger sequencing on genomic DNA (gDNA) (*Online Supplementary Table S3*). In six cases with available material, germline gDNA (extracted from saliva) was analyzed to establish the somatic nature of the mutations.

In vitro assays were also performed to test the sensitivity of primary cells carrying identified molecular alterations to specific inhibitors, as detailed in the *Online Supplemental Methods.*

Validation cohort

Candidate lesions validated in the discovery cohort were screened by Sanger sequencing of diagnostic gDNA in an independent cohort of 49 T-ALL patients. The validation cohort included refractory, relapsed and responsive cases enrolled in the multicenter GIMEMA LAL 2000 and LAL 0904 protocols (NCT00537550 and NCT00458848, respectively).

Two age-cohorts were considered: adolescents and young adults (15-35 years, n=22) and adults (36-60 years, n=27).

Overall, there were 36 males and 13 females with a median age of 37 years (range, 15-59). Molecular analysis detected the *STIL-TAL1* fusion in six cases, *MLLT10* fusions in three, *NUP98-PSIP1* and *NUP214-ABL1* in single cases, while the remaining 38 cases were negative (*Online Supplementary Table S4*).

The experimental strategy used to study the discovery and validation cohorts is detailed in *Online Supplementary Figure S2.*

Statistical analyses

The prognostic impact of the mutated genes was assessed in the validation cohort. The statistical methods are detailed in the *Online Supplementary Methods*.

Results

Fusion transcript and gene expression findings in the discovery cohort

RNAseq enabled an average 123x10^6 reads per sample, leading to an average coverage of 80X (*Online Supplementary Table S5*). We identified 183 predicted fusion transcripts (median 6/sample; range, 0-36) (*Online Supplementary Table S6A*) predominantly involving genes localized next to each other on the same chromosome, and likely representing read-through of transcription.[15,20] For example, two fusion transcripts involving adjacent genes, namely *SMG5-PAQR6* (n=2) and *TTTY15-USP9Y* (n=2), previously described in prostatic cancer[21-23] and also identified in two cases of our validation cohort, were detected in normal thymus cells from healthy donors.

After 'noise' removal, several known and novel fusion transcripts were identified and further validated by Sanger sequencing of RT-PCR products (Table 1). Fusion transcripts were identified also in cases with normal or failed cytogenetics. In addition to the *STIL-TAL1* (R24) and *SET-NUP214* fusions (R20, R21, R28), we identified and validated four fusions involving T-cell receptor genes (*TCR*). In three cases, the *TCR* was fused to known oncogenes - *TAL1* (R11), *LMO2* (R19) or *HOXA10-AS* (R23) - and induced overexpression of the partner genes (Figure 1A and *Online Supplementary Figure S3A*). A novel rearrangement joining the *TRAC* to *SOX8* on chromosome 16p13 was identified in the remaining case, also harboring *STIL-TAL1* (R24). This was associated with the transcriptional activation of *SOX8* (Figure 1B). The fusion was confirmed by FISH (*Online Supplementary Figure S3B*) and was also detected at relapse. Furthermore, a novel fusion juxtaposing *HOXA11-AS* to *MIR181A1HG* was documented in a patient showing overexpression of *HOXA13*, *HOXA11* and *HOXA10* (R27). Indeed, FISH confirmed a rearrangement between the HOXA cluster and chromosome region 1q31-1q32 (*Online Supplementary Figure S3C*).

We also identified out-of-frame fusions generated by deletions or inversions, predicted to cause inactivation of transcriptional regulators, i.e. *ETV6-SLC15A5* (R27), *GATA3-GS1-756B1.2* (R11) and *WT1-THEM7P* (R28), or inactivation of *PTEN* in two cases harboring the *PTEN-FAS* (R13) or *MAST3-C19orf10* (R19) transcripts. In the patient harboring the *MAST3-C19orf10* fusion, RNAseq also documented the *GLT25D1-AC020911.1* out-of-frame fusion generated by an inversion on chromosome 19p13. FISH analysis documented an amplification of the 19p13 region, and RNAseq data further indicated increased expression of *NOTCH3* and *JAK3*, both localized in this region (Figure 1C and *Online Supplementary Figure S4A-C*).

Paired diagnosis and relapse RNAseq analysis was performed in three cases. These cases retained the same fusions at the later stage of the disease, namely the *SET-NUP214* (R20 and R21) and the *TRBC2-HOXA10-AS* fusion (R23). Importantly, in all three cases the number of reads carrying the fusion transcripts was higher at relapse than in the diagnostic samples, suggesting clonal maintenance and expansion (*Online Supplementary Figure S5*).

Table 1. Summary of the lesions detected by RNAseq. The lesions listed below were validated by RT-PCR and Sanger sequencing (*), FISH (†) or Sanger sequencing on gDNA (§).

ID	Karyotype	Fusion transcript	JAK/STAT lesion(§)	RAS/PTEN lesion(§)	NOTCH1/FBXW7 lesion(§)
R11	t(1;14)(p32;q13)	TAL1-TRDC (*) GATA3-GS1-756B1.2 (*)	-	PTEN S226fsX23	-
R12	46, xy [20]	-	-	NRAS G12D KRAS G12V	-
R13	NA	PTEN-FAS(*)	-	-	-
R14	46, xy [20]	-	JAK1 R724H PTPRC 1721-2A>G	-	NOTCH1 1693_1695 FQS>LG FBXW7 R689W
R15	47-48,XY,+8,-9,der(11)t(9;11)(q13;p15),-19,-19,-22,+4mar[cp]	MAST3-C19orf10 (*;†) GLT25D1- AC020911.1(*;†)	JAK3 R657Q STAT6 E185K	KRAS G12D	NOTCH1 V1604E
R17	46, xy [20]	-	IL7R 243_245 LT>MICTL	-	FBXW7 R505C
R18	46, xy [20]	-	JAK1 604_606 delDYKinsRNDYN JAK3 R657W	PTEN R233fsX7 PIK3R1 R642X	NOTCH1 F1606LinsIQ FBXW7 R465H
R19	46+,XY,t(7;11)(q33;p15)[8]/46,idem, del(6)(q13q21)[5]	LMO2-TRBC2 (*)	-	-	-
R20	NA	SET-NUP214(*)	IL7R T244>ILCYPP	-	NOTCH1 L1585P NOTCH1 V2473X NOTCH3 G438R
R21	NA	SET-NUP214(*)	TYK2 R527Q	ITPR1 A1205V	-
R22	46, xy [20]	-	-	PTEN M239fsX14	-
R23	46, xx [20]	TRBC2-HOXA11-AS(*;†)	JAK3 V674A STAT5A T628S		- -
R24	NA	TRAC-SOX8(*;†) STIL-TAL1(*)	-	-	-
R25	NA	-	JAK1 R724H JAK3 R657Q JAK3 S1000L	-	NOTCH1 1578del V NOTCH1 V2443fsX35
R26	48,XY,+2mar[3]/46,XY[22]	-	-	-	NOTCH1 L1574P
R27	46, xx [15]	MIR181A1HG-HOXA11-AS(*;†) ETV6-SLC15A5(*)	JAK3 M511I STAT5A G472S	-	NOTCH1 L1709P NOTCH2 F1167V
R28	NA	WT1-THEM7P(*) SET-NUP214(*)	-	ITPKB R590Q	NOTCH1 V1722M NOTCH1 Y2490X

JAK/STAT and RAS/PTEN pathway mutations in the discovery cohort

RNAseq analysis identified 1,527 protein-altering single nucleotide variants (median 78/sample; range, 17-157) and 1,115 INDEL (median 59/sample; range, 30-82) across the 19 patients with refractory/relapsed T-ALL (*Online Supplementary Tables S6A* and *S7*). Two hundred and twenty-nine genes were recurrently affected in at least two samples and involved specific pathways (Table 1, *Online Supplementary Tables S8* and *S9*, Figure 2A).

Overall, mutations in the JAK/STAT pathway were identified in nine of 19 cases (47%). Mutations were found in all the subtypes, including two out of five cases of early T-cell precursor leukemia. The most frequently mutated gene was *JAK3* (n=5), followed by *JAK1* (n=3), *IL7R* (n=2), *STAT5A* (n=2), *STAT6* (n=1) and *TYK2* (n=1). Interestingly, *JAK1* and *JAK3* mutant cases concomitantly harbored another lesion in the same pathway, while no additional lesions were detected in the *IL7R* and *TYK2* mutant cases. Furthermore, we identified one case with abnormal splicing of *PTPRC*, encoding the tyrosine phosphatase CD45, a negative regulator of the JAK/STAT pathway.[24] RT-PCR and Sanger sequencing confirmed that mRNA retained *PTPRC* introns 15 and 16, whereas sequencing of the gDNA documented the presence of a splice site mutation, as previously reported.[25]

The RAS/PTEN pathway was also recurrently affected (8/19 cases, 42%). Cases carrying mutations in RAS or PI3K/AKT signaling were grouped together because of the biological convergence of the two pathways in mTOR activation.[26] *PTEN* was affected by out-of-frame fusions leading to potential inactivation (n=2, see above) and frameshift mutations resulting in downstream premature stop codons (n=3). Furthermore, we identified mutations in *K-RAS* (n=2) or *N-RAS* (n=1) as well as in driver genes involved in other cancers but so far not reported in T-ALL, namely *ITPKB* (n=1), *ITPR1* (n=1) and *PIK3R1* (n=1). A double hit within the pathway was found in three cases. Concomitant JAK/STAT and RAS/PTEN mutations were observed in three cases (2 of them with a double hit in both pathways). In addition, altered expression of *PTEN*, *PTPN11* and *FLT3* was documented by gene expression analysis (Figure 2B).

NOTCH1/FBXW7 was also recurrently mutated (9/19 cases, 47%). Six *NOTCH1*-mutated cases harbored either a double mutation (n=3, one of them also had a mutation in *NOTCH3*), or a mutation in another gene of the same pathway, namely *FBXW7* (n=2) or *NOTCH2* (n=1). Eight of the nine *NOTCH1/FBXW7*-positive cases harbored concomitant alterations in JAK/STAT or RAS/PTEN.

RNAseq analysis on matched diagnostic and relapse samples revealed that *NOTCH1* and *FBXW7* status could differ between diagnosis and relapse, with the *NOTCH1* mutation being lost (n=1) and the *FBXW7* mutation being

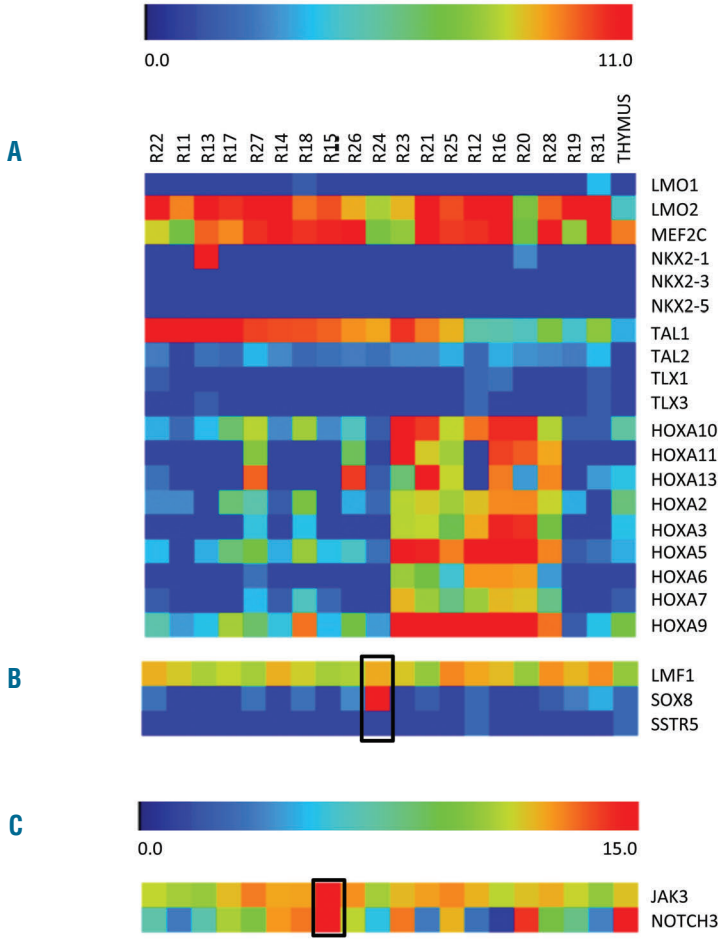

Figure 1. Heatmap of selected genes: expression levels. (A) Expression levels of transcription factors known to be overexpressed in T-ALL. *TAL1*-overexpressing cases included sample R24, harboring the *STIL-TAL1* fusion, and sample R11, carrying the *TAL1-TCR* rearrangement, whereas within *LMO2*-overexpressing cases we identified sample R19 harboring the *LMO2-TCR* fusion. *HOXA*-overexpressing cases included samples R20, R21, R28 harboring the *SET-NUP214* fusion, sample R23 with the *TRBC2-HOXA10-AS* rearrangement and sample R27 carrying the *MIR181A1HG-HOXA11-AS* fusion. The majority of samples showed overexpression of *MEF2C* reflecting the immature phenotype. (B) The heatmap illustrates the expression patterns of *SOX8*, together with its immediately upstream and downstream flanking genes in the genome. It shows strong overexpression (red) of *SOX8* in the R24 case harboring the *TRAC-SOX8* fusion. (C) The heatmap shows overexpression of *NOTCH3* and *JAK3* in the R15 case harboring rearrangements and amplifications on 19p13. The heatmaps are plotted with the normalized $\log_2$ (count) values.

acquired (n=1) at relapse. In contrast, mutations in JAK/STAT and RAS/PTEN pathways were retained at the later stage of the disease.

Mutations in chromatin modifier and transcription regulators - including *PHF6* (n=5), *KDM3A* (n=2), *EZH2* (n=2), *H3F3A* (n=1), *HDAC6* (n=1), *MLL1* (n=1), *MLL5* (n=1), *CNOT3* (n=1), *SF3B1* (n=1) and *NCOR1* (n=1) - were also identified. Finally, mutations in genes involved in purine synthesis or glucose metabolism (*NT5DC1*, *GMPS*, *GMPR* and *DOK2*) were found in single cases.

Incidence of JAK/STAT, RAS/PTEN *and* NOTCH1/FBXW7 *mutations in the validation cohort*

To assess the incidence of mutations in the JAK/STAT, RAS/PTEN and NOTCH1/FBXW7 pathways, Sanger sequencing was performed in a total of 49 gDNA samples from additional, newly diagnosed T-ALL patients (i.e. the validation cohort) older than 15 years, enrolled in two consecutive, multicenter GIMEMA protocols and including responsive, refractory and relapsed cases. The results are summarized in Figure 3 and *Online Supplementary Table S10*. Eight patients (16%) harbored JAK/STAT pathway mutations. We identified missense point mutations affecting a residue of the pseudokinase and kinase domains of *JAK1* (n=2), *JAK3* (n=2) or both (n=1), the DNA binding and SH2 domain of *STAT5B* (n=2), and in-frame INDEL in exon 6 of *IL7R*, coding for the transmembrane domain (n=3). All *STAT5B* mutants harbored concomitant mutations in *JAK3* (1 of them also had a *JAK1* mutation), whereas the *IL7R*-positive patients had no additional mutations of the same pathway. These lesions were localized in hotspot residues already reported as drivers or predicted to have a damaging effect. In addition, the *JAK3* P151R and *JAK3* V722I were observed in three patients; however, given their distribution also in the normal population, they were not taken into account. No statistically significant differences were observed in white blood cell count, gender and age distribution between cases harboring the above-mentioned mutations and those not carrying them. The *PICALM-MLLT10* fusion was identified in a single case, while the remaining cases were negative for recurrent fusion genes.

Alterations in the RAS/PTEN pathway were found in ten patients (20%) including: five *PTEN* INDEL, four *K/N-RAS* mutations affecting hotspot residues and one missense point mutation affecting the tyrosine kinase domain of *FLT3*, this last found in a case of early T-cell precursor leukemia. *PTEN* mutations were associated with younger age (P=0.04), whereas no significant differences were observed in gender and white blood cell count. Molecular analysis identified a *STIL-TAL1* fusion in three cases harboring *PTEN* mutations and proved negative in the remaining seven cases.

NOTCH1/FBXW7 mutations were found in 28 cases (57%), in 16 without additional alterations, while 12 carried a concomitant mutation in *JAK/STAT* (n=8) or *K/N-RAS* (n=4).

ITPKB, *ITPR1*, *PIK3R1*, *STAT5A* and *STAT6* hotspots were screened: no additional positive cases were identified in this cohort.

Correlation between genetics, response to chemotherapy and outcome in the validation cohort

Patients included in the validation cohort were grouped

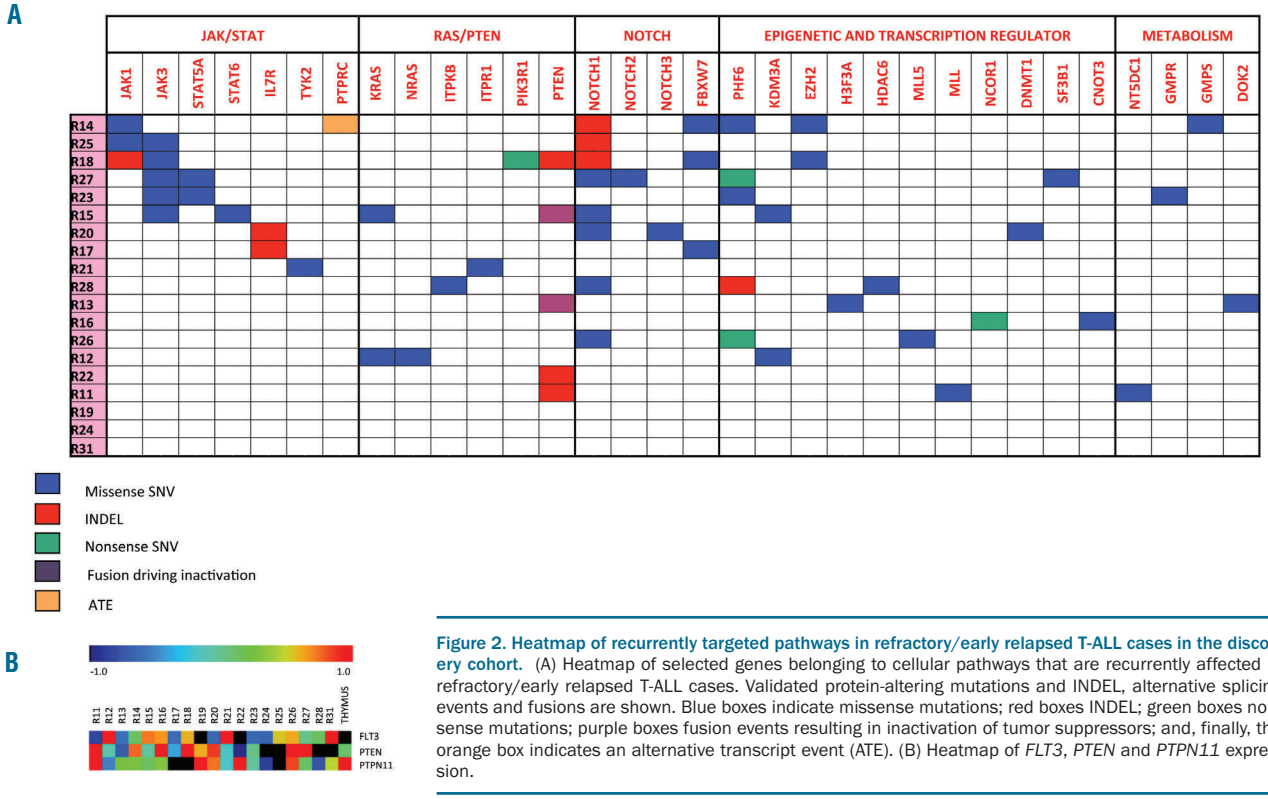

Figure 2. Heatmap of recurrently targeted pathways in refractory/early relapsed T-ALL cases in the discovery cohort. (A) Heatmap of selected genes belonging to cellular pathways that are recurrently affected in refractory/early relapsed T-ALL cases. Validated protein-altering mutations and INDEL, alternative splicing events and fusions are shown. Blue boxes indicate missense mutations; red boxes INDEL; green boxes nonsense mutations; purple boxes fusion events resulting in inactivation of tumor suppressors; and, finally, the orange box indicates an alternative transcript event (ATE). (B) Heatmap of *FLT3*, *PTEN* and *PTPN11* expression.

into three subtypes according to their mutational profile - JAK/STAT-positive, RAS/PTEN-positive and NOTCH1/FBXW7-positive only (i.e. without JAK/STAT and/or RAS/PTEN mutations) - and evaluated for response to therapy and survival.

Of the eight JAK/STAT-positive patients, five obtained a complete remission after induction whereas three did not because of refractoriness (n=2) or death (n=1). Notably, all patients but one relapsed shortly after achieving complete remission (median time: 10 months; range, 1-11). Eight of the ten RAS/PTEN-positive patients obtained a complete remission after induction, whereas two died during induction treatment. Seven of the eight responsive patients (87.5%, i.e. 4 *K/N-RAS*- and 3 *PTEN*-positive patients) experienced an early relapse (median time: 4 months; range, 1-11). Fifteen of the 16 patients with NOTCH1/FBXW7 mutations only were evaluable for response to therapy: 11 obtained a complete remission and four did not because of refractoriness (n=3) or death (n=1). Of the 11 patients who achieved complete remission, five (45.5%) have relapsed (median time from complete remission: 31 months; range, 6-41) and six (54.5%) are in long-term complete remission at a median follow-up of 73 months (range, 51-96).

While no significant difference in achievement of complete remission was found between the three subtypes, a significantly higher cumulative incidence of relapse was observed in JAK/STAT- and RAS/PTEN-positive cases when compared to negative patients or to cases harboring NOTCH1/FBXW7 mutations only (P=0.002, P=0.0001 and P=0.0015, respectively). Along this line, significantly shorter overall survival, disease-free survival and event-free survival were observed in patients harboring JAK/STAT mutations compared to patients without alterations in the pathways (Figure 4A-C). In detail, the overall, disease-free and event-free survival probabilities were 0% at 20 months in patients carrying JAK/STAT mutations (median overall survival 15.7 months; median disease-free survival 11 months; median event-free survival 3.3 months) compared to 69.1% (overall survival, 95% CI: 88.2%-54.1%; median, not reached, P=0.0045), 70% (disease-free survival, 95% CI: 93.3%-52.5%; median not reached, P=0.002) and 48.3% (event-free survival, 95% CI: 70.4%-33.1%; 17.3 months, P=0.027) in wild-type patients.

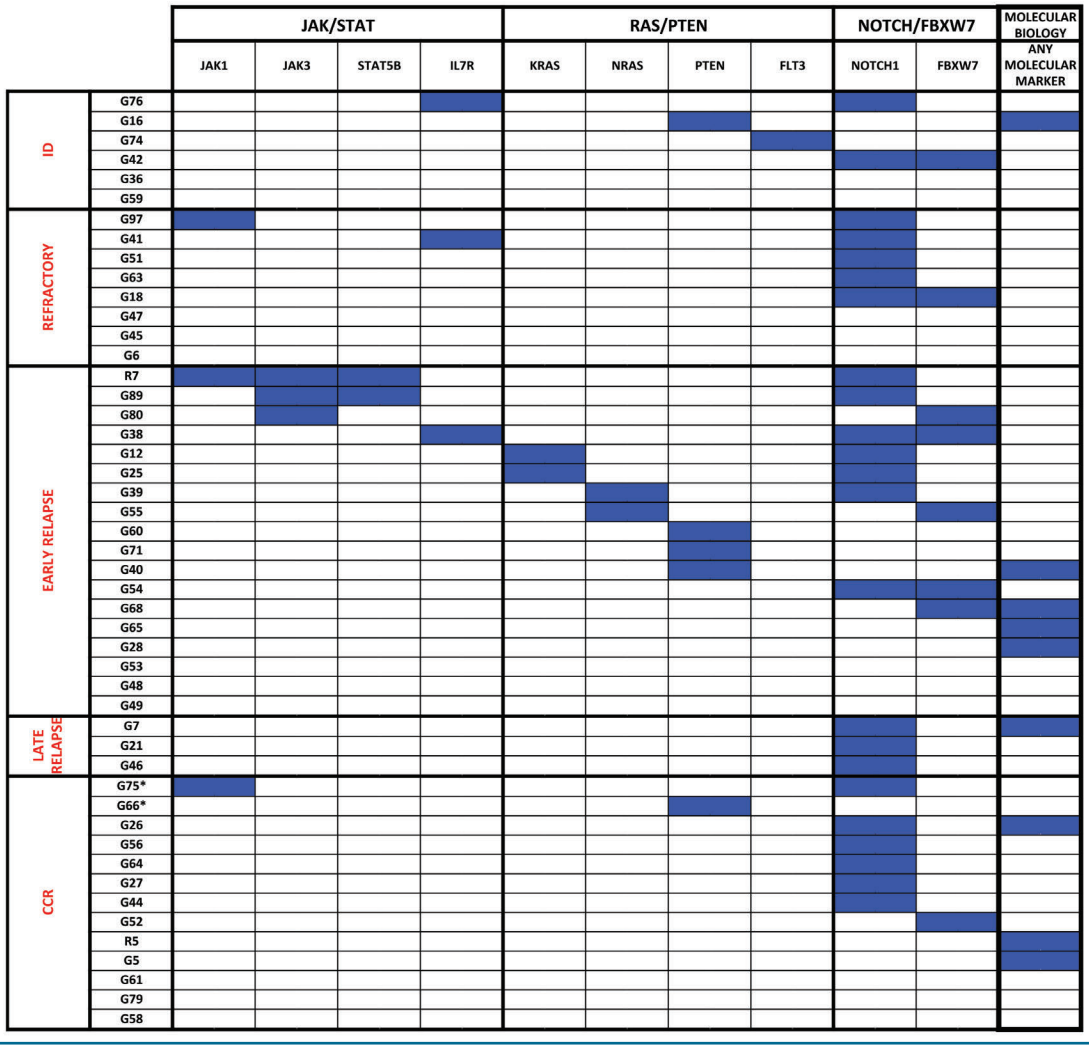

Figure 3. Heatmap of selected targeted pathways in the validation cohort. Heatmap of selected genes belonging to cellular pathways in the validation cohort. Patients are divided according to response to therapy. Blue boxes indicate the presence of a lesion. ID: induction death; CCR: continuous complete remission; *Patient lost from follow-up.

Similarly, a significantly shorter overall survival (15% at 20 months, 95% CI: 80.4%-2.8%, median 7.9 months, *P*=0.0032) and poorer disease-free survival (12.5% at 20 months, 95% CI: 78.2%-2%; median 4.3 months; *P*=0.0001) and event-free survival (10% at 20 months, 95% CI: 64.2%-1.6%; median 3.3 months; *P*=0.027) were observed in patients harboring RAS/PTEN alterations compared to wild-type patients (Figures 5A-C).

Conversely, patients harboring NOTCH1/FBXW7 mutations only had significantly better overall, disease-free and event-free survivals compared to those of wild-type patients or to patients with concomitant mutations in JAK/STAT or RAS/PTEN (Figure 6A-C). In particular, the overall survival probability was 73.3% at 20 months in patients carrying NOTCH1/FBXW7 mutations alone (95% CI: 99.5%-54%; median not reached; *P*=0.0325) compared to 50.1% (95% CI: 78.3%-32.1%; median 27.3 months) in wild-type patients and 0% (median 15.7 months) in patients with concomitant mutations in JAK/STAT or RAS/PTEN. Likewise, the disease-free and event-free survival probabilities were 81.8% (95% CI: 100%-61.9%; median not reached; *P*=0.0015) and 60% (95% CI: 90.7%-39.7%; median 36.8 months; *P*=0.03724) at 20 months in patients carrying NOTCH1/FBXW7 mutations alone compared to 46.2% (95% CI: 83%-25.7%; median 14.5 months) and 30% (95% CI: 58.6%-15.4%; median 7.3 months) in wild-type patients and 0% (median 9.8 months) and 0% (median 6.3 months) in patients with concomitant mutations in JAK/STAT or RAS/PTEN. Thus, these latter findings indicate that the favorable impact of NOTCH1/FBXW7 was overuled by the concomitant presence of JAK/STAT or RAS/PTEN mutations.

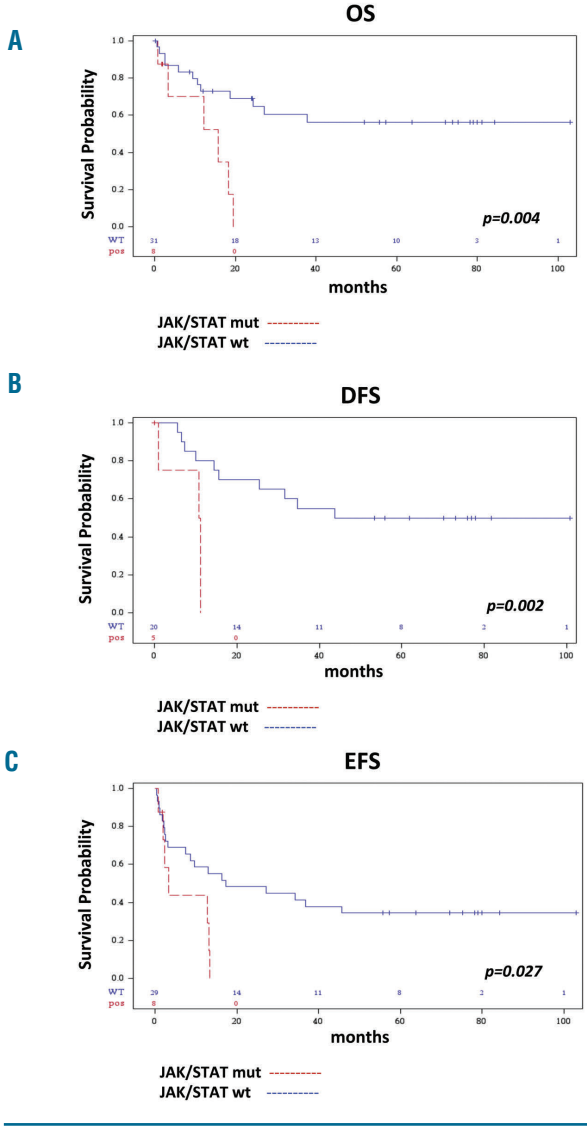

Figure 4. Clinical relevance of JAK/STAT mutations in the T-ALL validation cohort. Kaplan-Meier estimates of (A) overall survival (OS), (B) disease-free survival (DFS) and (C) event-free survival (EFS) in the validation cohort, according to JAK/STAT mutation status. Significantly shorter OS, DFS and EFS were observed in JAK/STAT-positive patients than in JAK/STAT-negative patients.

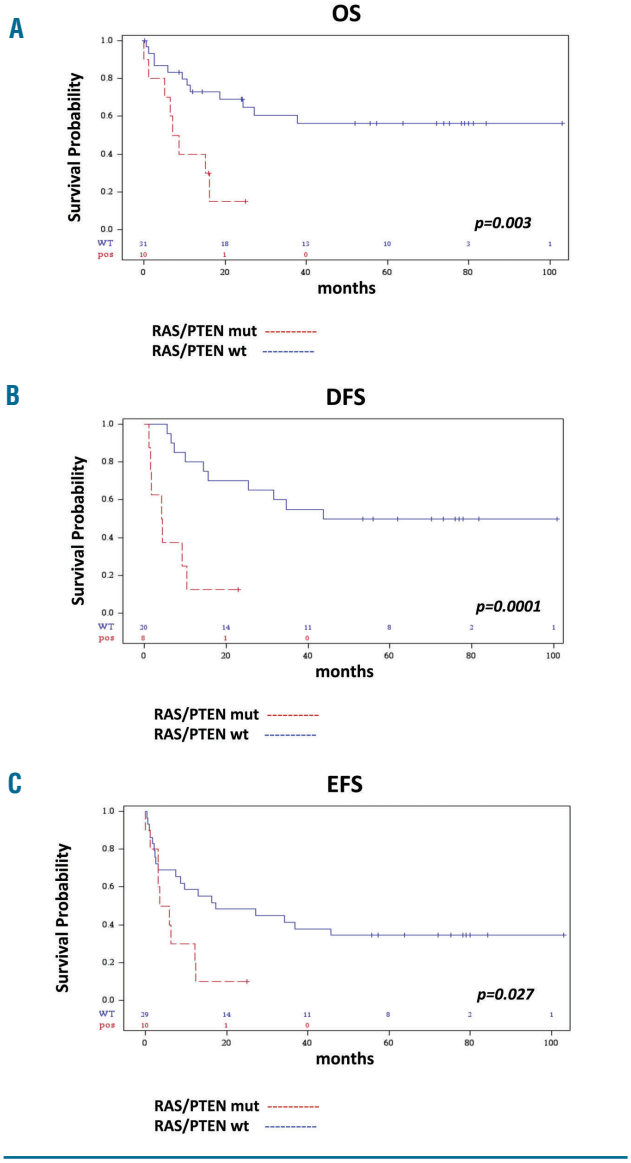

Figure 5. Clinical relevance of RAS/PTEN mutations in the T-ALL validation cohort. Kaplan-Meier estimates of (A) overall survival (OS), (B) disease-free survival (DFS) and (C) event-free survival (EFS) in the validation cohort, according to the RAS/PTEN mutational status. Significantly shorter OS, DFS and EFS were observed in RAS/PTEN-positive patients than in RAS/PTEN-negative patients.

Efficacy of target specific inhibition in refractory/relapsed cases

The *in vitro* effects of specific inhibitors were evaluated on primary cells from T-ALL patients with targetable genetic lesions, as detailed in the *Online Supplementary Results*. These experiments documented a specific effect on cell proliferation and viability of ruxolitinib in three cases with *JAK1* mutations; this effect was less pronounced in cases harboring more than one mutation in the pathway.

We also tested the sensitivity of primary cells to crenolanib and quizartinib (FLT3/PDGFR inhibitors) and observed a decreased cell survival in the case overexpressing *FLT3*.

Finally, decreases in proliferation and viability rate were observed in the single case with a *PTEN* frameshift mutation upon exposure to the PI3K/mTOR inhibitor BEZ235 or rapamycin.

Discussion

A more refined genetic characterization at diagnosis of adult T-ALL may optimize the prognostic stratification and may enable the design of more targeted anti-leukemic strategies. To shed light onto the genome of chemorefractoriness and to identify biological pathways responsible for and/or predictive of drug resistance, we used RNAseq to analyze a series of refractory/early relapsed cases of T-ALL, sampled at diagnosis. RNAseq is a powerful technique since it provides information on fusion genes, gene expression levels and point mutations, and it has been successfully applied to study T-ALL samples.[15] In the present cohort of refractory/relapsed cases we identified a high number of lesions, strengthening the previous observation that the genetic complexity is correlated with an increased likelihood of drug resistance.[9,27,28] Besides *SET-NUP214* fusions, out-of-frame fusions resulting in deregulated expression or in inactivation of transcriptional regulators or tumor suppressor genes were detected, including four TCR fusions. A novel rearrangement joining *TRAC* to *SOX8* on 16p13 was identified. This fusion was associated with transcriptional activation of *SOX8* and points to *SOX8* being a novel driver in T-cell leukemogenesis.

RNAseq analysis also revealed other mechanisms involved in gene expression deregulation. In fact, a novel non-TCR translocation joining *HOXA11-AS* to *MIR181A1HG* on chromosome 1q32 was associated with overexpression of *HOXA* genes; interestingly, the 1q32 region was recently described to be rearranged with *MYC* in a case of T-ALL[29] and might represent a novel region of chromosomal rearrangement holding actively transcribed sequences. In addition, RNAseq allowed the identification of complex intrachromosomal 19p13 rearrangements and amplifications producing an out-of-frame *MAST3-C19orf10* fusion, probably causing PTEN inactivation, and amplification and overexpression of *NOTCH3* and *JAK3*, both located on 19p13. Importantly, most of the validated fusions were not detected by conventional cytogenetics but were later confirmed by FISH, thus corroborating the power of RNAseq to identify fusion transcripts even in cases with uninformative or normal kariotyping.

Mutational analysis of refractory/relapsed T-ALL cases revealed the frequent co-occurrence of lesions affecting the same pathway and/or different pathways, suggesting that different lesions might cooperate in the acquisition of an aggressive phenotype. We observed a higher frequency of mutations resulting in aberrant JAK/STAT and RAS/PTEN signaling (47% and 42%, respectively). In contrast, the incidence of *NOTCH1/FBXW7* mutations (47%) was lower in refractory/relapsed T-ALL than in the general unselected T-ALL population and these mutations were mostly found in combination with a second lesion in the pathway or different pathways, in line with their association with a favorable prognosis.[30-33] Moreover, we observed that *NOTCH1/FBXW7* status could differ between matched diagnostic and relapse samples, indicating that *NOTCH1/FBXW7* mutations might be secondary events in T-ALL.

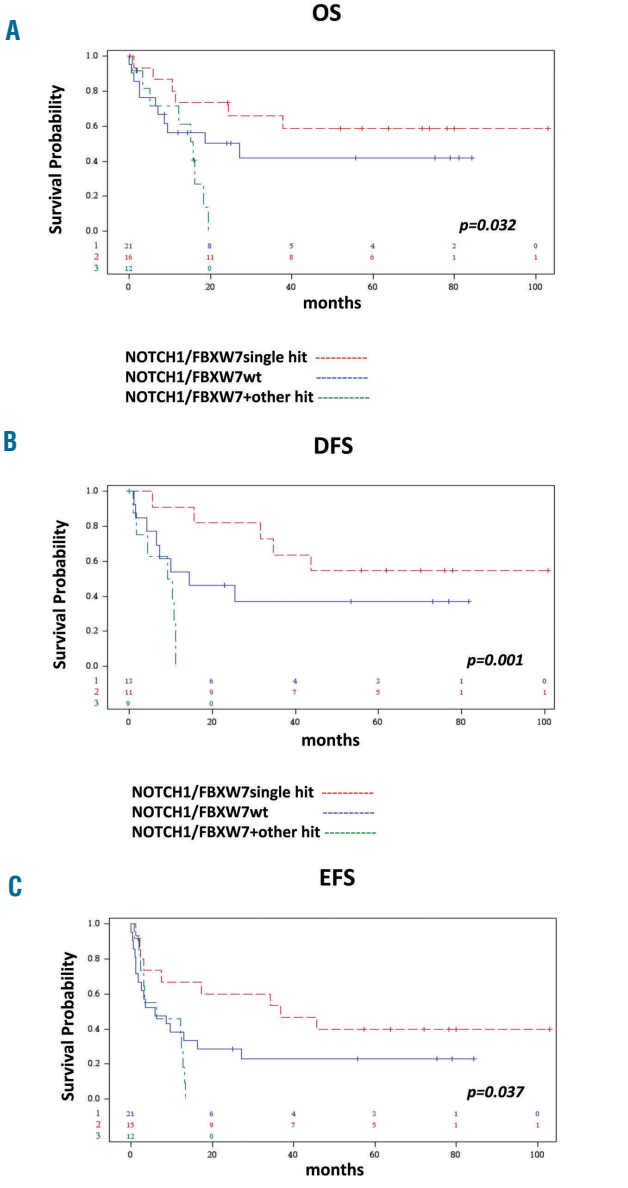

Figure 6. Clinical relevance of NOTCH1/FBXW7 mutation in the T-ALL validation cohort. (A) Kaplan-Meier estimates of overall survival (OS), (B) disease-free survival (DFS) and (C) event-free survival (EFS) in the validation cohort, according to the NOTCH1/FBXW7 mutation status. Significantly better OS, DFS and EFS were observed in patients harboring NOTCH1/FBXW7 mutations alone than in NOTCH1/FBXW7 negative patients or patients with concomitant mutations in K/N-RAS or JAK/STAT.

Activating mutations of *JAK1*, *JAK3* or *IL7R* have been reported in both B- and T-ALL, in up to 25% of cases.[34-40] It has been shown that *JAK3* and *IL7R* mutants promote cell transformation and tumor formation, and that the use of selective JAK inhibitors can reduce cell viability and tumor burden.[36,41,42] Several studies have described an enrichment of mutations in genes mediating JAK/STAT signaling in early T-cell precursor leukemia, a subgroup of ALL associated with a poor response to standard chemotherapy.[39,42] However, it is unclear whether JAK/STAT mutations affect T-ALL outcome. Contradictory results have been reported for *JAK1* mutations in adults, probably due to different chemotherapeutic approaches.[34,43] Zenatti *et al.* found no association between *IL7R* mutational status and clinical outcome in childhood.[36] Conversely, Bandapalli *et al.* identified a high rate of STAT5B mutations in relapsed pediatric patients.[44]

In the present study, RNAseq analysis revealed a high incidence of mutations in the JAK/STAT pathway in refractory/relapsed cases, indicating that these lesions are a hallmark of very poor prognosis in T-ALL. The majority of mutations detected involve *JAK3*, in line with the observation that *JAK3* mutations are drivers of T-ALL.[41]

All cases carrying *JAK1* and *JAK3* mutations harbored at least one other lesion in the same pathway or another pathway. Although conducted in a small number of cases, *in vitro* experiments showed that primary *JAK1*-mutated T-ALL cells may be susceptible to the anti-*JAK1-2* inhibitor ruxolitinib. Moreover, cells with a *JAK1* mutation alone were more sensitive than cases harboring additional mutations in the pathway, as observed in cell lines.[45]

As already noted for JAK/STAT, *RAS/PTEN* alterations are a common feature of T-ALL; their prognostic impact is still controversial in childood.[46-49] In particular, in their study of children treated with the Berlin-Frankfurt-Munster protocol, Bandapalli *et al.* reported that patients with *PTEN* and *NOTCH1* mutations had a marked sensitivity to induction treatment and excellent long-term outcome, similar to that of patients with *NOTCH1* mutations only and more favorable than that of patients with *PTEN* mutations only.[48] The study of Jenkinson *et al.* on pediatric patients treated on the Medical Research Council UKALL2003 trial showed that neither *PTEN* nor *RAS* genotypes have a significant impact on response to therapy or long-term outcome; it was also shown that neither *PTEN* nor *RAS* genotypes affect the highly favorable outcome of patients with concomitant *NOTCH1/FBXW7* mutations.[49] Conversely, the GRAALL group reported that the favorable prognostic significance of *NOTCH1/FBXW7* mutations was restricted to adult patients without *RAS/PTEN* abnormalities. In fact, *K/N-RAS* mutations and/or *PTEN* gene alterations have been associated with poor prognosis.[33,50] In line with this, we identified a high rate of alterations involving the RAS/PTEN pathway in the discovery cohort of highly unfavorable cases.

To evaluate the prognostic impact of these lesions, the mutational analysis was extended to a validation cohort of 49 adolescent, young adult and adult patients enrolled in two consecutive GIMEMA protocols, in which the induction treatment was similar. The series included refractory, relapsed and responsive cases. The overall incidence of JAK/STAT and RAS/PTEN alterations within the validation cohort was 16% and 20%, respectively. In the validation cohort, JAK/STAT and RAS/PTEN alterations were again associated with a significantly higher risk of relapse (P=0.002 and P=0.0001, respectively) and inferior 20-month overall survival (P=0.0045 and P=0.0032, respectively), disease-free survival (P=0.002 and P=0.0001, respectively) and event-free survival (P=0.027 and P=0.027, respectively). In contrast, the presence of NOTCH1/FBXW7 mutations was associated with a better response to treatment and a reduced risk of relapse. Notably, the favorable impact of NOTCH1/FBXW7 mutations was lost in the presence of concomitant JAK/STAT or RAS/PTEN mutations, suggesting that mutations activating these pathways are important for T-ALL progression and prognosis, in agreement with the GRAAL report.[50] Thus, the screening confirmed that the genetic profile is a predictor of outcome in T-ALL, at least when using standard chemotherapy regimens. Recently, no adverse effect on prognosis was observed in the UKALL2003 trial for T-ALL patients with *JAK1*, *JAK3* or *IL7R* mutations:[40] it must be underlined however, that the patients were treated with very intensive chemotherapy and very few relapses were observed in general.

In conclusion, we provide a comprehensive overview of recurrent lesions present in cases of refractory/early relapsed T-ALL and have identified pathways with a prognostic role, which could be targeted by novel therapeutic agents. We also describe a high rate of *JAK3* mutations in refractory/relapsed T-ALL cases, as well as a frequent, often concomitant, deregulation of the JAK/STAT, RAS/PTEN and NOTCH1/FBXW7 pathways; the presence of additional JAK/STAT/RAS/PTEN mutations appears to be noxious. Thus, accurate genetic characterization of T-ALL at diagnosis is recommended in order to define individual patients' risk optimally and to identify patients who might benefit from more intensive treatment or combined targeted approaches. Preliminary *in vitro* experiments suggest that the use of specific inhibitors might be clinically valuable depending on the underlying lesions and that a suboptimal response might be sustained by the presence of a second hit. Finally, the marked overlap of mutations suggests that combinations of specific inhibitors targeting different pathways might prove useful for these patients and prospective studies are currently underway to address this issue.

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