Targeted sequencing identifies associations between IL7R-JAK mutations and epigenetic modulators in T-cell acute lymphoblastic leukemia

Carmen Vicente,[1,2] Claire Schwab,[3] Michaël Broux,[1,2] Ellen Geerdens,[1,2] Sandrine Degryse,[1,2] Sofie Demeyer,[1,2] Idoya Lahortiga,[1,2] Alannah Elliott,[3] Lucy Chilton,[3] Roberta La Starza,[4] Cristina Mecucci,[4] Peter Vandenberghe,[1] Nicholas Goulden,[5] Ajay Vora,[6] Anthony V. Moorman,[3] Jean Soulier,[7] Christine J. Harrison,[3,8] Emmanuelle Clappier,[7,8] and Jan Cools[1,2,8]

[1]Center for Human Genetics, KU Leuven, Belgium; [2]Center for the Biology of Disease, VIB, Leuven, Belgium; [3]Leukaemia Research Cytogenetics Group, Northern Institute for Cancer Research, Newcastle University, Newcastle-upon-Tyne, UK; [4]Hematology Unit, University of Perugia, Polo Unico S.M. Misericordia, Italy; [5]Department of Haematology, Great Ormond Street Hospital, London, UK; [6]Department of Haematology, Sheffield Children's Hospital, Sheffield, UK; and [7]U944 INSERM and Hematology Laboratory, St-Louis Hospital, APHP, Hematology University Institute, University Paris-Diderot, Paris, France

ABSTRACT

T-cell acute lymphoblastic leukemia is caused by the accumulation of multiple oncogenic lesions, including chromosomal rearrangements and mutations. To determine the frequency and co-occurrence of mutations in T-cell acute lymphoblastic leukemia, we performed targeted re-sequencing of 115 genes across 155 diagnostic samples (44 adult and 111 childhood cases). *NOTCH1* and *CDKN2A/B* were mutated/deleted in more than half of the cases, while an additional 37 genes were mutated/deleted in 4% to 20% of cases. We found that *IL7R-JAK* pathway genes were mutated in 27.7% of cases, with *JAK3* mutations being the most frequent event in this group. Copy number variations were also detected, including deletions of *CREBBP* or *CTCF* and duplication of *MYB*. *FLT3* mutations were rare, but a novel extracellular mutation in FLT3 was detected and confirmed to be transforming. Furthermore, we identified complex patterns of pairwise associations, including a significant association between mutations in *IL7R-JAK* genes and epigenetic regulators (*WT1*, *PRC2*, *PHF6*). Our analyses showed that *IL7R-JAK* genetic lesions did not confer adverse prognosis in T-cell acute lymphoblastic leukemia cases enrolled in the UK ALL2003 trial. Overall, these results identify interconnections between the T-cell acute lymphoblastic leukemia genome and disease biology, and suggest a potential clinical application for JAK inhibitors in a significant proportion of patients with T-cell acute lymphoblastic leukemia.

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) comprises a group of aggressive hematologic malignancies accounting for 10%-15% of cases of pediatric ALL and 25% of adult ALL cases; these disorders are more frequent in males than females.[1-3] The development of T-ALL is a multi-step process in which different genetic lesions accumulate and alter the mechanisms controlling proliferation, survival, cell cycle and differentiation of T cells. Loss of the *CDKN2A (p16)* locus and aberrant *NOTCH1* signaling constitute the most predominant oncogenic lesions involved in the pathogenesis of T-ALL. Deletions of the *CDKN2A* locus in chromosome band 9p21 are present in up to 70% of T-ALL,[4] while up to 60% carry *NOTCH1* activating mutations.[1] *NOTCH1* mutations lead to ligand-independent cleavage and activation of the intracellular NOTCH1 part (ICN) and/or to stabilization of active protein.[2] NOTCH1 is an essential protein for T-cell development and aberrant activation of NOTCH1 in T-ALL affects many different pathways including the cell cycle, NFκB and PI3K/AKT pathways. In addition, T-ALL cases harbor chromosomal rearrangements that result in aberrant expression of transcription factor genes, such as *TLX1*, *TLX3*, *TAL1*, *LMO2*, and *HOXA*.[5-10] These chromosomal aberrations are often used to classify T-ALL into subclasses associated with expression of one of these transcription factors, which in many cases also resembles a specific block in differentiation.[11]

The mutational landscape of T-ALL also includes somatic mutations of *IL7R*, *JAK3*, *JAK1*, *PTEN*, and *NRAS* signaling proteins.[12-15] Activating mutations in *JAK1*, *JAK3* or *IL7R* lead to activation of the JAK/STAT pathway, resulting in stimulation of proliferation and survival pathways in the leukemic cells. Recent next-generation sequencing studies identified further recurrent mutations in genes encoding for proteins involved in mRNA degradation and translation (*CNOT3* and *RPL10*) and proteins implicated in the regulation of chromatin structure, including histone demethylases (*KDM6A/UTX*), and members of the polycomb repressive complex 2 (PRC2: genes *EZH2*, *SUZ12* and *EED*).[16-19] Sequencing studies have suggested that on average 10 to 20 protein-altering mutations can be detected in T-ALL cells,[18-20] but their exact frequency and patterns of co-occurrence have not been investigated in detail in large T-ALL cohorts.

Here, we used Haloplex targeted DNA capture followed by Illumina massive parallel sequencing to investigate the coding sequence of 115 recurrently mutated genes in 155 T-ALL samples. Our results revealed that 40 genes had genetic alterations (combining sequence mutations with copy number variations) in more than 4% of cases. Our comprehensive sequence analysis identified mutations/copy number variations of the IL7R-

 doi:10.3324/haematol.2015.130179
The online version of this article has a Supplementary Appendix.
Manuscript received on May 8, 2015. Manuscript accepted on July 9, 2015
Correspondence: jan.cools@cme.vib-kuleuven.be, christine.harrison@newcastle.ac.uk, emmanuelle.clappier@sls.aphp.fr

JAK signaling pathway members in 27.7% of T-ALL samples screened; an observation with therapeutic potential. Statistically significant pairwise associations were found between different mutations, indicating the presence of functional interactions among different pathways in T-ALL pathogenesis. Of significance, we found a mutually exclusive relationship between IL7R-JAK mutations and the presence of *TAL1/LMO2* rearrangements. We also identified positive correlations among IL7R-JAK mutations and mutations/deletions in *PHF6* and members of the PRC2 complex. Our findings begin to unravel the diversity of genetic lesions that are implicated in the development of T-ALL.

Methods

DNA samples

T-ALL samples from patients (n=155: 111 children, 44 adults) were collected from various institutions (*Online Supplementary Table S1*). The diagnosis of T-ALL was based on morphology, cytochemistry and immunophenotyping according to World Health Organization criteria. Genomic DNA was isolated from bone marrow (either fixed or fresh bone marrow cells).[5,21,22]

To investigate the prognostic relevance of *IL7R*, *JAK1* and *JAK3* mutations, Sanger sequencing was used to screen for mutations in these three genes in an independent cohort of 78 T-ALL patients. Those patients were all enrolled into the United Kingdom (UK) Children's Cancer and Leukaemia Group (CCLG) ALL2003 trial.[23] This study was approved by the ethics committees of the institutes involved and informed consent was obtained from the participants. Samples and clinical data were stored in accordance with the declaration of Helsinki.

Capture design

SureDesign software was used to design two slightly different Haloplex capture assays (Table 1). The total amplicon number for design A was 23,127 with a region size of 472.006 kbp and a predicted target coverage of >99%. For design B the total amplicon number was 19,694 with a region size of 418.373 kbp and a predicted target coverage of >99%. For this study, 80 samples were processed with design A and 75 samples with design B. In both assays, the coding exons of selected genes (based on RefSeq, CCDS and VEGA databases) were targeted with an extra ten bases upstream and downstream. Targeted regions comprised the coding sequence of genes that were either recently identified as recurrently mutated in ALL or other hematologic malignancies (known driver genes) or were similar to known oncogenes (candidate driver genes) to be sequenced.[18,19,24,25] For statistical analyses we only considered the 115 genes that were sequenced in both Haloplex designs (*Online Supplementary Table S2*). Library preparation and sequencing were performed as described in the *Online Supplementary Material*.

Data analyses

In NextGENe software (v2.2.1, Softgenetics, State College, PA, USA), we performed the following steps: (i) the fastQ output file was converted into a FASTA file to eliminate reads that were not "paired" and that did not meet the criteria of the default settings; (ii) reads from the converted unique FASTA file were aligned to the reference genome (Human_v37.2). After alignment a *.pjt file was created and opened in the NextGENe Viewer; (iii) a mutation report was created using the coordinates from the targeted enrichment kit as a *.bed file to enable calling of single nucleotide variants and small insertion/deletions (indels) in the regions of interest; and (iv) an expression report was created from which the mean, minimal and maximal coverage per target and targeted nucleotide were calculated. The coverage was defined as the average number of reads representing a given nucleotide in the reconstructed sequence. To interpret the data, additional custom-filtering criteria were imposed to minimize false-positive rates (*Online Supplementary Material*). Polymorphisms annotated in the dbSNP138 or 1000 Genomes databases were excluded from the analyses. For variant calling we also required a minimum read depth of 20 and an allele frequency of at least 15%.

In vitro cell experiments

JAK3 wild type cDNA and mutants were generated by GenScript, and were cloned into the MSCV-GFP vector. Viral vector production, retroviral transduction and culture of Ba/F3 cells were performed as previously described.[15] Western blot analyses were performed as described in the *Online Supplementary Material*.

Results

Sequencing metrics and validation of the gene panel

Next-generation sequencing studies have contributed significantly to our understanding of the genomic landscape of T-ALL. We recently profiled two cohorts of T-ALL using exome sequencing (67 cases)[18] and RNA-sequencing (31 cases),[24] while Zhang and colleagues profiled immature T-ALL cases (also known as early thymic or T-cell precursor T-ALL, ETP-ALL) using whole genome sequencing (12 cases)[19] and targeted re-sequencing in 94 childhood T-ALL cases. To determine the spectrum of mutations present in both adult and childhood T-ALL, we selected 155 T-ALL cases (44 adult/111 childhood) to be analyzed by targeted re-sequencing. These cases were derived from two different cohorts from France (n=80) and the UK (n=75). Based on all available sequence data, we selected 115 genes that were recurrently mutated in ALL or other hematologic malignancies.

Table 1. Summary of targeted sequencing of 155 T-ALL samples.

	Design A (n=80 T-ALL cases)	Design B (n=75 T-ALL samples)
Design information		
Number of genes in design	149	141
Region size of the design (kbp)	472.006	418.373
Total amplicons in design	23127	19694
Minimum sequencing required (Mbp)	247.7	229.35
Sequence information		
Reads passing filtration (Gb)	**8.7** (3.0 - 25.0)	**4.2** (1.4 - 14.5)
Reads mapped (%)	**98.1** (90.4 - 99.1)	**79.6** (41.4 - 96.2)
Capture efficiency (%)*	**60.8** (55 - 66.3)	**45.5** (23.2 - 56.5)
Mean depth	**2312** (733 - 6580)	**959** (199 - 3586)
≥ 10x coverage (%)†	**97.9** (94.9 - 99.0)	**97.0** (91.2 - 98.6)
≥ 20x coverage (%)†	**97.1** (92.6 - 98.8)	**95.6** (85.6 - 98.2)
≥ 50x coverage (%)†	**94.9** (87.0 - 98.0)	**91.8** (73.1 - 97.2)
≥ 100x coverage (%)†	**91.9** (79.7 - 94.3)	**85.3** (57.5 - 96.2)
≥ 200x coverage (%)†	**86.6** (66.7 - 94.3)	**73.5** (31.1 - 94.4)

*Data presented are median (0.25~0.75 quartile). *Capture efficiency (%), the ratio of the read number that mapped to the targeted region to the total read number. †≥ 10x, 20x, 50x, 100x, 200x coverage (%), the ratio of target regions covered 10, 20, 50, 100 or 200x the total number of target regions.*

We used Haloplex enrichment to capture all exons of the selected genes, followed by Illumina sequencing. Two slightly different designs were used to profile the two T-ALL cohorts, which yielded a total of 12.9 Gb of sequence, with capture efficiencies of 45% to 60%. The average coverage per sample was 209X. All genes were covered at >20X for at least 95% of their coding regions (Table 1). After excluding sequencing/mapping errors and known polymorphisms, 685 single nucleotide variants or small indels were identified in 103 genes as high-probability changes.

In order to validate the accuracy of our Haloplex assay and bioinformatics analyses (which we refer to as Haloplex analyses), we sequenced 158 variants across 33 genes using Sanger sequencing (*Online Supplementary Table S3*). We confirmed 137 of the 158 predicted single nucleotide variants and indels (86.7%). Furthermore, we also found that 124 additional single nucleotide variants and indels (although not validated here by Sanger sequencing), were already described and documented in the COSMIC database.[26] To determine the sensitivity of Haloplex for the detection of single nucleotide variants and indels, we examined sequence mutations in *CNOT3* (exon 5, n=3/79) and *RPL10* (exon 4, n=2/79) and indels in *IL7R* (n=12/155) and *FLT3* (n=1/155) genes, for which Sanger sequencing data were available. Haloplex identified all known point mutations in *CNOT3* and *RPL10*, but failed to detect six of 12 *IL7R* indels and one of one *FLT3*-ITD (*Online Supplementary Table S3*).

Data from Haloplex target enrichment can also be used to identify copy number variations at the captured loci.[27] To this end, we normalized the coverage of all genes against the on-target mapped nucleotides across samples. First we looked at X chromosome genes (*MTMR8*, *KDM6A*, *PHF6*, *MAGEC3*, *RPL10* and *USP9X*). As shown in Figure 1A, all samples except one (TL91) showed read depths for X chromosome genes consistent with the patients' gender, with females showing an approximately 2-fold higher coverage than males. No copy number aberrations of *MAGEC3* or *USP9X* genes were found. There was evidence for deletion of *KDM6A* (1 sample), *MTMR8* (1 sample) and *PHF6* (4 samples), with possible duplication of *RPL10* (2 samples) or *PHF6* (1 sample) (Figure 1A, *Online Supplementary Table S3*).

To determine the accuracy of Haloplex data for the

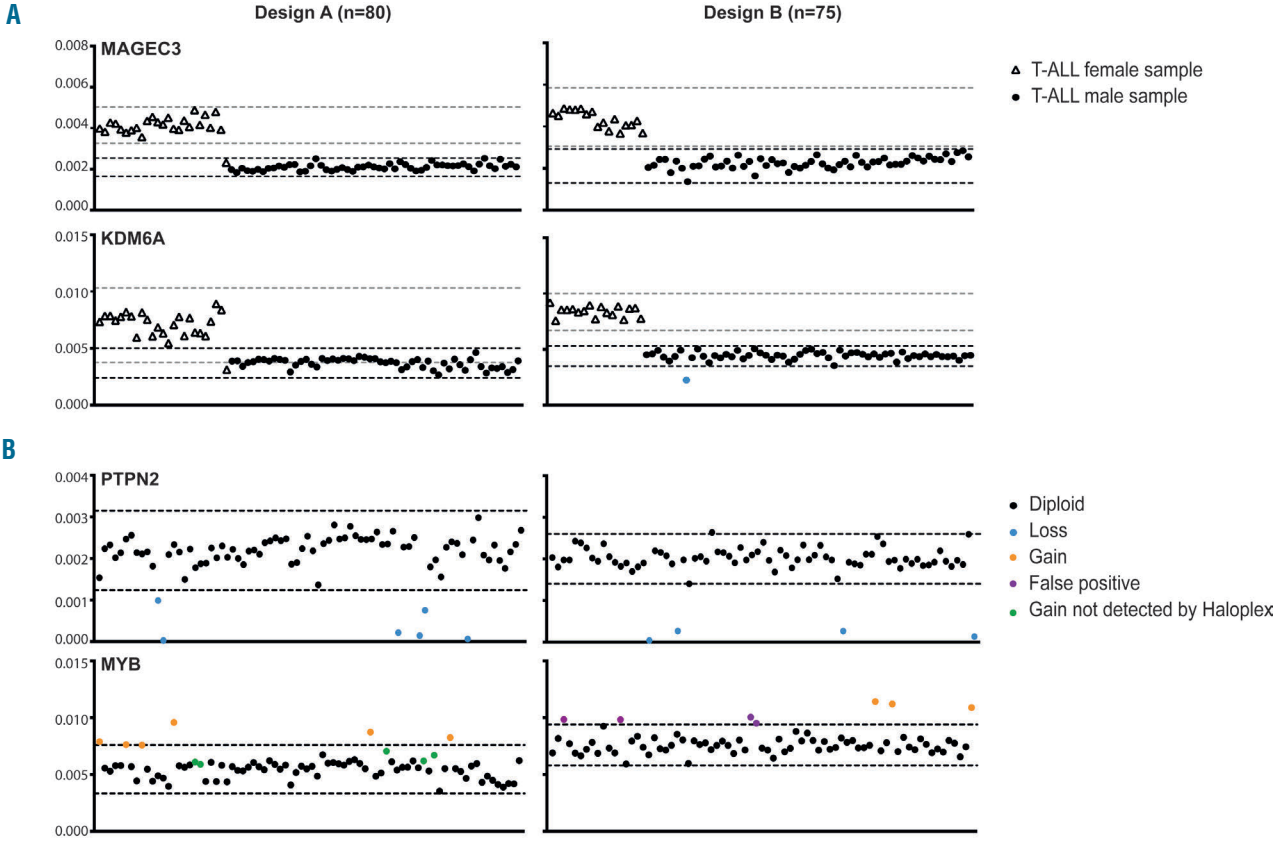

Figure 1. Haloplex target enrichment analyses can be used to identify copy number variations. For *MAGEC3*, *KDM6A*, *MYB* and *PTPN2* genes, the normalized coverage against the on-target mapped nucleotides is plotted for all patients on an arbitrary linear scale. In order to determine real copy number variations, for each gene we calculated the first quartile (Q1), third quartile (Q3) and the interquartile range (IQR= Q1 – Q3) from the normalized coverage values. Samples with a normalized coverage for a particular gene greater than Q3+1.5*IQR were considered to have a duplication/amplification in that gene. Conversely, samples with a normalized coverage less than Q1-1.5*IQR, were considered to carry a deletion. The calculated values for each gene are represented in the graphs as dotted lines. For X-chromosome genes, we did those calculations taking along only male or female samples. (A) Read depths for *MAGEC3* are consistent with the patients' gender (except case number TL91), with females showing an ~2-fold higher coverage than males. While no copy number variations were found in *MAGEC3*, there was evidence for deletion of *KDM6A* in case number 4139. (B) By using Haloplex we were able to confirm the presence of *PTPN2* deletions in all (6/6) *PTPN2*-positive cases (shown as blue dots in the graphs). *MYB* duplications were confirmed in 64% (9/14) of *MYB*-positive cases (orange dots). Haloplex analyses predicted four additional cases carrying *MYB* duplication (purple dots), which were false positive results.

detection of copy number variations, we examined copy number variations at the *MYB* (n= 155), *CDKN2A* (n=95) and *PTPN2* (n=80) loci for which fluorescence *in situ* hybridization, multiplex ligation probe amplification or array comparative genomic hybridization data were available. Overall, 64% (9/14) of cases with *MYB* duplication, 93% (69/74) of cases with *CDKN2A* deletion and 100% (6/6) of cases with *PTPN2* deletion were identified by our Haloplex copy number analysis (Figure 1B, *Online Supplementary Table S4*). Haloplex data predicted four additional cases with *MYB* duplication that had a lower normalized coverage when compared with those samples

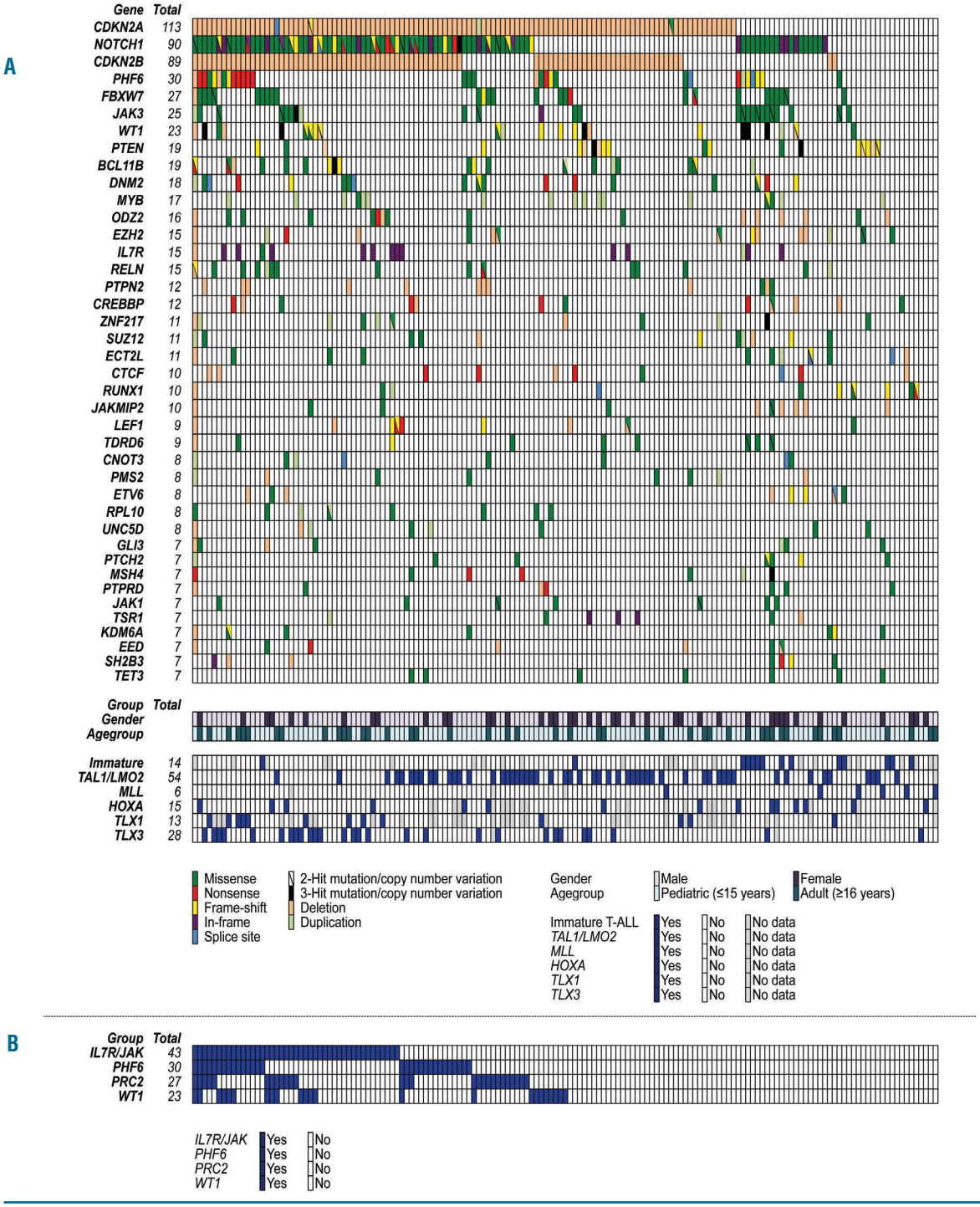

Figure 2. Overview of the genetic lesions identified in the most frequently mutated genes in T-ALL. (A) Single nucleotide variants, indels and copy number variations are shown for the 40 genes we found mutated in more than 4% of the cases in our series. Each type of mutation is indicated by a different color. Age, gender and T-ALL characteristics are also shown for each patient (bottom table). (B) Spectrum of genetic lesions in IL7R-JAK signaling members (*IL7R*, *JAK3*, *JAK1* and *STAT5* genes), *PHF6*, PRC2 complex members (*SUZ12*, *EZH2* and *EED*) and *WT1*. The cases with *PHF6*, PRC2 and *WT1* mutations were more frequently found among IL7R-JAK-positive cases.

with true *MYB* duplications. However, as they were not confirmed by any other method, we concluded that these were false positive results. Together, our data indicated that Haloplex re-sequencing data can be used for the identification of single nucleotide variants and copy number variations with high confidence, with deletions being easier to detect than duplications. The detection of larger indels is difficult, especially for longer insertions.

Landscape of genetic alterations in T-cell acute lymphoblastic leukemia

In total, 153 of the 155 patients' samples (98.7%) harbored at least one sequence mutation/copy number change, with a median number of five (range, 1–65) genetic lesions per sample. Known T-ALL tumor suppressor genes such as *CDKN2A/B*, *PHF6*, *PTEN* and *PTPN2* were frequently identified as common targets of gene deletions. From the 115 genes analyzed, 40 genes showed genetic alterations (single nucleotide variants, indels or copy number variations) in more than 4% of cases (Figure 2). Only *NOTCH1*, *CDKN2A* and *CDKN2B* aberrations were found in more than 50% of cases. Mutation frequencies of *PHF6* (19.4%), *FBXW7* (17.4%), *WT1* (14.8%), *PTEN* (12.3%), *BCL11B* (12.3%) and *PTPN2* (7.7%) were in the range of previously reported frequencies.[2] Our data confirmed that *JAK3* was frequently mutated in T-ALL (16.1%).[19] Other recently identified mutations were also found, including *CREBBP* (7.7%),[28] *CNOT3* (5.2%)[18] and *RPL10* (5.2%).[18] In contrast to the situation in acute myeloid leukemia, *FLT3* mutations were rare among the T-ALL cases, although we identified one new mutation in the extracellular domain of FLT3 (S471C), which was confirmed to be a transforming mutation (*Online Supplementary Figure S1*).

Genes with identified genomic lesions were grouped into functional pathways linked to the pathogenesis of T-ALL (*Online Supplementary Table S2*). The most frequently affected pathway was transcriptional regulation, with mutations observed in as many as 81.9% of cases, followed by genes associated with cell cycle regulation (74.2%), chromatin modification (38.1%), genes encoding kinases (29.7%) and phosphatases (26.5%), and DNA repair pathways (18.7%).

The mean number and spectrum of some of the identified genetic lesions closely correlated with patients' age and T-ALL subtype. Alterations in *PTPN2* were more prevalent among adult cases (18.2% *versus* 3.6%; *P*=0.005), as were those in *MYB* (22.7% *versus* 6.3%; *P*=0.008) and *CREBBP* (15.9% *versus* 4.5%; *P*=0.039). In contrast, *EZH2* and *RPL10* aberrations were found exclusively in children (*EZH2*, 13.5%; *P*=0.006: *RPL10*, 7.2%; *P*=0.106) although the trend did not reach statistical significance for *RPL10*.

We also found new and previously reported correlations between genetic lesions and T-ALL subgroups. Among immature T-ALL cases there were lower incidences of *CDKN2A* (13.3% *versus* 81.9%; *P*<0.001) and *CDKN2B* (13.3% *versus* 66.4%; *P*<0.001,) deletions,[19] and significantly higher incidences of mutations in *JAK3* (53.3% *versus* 12.9%; *P*=0.001),[19] *JAKMIP2* (26.7% *versus* 4.3%; *P*=0.010), *RUNX1* (20% *versus* 2.6%; *P*=0.020)[19] and *IL7R* (26.7% *versus* 7.8%; *P*=0.043).[19] Aberrations of *PHF6* (53.8% *versus* 16.8%; *P*=0.006),[29] *PTPN2* (30.8% *versus* 6.5%; *P*=0.018),[22] and *CDKN2B* (92.3% *versus* 55.1%; *P*=0.014) were more frequently found among *TLX1*-positive cases. *TLX3*-positive cases had significantly more lesions in *CDKN2A* (92.9% *versus* 68.8%; *P*=0.001), *CDKN2B* (85.7% *versus* 52%; *P*=0.001), *DNM2* (28.6% *versus* 8%; *P*=0.006), *WT1* (42.9% *versus* 8.8%; *P*<0.001)[30] and *JAK1* mutations (17.9% *versus* 0.8%; *P*=0.001). The following lesions were under-

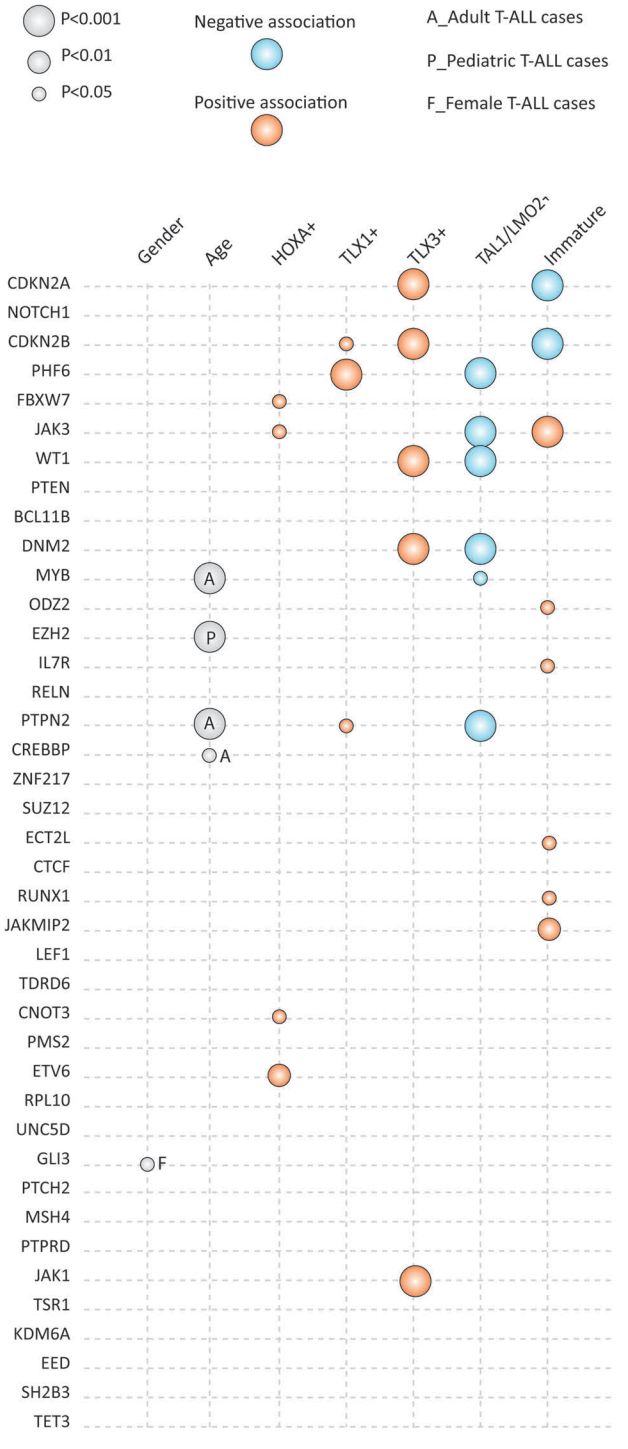

Figure 3. The spectrum of some of the identified genetic lesions closely correlated with patients' characteristics and T-ALL subtypes. Associations between the most frequently mutated genes (top 40 genes, ranked from the most frequently targeted gene) and patients' characteristics/T-ALL subtypes are shown. Associations with *P*-values lower than 0.05, 0.01 and 0.001 are indicated with circles of different sizes. Positive associations are shown in orange, negative associations are shown in blue. Associations that could not be considered as positive or negative are shown in gray. A = adult group, P = pediatric group, F = female group.

represented in *TAL1/LMO2*-positive patients: *PHF6* (7.4% *versus* 26%; *P*=0.005),[29] *JAK3* (3.7% *versus* 23%; *P*=0.002), *DNM2* (18%; *P*<0.001), *PTPN2* (12%; *P*=0.009) and *WT1* (1.9% *versus* 22%; *P*=0.001) (Figure 3).

The IL7R-JAK axis is an important oncogenic pathway in T-cell acute lymphoblastic leukemia

The IL7R-JAK signaling cascade is an essential signaling pathway in hematopoiesis, and somatic mutations in *IL7R*,

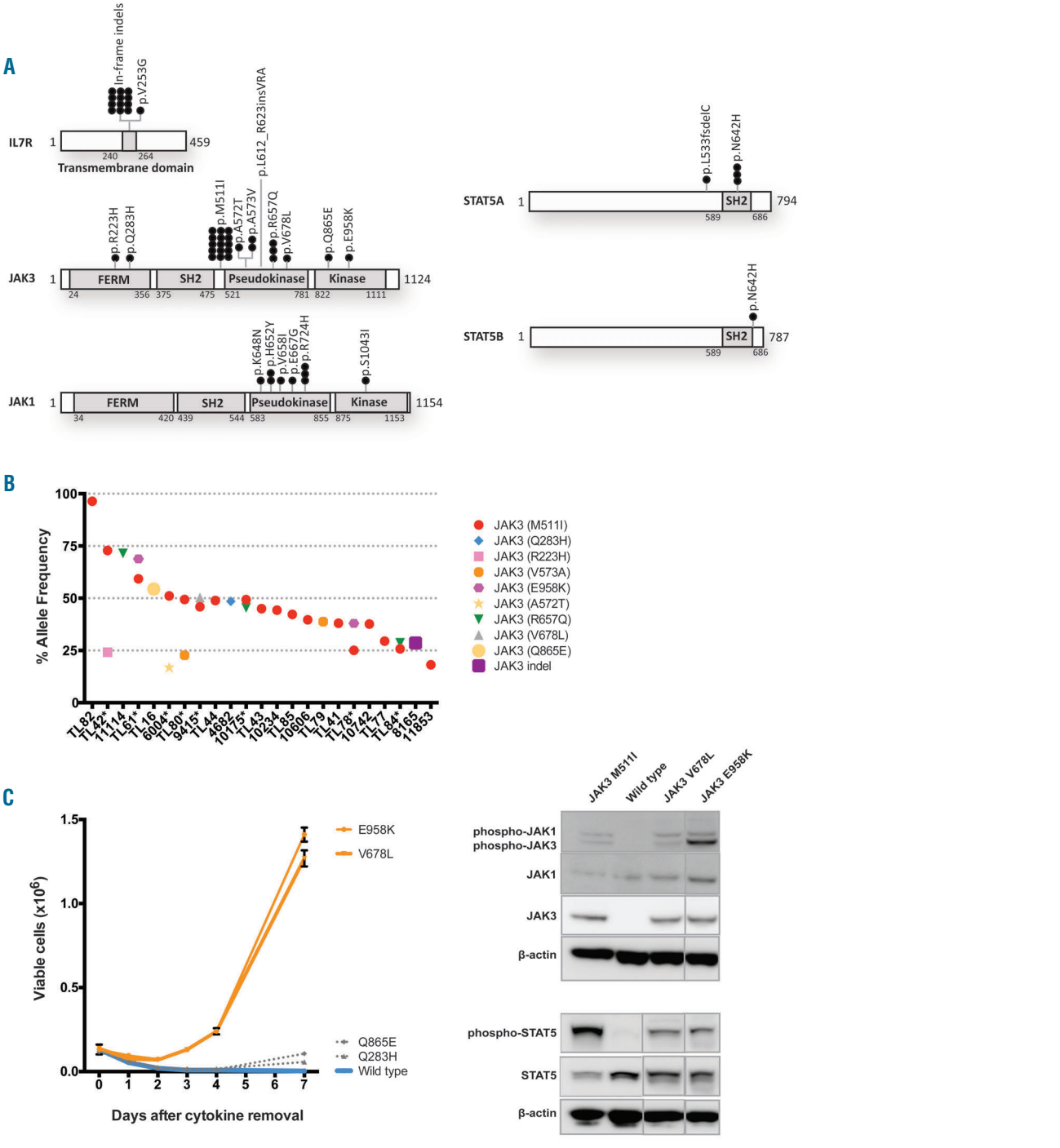

Figure 4. Overview of the identified mutations in the IL7R-JAK signaling pathway members. (A) Schematic view of the IL7R, JAK3, JAK1, STAT5A and STAT5B proteins and their main protein domains. The mutations found in each of those proteins are shown. (B) The variant allele frequencies for *JAK3* mutations are shown in the graph. In eight out of the 23 cases carrying *JAK3* mutations (case numbers indicated with asterisks), the M511I mutation was detected together with a second mutation of *JAK3*. (C) Proliferation curve of Ba/F3 cells expressing various *JAK3* mutants or the empty vector, in the absence of cytokines. Mutations that did not stimulate proliferation more than the empty vector were considered as not transforming mutants. (D) Western blot analysis of whole cell lysates of Ba/F3 cells transformed by the JAK3 mutants V678L and E958K, or empty vector. A protein lysate of Ba/F3 cells transformed by the JAK3 M511I mutant (previously described as transforming) was included as a positive control. Phosphorylation of JAK1 and JAK3 was detected for all JAK3 mutants. JAK3 protein expression was detected with a human specific antibody, not recognizing the endogenous JAK3 expression. The transforming JAK3 mutants were able to phosphorylate STAT5.

JAK1, *JAK3* and *STAT5* have been reported previously in T-ALL.[12,15,18,31] However, the exact frequency of mutations for all members of the signaling pathway is unclear. In our series, sequence mutations/copy number variations of the *IL7R*, *JAK1*, *JAK3* and *STAT5* genes were found in 27.7% of cases, confirming that this signaling pathway is an important oncogenic axis in T-ALL (Figure 4A). Incidences of cases with genetic lesions of *IL7R*, *JAK1*, *JAK3* and *STAT5* genes were 9.7%, 4.5%, 16.1% and 4.5%, respectively. We found that 5.8% of cases carried combinations of these mutations (*Online Supplementary Table S2*).

We identified *JAK3* as the most common targeted member of the pathway (16.1% of T-ALL cases). Most mutations were located within the pseudokinase domain, which has a regulatory function on kinase activity (Figure 4A). The M511I mutation was the most frequent. In 34.7% of JAK3 mutant cases the M511I mutation was detected together with a second mutation of *JAK3* (Figure 4B). Seven patients had mutations/duplications of *IL7R* (n=2), *JAK1* (n=3) or *STAT5* (n=2) in addition to a *JAK3* mutation.

The majority of *JAK3* mutations identified in T-ALL have been confirmed as activating mutations,[15] but the transforming capacity of four JAK3 mutations identified in this study (Q283H, V678L, Q865E and E958K) was not studied previously. We expressed these mutants in Ba/F3 cells and confirmed that JAK3 V678L and Q865E were capable of transforming the cells to IL3-independent growth (Figure 4C). The JAK3 Q283H and Q865E mutants were not transforming (Figure 4C), suggesting that these may be passenger mutations. In summary, our results show that the IL7R-JAK signaling pathway is frequently mutated in T-ALL, with *JAK3* being the most frequently altered gene (14.9% of T-ALL cases harbor transforming *JAK3* mutations).

Table 2. Overall clinical, immunophenotypic and molecular cytogenetic characteristics of T-ALL patients with mutations in the IL7R-JAK signaling pathway.

	IL7R-JAK signaling pathway			
Clinical	Wild-type (n=112)	Mutated (n=43)	*P*	Type of Association
Gender			0.586	
Male	83 (74.1%)	30 (69.8%)		
Female	29 (25.9%)	13 (30.2%)		
Median age (range)	11 (1-63)	12 (2-66)	0.767†	
T-ALL clusters	**Wt**	**Mut**	***P***	
HOXA+ (n=15)	6 (5.4%)	9 (20.9%)	**0.006***	Positive
TLX1+ (n=13)	10 (11.2%)	3 (9.7%)	1.000*	
TLX3+ (n=28)	15 (13.5%)	13 (31%)	**0.013**	Positive
TAL1/LMO2+ (n=54)	48 (43.2%)	6 (14%)	**0.001**	Negative
Immature T-ALL+ (n=14)	4 (4.4%)	10 (25%)	**0.001***	Positive
Del9p21 status	**Wt**	**Mut**	***P***	
Wild-type	27 (24.1%)	13 (30.2%)		
Mutant	85 (75.9%)	30 (69.8%)	0.435	
***PHF6* status**	**Wt**	**Mut**	***P***	
Wild-type	97 (86.6%)	28 (65.1%)		
Mutant	15 (13.4%)	15 (34.9%)	**0.002**	Positive
***PRC2* status**	**Wt**	**Mut**	***P***	
Wild-type	97 (86.6%)	31 (72.1%)		
Mutant	15 (13.4%)	12 (27.9%)	**0.033**	Positive
***WT1* status**	**Wt**	**Mut**	***P***	
Wild-type	102 (91.1%)	30 (69.8%)		
Mutant	10 (8.9%)	13 (30.2%)	**0.001**	Positive

*P: P value. Median age indicated in years. Significant P values are indicated in bold; all P values were calculated using the Pearson's χ^2 test, unless indicated otherwise: *Fisher exact test; †Mann-Whitney U test. The* HOXA+, TLX1+ *and* TLX3+ *and* TAL1/LMO2+ *groups were based on the presence of* HOXA, TLX1, TLX3, TAL1 *or* LMO2 *rearrangements or by having a* HOXA, TLX1, TLX3 *or* TAL1/LMO2 *expression signature. The immature TALL group was defined immunophenotypically or based on gene expression cluster analysis.*

Mutations of the IL7R-JAK axis and epigenetic modulators are associated in T-cell acute lymphoblastic leukemia

Having observed that the components of the IL7R-JAK axis were frequently mutated in T-ALL, we analyzed the possible associations between these mutations and the different T-ALL subgroups (Table 2). Cases with IL7R-JAK mutations were more frequently found among *TLX3*-positive cases (31% *versus* 13.5; *P*=0.013), immature T-ALL cases (25% *versus* 5.5%; *P*=0.002),[19] or *HOXA*-positive cases (20.9% *versus* 5.4%; *P*=0.006), but underrepresented among *TAL1/LMO2*-positive cases (14% *versus* 43.2%; *P*=0.001). Of importance, we observed that mutations of IL7R-JAK were positively associated with mutations in epigenetic factors (42.4% *versus* 18.8%; *P*=0.001). More detailed analyses revealed specific associations between mutations in IL7R-JAK and *PHF6* (34.9% *versus* 13.4%; *P*=0.002), *WT1* (30.2% *versus* 8.9%; *P*=0.001), and members of the PRC2 complex (*EZH2*, *SUZ12* and *EED*) (27.9% *versus* 13.4%; *P*=0.033) (Table 2). Taking into account the two *JAK3* mutations (Q283H and Q865E) that lack transforming potential, the significance of these associations is not altered (*Online Supplementary Material*). Together, these results indicate that functional interactions are present between the IL7R-JAK signaling pathway and epigenetic modifiers (*WT1*, *PHF6*, *PRC2*).

Co-occurring mutations and analysis of clonality in cases with IL7R-JAK mutations

Clonal evolution has been documented in a range of hematologic malignancies.[32] The proportion of sequencing reads reporting a given mutation can be used to identify whether mutations are present in the major clone or a subclone at the time of diagnosis.

Overall, we did not observe any specific genes that were more frequently mutated in subclones. As we had detected a high frequency of *JAK3* mutations, we analyzed the 23 cases with *JAK3* mutations in more detail (Figure 5). Of these cases, 82.6% showed the presence of the *JAK3* mutation in more than 35% of cells, indicating that *JAK3* mutations occur predominantly in the major clone at diagnosis. One of these cases carried a *JAK3* M511I mutation at an allele frequency of 96.4%. As there were no copy number variations and the allele frequency for the single nucleotide polymorphisms present in that genomic region was around 100%, we conclude that copy number neutral loss of heterozygosity was responsible for this homozygous *JAK3* mutation. In cases with two *JAK3* mutations or with *JAK3* and *JAK1* mutations together, one *JAK3* mutation was typically present in the major clone (>40% variant allele frequency in 9 out of 12 cases), with a minor clone having the second mutation (or both mutations). These data confirm that a single *JAK3* mutation can contribute to the development of leukemia, while the acquisition of an additional

JAK3 or *JAK1* mutation is likely to be of benefit for driving the growth of subclones during disease progression.

Mutations of IL7R, JAK3 and JAK1 genes do not have an adverse prognostic impact

To gain insight into the clinical relevance of *IL7R*, *JAK3* and *JAK1* mutations, we analyzed their prognostic significance in a cohort of 78 T-ALL cases treated on the UKALL2003 protocol.[23] Sanger sequencing analyses revealed that ten of the 78 cases (12.8%) carried mutations in IL7R-JAK genes. The seven males and three females were aged 2 to 22 years old and seven were classified as high risk according to National Cancer Institute criteria. All patients achieved complete remission within 28 days but four out of eight had minimal residual disease at this time point. Eight of the ten patients (80%) remain in first complete remission 3.7-6.9 years from diagnosis. The other two patients (both carrying *IL7R* indels) relapsed. One male who presented before his second birthday and who had a very high white blood cell count ($497x10^9$/L) experienced an isolated central nervous system relapse after 9 months and subsequently died. The second patient, a 13-year-old male, experienced an isolated bone marrow relapse after 18 months, but remains alive 3.3 years after an HLA-matched unrelated donor transplant despite failing induction after relapse. In conclusion, this cohort of T-ALL patients fared quite well, and there is no evidence that IL7R-JAK mutations are associated with a poor prognosis.

Discussion

We performed targeted sequencing of a large cohort of T-ALL cases, coupled with a comprehensive study of their genetic background, in order to elucidate molecular lesions and their co-occurrence in patients' samples. We used Haloplex enrichment to sequence the entire coding regions of 115 genes (known T-ALL driver genes or candidate driver genes). We showed that Haloplex sequencing is highly specific and sensitive for the detection of single nucleotide variants. Deletions of entire genes were also detected with a high degree of accuracy. In contrast, the detection of gene duplications and indels, such as *MYB* duplications and insertions into the *IL7R* and *FLT3* genes was less reliable, leading to false positive detection of duplications and false negative detection of indels. Similar findings were recently made for targeted re-sequencing in acute myeloid leukemia, and it was shown that increased coverage could

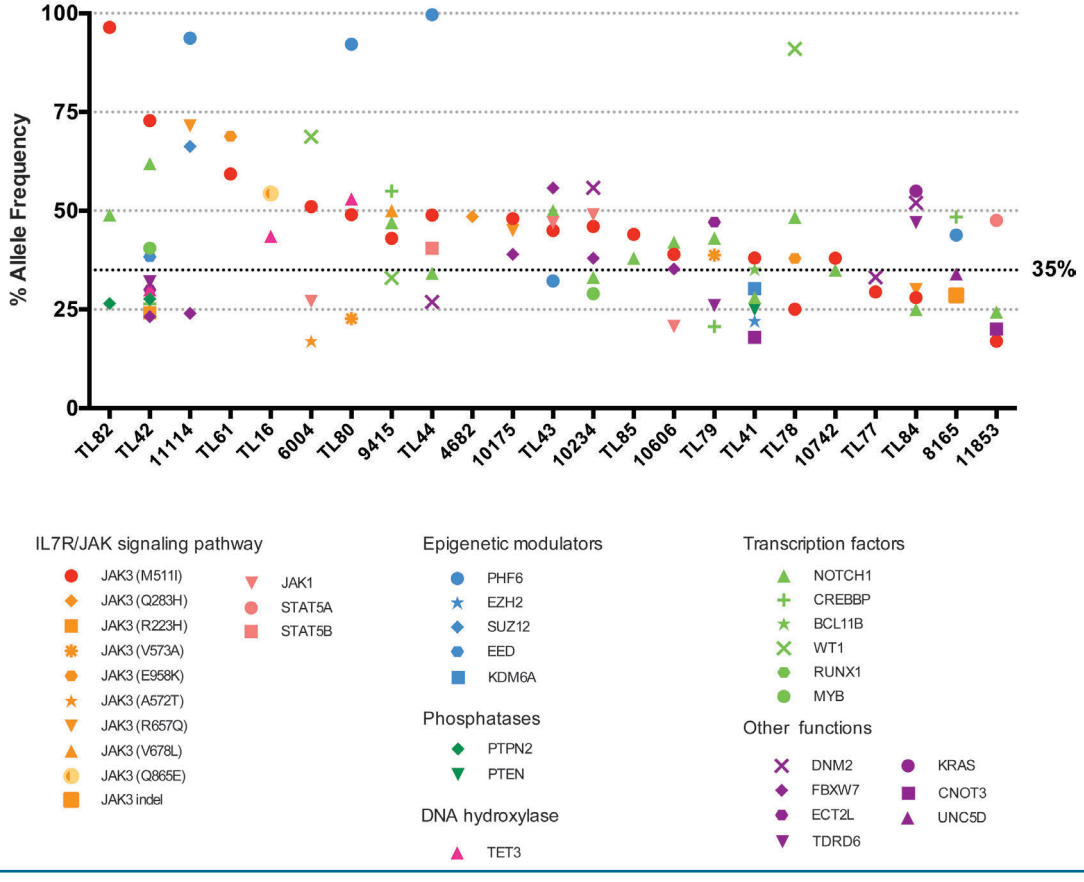

Figure 5. ***JAK3*** **mutations occur predominantly in the major clone at diagnosis. Variant allele frequencies of the mutated genes identified in the 23 cases with** ***JAK3*** **mutations. For the purpose of clarity, only those genes mutated in more than 4% of T-ALL cases are shown.** ***JAK3*** **mutations are predominantly present in the major clone when two** ***JAK3*** **mutations or** ***JAK3*** **and** ***JAK1*** **mutations occur together, while a minor clone has the second mutation.**

improve the identification of copy number alterations in Haloplex assays.[27] Haloplex analyses should not be preferred over array comparative genomic hybridization or multiplex ligation probe amplification techniques for the detection of copy number variations, especially for the detection of duplications.

Visual inspection of the reads for *IL7R* and *FLT3* identified mutant alleles with indels, which had been missed by the analysis software. However, in most cases the mutant reads were missing, suggesting that the mutant exons had not been adequately captured during the target enrichment step. These observations highlight the need for further refinements to the alignment algorithms and the capture design for cancer samples, which will greatly increase the ability to detect large indels such as those present in *IL7R* and *FLT3*.

Once the Haloplex method had been successfully validated, we used this approach to characterize the mutational profile of a cohort of 155 T-ALL cases. Targeted resequencing was performed in the absence of germline/remission matched DNA and this should be considered. However, the fact that we identified known gene mutations/copy number variations at the same frequencies in our T-ALL cohort as those reported in the literature, confirms both the validity of our approach and our cohort. Similar to observations in other tumors, only a few genes (*NOTCH1*, *CDKN2A/B*) were mutated or deleted in >50% of the T-ALL cases, while a large number were mutated in <20% of the cases. These observations corroborate the complexity and variation of events underlying T-ALL malignant transformation.

We observed that the IL7R-JAK signaling pathway was targeted in 27.7% of T-ALL cases. These findings have potential therapeutic implications, as JAK kinase inhibitors are known to target these alterations.[15,33-38] *JAK3* is the most frequently mutated gene (16.1% of T-ALL cases) within the IL7R-JAK signaling pathway. Based on the results described in this article and our previously published work,[15] the transforming capacities of a total of 16 JAK3 mutants have been tested using the Ba/F3 *in vitro* cell system. Six of these 16 JAK3 mutants (R272H, Q283H, R403H, Q865E, R925S and E1106G) lacked transforming potential, illustrating that results from sequencing need to be confirmed by functional assays to distinguish driver mutations from passenger mutations. If we exclude the *JAK3* mutations that lack transforming potential, then the frequency of cases with *JAK3* mutations in our series is 14.9% (instead of 16.1%).

Some T-ALL cases harbored two transforming *JAK3* mutations, which suggests that those T-ALL cells that are dependent on a *JAK3* mutation may gain proliferative advantage by mutating the second *JAK3* allele. In line with this hypothesis, our clonality analysis of *JAK3* mutated cases showed that in those with two *JAK3* mutations or a *JAK3* and *JAK1* mutation together, one *JAK3* mutation was typically present in the major clone, with the second mutation found in a lower percentage of the cells, most likely representing a minor clone.

We also investigated the prognostic impact of mutations of *IL7R, JAK1* and *JAK3*, using data from the UKALL2003 trial, in particular because these mutations occur more frequently in immature T-ALL, a subgroup of T-ALL initially associated with a poor outcome in some studies.[39]

Despite the small subset of patients with *IL7R*, *JAK1* or *JAK3* mutant T-ALL (n=10) in this cohort, it appeared that in general these patients fared well. It should be noted that the outcome of patients with immature T-ALL has improved since initial reports on this subtype, and that more recent data from UKALL2003 and COG AALL0434 suggest patients with immature T-ALL may not have worse outcome when treated with intensified therapy.[23,40] In fact, seven of the ten patients with mutations in the IL7R-JAK pathway received very intensive chemotherapy based on risk stratification. Our data provide no evidence that *IL7R*, *JAK1* and *JAK3* mutations are a consistent marker of poor prognosis. Nevertheless, it might be that immature T-ALL cases harboring IL7R-JAK mutations would benefit from the use of JAK inhibitors in T-ALL trials, since this might reduce the toxic side effects of high-dose chemotherapy. The efficacy of ruxolitinib (a JAK1/2 inhibitor) was recently demonstrated in murine xenograft models of immature T-ALL.[41] Of interest, both JAK/STAT pathway activation and ruxolitinib efficacy were independent of the presence of JAK/STAT pathway mutations, raising the possibility that the therapeutic potential of ruxolitinib in T-ALL extends beyond those cases with JAK mutations.[41] Further studies are warranted to clarify this issue.

Importantly, we found statistically significant associations between gene mutations and signaling pathway-groups or T-ALL patients' characteristics. These include previously described associations between immature T-ALL and *JAK3* mutations,[19] and between *TLX1*-positive cases and *PHF6* mutations/deletions.[29] Our current study points out that mutations in *PHF6*, *WT1* and PRC2 complex members are particularly prevalent in T-ALL cases harboring mutations of the IL7R-JAK signaling pathway. We speculate that such epigenetic mutations could result in chromatin changes that affect the accessibility of STAT5 target genes for transcription, cooperating in this way with the activation of the IL7R-JAK-STAT signaling pathway. Functional studies are needed to understand the underlying mechanism of this association.

Acknowledgments

This work was supported by grants from the FWO-Vlaanderen, the Foundation against Cancer, an ERC-starting grant, the Interuniversity Attraction Poles granted by the Federal Office for Scientific, Technical and Cultural Affairs, Belgium, the Belgian Government Cancer Action Plan, and by the FP7 programme of the European Commission (NGS-PTL, Grant 306242). CV is supported by the European Hematology Association (EHA Research Fellowship Junior Non-clinical award). SD is supported by the Agency for Innovation by Science and Technology in Flanders. MB is supported by an Emmanuel Van der Schueren fellowship, Vlaamse Liga Tegen Kanker. JS acknowledges the financial support of the Canceropole Ile de France, the European Research Council St. Grant Consolidator 311660 and the Saint-Louis Institute program ANR-10-IBHU-0002. CJH and AVM acknowledge the financial support of the Leukaemia & Lymphoma Research and member laboratories of the UK Cancer Cytogenetic Group (UKCCG) for providing data. Primary childhood leukemia samples from the ALL2003 trial were provided by the Leukaemia and Lymphoma Research Childhood Leukaemia Cell Bank.

Authorship and Disclosures

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

References

1. Weng AP, Ferrando AA, Lee W, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science. 2004;306(5694):269-271.
2. Van Vlierberghe P, Ferrando A. The molecular basis of T cell acute lymphoblastic leukemia. J Clin Invest. 2012;122(10):3398-3406.
3. Aifantis I, Raetz E, Buonamici S. Molecular pathogenesis of T-cell leukaemia and lymphoma. Nat Rev Immunol. 2008;8(5):380-390.
4. Hebert J, Cayuela JM, Berkeley J, Sigaux F. Candidate tumor-suppressor genes MTS1 (p16INK4A) and MTS2 (p15INK4B) display frequent homozygous deletions in primary cells from T- but not from B-cell lineage acute lymphoblastic leukemias. Blood. 1994;84(12):4038-4044.
5. Soulier J, Clappier E, Cayuela J-M, et al. HOXA genes are included in genetic and biologic networks defining human acute T-cell leukemia (T-ALL). Blood. 2005;106(1):274-286.
6. Ferrando AA, Neuberg DS, Dodge RK, et al. Prognostic importance of TLX1 (HOX11) oncogene expression in adults with T-cell acute lymphoblastic leukaemia. Lancet. 2004;363(9408):535-536.
7. Bernard OA, Busson-LeConiat M, Ballerini P, et al. A new recurrent and specific cryptic translocation, t(5;14)(q35;q32), is associated with expression of the Hox11L2 gene in T acute lymphoblastic leukemia. Leukemia. 2001;15(10):1495-1504.
8. Aplan PD, Lombardi DP, Ginsberg Am, et al. Disruption of the human SCL locus by "illegitimate" V-(D)-J recombinase activity. Science. 1990;250(4986):1426-1429.
9. Van Vlierberghe P, van Grotel M, Beverloo HB, et al. The cryptic chromosomal deletion del(11)(p12p13) as a new activation mechanism of LMO2 in pediatric T-cell acute lymphoblastic leukemia. Blood. 2006;108(10):3520-3529.
10. Speleman F, Cauwelier B, Dastugue N, et al. A new recurrent inversion, inv(7)(p15q34), leads to transcriptional activation of HOXA10 and HOXA11 in a subset of T-cell acute lymphoblastic leukemias. Leukemia. 2005;19(3):358-366.
11. Dadi S, Le Noir S, Payet-Bornet D, et al. TLX homeodomain oncogenes mediate T cell maturation arrest in T-ALL via interaction with ETS1 and suppression of TCR gene expression. Cancer Cell. 2012;21(4):563-576.
12. Zenatti PP, Ribeiro D, Li W, et al. Oncogenic IL7R gain-of-function mutations in childhood T-cell acute lymphoblastic leukemia. Nat Genet. 2011;43(10):932-939.
13. Palomero T, Sulis ML, Cortina M, et al. Mutational loss of PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia. Nat Med. 2007;13(10):1203-1210.
14. Bar-Eli M, Ahuja H, Foti A, Cline MJ. N-RAS mutations in T-cell acute lymphocytic leukaemia: analysis by direct sequencing detects a novel mutation. Br J Haematol. 1989;72(1):36-39.
15. Degryse S, de Bock CE, Cox L, et al. JAK3 mutants transform hematopoietic cells through JAK1 activation causing T-cell acute lymphoblastic leukemia in a bone marrow transplant mouse model. Blood. 2014;124(20):3092-3100.
16. Van der Meulen J, Sanghvi V, Mavrakis K, et al. The H3K27me3 demethylase UTX is a gender-specific tumor suppressor in T-cell acute lymphoblastic leukemia. Blood. 2015;125(1):13-21.
17. Ntziachristos P, Tsirigos A, Van Vlierberghe P, et al. Genetic inactivation of the polycomb repressive complex 2 in T cell acute lymphoblastic leukemia. Nat Med. 2012;18(2):298-301.
18. De Keersmaecker K, Atak ZK, Li N, et al. Exome sequencing identifies mutation in CNOT3 and ribosomal genes RPL5 and RPL10 in T-cell acute lymphoblastic leukemia. Nat Genet. 2013;45(2):186-190.
19. Zhang J, Ding L, Holmfeldt L, et al. The genetic basis of early T-cell precursor acute lymphoblastic leukaemia. Nature. 2012;481(7380):157-163.
20. Holmfeldt L, Wei L, Diaz-Flores E, et al. The genomic landscape of hypodiploid acute lymphoblastic leukemia. Nat Genet. 2013;45(3):242-252.
21. Clappier E, Cuccuini W, Kalota A, et al. The C-MYB locus is involved in chromosomal translocation and genomic duplications in human T-cell acute leukemia (T-ALL), the translocation defining a new T-ALL subtype in very young children. Blood. 2007;110(4):1251-1261.
22. Kleppe M, Lahortiga I, Chaar El T, et al. Deletion of the protein tyrosine phosphatase gene PTPN2 in T-cell acute lymphoblastic leukemia. Nat Genet. 2010;42(6):530-535.
23. Patrick K, LaFave LM, Wade R, et al. Outcome for children and young people with early T-cell precursor acute lymphoblastic leukaemia treated on a contemporary protocol, UKALL 2003. Br J Haematol. 2014;166(3):421-424.
24. Atak ZK, Gianfelici V, Hulselmans G, et al. Comprehensive analysis of transcriptome variation uncovers known and novel driver events in T-cell acute lymphoblastic leukemia. PLoS Genet. 2013;9(12):e1003997.
25. Dagklis A, Pauwels D, Lahortiga I, et al. Hedgehog pathway mutations in T-cell acute lymphoblastic leukemia. Haematologica. 2015;100(3):e102-5.
26. Forbes SA, Beare D, Gunasekaran P, et al. COSMIC: exploring the world's knowledge of somatic mutations in human cancer. Nucleic Acids Res. 2015;43 (Database issue):D805-811.
27. Bolli N, Manes N, McKerrell T, et al. Characterization of gene mutations and copy number changes in acute myeloid leukemia using a rapid target enrichment protocol. Haematologica. 2015;100(2):214-222.
28. Mullighan CG, Hanna DMT, Zhang J, et al. CREBBP mutations in relapsed acute lymphoblastic leukaemia. Nature. 2011;471(7337):235-239.
29. Van Vlierberghe P, Palomero T, Khiabanian H, PHF6 mutations in T-cell acute lymphoblastic leukemia. 2010;42(4):338-342.
30. Tosello V, Mansour MR, Barnes K, et al. WT1 mutations in T-ALL. Blood. 2009;114(5):1038-1045.
31. Bandapalli OR, Schuessele S, Kunz JB, et al. The activating STAT5B N642H mutation is a common abnormality in pediatric T-cell acute lymphoblastic leukemia and confers a higher risk of relapse. Haematologica. 2014;99(10):e188-192.
32. Landau DA, Carter SL, Getz G, Wu CJ. Clonal evolution in hematological malignancies and therapeutic implications. Leukemia. 2014;28(1):34-43.
33. Flanagan ME, Blumenkopf TA, Brisette WH, et al. Discovery of CP-690,550: a potent and selective Janus kinase (JAK) inhibitor for the treatment of autoimmune diseases and organ transplant rejection. J Med Chem. 2010;53(24):8468–8484.
34. Quintás-Cardama A, Vaddi K, Liu P, et al. Preclinical characterization of the selective JAK1/2 inhibitor INCB018424: therapeutic implications for the treatment of myeloproliferative neoplasms. Blood. 2010;115(15):3109-3117.
35. LaFave LM, Levine RL. JAK2 the future: therapeutic strategies for JAK-dependent malignancies. Trends Pharmacol Sci. 2012;33(11):574-582.
36. Wilkins BS, Radia D, Woodley C, et al. Resolution of bone marrow fibrosis in a patient receiving JAK1/JAK2 inhibitor treatment with ruxolitinib. Haematologica. 2013;98(12):1872-1876.
37. Hanna DM, Fellowes A, Vedururu R, Mechinaud F, Hansford JR. A unique case of refractory primary mediastinal B-cell lymphoma with JAK3 mutation and the role for targeted therapy. Haematologica. 2014;99(9):e156-158.
38. Chase A, Bryant C, Score J, et al. Ruxolitinib as potential targeted therapy for patients with JAK2 rearrangements. Haematologica. 2013;98(3):404-408.
39. Coustan-Smith E, Van Vlierberghe P, Mullighan CG, et al. Early T-cell precursor leukaemia: a subtype of very high-risk acute lymphoblastic leukaemia. Lancet Oncol. 2009;10(2):147-156.
40. Wood BL, Winter SS, Dunsmore KP, et al. T-lymphoblastic leukemia (T-ALL) shows excellent outcome, lack of significance of the early thymic precursor (ETP) immunophenotype, and validation of the prognostic value of end-induction minimal residual disease (MRD) in Children's Oncology Group (COG) study AALL0434. Blood. 2014;124 (21):1.
41. Maude SL, Dolai S, Delgado-Martin C, et al. Efficacy of JAK/STAT pathway inhibition in murine xenograft models of early T-cell precursor (ETP) acute lymphoblastic leukemia. Blood. 2015;125(11):1759-1767.