RELEVANCE OF SIGNAL TRANSDUCTION PATHWAY MUTATIONS IN PEDIATRIC T-ALL

Lidwina Catharina Zuurbier
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Cover: represents the T-ALL puzzle that needs to be solved. The nucleotide letters encode the 5-HTTLPR polymorphism in the promoter of the SLC6A4 gene. The protein letters encode the first part of the SLC6A4 gene that is translated. Literature describes that people having this 5-HTTLPR long variant, are twice as happy and satisfied than people lacking this polymorphism (de Neve et al., 2011, Journal of Human Genetics).


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Lidwina Catharina Zuurbier
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Promotor: Prof.dr. R. Pieters

Leescommissie: Prof.dr. J.J. Cornelissen
Prof.dr. D.F.E. Huylebroeck
Prof.dr. M.L. den Boer

Co-promotor: Dr. J.P.P. Meijerink
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CHAPTER 1

Introduction
HEMATOPOIESIS & T-CELL DEVELOPMENT

The functions of our blood are coagulation, oxygen and nutrient transportation, toxics and waste disposal and immunity. These tasks are achieved by multiple cell types (platelets, erythrocytes and leukocytes) that continuously develop from a common ancestor (hematopoiesis); i.e. the hematopoietic stem cell (HSC). HSCs reside in the bone marrow and represent only a minor fraction of adult blood cells; 0.01-0.05%. Differentiation of HSCs into mature blood cells occurs in the bone marrow and/or secondary lymphoid organs including thymus and spleen following specific stimuli (Figure 1).

During hematopoiesis, HSCs first differentiate into lineage restricted immature myeloid or lymphoid precursor cells (blasts). These can then develop further into erythrocytes (red blood cells), thrombocytes (platelets), or leukocytes (white blood cells). Leukocytes include granulocytes and macrophages (i.e. the myeloid cells) and B- and T lymphocytes.

During T lymphocyte (T-cell) maturation, HSCs first develop into early thymic progenitor (ETP) cells. These cells differentiate into either T- or B lymphocytes. Precursor (pro) T-cells rearrange the TCR gene elements by V(D)J rearrangement to form distinct γδ or αβ TCRs. αβ TCRs are functionally tested during beta-selection, positive selection and negative selection. At these selection points, the functionality of TCRs and their ability to bind major histocompatibility complex (MHC) molecules and to recognize MHC-bound self-peptides, is verified. αβ-Positive thymocytes further differentiate into mature CD4-positive T-cell helper subsets or into a CD8-positive cytotoxic T-cell subset. Both with different functions.

Different stages of T-cell development can be distinguished by specific protein markers on the cell surface. These include CD (cluster of differentiation) markers such as CD44, CD25, CD4, CD8 or CD3, c-kit and the αβ or γδ T-cell receptors (TCR) (Figure 2). This also involves the activation of specific intracellular signaling pathways and transcription factors like NOTCH1 and interaction with the microenvironment.
LEUKEMIA

Leukemia is the outgrowth of leukocytes and can either develop in a very short period of time (acute leukemia) or develop more slowly (chronic leukemia). Children most often are presented with the acute form of leukemia in which immature blasts are arrested in differentiation and expand uncontrollably. Depending on the cell lineage involved, acute leukemia is defined as acute myeloid leukemia (AML) or acute lymphoid leukemia (ALL). In case of lymphoid cells, these can be further specified in precursor B- or T-cells (B-ALL or T-ALL).

As the bone marrow of acute leukemic patients is packed with leukemic cells, the development of normal erythrocytes, thrombocytes and mature leukocytes has become repressed. This causes the majority of symptoms when the disease presents itself. Patients are pale and show signs of fatigue (lack of erythrocytes), bruise easily, have spontaneous bleedings (lack of coagulation due to low thrombocyte numbers) and are susceptible to infections and fever (shortage of mature leukocytes). Furthermore, leukemic blasts can infiltrate organs, lymph nodes and the central nervous system and can also form solid masses resulting in more severe symptoms like hepatosplenomegaly, adenopathy or stroke.

Pediatric T-cell acute lymphoblastic leukemia (T-ALL)
The incidence of ALL in children peaks between 2 to 5 years of age. Most acute leukemias are of lymphoid origin (80%) of which the majority are B cell lineage malignancies. About 15% of ALL is caused by T-cell lineage ALL (T-ALL). This corresponds to 15-20 new patients each year in the Netherlands of which the majority is generally male. The survival rates of pediatric T-ALL patients have increased over the last years and according to the most recent published
DCOG ALL9 protocol, the 5yrs event-free survival for T-ALL is 72%. About a quarter of T-ALL patients relapse. This relapse is in general more aggressive than the primary disease due to acquired therapy resistance, and these patients almost all ultimately die. All patients receive combinational chemotherapy including prednisone, dexamethasone, vincristine, asparaginase, daunorubicine, methotrexate, cytarabine, and mercaptopurine. Until now, the only prognostic factors in pediatric T-ALL are age and white blood cell count. However, no uniform genetic aberration that is correlated to poor or good outcome has been defined for T-ALL. Whereas TAL1 rearrangements were associated with a good prognosis in the ALL7/8/9 cohorts, this could not be confirmed by others. CALM-AF10 seems to be associated with poor prognosis. This, however, was reported by only two studies.

Improving survival
To improve outcome of T-ALL patients, we need to investigate disease biology that might contribute to the development of more effective (and specific) treatment. With children in early childhood, side-effects of therapy can affect developing organs resulting in negative effects later in life. To reduce side-effects we aim for personalized treatment and stratification of patients, to prevent over-treatment in patients who respond very well on relatively lighter regimens. For this, predictive biomarkers for therapy response and prognostic biomarkers for outcome (before or during therapy) are essential.

T-ALL GENETICS

T-ALL is a result of accumulated genetic defects in developing T-cells. These defects lead to aberrant gene expression and uncontrolled self-renewal of cells which have been arrested in differentiation. Abnormal expansion of cells is due to accelerated growth factor signaling and resistance to apoptosis. Genetic defects include numerical chromosomal aberrations (i.e. gain or loss of entire chromosomes), deletions of entire chromosomal arms, translocations that result in the exchange of chromosomal domains between chromosomes, inversions, single nucleotide point and missense mutations, insertion/deletion mutations or local amplifications or deletions that involve few genes.

Type A mutations
The knowledge of genetic changes occurring in T-ALL is expanding. Several genetic mutations in T-ALL that lead to the activation of specific oncogenes occur in a mutually exclusive manner and T-ALL subgroups are defined based on these mutations (type A mutations). These aberrations lead to the expression of the oncogenes; TAL1 or TAL2, LMO1, LMO2 or LMO3, TLX1 (HOX11), TLX3 (HOX11L2), HOXA, MEF2C, NKX2.1 or NKX2.2. Most are transcription factors involved in the control of gene expression regulating the development of blasts. There are still patients without any (so far detected) aberration affecting the expression of these genes (unknowns),
and the genetic background of these patients is currently under extensive investigation. Forty patients of the COALL-97 and DCOG ALL7/8/9 pediatric T-ALL cohorts which have been used for the research described in this thesis, have a type A mutation in the \textit{TAL1}, \textit{TAL2}, \textit{LMO1}, \textit{LMO2} or \textit{LMO3} gene or genes that have a similar function (TALLMO-like). The second largest group of patients is characterized by an aberration in the \textit{TLX3} gene (21%). The remainder of the “known” patients have \textit{HOXA}, \textit{TLX1}, \textit{NKX2.1} or \textit{NKX2.2} and \textit{MEF2C} abnormalities (Figure 3)\textsuperscript{[8]}. 

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3.png}
\caption{Distribution of chromosomal rearrangements (type A mutations) within COALL-97 and DCOG ALL7/8/9. This includes HOXA, MEF2C-activating, NKX2.2, TLX1, TLX3, TAL/LMO or TAL/LMO-like aberrations. Also a minority of patients have yet unidentified type A mutations.}
\end{figure}

\textbf{Type B mutations}  
In addition to type A mutations, T-ALL patients can have multiple type B mutations, scattered among all T-ALL subgroups. Type B mutations often occur in other cancers as well and affect genes involved in cell cycle, proliferation and apoptosis. The most prevalent type B mutation in T-ALL abolishes the cyclin-dependent kinases \textit{CDKN2B} and/or \textit{CDKN2A} gene expression (~80\% of patients). Another type B-mutated protein frequently increased in activity and more specific for T-ALL, is the \textit{NOTCH1} receptor (~38-63\% of patients). Increased \textit{NOTCH1} activity can be a result of genetic mutations in the \textit{NOTCH1} gene itself and/or mutations in the \textit{FBXW7} gene. These mutations affect \textit{NOTCH1} protein localization and stability. Other type B mutations involved in T-ALL affect, among others, \textit{PTEN}, \textit{WT1}, \textit{RAS}, \textit{IL7R} and \textit{PHF6} expression\textsuperscript{[9]}. Dysregulation of these genes can affect whole protein cascades (pathways) that are involved in signal transduction. Examples are the \textit{NOTCH1}, PI3K/AKT, and RAS/MEK/ERK pathway which are canonical pathways that are interconnected. Hyperactivation of these pathways in T-ALL can be blocked/dampened by (specific) therapy. For example, gamma-secretase inhibitors can inhibit the activation of the \textit{NOTCH1} signaling but are, unfortunately, so far proven to be too toxic in clinical trials.
AIMS OF THIS THESIS

In this thesis, we investigated the role of type A and type B mutations and their relation to outcome and other clinical and biological parameters in pediatric T-ALL, with a focus on type B mutations. The research in this thesis has predominantly been performed using samples from 146 pediatric T-ALL patients that were treated according to the German COALL-97 cohort and the Dutch DCOG ALL7, ALL8 or ALL9 cohort.

Many reviews highlight the incidence and important role of chromosomal rearrangements (so called type A mutations) in T-ALL. Since the focus of this thesis is on type B mutations, chapter 2 gives an overview of all type B mutations known in pediatric T-ALL. This chapter focuses on specific signal transduction pathways that may be dysregulated in T-ALL as a result of type B mutations. These pathways are normally involved in cell cycle, proliferation, or apoptosis.

In chapter 3, we explored the incidence of various types of mutations that activate the NOTCH1 transcription factor. NOTCH1 is a transmembrane receptor that is normally activated by various proteolytic cleavages including cleavage at the intracellular domain that is executed by the γ-secretase complex upon ligand binding. Cleaved NOTCH1 (intracellular NOTCH or ICN) is translocated to the nucleus and initiates transcription. Until now, mutations in the NOTCH1 gene itself have been described as well as inactivating mutations in other genes such as FBXW7, a ubiquitin ligase that normally targets intracellular NOTCH1 for ubiquitin-mediated proteolysis. Their prognostic value varies among study cohorts.

PTEN, PI3K and AKT mutations are type B mutations in pediatric T-ALL patients that have been suggested to hyperactivate the PI3K/AKT pathway and subsequent mTOR pathway. PTEN-inactivating mutations were initially proposed to follow NOTCH1-activating mutations as a γ-secretase inhibitor resistance mechanism. In chapter 4, we examined the incidence of PTEN, PI3K and AKT mutations and their association with clinical and biological parameters as well as with PTEN, AKT and further downstream protein expression. In addition, we investigated a potential role of epigenetic silencing and defective RNA splicing of PTEN in T-ALL as well as PTEN promoter mutations as alternative mechanisms to inactivate PTEN.

In this thesis, research about the PTEN gene was extended by exploring the cause of the newly discovered PTEN splice-defects resulting in micro-deletions (chapter 5).

In chapter 6, we extended our screening by identifying IL7R (IL7 receptor), N-RAS, K-RAS, JAK1 and JAK3 (Janus Kinases) mutations within our cohorts and verified how these mutations co-occurred with each other and our previously identified NOTCH1-activating and PI3K/AKT mutations. Mutations in the IL7Rα-chain have recently been identified as an additional type B mutation in ~9% of T-ALL pediatric patients, resulting in IL7-independent signaling. The IL7R can signal downstream through JAK-STAT, RAS-MEK-ERK or PI3K/AKT pathways. For some of the detected mutations, we determined their transformation capacity using IL3-dependent Ba/F3 cell lines and measured activated downstream signaling. In addition to this, we tested clinically relevant inhibitors for their ability to block the activation of these pathways.

In 2011, we described an immature T-ALL subtype revealed by gene expression profiling
analysis, that is characterized by early T-cell developmental markers and frequent expression of myeloid markers and MEF2C-activating aberrations. Comparable immature T-ALL entities, i.e. early thymic progenitor ALL (ETP-ALL) or the ABD (absence of bi-allelic deletion) T-ALL subgroups were identified by others. In chapter 7, we analyzed whether features for all three entities identified would point to one and the same disease entity.

In chapter 8, we have performed a proteome screening using reverse-phase protein arrays (RPMAs) to identify the activation status of a set of molecules that act in signal transduction pathways. We explored differential activation of pathways between different T-ALL subgroups that are characterized by specific type A or B mutations such as NOTCH1-activating, PTEN/AKT or WT1-inactivating mutations. RPMA data was combined with global test analyses of mRNA gene expression data. The study was performed to contribute to the understanding of T-ALL biology and to diagnostic or prognostic biomarker discovery or druggable targets. The analysis of these protein arrays reveals various suggestions of important pathways that play a role in particular subgroups that should be further validated.
REFERENCES

CHAPTER 2

Signal transduction pathways affected by mutations and their druggable potential in T-cell acute lymphoblastic leukemia

Linda Zuurbier¹, Rob Pieters¹,² and Jules P.P. Meijerink¹

From the ¹Department of Pediatric Oncology/Hematology, Erasmus MC Rotterdam-Sophia Children’s Hospital, Rotterdam, The Netherlands; ²Princess Maxima Center for pediatric Oncology, Utrecht, The Netherlands.

Review
ABSTRACT

T-cell acute lymphoblastic leukemia (T-ALL) is genetically a very heterogeneous disease involving type A mutations reflecting T-ALL genetic subgroups and additional type B mutations. Multiple type B mutations affect a limited number of signal transduction pathways, including the IL7-JAK-STAT, RAS-MEK-ERK, PI3K-AKT and NOTCH1 signaling. The importance of these pathways for regulation of cellular proliferation and survival, regulation of DNA damage, gene transcription and translation has been demonstrated. Although some mutations have been related to the very immature early T-cell progenitor ALL, no clear and consistent relation is observed between mutations and clinical parameters in T-ALL yet. Drugs that specifically target a pathway are under development and single or combinational therapy with these therapeutics may be beneficial for patients that have hyperactivating mutations in these pathways.
Acute Lymphoblastic leukemia

Acute Lymphoblastic leukemia (ALL) is a malignant blood disease affecting one in 1500 children before the age of 18. Leukemia develops following genetic mutations leading to deregulation of various cellular processes including signal transduction pathway activities. Enhanced cellular proliferation and maintenance, arrest in differentiation and increased resistance to apoptosis transform normal cells into leukemic cells.

Genetic changes in T-cell acute lymphoblastic leukemia (T-ALL) can be classified into so called type A and type B aberrations. Type A mutations are mostly chromosomal rearrangements that result in a differentiation arrest and define patient subtypes. Type B abnormalities are scattered among all “type A” patient groups and frequently result in changes in activities of signal transduction pathways. An elegant study by Clappier and colleagues (2011) showed the importance of type B abnormalities during tumor progression. They investigated clonal selection of genetic subclones as present in diagnosis samples of T-ALL patient biopsies using a xenograft transplantation model and found multiple leukemic subclones that harbor a different landscape of type B mutations, according to the neo-Darwinian model. They suggested that specific type B mutations could be drivers of leukemic selection towards highly aggressive subclones. One example of clonal selection of leukemia subclones comprising such type B abnormalities was demonstrated and affected inactivating mutations in the PTEN tumor suppressor gene that was responsible for the outgrowth of a particular leukemic subclone during relapse. Only a limited number of signal transduction pathways are altered by a variety of type B abnormalities. In T-ALL, most frequent abnormalities affect the PI3K/AKT and NOTCH pathway activity and cell cycle regulators such as deletions of the CDKN2A/2B genes. However, aberrant activation of the T-cell receptor (TCR) signaling, RAS-MEK-ERK, IL7R-JAK-STAT and other signal transduction pathways have frequently been implicated in the development of T-ALL.

In this review, we give an overview of signal transduction pathways that are altered as a result of type B abnormalities and have a converged role in T-ALL leukemogenesis. Furthermore, we highlight drugs that may be suitable to specifically counteract the activity of affected pathways.

T-cell Receptor signaling

The T-cell receptor (TCR) has a pivotal role in the function of T-cells and provides specificity to cellular response epitopes in the context of major histocompatibility complex I or II molecules. To fulfill these actions, the TCR signals through many different pathways to ultimately respond to cellular and environmental changes (Figure 1). This results in cytokine production, T-cell-supported B-cell responses or cellular lysis by natural-killer or cytotoxic T-cells. The TCR signaling cascade is indispensable for T-cell survival and proliferation.

Pre-TCR and TCR signal through similar downstream molecules upon activation, in which the Syk family, Src family and Tec family tyrosine kinases are involved. The TCR is non-covalently linked to the CD3 complex on the membrane. Other essential molecules present on the membrane are co-receptors like CD4, CD8 or CD28. The Src family tyrosine kinases LCK (lymphocyte-specific protein tyrosine kinase) or Fyn are constitutively bound to the cytoplasmic tails of the CD4/CD8
or CD28 molecules and can become dephosphorylated (inactivated) by CD45 (PTPRC). Upon TCR activation, LCK and Fyn phosphorylate certain ITAMs (Immune receptor tyrosine activation motifs) located on the cytoplasmic tails of CD3 and TCR. This enables the recruitment of Syk tyrosine kinases including ZAP-70 (zeta-chain associated protein kinase of 70 kDa) to the membrane, which also becomes phosphorylated by LCK/Fyn and thereby allows downstream signaling. ZAP-70 phosphorylates T-cell specific adapter proteins like LAT (linker of activated T-cells) and SLP76 (SH-2 domain containing lymphocyte protein of 76 000 MW). Misschien is hier een plaatje handig! The T-cell specific adapters form complexes with various molecules, for example Tec family kinases, and thereby link TCR molecules to various downstream routes including the PI3K/AKT pathway, the RAS-MEK-ERK pathway, and NFAT (nuclear factor of activated T-cells). The PI3K/AKT and RAS-MEK-ERK pathways will be described separately in the review. NFAT initiates transcriptional activation of cytokine genes through Jun/Fos transcription factors. For this, NFAT translocates to the nucleus upon dephosphorylation by the Calcineurin (PPP3CC) phosphatase, which is activated following calcium efflux from mitochondria. This calcium release is regulated by activated PLCγ (phospholipase Cγ) via LAT following TCR activation. PLCγ can hydrolyze PtdIns(4,5)P₂ into IP₃ (inositol 1,4,5-triphosphate) and DAG (diacylglycerol). IP₃ binds to the IP₃ receptor on mitochondria, thereby eliciting a calcium efflux.

Mutations in the TCR cascade that result in immunodeficiency are known. Regarding its important role, it is surprising that aberrant hyperactivation due to genetic changes within the initial TCR signaling are relatively infrequent or unknown in T-cell leukemias. Aberrations in the TCR signaling cascade found in T-cell malignancies are SYK translocations in a few T-cell lymphoma patients, in which SYK is fused to Itk. Itk is a member of the Tec family kinases. Also in cutaneous T-cell lymphoma, mutations in the PLCG1 gene were found in 19% of patients. In T-ALL, hyperactivation of LCK as a result of translocation to the TCRB gene has been rarely observed in patients and is observed in the T-ALL cell line HSB2. Furthermore, loss-of-function mutations in the PTPRC phosphatase are occasionally found in T-ALL patients.

Mutations in the initial TCR pathway are not frequently found. On the other hand, downstream TCR pathways like the RAS-MEK-ERK and PI3K/AKT pathway are often mutated (described in this review). Preliminary data of our pathway analyses indicate that the initial TCR pathway seems active in many T-ALL patient samples. Although this may be a consequence of the arrest at specific T-cell development stages for particular T-ALL subtypes, therapeutic approaches targeting this pathway might theoretically be effective for T-ALL patients like Calcineurin inhibitors such as cyclosporin A or the Src kinase inhibitors imatinib and dasatinib.

**IL7 receptor signaling**

The IL7 receptor (IL7R) signaling is essential for the development and homeostatic maintenance of T and B lymphocytes and other hematopoietic cell lineages. It is activated through IL7R-IL7 cytokine binding. The receptor is composed of two heterodimeric chains; the IL7Rα chain and the IL2 common y-chain. Both chains are shared among different cytokine receptors which interaction is influenced by the presence of cytokines: the IL7Rα-TSLPR (thymic stromal
lymphopoietin receptor) dimer binds TSLP on T-cells, pre-B cells en dendritic cells and the IL7Rα-IL7Rγ-c dimer binds IL7 on T-cells specifically. The IL7R receptor has several downstream substrates that can activate various pathways (Figure 1). The major downstream cytoplasmic interactors are JAK kinases. Each JAK family member has specificity for a receptor chain.

Hereditary recessive loss-of-function mutations in IL7R are correlated with SCID and a polymorphism in the IL7R gene promotes exon 6 skipping in multiple sclerosis patients resulting in higher soluble IL7Rα levels. The same polymorphism was also associated with rheumatoid arthritis. Gain-of-function mutations were recently found in B-ALL and in 9-12% of T-ALL patients, predominantly by in-frame insertion/deletion (INDEL) mutations but also point mutations. Most of these mutations result in the introduction of a cysteine in the juxtamembrane/transmembrane region. This results in constitutive IL7Rα homodimerization and ligand independent JAK-STAT and PI3K/AKT pathway activation. IL7R mutations initiated tumor formation in mice and exhibited oncogenic transformation capacity in IL3-dependent Ba/F3 cells and IL7-dependent D1 thymocytes, which was abrogated when the unpaired cysteines were substituted by serine/alanine/glycine. Recently, IL7R mutations are also detected by next generation sequencing of children with early T-cell progenitor ALL (ETP-ALL). This reflects leukemia with a very immature T-cell differentiation arrest with retained myeloid features. These mutations induce ETP-ALL in mice transplanted with primitive transduced thymocytes from p19Arf−/− that can be inhibited with JAK-STAT inhibitors.

JAK-STAT signaling

The JAK (Janus kinase) family comprises four non-receptor tyrosine kinases; JAK1, JAK2, JAK3 and TYK2. They mainly signal through STATs (signal transducers and activators of transcription proteins) to activate transcription following stimulation of the TCR or other receptors (Figure 1). Loss-of-function mutations affecting JAK-STAT signaling can result in autosomal severe combined immune deficiency (SCID). On the other hand, gain-of-function mutations can result in malignant transformation.

JAK family members are constitutively bound to the intracellular part of various transmembrane receptors (e.g. IL7 receptor), which do not have intrinsic catalytic activity on their own but exert their function through proteins like JAK kinases. Depending on the type of receptor, different homo- or heterodimer JAK-complexes are attached to the receptor, each resulting in specific downstream signaling events when activated. Activation occurs upon extracellular ligand-receptor binding by various cytokines, interferons or growth hormones, resulting in either dimerization of receptor subunits or conformational changes of receptors. As a result, two receptor-bound JAK molecules come in close proximity to each other, which allows transphosphorylation of specific amino acids within the JAK activation loop and activation of JAK. First, activated JAK proteins phosphorylate their neighboring receptor, enabling recruitment of STAT proteins to the receptor. Consequently, JAK proteins can phosphorylate STAT proteins, leading to the formation of STAT dimers or other STAT complexes. These complexes then translocate to the nucleus to initiate transcription. Activation of JAK proteins also results in the activation of
downstream RAS-MEK-ERK and PI3K/AKT pathways. STAT proteins can also become activated by other tyrosine kinases than JAK kinases. Negative regulators of the JAK-STAT pathway are various phosphatases (including CD45, PTPN2 and SHP1), PIAS (Protein inhibitor of activated STAT) and SLIM (Stat-interacting LIM) proteins and members of the SOCS family comprising CIS (cytokine-inducible SH2 protein) and SOCS (suppressor of cytokine signaling) motifs. Phosphatases can counteract the function of JAK. PIAS proteins target activated STAT that act as transcription factors in the nucleus by preventing binding to DNA and promoting STAT protein sumoylation. In addition, they can recruit transcriptional corepressors such as histon acetylases. SLIM proteins are E3 ligases and can target STAT for proteasomal degradation. SOCS proteins physically interact with JAK and thereby block JAK-STAT-binding, inhibit JAK kinase activity or induce JAK or STAT ubiquitination.

Various members of the JAK-STAT pathway can be affected by mutations causing malignant transformation of cells. Mutations have rarely been observed in solid tumors but more frequently in hematopoietic diseases. The JAK2 V617F mutation is predominantly observed in myeloproliferative disorders (MPDs) and Down syndrome-associated ALL patients. Also some pediatric B-ALL patients are characterized by JAK mutations. Mutations in JAK family members have been rarely reported in pediatric T-ALL patients. Different studies report an incidence of 4-27% JAK1 mutations in adult T-ALL patients and only 2% in pediatric T-ALL. A study that did not discriminate adult and pediatric patients identified 1 and 3 out of 42 T-ALL patients with JAK1 or JAK3 mutations, respectively. JAK1 and JAK3 mutations were also identified by whole genome sequencing of ETP-ALL children and exome screening of adult/pediatric T-ALL patients. Moreover, various JAK fusion proteins are described in leukemia, of which only ETV6-JAK2 and TEL-JAK2 have been occasionally found in T-ALL, but are more common in B-ALL. Mutations in STAT have never been identified in cancer whereas their expression has been reported to be frequently upregulated in various tumors. Negative regulators of the JAK-STAT pathway are known to be hypermethylated or mutated in hematological malignancies. SOCS1, SHP1 and PTPN2 deletions/mutations occur in 6-8% of T-ALL. One patient with a PTPN11 mutation has been reported. Interestingly, PTPN2 loss results in increased phosphorylation of JAK1 and STAT1/5 as well as the inability to dephosphorylate the NUP214-ABL1 fusion protein. The JAK-STAT pathway is also one of the inhibitory targets of PTPRC (CD45), a gene that is occasionally mutated in T-ALL patients, as discussed before. The prognostic significance of mutations affecting the JAK-STAT pathway in T-ALL has not been thoroughly investigated yet.

Inhibitors targeting the JAK-STAT pathway are JAK-specific kinase inhibitors. Besides general protein tyrosine kinase inhibitors like dasatinib, various specific JAK inhibitors are already in clinical trial regarding autoimmunity diseases like rheumatoid arthritis and psoriasis, immunosuppressors during kidney transplantation and myeloproliferative disorders. These drugs may also be a future option for the treatment of T-ALL patients with mutations in the JAK-STAT pathway.
The RAS-MEK-ERK pathway

RAS uses multiple factors to mediate transcription in T-cells. It has a general role in proliferation and differentiation during development of T-cells (positive selection) and peripheral lymphocyte activation by processes like cytokine production. The RAS-MEK-ERK pathway is aberrantly expressed in various tumors including T-ALL.

RAS is a small GTPase that is constitutively present in a silent state and anchored to the intracellular part of the cell membrane. RAS is member of the RAS superfamily that further includes related RHO, RAB, ARF, RAC and RAN family members. Three different highly homologous RAS genes are known: H-RAS (Harvey-RAS), N-RAS (Neuroblastoma-RAS), and K-RAS (Kirsten-RAS) that has two different splice forms (K-RAS4A and K-RAS4B). The N-terminals are identical, but the GTP-binding domains differ among these three RAS proteins. RAS proteins cycle between GDP-bound (inactive) and GTP-bound (active) states, a mechanism controlled by GEFs (guanine-exchanging factors) and GAPs (GTPase-activating proteins). SOS (Son of Sevenless) or GRP (T-cell specifically expressed guanine nucleotide releasing protein) are GEF’s that can exchange RAS-bound GDP for GTP, thereby activating RAS. The activation of GEF’s takes place via RAS-adaptor molecules, mainly following ligand-activation of extracellular transmembrane receptor, for example via LAT upon TCR or cytokine receptors (Figure 1). The most common RAS-adaptor molecules are Shc and Grb2 (Growth factor receptor-bound protein 2). GTP-bound RAS undergoes a conformational change that results in the dissociation and replacement of GAPs by GEFs to become fully activated, but also to specify downstream interactions. RAS can activate many downstream signaling pathways of which the MEK-ERK pathway and PI3K/AKT pathway are most often described. RAS contains intrinsic GTPase activity that can hydrolyze GTP into GDP and consequential inactivation of RAS. GTPases like NF1 (neurofibromin 1), GAP or p120GAP can accelerate this reaction. Furthermore, RAS can directly activate PI3K that activates the PI3K/AKT pathway.

Missense mutations in RAS are well known to occur in cancer including AML and B-ALL. N-RAS point mutations have been identified in 4-9% of pediatric T-ALL patients. H-RAS mutations are investigated but not found in pediatric T-ALL patients. K-RAS mutations are detected in two pediatric T-ALL patients studies in 2% and 9.5% of patients. Recently, RAS signaling mutations are found to be associated with the myeloid-like ETP ALL. RAS mutations have predominantly been found in codon 12 and 13 but also 61, 63, 117, 119 and 146. These mutations impair the intrinsic RAS GTPase capacity, rendering mutant RAS proteins constitutively active. These mutations may sterically hinder GTPase activity due to a consequent conformational change or a decrement in GDP nucleotide affinity. Von Lintig and colleagues (2000) determined the activity of the RAS protein in 18 T-ALL children using an enzyme-based method. They observed that half of the patients had an enhanced RAS activity compared to healthy subjects, but a mutation analysis had not been performed in this context. Given the fact that RAS mutations are generally detected in only a minority of pediatric T-ALL patients, increased RAS activation in these patients is most likely caused by other factors upstream of RAS, e.g. by IL7R, JAK or NF1 mutations. Also other members of the RAS-MEK-ERK pathway are frequently mutated in cancer, but have not
been observed in T-ALL so far.

**FLT3** (FMS-like tyrosine kinase 3 gene) encodes for a tyrosine kinase receptor that dimerizes upon ligand binding. Subsequent autophosphorylation initiates downstream signaling, including the RAS-MEK-ERK pathway, PI3K/AKT pathway or Src tyrosine family kinases involved in TCR signaling. Cells with FLT3-ITD also activate STAT5 molecules. FLT3 is altered in one-third of AML patients by in frame ITDs (internal tandem duplications) or point mutations, resulting in a constitutive activation of the receptor. FLT3 ITDs and point mutations are also found in ALL patients, although less frequently. In 2004, *Paietta and colleagues* reported three adult T-ALL patients that had FLT3 ITD or point mutations. In three separate studies, 3-7% of pediatric T-ALL patients were identified with FLT3-ITD mutations. Very recently, FLT3 mutations were also shown to be enriched in adult early ETP-ALL. Deletions and mutations in the *NF1* gene also result in activated RAS. We described mutations and/or deletions in *NF1* in 3% of pediatric T-ALL patients, including 2 patients that contained an *NF1* deletion thereby inactivating both *NF1* alleles.

RAS mutations are associated with a poor survival in AML and *MLL*-rearranged B-ALL. In T-ALL, *N-RAS* mutations have been associated with a higher risk for relapse. Furthermore, they have been associated with relapsed T-ALL as well as with the poor prognostic ETP-ALL subtype.

RAS specific inhibitors are still awaiting. Drugs were developed targeting the posttranslational farnesylation event of RAS proteins, essential to anchor RAS to the plasma membrane. Tipifarnib is one such a drug that showed a good response in AML and T-ALL cell lines *in vitro*. Although farnesyl transferase inhibitors (FTI’s) are effective in clinical trial, the efficacy appeared not related to RAS mutations and leukemic cells often acquire an increased resistance to these compounds. Therefore, studies focuses more on drugs targeting RAS downstream molecules like B-RAF or MEK1/2.

**PI3K/AKT pathway**

The PI3K/AKT pathway contributes to many cellular processes and can become activated through activated transmembrane receptors like the TCR (*Figure 1*). Hyperactivation of this pathway by mutations is shown to be oncogenic in various tumors.

The PI3K/AKT pathway signals via direct interaction of the PI3K (phosphatidylinositol 3-kinase) regulatory subunit with an upstream membrane bound receptor. Apart from direct receptor binding, the PI3K/AKT pathway can be activated through the T-cell specific adapter complex or through RAS, which can directly bind and activate PI3K. Activated PI3K phosphorylates PtdIns(3,4)P$_2$ (PIP$_2$) to form PtdIns(3,4,5)P$_3$ (PIP$_3$), which activates PDK1 at the membrane. Activated PDK1 allows recruitment and phosphorylation of AKT on Ser473 and Thr308 positions, in concert with a still unknown kinase. AKT activates or inhibits various downstream processes through protein phosphorylation. Best known examples are a) the synthesis of proteins and initiation of cell growth by activation of the downstream mTOR pathway through direct inhibition of the mTOR inhibitor TSC2 b) through nuclear export of Forkhead transcription factors.

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factors that transcribe the cell cycle inhibitor p27, among others, c) stimulation of the cell cycle by delocalization of the cell cycle inhibitor p27 through direct phosphorylation or stabilization of cyclin D1 d) prevention of apoptosis by the phosphorylation and inactivation of pro-apoptotic proteins including BAD or pro-caspase 9\(^87\). PI3K/AKT activity is counteracted by the phosphatases PTEN (phosphatase and tensin homologue deleted on chromosome 10) or SHIP1/2 (SH2-containing inositol polyphosphate 5-phosphatase), which dephosphorylates PIP\(_3\) at the 3\(^{rd}\) or 5\(^{th}\) position of the inositol ring, respectively\(^86\). PTEN also directly interacts with p53 thereby stabilizing the protein and changing its DNA binding potential, or interacts with other nuclear proteins that control DNA damage like MDM2 and Chk1\(^87\). It has recently been shown that PTEN can be transcriptionally silenced by the HES1 repressor following activation of the NOTCH1 pathway in T-ALL cell lines. Although we could not confirm it, it has been postulated that this results in elevated AKT activity. Oncogenic loss of PTEN has also been associated with resistance to NOTCH inhibitors like gamma-secretase inhibitors\(^88\), however as our data indicates a nearly mutual exclusive pattern of PTEN/AKT and NOTCH1-activation mutations we doubt the clinical relevance of this\(^89\).

The PI3K/AKT pathway is deregulated in a broad range of cancers in which loss of PTEN function is the most prevalent cause. PTEN germ-line mutations result in various hereditary diseases that predispose to cancer, whereas somatic PTEN inactivation has been documented in various forms of cancer including glioblastoma, lung, breast, prostate, melanoma, bladder, endometrial and renal cancer, among others\(^90-95\). The PTEN locus is mostly inactivated through chromosomal deletions, although point-or frame shift mutations in the PTEN coding region or PTEN promoter as well as promoter hypermethylation are commonly detected\(^96-98\). Alternative to PTEN mutations, we found several patients with PTEN splice defects resulting in loss of PTEN protein\(^88,99\). PTEN phosphorylates various downstream targets and several studies showed that the AKT pathway is mostly affected by PTEN loss in cancer\(^88,96,100\). PTEN is a haploinsufficient tumor suppressor gene, indicating that loss of a single gene copy results in a cellular phenotype\(^98\). Bi-allelic inactivation of PTEN has been associated with disease progression or an aggressive cancer phenotype\(^101,102\). Our study supports the idea of PTEN acting as a haploinsufficient tumor suppressor, as we noticed a difference in PTEN expression levels between mono-allelic and bi-allelic-mutated patients, and the ongoing pressure on leukemic cells that have lost already one allele to lose or mutate the remaining wild-type allele\(^103,108\). In pediatric T-ALL, PTEN mutations have been detected in 9-17\% of patients\(^65,104\). Smaller studies reported variable incidences between 5 and 63\%\(^103,105\). Besides PTEN, also PI3K and AKT are mutated in cancers including colorectal, gastric, breast and ovarian cancer\(^106-108\). In our study, AKT1 mutations were present in only 2\% of the patients while no PIK3RI and PIK3CA mutations were observed in contrast to earlier observations\(^109\). In that study, Gutierrez et al (2009) determined the frequency of PI3K, AKT or PTEN mutations/deletions in a small cohort and found a total incidence of 48\%. In addition, other mechanisms to hyperactivate the PI3K/AKT pathway are observed in T-ALL. This is a decrease in PTEN activity as a result of CK2 (casein kinase 2) overexpression or high levels of ROS\(^109\).

Elevated AKT levels have been associated with poor prognosis in acute myeloid leukemia
Regarding pediatric T-ALL, five studies have correlated aberrations of the PI3K/AKT pathway with clinical outcome. In three of these studies, a correlation with long-term survival was noticed in subgroups of patients only\(^{104,111-113}\). In one study, also NOTCH1-activating mutations were taken into account. Patients with both PTEN and NOTCH1-activating mutations have favorable MRD (multiple residual disease) levels (alike single NOTCH1-activating mutations) at day 78 but not day 33. Moreover, these patients had improved long-term survival rates\(^ {104}\). PTEN/AKT-mutated patients of the Dutch cohorts were associated with negative long-term survival rates when NOTCH1-activating mutations were taken into account\(^ {89}\).

The many feedback and feed-forward loops in the PI3K/AKT pathway make it a very complex pathway. Therefore, therapeutically targeting the PI3K/AKT pathway might be most effective when targeting molecules at multiple levels in this pathway. Various inhibitors have been developed, including PI3K inhibitors including Wortmannin and LY294002, a PDK1 inhibitor such as GSK2334470, AKT inhibitors as Triciribine, AKT-blocking agents as D-3-deoxy-phosphatidylinositol analogues or Perifosine or the mTOR inhibitor Rapamycin\(^{114-120}\). In T-ALL, single-agent treatment as well as targeting multiple levels of the pathway works effectively or even synergistically in vitro. Similar effects are achieved when combined with glucocorticoids\(^ {116,121,122}\).

For example, the AKT inhibitor Triciribine induces cell cycle arrest and apoptosis in T-ALL cell lines\(^ {123}\). Also, a dual inhibitor targeting both PI3K and mTOR induces cell cycle arrest and apoptosis in T-ALL cell lines and patient samples\(^ {124,125}\). In xenografts, rapamycin induces apoptosis of T-ALL\(^ {126}\). In addition, since the PI3K/AKT pathway might intertwine with the NOTCH pathway, it is postulated that a combination of inhibitors targeting both pathways would be effective. Indeed, rapamycin may have an additional effect when combined with NOTCH inhibitors in mice and synergism between NOTCH1 and mTOR inhibitors or PI3K inhibitors was observed in T-ALL cells in vitro\(^ {127,128}\).

**NOTCH signaling**

One of the most prevalent type B mutations in T-ALL affect genes of the NOTCH signaling pathway including NOTCH1 and the E3-ubiquitin ligase FBXW7 (F-BOX domain containing protein 7), resulting in a constitutively active NOTCH1 pathway. Cellular changes of active NOTCH signaling differ among tissue types, but NOTCH activation in developing T-cells affects differentiation and proliferation. NOTCH has also a prominent role in hematopoietic stem cells (HSCs) during embryonic development, adult HSC maintenance and cell fate decisions\(^ {129}\).

NOTCH proteins are transmembrane receptor proteins (Figure 1) that transmit extracellular signals towards the nucleus upon interaction with a variety of NOTCH1 ligands including jagged1, jagged2, delta1, delta3 or delta4 (Delta/Serrate/Lag-2 family) that are expressed on neighboring cells. The NOTCH receptors exist of two non-covalently bound heterodimeric parts, which are sequentially processed from a larger NOTCH molecule following a furin-cleavage (S1-cleavage) in the golgi apparatus. Activation of the receptors upon ligand-binding triggers another cleavage (S2-cleavage) by metalloproteases, that results in a conformational change of the Lin12/NOTCH repeats (LNR) and a consequential conformational change in the heterodimerization domain.
Type B mutations in signal transduction pathways in T-ALL

that facilitates a third cleavage (S3-cleavage) by the γ-secretase complex in the cytoplasm. This results in the release of an intracellular part of the NOTCH receptor (ICN) that migrates toward the nucleus where it acts as a transcription factor. Here, it binds the DNA-binding protein CBF1 (C promoter binding factor 1) while displacing its co-repressors. The ICN transcription complex further involves proteins like MAML (mastermind-like), p300 and deltex. NOTCH has many potential transcriptional targets and the choice of gene transcription depends on the level of NOTCH activation but also on tissue type, differentiation state and type of ligand binding. Well-known NOTCH targets in T-cells are members of the HES (hairy enhancer of split) family, MYC and PTCRA (pre-T-α). Although CBF1-binding is indispensable for NOTCH function, a non-canonical CBF1-independent NOTCH pathway is known.

Several proteins are known to regulate the NOTCH expression levels and activity. The turnover of ICN is regulated by phosphorylation of so-called phospho-degron motifs located in the C-terminal PEST domain. Phosphorylation by CDK8 (cyclin-dependent kinase 8) is followed by subsequent ubiquitination by the E3 ubiquitin ligase FBXW7. Another cytoplasmic NOTCH inhibitor is Numb that prevents ICN to translocate to the nucleus. In turn, expression of Numb can be inhibited by Musashi. Furthermore, Spen (Mint) can corepress NOTCH activity in the nucleus by binding to CBF1 and recruitment of histon deacetylases.

In humans, four different NOTCH genes (NOTCH1-4) are known. Apart from enhanced NOTCH expression in several solid tumors as breast, melanoma, medulloblastoma and ovarian cancer, aberrations in NOTCH genes or regulators are not commonly found in cancer. In pediatric T-ALL, the incidence of mutations in the NOTCH pathway varies among study populations and with an average of 50% in the NOTCH1 gene only and 55% in NOTCH1 and/or FBXW7. Reduced numbers of NOTCH1 mutations have been observed in ETP-T-ALL, possibly as an effect of their maturational arrest before definite T-cell commitment. In T-ALL most NOTCH1 mutations are present in the heterodimerization (HD) domain or the proline glutamic serine threonine (PEST) domain. HD-domain mutations, but also mutations in the transmembrane (JM), result in ligand-independent exposure of the S3-cleavage site that provokes cleavage by the γ-secretase complex and release of ICN. PEST domain mutations abrogate FBXW7-binding and NOTCH ubiquitination, prolonging the half-life of ICN. Inactivating mutations in FBXW7 phenocopy NOTCH1 PEST-domain mutations, and also result in increased half-life of ICN. As expected, mutations in the NOTCH1 PEST domain and FBXW7 are mutually exclusive. NOTCH1 HD and PEST mutations in cis or either of these mutations in combination with FBXW7 act synergistically in activating the NOTCH1 pathway. Single NOTCH1 HD or PEST mutations are weak leukemia-initiating mutations. In contrast, combinations of NOTCH1 HD and PEST domain mutations, or HD mutations in combination with mutant FBXW7 or single NOTCH1 JM mutations act as strong activating mutations. Sporadic mutations flanking the LNR or mutations located in the ANK and TAD domains have also been reported. LNR mutations result in the destabilization of the heterodimers of the HD-domain and continuously exposure of the S3-cleavage site. Although the exact mechanism of ANK and TAD mutations in T-ALL development is not completely understood, it is hypothesized that they interfere with
Chapter 2

NOTCH1 protein binding. Furthermore, rare TCR-recombination driven NOTCH1 translocations are described for T-ALL placing part of the NOTCH1 gene under control of T-cell receptor regulatory elements\(^{156-158}\). Most known is the translocation in which the intracellular part of NOTCH1 is juxtaposed to the joining segment of TCRB thereby producing constitutively active truncated NOTCH1 protein (TAN1)\(^{156}\). Also, an alternative mechanism of Notch activation in leukemogenesis was discovered. Deletion of the 5’ end of NOTCH1 accelerates spontaneous leukemogenesis in Ikaros-knockout mice. These mice lack the normal NOTCH1 promoter and use an alternative NOTCH1 promoter located in an intron upstream of the HD-domain encoding exons that drives expression of ICN\(^{159}\). This new mechanism of NOTCH1 activation was earlier discovered in radiation-induced mouse thymic lymphomas and needs further investigation regarding T-ALL oncogenesis\(^{160}\). Strong activating NOTCH1 mutations are especially associated with TLX3 rearranged T-ALL\(^2\).

The prognostic relevance of NOTCH1-activating mutations in pediatric T-ALL patients is still under debate. Some studies reported favorable survival rates or treatment responses for NOTCH1-mutated patients while other studies reported no difference or even unfavorable survival rates or treatment response for NOTCH1-mutated patients\(^{2,65,111,139,140,142,143,146,147,149,151,161}\). This variation can be a consequence of the heterogeneity within NOTCH1-activating mutations, reflecting different types of weak and strong mutations, which are differently distributed among cohorts. Also, little attention has been given to the types of NOTCH1-activating mutations in relation to outcomes in these studies, or their association with other type B mutations. Comparison of study results is further hampered by the fact that not every study screens for both NOTCH1 and FBXW7 mutations and that different treatment protocols exist among study cohorts that may result in a different treatment response.

Due to the high mutational rate of this pathway in pediatric T-ALL, therapeutic inhibition of the NOTCH pathway is particularly attractive. The NOTCH pathway can be targeted on various levels; best examined are g-secretase inhibitors (GSIs) that inhibit S3-cleavage of NOTCH and thereby release of ICN. GSIs were initially developed to treat Alzheimer’s disease. A phase I trial with GSI MK-0752 in relapsed T-ALL and acute myeloid leukemia patients resulted in major gastrointestinal toxicity\(^{162}\). Nevertheless, GSIs may still be useful as in vitro and pre-clinical studies showed the successively use of GSI in combination therapy\(^{126,128}\). An important finding is the resensitization to steroids of otherwise glucocorticoid-resistant T-ALL cells by GSI\(^{163}\). Vice versa, steroids seem to be able to overcome gastro-toxicity effects by GSIs\(^{163}\). The proteasome inhibitor bortezomib is an alternative approach to block NOTCH1 activity and looks most promising. Most likely it inhibits the NOTCH-transactivator SP1. Addition of bortezomib resulted in downregulation of ICN levels, which is probably due to dissociation of the SP1 transcription factor from the NOTCH promoter and consequential downregulation of NOTCH targets\(^{164}\). Currently, other models that silence the NOTCH pathway have been successively studied in vitro and in vivo and are awaiting for clinical trial. Examples are antagonists targeting distinct NOTCH receptors\(^{165}\) or MAML1\(^{166}\) and NOTCH1 antibodies\(^{167}\).
Cell cycle and DNA repair

Cyclins and CDKs (cyclin-dependent protein kinases) heterodimeric complexes are master regulators of cell cycle. Various different cyclin and CDK proteins are known that act at specific phases of cell cycle, and the nature of the heterodimeric complex determines the activation of particular downstream proteins. The action of cyclin-CDK complexes is blocked by various members of the INK/ARF (p15, p16, p18 and p19) and CIP/KIP (p21, p27 and p57) cell cycle inhibitor gene families. The regulation and expression of these genes are regulated by upstream signaling cascades, including the PI3K/AKT and RAS-MEK-ERK pathways. Also other kinases like ATM/ATR kinases become active upon DNA damage, together with other essential enzymes and proteins including p53. Among these are Chk1 and Chk2 proteins and the tyrosine kinase Abl1. After repair, the block in cell cycle progression is relieved by the activation of Cdc25 and the inactivation of particular kinases and proteins.

The chromosomal 9p21 deletion is a frequent genetic lesion in cancer and the most commonly detected abnormality in pediatric T-ALL. The deletion frequently includes the CDKN2A gene, encoding for p16/p14, as well as the CDKN2B gene encoding for p15. The incidence of CDKN2A/B deletions is highly variable among study cohorts but comprises 60% of pediatric T-ALL patients on average. Inactivating mutations have also been described for CDKN2A/B, with frequencies between 0-13%. Both genes can further become inactivated by hypermethylation of their promoter regions, which occurs in less than 5% of patients for CDKN2A but is more prevalent for CDKN2B, with reported incidences of 38-47% of pediatric T-ALL patients. Another member of the INK/ARF family is p18 and deletions are described in a single study so far with a reported incidence of 14% of pediatric T-ALL patients. The p27 cell cycle inhibitor gene may comprise both mutations and deletions in 60-67% of pediatric T-ALL patients. Also overexpression of Cyclins itself are potential oncogenic targets in T-ALL. Rare Cyclin D2 translocations are described in 5 patients.

Apart from interference with direct cell cycle molecules, outbalancing the apoptotic system or creating genetic instability by inactivation of the DNA-repair system can deregulate the cell cycle. In pediatric T-ALL, around 5% of patients have p53-inactivating mutations/deletions at diagnosis and it is hypothesized that p53 aberrations are associated with relapse and poor survival rates after relapse. Also 5 and 10% of somatic ATM mutations/deletions are reported in two studies and furthermore, ATM germ-line mutations are suggested to be associated with T-ALL.

Other type B abnormalities

Besides mutations that deregulate specific pathways, T-ALL also carries mutations of which the oncogenic mechanism remains unclear. These include ABL1 fusion proteins, inactivating WT1 mutations/deletions, Ikaros deletions, LEF1 mutations and PHF6 mutations.

ABL is a predominantly nuclear protein that has been connected to regulate a variety of processes including cellular shape, motility and adhesion, cell cycle, DNA repair and apoptosis. ABL1 fusion proteins as consequence of chromosomal rearrangements are observed in a small
percentage of pediatric T-ALL patients with ETV6, EML1, NUP214 and BCR as fusion partners. The ETV6-ABL1 and EML1-ABL1 fusions have only been described for single pediatric T-ALL cases. The NUP214-ABL1 fusion as a result of the 9q34.11-9q34.13 deletion is the most commonly observed ABL1 fusion protein in T-ALL and present in ~5% of pediatric T-ALL patients. BCR-ABL1 translocations are known to occur in B-ALL patients but are exceptionally rare in T-ALL. Whereas ABL1 phosphorylates many downstream targets, based on in vitro and in vivo models, the BCR-ABL1 and ETV6-ABL1 fusion protein seem to activate RAS-MEK-ERK and PI3K/AKT pathways. For this, BCR-ABL1 has been demonstrated to bind to GRB2 (growth factor receptor–bound protein 2), which can phosphorylate the docking protein GAB2 (GRB2-associated binding protein 2) that allows recruitment of the GDP to GTP exchange factor SOS (son of seventhless) to activate membrane-bound RAS proteins. Also recruitment of the protein-tyrosine phosphatase PTPN11 (SHP2) further enhances RAS-MEK-ERK and PI3K/AKT signaling. BCR-ABL1 can also interact with the adapter molecule SHC or directly activate the RAS MAPK pathway through GRB2-binding.

In addition, JAK2 and STAT1 or STAT5 molecules are known phosphorylation targets of BCR-ABL1. Imatinib is a tyrosine kinase inhibitor that is successfully used in BCR-ABL1 patients. However, many BCR-ABL1 translocated patients develop resistance against imatinib by acquiring mutations in the imatinib binding pocket, and second generation kinase inhibitors have been developed like dasatinib, nilotinib and ponatinib. Patients with other ABL1 fusion proteins are also sensitive for ABL1 kinase inhibitors but also in these patients resistance may develop.

WT1 (Wilms tumor 1) is another gene regularly mutated/deleted in acute myeloid leukemia but more recently also found to play a role in occasional pediatric T-ALL patients. Mutations are often heterozygous frame-shift mutations that result in the truncation of the WT1 transcription factor rendering it incapable to bind to DNA. In two studies WT1 mutations were identified in 10-13% of pediatric T-ALL patients. The consequence of WT1 alterations in malignant transformation is not exactly clear. WT1 acts as a haploinsufficient tumor suppressor gene. It can probably inhibit wild-type WT1 proteins in a dominant-negative fashion. During hematopoiesis WT1 confers the regulation of differentiation, apoptosis and proliferation. Treatment options that specifically target WT1 or downstream pathways have not been identified yet.

Ikaros is a DNA-binding protein and loss of this protein is important in the development of B-ALL, though the involvement of Ikaros in human T-cell leukemogenesis is rare. In contrast, loss of IKZF1 (Ikaros gene) is frequently observed in mice developing T-cell lymphoma/leukemia. So far, around 5% of adult/pediatric T-ALL patients have been identified to carry IKZF1 deletions. Ikaros aberrations were described for ~10% of T-ALL patients by next generation sequencing. The function of Ikaros in T-cells needs to be established, but a strong association is observed between NOTCH1 expression and IKZF1 mutations in lymphoma/leukemia mice. Ikaros can bind to the same DNA-binding sites as NOTCH1 and may therefore be able to suppress NOTCH target gene expression. Furthermore, a recent study suggests that Ikaros is regulated by CK2 kinases during cell cycle in both T-ALL and B-ALL cell lines. Ikaros is induced by a retinoid receptor agonist in BCR-ABL1 cells which increased sensitivity to conventional tyrosine kinase inhibitor therapy.
LEF1 (lymphoid enhancer-binding factor 1) is a transcription factor of the Wnt signaling. Microdeletions and inactivating mutations have been found in LEF1 in ~5%-7% of pediatric T-ALL patients\textsuperscript{224}. The authors showed that LEF1 inactivation was associated with high MYC levels. It was hypothesized that LEF1 may act in concert with NOTCH1 function to achieve high MYC levels. However, the exact role of LEF1 in T-cell leukemogenesis is not clear and needs further investigation. Very recently, mutations in the TCF7 transcription factor are found in a small number of early T-cell progenitor ALL patients which interferes with LEF1 function and can thereby cooperate with LEF1 mutations\textsuperscript{225}. Treatment options that specifically target LEF1 or downstream pathways have not been identified yet.

Another mutated gene in T-ALL is PHF6. Although the function of PHF6 is unclear it is postulated that it is involved in cell cycle regulation and DNA repair. Inactivating PHF6 mutations and deletions are detected in 16% and 38% of pediatric T-ALL patients, respectively\textsuperscript{226}, and in 28% of a mixed adult/pediatric Korean cohort\textsuperscript{20}. In response to these findings, Yoo et al sequenced PHF6 in several tumors including T-ALL and detected that 35% adult/pediatric T-ALL patients have mutations\textsuperscript{227}. Later, Wang et al reported 19% of PHF6 mutations in adult and 5% in pediatric T-ALL\textsuperscript{228}.

**Targeting epigenetic regulators**

When proper transcription factors like ETV6 or RUNX1 are available, RNA polymerases are able bind to a core sequence within the DNA promoter to initiation of RNA transcription. Ribosomal RNA represent ribosome components for mRNA translation into protein. Deadenylation is crucial in the degradation of mRNA. Transcription factors and RNA polymerases are only able to bind when the genetic material harbors an accessible configuration. This is regulated by DNA- and histone modifications. This is, for instance, exhibited by DNA methyltransferases (DNMTs) that catalyze the methyl-group transfer to the DNA, histone acetyltransferase or methyltransferase enzymes. The latter enzyme complex is represented by polycomb complexes.

Whole exome sequencing of adult and pediatric T-ALL samples revealed CNOT3 mutations in 8% and RPL5 and RPL10 mutations in 10%\textsuperscript{229}. CNOT3 is part of a deadenylase complex that is involved in the shortening of the mRNA 3’poly(A) tail. Silencing of this gene in the Drosophila eye model results in a marked increase in tumor incidence. Loss-of-function mutations in the ribosomal protein RPL10 result in impaired proliferation and ribosome biogenesis effects.

The DNA-methyltransferase DNMT3 that is involved in transferring methyl-groups to cytosine residues in the DNA is frequently mutated in acute myeloid leukemia. It is recently identified by next generation sequencing to be mutated in 16-17% of adult T-ALL patients but was not identified in pediatric T-ALL\textsuperscript{77,230-235}. The genes EED, EZH2 and SUZ12 that encode the polycomb repression complex 2 (PRC2), which is involved in the epigenetics of the chromatin, are mutated in adult and pediatric T-ALL and predominantly in ETP-ALL patients\textsuperscript{25,232,236-238}. Also inactivating mutations in the transcriptional repressor ETV6 are recently identified in adult T-ALL. In this same study, inactivating point mutations and truncating frame shift mutations in the RUNX1 transcription factor were identified. Both mutations are also detected by next generation
sequencing of 12 ETP-ALL children and in a cohort of 77 additionally T-ALL children (RUNX1; 16% of T-ALL patients, ETV6 in 33% of ETP-ALL patients). RUNX1 regulates various genes involved in cell differentiation including the granulocyte-macrophage colony-stimulating factor. Mutant alleles of RUNX1 and ETV6 are suggested to act in a dominant negative fashion.

Demethylating agents alter the level of DNA methylation and are predominantly represented by cytidine analogs like azacitidine. Azacitidine is FDA-approved to treat patients with myelodysplastic syndromes and adult AML. Also targeting chromatin-remodeling events to prevent DNA exposure is therapeutically proven. Treatment with histon deacetylase (HDACs) inhibitors result in retained hyperacetylated histon lysine residues and accessible chromatin. This affects the gene expression of a small number of tumor suppressor or oncogenes by activation or repression. Inhibitors Vorinostat and Romidepsin have been FDA-approved for the treatment of subcutaneous T-cell lymphoma. Various alternatives are currently in clinical trial. Also RNA polymerases can be targeted by drugs like BMH-21, which was initially a p53 activator but also degrades the RNA polymerase I subunit and thereby reduces rRNA synthesis. In addition, transcriptional processes can be indirectly reduced by the interference of associated transcriptional complexes like TFIH. Interference with TFIH enables the opening of double stranded DNA and thereby transcription. One such a drug is Triptolide that is currently used in cancer treatment with positive effects.

CONCLUSION

Many mutations are known that make T-ALL a very heterogeneous disease. Type B mutations in T-ALL affect a various signal transduction pathways. To a minor extent, this includes the TCR signaling pathway. More frequently, the IL7R/JAK/STAT, RAS-MEK-ERK, PI3K/AKT and NOTCH signaling pathways are affected. Also general cellular processes like cell cycle, DNA repair, transcription and translation are frequently dysregulated by mutations in T-ALL. Type B mutations are, in contrast to type A driving oncogenic events, frequently present on the near clonal or subclonal level, making them difficult targets for targeted drug strategies. Also, the multitude of different type B mutations may hamper to analyze the prognostic significance of single type B mutations in relative small patient cohorts of pediatric T-ALL patients. It may therefore be important to group genetic alterations to their common involvement in signal transduction pathways or cellular processes. This may help to recognize relevant prognostic disease subtypes that may be stratified towards a specific treatment strategy.
Figure 1 | Aberrantly expressed signal transduction pathways by type B mutations in T-ALL. This includes the TCR (T-cell receptor), IL7R, JAK-STAT, RAS-MEK-ERK, PI3K/AKT and NOTCH1 signaling. Moreover this affects cell cycle, DNA damage control and epigenetic regulation. Known mutated proteins in T-ALL are indicated by *.
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NOTCH1 and/or FBXW7 mutations predict for initial good prednisone response but not for improved outcome in pediatric T-cell Acute Lymphoblastic Leukemia patients treated on DCOG or COALL protocols

Linda Zuurbier¹, Irene Homminga¹, Valerie Calvert², Mariël L. te Winkel¹, Jessica G.C.A.M. Buijs-Gladdines¹, Clarissa Kooi¹, Willem K. Smits¹, Edwin Sonneveld¹, Anjo J.P. Veerman,³ Willem A. Kamps,¹,⁴ Martin Horstmann⁵,⁶, Emanuel F. Petricoin III²,⁷, Rob Pieters¹, and Jules P.P. Meijerink¹

From the ¹Department of Pediatric Oncology/Hematology, Erasmus University Medical Center-Sophia Children’s Hospital, Rotterdam, the Netherlands; ²Center for Applied Proteomics and Molecular Medicine, George Mason University, Manassas, VA, USA; the ³Dutch Childhood Oncology Group (DCOG), the Hague, the Netherlands; the ⁴Department of Pediatric Oncology, University of Groningen-Beatrix Children’s Hospital, Groningen, the Netherlands; the ⁵German Cooperative Study Group for Childhood Acute Lymphoblastic Leukemia (COALL), Hamburg, Germany; ⁶the Research Institute Children’s Cancer Center Hamburg, Clinic of Pediatric Hematology and Oncology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; ⁷NCI-FDA Clinical Proteomics Program, Food and Drug Administration, Bethesda, MD, USA.

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CHAPTER 3

ABSTRACT

Aberrant activation of the NOTCH1 pathway by inactivating and activating mutations in NOTCH1 or FBXW7 is a frequent phenomenon in T-ALL. We retrospectively investigated the relevance of NOTCH1/FBXW7 mutations for pediatric T-ALL patients enrolled on Dutch DCOG ALL7/8 or ALL9 or the German COALL-97 protocols.

NOTCH1-activating mutations were identified in 63% of patients. NOTCH1 mutations affected the heterodimerization, the juxtamembrane and/or the PEST domains but not the RBP-J-kappa-associated module, the ankyrin-repeats or the transactivation domain. Reverse-phase protein microarray data confirmed that NOTCH1 and FBXW7 mutations resulted in increased intracellular NOTCH1 levels in primary T-ALL biopsies. Based on microarray expression analysis, NOTCH1/FBXW7 mutations were associated with activation of NOTCH1 direct target genes including HES1, DTX1, NOTCH3, PTCRA but not cMYC. NOTCH1/FBXW7 mutations were associated with TLX3 rearrangements, but were less frequently identified in TAL1 or LMO2-rearranged cases. NOTCH1-activating mutations were less frequently associated with mature T-cell developmental stage. Mutations were associated with a good initial in vivo prednisone response, but were not associated with a superior outcome in the DCOG and COALL cohorts.

Comparing our data with other studies, we conclude that the prognostic significance for NOTCH1/FBXW7 mutations is not consistent and may depend on the treatment protocol given.
INTRODUCTION

T-cell acute lymphoblastic leukemia (T-ALL) accounts for approximately 10-15% of all leukemias in children. Despite improved therapy, still 30% of these cases relapse and ultimately die.\(^{1,2}\)

Various chromosomal aberrations are known in T-ALL and some have been associated with prognosis.\(^{3-5}\) *NOTCH1* may be important for T-ALL pathogenesis and was initially identified as part of rare t(7;9) translocations.\(^{6,7}\) A role for NOTCH1 is now more clear as nearly 60 percent of T-ALL cases have *NOTCH1* mutations affecting the heterodimerization (HD), the juxtamembrane domain (JM) or the proline, glutamic acid, serine, threonine rich (PEST) domains.\(^{8,9}\) HD or JM mutations result in a ligand-independent proteolytical cleavages (reviewed in\(^{10}\)), resulting in the release of intracellular NOTCH1 (ICN). ICN is a transcription factor that regulates differentiation and proliferation through the activation of various target genes including *cMYC*, *HES1*, *PTCRA*\(^{10-12}\).

As an alternative NOTCH1 activation mechanism, inactivating mutations in the F-Box WD40 domain containing protein 7 gene (*FBXW7*) were identified in 8%-30% of T-ALL patients.\(^{13-16}\) *FBXW7* is part of the E3 ubiquitin ligase complex that controls the turnover of various proteins including ICN. *FBXW7* interacts with phosphodegron domains located in the PEST domain of ICN. Therefore, inactivating mutations in *FBXW7* or loss of the phosphodegron domains through truncating *NOTCH1* PEST mutations both result in the stabilization of ICN in the nucleus. Mutations in *FBXW7* and *NOTCH1* PEST mutations are mutually exclusive\(^{13,14,16}\), indicating that they seem to exert an equivalent oncogenic effect.

Mutations in *NOTCH1* or *FBXW7* may have prognostic relevance in T-ALL. Breit *et al* (2006) reported that *NOTCH1* mutant pediatric patients in the German ALL-BFM 2000 study demonstrate a good *in vivo* prednisone response and have an improved event free survival (EFS).\(^{17}\) In contrast, Zhu *et al* (2006) published an unfavorable outcome for *NOTCH1*-mutated adult T-ALL patients, but not for pediatric patients.\(^{18}\) We could not confirm a favorable prognostic effect for *NOTCH1*-mutated pediatric T-ALL patients treated on DCOG protocols,\(^{19}\) and this was confirmed by children treated on POG protocols for which no relation was identified between the presence of *NOTCH1* mutations and relapse.\(^{20}\) These initial studies investigated the relevance for *NOTCH1* HD and PEST mutations\(^ {17-19}\), but did not include *NOTCH1* JM mutations or *FBXW7* mutations. We now extended our initial study by examining the prognostic effect of *NOTCH1* and *FBXW7* mutations in 141 pediatric T-ALL patients treated on Dutch DCOG or German COALL-97 protocols. The functional consequences of *NOTCH1*/*FBXW7* mutations in relation to ICN levels and activation of target genes in primary leukemia samples were investigated.

MATERIAL AND METHODS

Patient samples
This study comprised 146 primary pediatric T-ALL patients, of which 72 were treated on Dutch
Childhood Oncology Group (DCOG) protocols ALL-7/8 (n=30) or ALL-9 (n=42) were compared. This cohort had a median follow up of 67 months, and included 51 male and 21 female patients. Because the overall disease free survival for patients treated on these DCOG protocols are comparable, these patients will be analyzed as one cohort as done before. Seventy of these patients were part of our previous study. For ALL7/8 patients, in vivo prednisone response was monitored at day 8 following 7 days of BFM-like prednisone monotherapy and one intrathecal dose of methotrexate. A clearance to less than 1000 blasts per micro liter blood at day 8 was considered as a good initial prednisone response (GPR). Seventy-four patients were enrolled in the German Co-Operative Study Group for Childhood Acute Lymphoblastic Leukemia study (COALL-97) protocol with a median follow up of 52 months. This cohort included 49 male and 25 female patients. The patients’ parents or legal guardians provided informed consent to use leftover diagnostic biopsies for research in accordance with the Institutional Review Board and the Declaration of Helsinki. Isolation of leukemia cells from blood or bone marrow samples has been described before and all samples contained >90% of leukemic blasts. Clinical and immunophenotypic data were supplied by both study centers. Classification into T-cell development stages was based on EGIL criteria: pro-/pre- (CD7+, CD2+ and/or CD5+ and/or CD8+), cortical (CD1+) or mature T-cell stage (sCD3+/CD1-).

Genomic DNA and RNA extraction
Isolation of genomic DNA and RNA from 5*10^6 leukemic cells using the Trizol reagent (Invitrogen, Breda, The Netherlands) and copy-DNA synthesis were done as described before.

Mutational detection
NOTCH1 exons 25-34 were screened for mutations that include all relevant domains (Table S1). For FBXW7, the F-box and WD40 domains (exon 5, exons 7-11) were amplified, covering all FBXW7 mutations as published so far. PCR reactions were done as described before. Primers are displayed in Table 1. PCR products were sequenced using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems) on a 3130 DNA Analyzer (Applied Biosystems).

Identification of recurrent rearrangements by FISH, RQ-PCR or array-CGH
SIL-TAL, CALM-AF10 or rearrangements of LMO2, TLX1, TLX3, TAL1, CALM-AF10, SET-NUP214, HOXA, or MLL were determined with fluorescence in-situ hybridization (FISH) as previously described. NOTCH1 translocations were detected using bacterial artificial chromosomes (BACs) clones RP11-769N4, RP11-1008C19, RP11-83N9 and RP11-662J2 covering both sides adjacent to the NOTCH1 locus. BACs were obtained from BAC/PAC Resource Center (Children’s Hospital, Oakland, CA). Expression levels of TLX1, TLX3, TAL1, LMO2 or HOXA or CALM-AF10 and SET-NUP214 fusion products were measured relative to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as described before. Array-CGH analysis was performed as previously described, on the human genome CGH Microarray 105K or 400K dual arrays (Agilent Technologies, Santa-Clara, USA), which consists of ~105,000 or ~400,000 60-mer
oligonucleotide probes that span both coding and non-coding sequences with an average spatial resolution of ~15 or 5kb, respectively. Microarray images were analyzed using feature extraction software (version 8.1, Agilent) and the data were subsequently imported into array-CGH analytics software v3.1.28 (Agilent).

**Gene expression array analysis**
RNA integrity testing, copy-DNA and ccRNA syntheses, hybridization and washing to Human Genome U133 plus2.0 microarrays (Affymetrix, Santa-Clara, CA, USA), extraction of probeset intensities from CEL-files and normalization with RMA or VSN methods were done as described before 28. Differentially expressed genes between NOTCH1 mutant versus wild-type T-ALL patients were determined by Wilcoxon statistics and corrected for multiple testing error 29 using the Bioconductor package “Multtest” in R. Heatmaps based on the TOP50 most significant differentially expressed genes were performed in Dchip software 30. Microarray data are available at http://www.ncbi.nlm.nih.gov/geo/.

**Reverse-phase Protein Microarray analysis (RPMA) and western blot**
Reverse-phase protein microarray construction and analysis was performed essentially as previously described 31,32. To isolate proteins from 10*10^6 leukemic cells, lysis was performed in 20µL Tissue Protein Extraction Reagent (TPER, Pierce Biotechnology, Rockford, IL, USA) with 300 nM NaCl, 1 mM orthovanadate and protease inhibitors. Cells were incubated at 4°C for 20’ and subsequently centrifuged at 10.000 rpm for 5’ in an Eppendorf centrifuge. Supernatants were stored at -80°C prior to printing on the microarrays. Lysates were diluted to 1.0 mg/ml protein concentration and mixed 1:1 with 2x SDS Tris-glycine buffer (Invitrogen) containing 5% 2-mercaptoethanol (Sigma, Zwijndrecht, the Netherlands) (FC = 0.5 mg/ml). Lysates were spotted at a concentration of 0.5 µg/µl (neat spot) and 0,125 µg/µl in duplicate with 350 micron pins on glass-backed nitrocellulose coated array slides (FAST slides, Whatman plc, Kent, UK) using an Aushon Biosystems 2470 (Aushon Biosystems, Billerica, MA, USA). Printed slides were stored at -20°C or directly used. The first of each 25 slides printed were subjected to Sypro Ruby Protein Blot staining (Invitrogen) to determine total protein amount. These slides were visualized on a NovaRay CCD fluorescent scanner (Alpha Innotech. San Leandro, CA, USA). The remaining slides were used for staining with a specific antibody. Prior to this, slides were incubated with 1x Reblot (Chemicon, Temecula, CA, USA) for 15’ and subsequently washed with PBS twice. This was continued with a blocking procedure for 5 hrs using 1gr l-block (Applied Biosystems) diluted in 500mL PBS with 0,5% Tween-20. Slides were stained with an automated slide stainer (Dako) according to manufacturer’s instructions using the Autostainer catalyzed signal amplification (CSA) kit (Dako). In each staining run, a negative control slide was stained with the secondary antibody only for background subtraction. Briefly, endogenous biotin was blocked for 10 minutes with the biotin blocking kit (Dako), followed by application of protein block for 5 minutes; primary antibodies were diluted in antibody diluent and incubated on slides for 30 minutes and biotinylated secondary antibodies were incubated for 15 minutes. Signal amplification involved
incubation with a streptavidin-biotin-peroxidase complex provided in the CSA kit for 15 minutes, and amplification reagent (biotinyl-tyramide/hydrogen peroxide, streptavidin-peroxidase) for 15 minutes each. A signal is generated using streptavidin-conjugated IRDye680 (LI-COR Biosciences, Lincoln, NE, USA). Slides were allowed to air dry following development. Stained slides were scanned individually on the NovaRay scanner (Alpha Innotech) and files were saved in TIF format in Photoshop 7.0. All slides were subsequently analyzed with the MicroVigene v2.8.1.0 program (Vigenetech, Carlisle, MA, USA). To screen for ICN protein levels, we have used and optimized the conditions for the ICN Val1744 antiserum (Cat#2421, Cell Signaling Technology, Beverly MA, USA). Slides were scanned in a NovaRay scanner (Alpha Innotech) and analyzed with the MicroVigene v2.8.1.0 program (Vigenetech, Carlisle, MA, USA). For western blot validation 28, protein loading was validated by staining for Actin (Sigma, Cat#2547).

Statistics
Statistics were performed using SPSS 15.0 software. The Pearson’s Chi-square test or the Fisher’s exact test was used to test differences in the distribution of nominal data as indicated. Statistical significance for continuous distributed data was tested using the Mann-Whitney-U test. Differences between patient populations in event free survival (EFS) and relapse free survival (RFS) were tested by using the log-rank test. For RFS, an event is defined as relapse or non-response towards induction therapy at day 56 (COALL) or at start of consolidation therapy (DCOG). An event for EFS is defined as relapse, non-response towards induction therapy, death in remission due to toxicity or development of a secondary malignancy. Data were considered significant when \( p \leq 0.05 \) (two-sided).

RESULTS

**NOTCH1 and/or FBXW7 mutations in pediatric T-ALL patients**
Bone marrow or blood DNA samples for 146 primary T-ALL patients were analyzed for NOTCH1 (exons 25-34) and/or FBXW7 mutations (exons 5, 7-11) and 141 samples were successfully amplified and sequenced. The locations of mutations in specific NOTCH1 or FBXW7 domains are shown in Figure 1A.

Heterozygous mutations in NOTCH1 were detected in 79 out of 141 cases (56%) whereas 23 T-ALL patients (16%) harbored a point mutation in FBXW7. In total, 89 patients (63%) contained NOTCH1 and/or FBXW7 mutations. Thirty-five patients (39%) had a missense mutation or an in-frame insertion/deletion in the HD-domain of NOTCH1 whereas nine (10%) and 13 (15%) patients harbored a combination of HD and PEST or FBXW7 mutations, respectively. Seventeen patients (19%) had a single NOTCH1 PEST mutation and ten (11%) had a single FBXW7 mutation (Figure 1B). We confirmed that NOTCH1 PEST domain mutations and FBXW7 mutations were nearly mutual exclusive 14,16, but one patient carried a FBXW7 and a NOTCH1 PEST mutation. Five patients had a mutation in the JM domain of NOTCH1 (5.6%) of which one also had a NOTCH1
HD mutation. It is not known whether these JM and HD mutations occurred in cis or affected different alleles.

Figure 1 | NOTCH1 and FBXW7 mutations in pediatric T-ALL patients. (A) Schematic representation of identified mutations in the heterodimerization (HD), juxtamembrane (JM) and PEST domains in NOTCH1 and in the WD40-repeats of FBXW7. Missense mutations are indicated by an open triangle, a silent mutation is indicated by a filled grey triangle, and nonsense mutations are indicated by a filled black triangle (B) The distribution of NOTCH1 and FBXW7 mutation types in the DCOG and COALL cohorts.

In total, 66 different NOTCH1 mutations were found and ten HD and nine PEST mutations were not reported before to the best of our knowledge (Figure S1). Ten different FBXW7 point mutations were found, five of which have not been observed before in T-ALL (Figure S2). These are H379L in exon 7, R465P in exon 8, and K622STOP, G687V and E693K in exon 11. The E693K mutation was previously identified in a gastric carcinoma patient 33.

NOTCH1 and/or FBXW7 mutations activate ICN and downstream target genes in primary T-ALL samples
As published for T-ALL cell lines8,9,11,12,14,16, we demonstrated by using reverse-phase protein microarrays (RPMA) that NOTCH1 and/or FBXW7 mutations result in enhanced levels of ICN in primary T-ALL cells. The specificity of the NOTCH1 antibody was validated on the T-ALL cell line HPB-ALL, and ICN detection was lost upon treatment with a g-secretase inhibitor (Figure 2A). NOTCH1 and/or FBXW7-mutated patients displayed about 2 fold higher ICN levels compared to wild-type patients (Figure 2B, p=0.0015). Strikingly, four wild-type patients also showed high ICN levels despite the absence of NOTCH1 and/or FBXW7 mutations (Figure 2B). Subsequent FISH and array-CGH analyses ruled out potential NOTCH1 translocations or other chromosomal NOTCH1 rearrangements in these four patients (data not shown).
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Figure 2 | NOTCH1/FBXW7 mutations activate the NOTCH1 pathway in primary T-ALL patient biopsies. (A) Western blot analysis of lysates from the HPBALL T-ALL cell line which is NOTCH1-mutated. Treatment for 96 hrs with g-secretase inhibitors including compound E (100 nM) or DAPT (5 µM), results in loss of activated intracellular NOTCH1 expression (ICN). Actin was used as loading control. (B) NOTCH1 ICN levels in wild-type and NOTCH1 and/or FBXW7-mutated T-ALL patients and T-ALL cell lines analyzed with Reverse-phase Protein microarray. NOTCH1/FBXW7 wild-type patient samples with high ICN levels are marked by an asterisk (C) Heatmap showing the TOP50 most differentially expressed genes between NOTCH1 and/or FBXW7 mutant patients versus wild-type patients. NOTCH1 direct target genes are indicated. Annotations indicated are genetic rearrangements, Gender and NOTCH1/FBXW7 mutation status. Genetic rearrangements indicated are: T, TAL1 or SIL-TAL1; L, LMO1 or LMO2 (includes del(11)(p12p13)); A, HOXA-activated (includes cases with SET-NUP214; CALM-AF10 or Inv(7)(p15q34)); 1, TLX1; 2, TLX2; 3, TLX3; O, Other; U, Aberration unknown. Gender is indicated F, Female or M, Male. NOTCH1/FBXW7 mutation status is indicated 0, wild-type and 1, NOTCH1 and/or FBXW7-mutated; NOTCH1/FBXW7 wild-type patients with high ICN levels are marked by an asterisk; NOTCH1/FBXW7 wild-type patients having a NOTCH1 signature that cluster with NOTCH1-activated patients based upon hierarchical clustering based on the TOP50 probeset are indicated with a filled triangle.

We investigated whether NOTCH1/FBXW7 mutations would result in the activation of specific genes. Expression array data \textsuperscript{28,34} was available for 111 T-ALL patients with a known NOTCH1/ FBXW7 mutation status. The TOP50 most significant and differentially expressed genes (probesets) between NOTCH1/FBXW7 mutant and wild-type patients comprised previous published and validated NOTCH1 direct target genes including HES1, HES4, DTX1, PTCRA, NOTCH3, PTPRC, CR2, L2TFL1, TASP1, SHQ1 and RHOU (Figure 2C) \textsuperscript{10,11}. Although cMYC is a NOTCH1 target gene in T-ALL cell lines, this gene did not appear in our TOP50 nor TOP200 gene lists (not shown). Eight wild-type patients also seemed to express genes from this NOTCH1 signature (Figure 2C and data not shown). For six out of these eight patients for which ICN levels were available, two patients were among the four wild-type cases having the highest ICN protein levels. Alike these two cases having a NOTCH1 signature and high ICN levels, none of the remaining six patients with a NOTCH1 signature carried NOTCH1 translocations or alternative chromosomal abnormalities based on FISH and array-CGH results (data not shown).
NOTCH1/FBXW7 mutations in relation to clinical, immunophenotypic and cytogenetic parameters

We did not observe a relationship between NOTCH1/FBXW7 mutations with gender, age or white blood cell counts (Table 1). For 23 patients, the in vivo prednisone response was known. NOTCH1-activated patients were correlated with a good in vivo prednisone response since 14 out of 16 patients with a good initial prednisone response (GPR) contained NOTCH1 mutations, in contrast to two out of seven cases with a poor response (p=0.01). This observation was stronger by including FBXW7 data where 15 out of 16 cases with a GPR had a NOTCH1/FBXW7 mutation in contrast to only two out of seven PPR cases (p=0.003, Table 1). Classification into T-cell development stages on EGIL criteria \(^{26}\) revealed that NOTCH1/FBXW7 mutations were less frequently identified in mature T-ALL cases (p=0.05, Table 1). In relation to molecular cytogenetic data, NOTCH1/FBXW7 mutations were identified in all cytogenetic T-ALL subgroups (Table 1). Considering TAL1 or LMO2 rearranged cases as a single TAL/LMO entity based on their identical expression profiles \(^{28,14}\), and including an additional 19 TALLMO-like patients with a TAL/LMO signature that lack TAL1 or LMO2 rearrangements \(^{28}\), NOTCH1 mutations were less frequent. Only 25 out of 60 TALLMO patients (42%) had a NOTCH1 mutation (p=0.002, Table 1). This remained significant when including FBXW7 mutations since only 30 out of 60 cases (50%) had a NOTCH1/FBXW7 mutation (p=0.004). NOTCH1 mutations were more prevalent in TLX3-rearranged cases, in which 21 out of 27 cases (86%) had a NOTCH1 mutation (p=0.02). This remained significant when taking FBXW7 mutations into account (p=0.01).

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Table 1 | Distribution of wild-type and NOTCH1 mutations within categorized subgroups of T-ALL patients. Abbreviations: Mut, mutant; PGR, prednisone good response; PPR, prednisone poor response; NE, not evaluable; T-ALL, T-cell acute lymphoblastic leukemia; WBC, white blood cell count; WT, wild-type. Only significant P-values are indicated in bold. #P-values calculated by the Fisher’s exact method; *P-values calculated by the Mann–Whitney-U method.

Prognostic relevance of NOTCH1 and/or FBXW7 mutations

We then investigated the relevance of NOTCH1 and/or FBXW7 mutations in relation to treatment outcome. For the DCOG cohort, mutations in NOTCH1 and/or FBXW7 trended towards poor treatment outcome. The 5 years event free survival (5 yrs EFS) rates for patients with NOTCH1 mutations only compared to wild-type patients were 57±8% versus 76±8% (p=0.08) for the
DCOG cohort but 63±8% versus 64±10% for the COALL cohort (p=0.99, Figure 3A,B). Inclusion of FBXW7 mutations resulted in 5 yrs EFS rates of 58±7% versus 74±9% (p=0.16) for the DCOG cohort and 63±8% versus 68±10% for the COALL cohort (p=0.90, not shown).

Events in both cohorts are summarized in Table 2. NOTCH1 mutations trended towards a lower relapse free survival (RFS) in the DCOG (p=0.068) and COALL cohorts (p=0.094) with 5 years RFS of 83±7 versus 62±8 percent for the DCOG cohort and 89±6 versus 70±8 percent for the COALL cohort for wild-type and NOTCH1-mutated patients, respectively (Figure 3C,D). These trends became less evident when including FBXW7 mutation data, with a RFS of 82±8 versus 62±8 percent in the DCOG cohort (p=0.101) and a RFS of 86±7 versus 70±8 percent in the COALL cohort (p=0.23) for wild-type or NOTCH1-activated patients, respectively (not shown).

Figure 3 | NOTCH1-activating mutations have no prognostic implication in pediatric T-ALL. Event Free Survival (EFS) (A,B) and Relapse Free Survival (RFS) (C,D) for the pediatric T-ALL patients treated on DCOG protocols (A,C) or the COALL protocol (B, D). Patients carrying NOTCH1 and/or FBXW7 mutations and wild-type patients have been indicated.

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Table 2 | Events in DCOG and COALL cohorts. Abbreviations: CNS, central nervous system; DCOG, Dutch Childhood Oncology Group; COALL, Childhood Acute Lymphoblastic Leukemia study; Mut, mutant for NOTCH1; WT, wild-type. *Includes CNS relapse.
We also investigated the effect for specific NOTCH1 and/or FBXW7 mutations on the activation of downstream target genes and outcome. As published by the group of Pear and co-workers, specific NOTCH1 mutations or combinations of NOTCH1/FBXW7 mutations may have strong NOTCH1-activating effects whereas others may only have modest activating effects. For this, we distinguished weak NOTCH1-activating mutations, i.e. NOTCH1 HD or PEST mutations or FBXW7 mutations, and strong NOTCH1-activating mutations, i.e. NOTCH1 JM mutations or combinations of NOTCH1 HD mutations with PEST mutations or FBXW7 mutations. Although ICN protein levels were significantly higher for NOTCH1 and/or FBXW7 mutated cases versus wild-type cases, there was relation to the types of NOTCH1-activating mutations investigated (Figure S3). To investigate differential activation of downstream target genes between patients with weak or strong NOTCH1-activating mutations, we first calculated the most significantly and differently expressed genes (probesets) between patients with strong NOTCH1-activating mutations versus wild-type patients, which again revealed mostly bonafide NOTCH1 target genes. However, these genes were expressed at intermediate levels for patients having weak NOTCH1-activating mutations (Figure S4) indicating that these types of mutations indeed differ in their potential to activate downstream target genes in primary leukemic samples. Distinction between these types of mutations may also have prognostic significance as patients from the DCOG cohort with strong NOTCH1-activating mutations had a significant poor outcome relative to wild-type patients (p=0.012) as well as to patients carrying weak NOTCH1-activating mutations (p=0.048) (Figure S5A). However, this observation could not be substantiated for COALL-97 T-ALL patients (Figure S5B). We also investigated whether ICN protein levels itself had prognostic significance. As 55 out of 66 patients for which ICN protein levels were available were treated on the COALL cohort, we divided these patients into quartiles and determined their RFS and EFS rates. However, no relationship between ICN protein levels and RFS nor EFS was present (p=0.98 and p=0.97, respectively).

**DISCUSSION**

Activation of NOTCH1 as a consequence of activating NOTCH1 mutations or inactivating FBXW7 mutations is a frequent phenomenon in T-ALL. We screened for NOTCH1 and FBXW7 mutations in 141 pediatric T-ALL patient samples and identified NOTCH1 mutations in 56% and FBXW7 mutations in 16% of the patients. In total, 63 percent of the patients had an aberrantly activated NOTCH1 pathway due to mutations. In line with previous studies, we observed that NOTCH1 PEST domain mutations and FBXW7 mutations occurred in a mutually exclusive fashion with the exception of one patient. This patient had a nonsense mutation in FBXW7 in contrast to missense mutations that are normally observed in FBXW7 mutated patients. This implies that mutant FBXW7 but not truncated FBXW7 proteins exert a dominant negative effect in the E3-ubiquitin ligase complex. Interestingly, Park et al (2009) also discovered a nonsense mutation due to a 5 bp insertion in FBXW7 in combination with a NOTCH1 PEST mutation in a non-Hodgkin’s lymphoma
The frequency of NOTCH1-activating mutations is in line with other studies also comprising adult T-ALL patient series. In adult studies, NOTCH1 and FBXW7 mutations were identified in 60-62% and 18-24% of the T-ALL patients, respectively. This indicates that the oncogenic role for NOTCH1/FBXW7 during T-cell oncogenesis remains conserved over age. We did not find evidence for mutations outside the NOTCH1 HD, JM and PEST-domains in any of the 141 pediatric T-ALL patients indicating that reported mutations in the LNR region, the RAM-, ANK-, and TAD-domains are very rare.

We found that NOTCH1 and FBXW7 mutations resulted in increased levels of cleaved NOTCH1 (ICN) in primary leukemia cells and was associated with the activation of NOTCH1 target genes, including HES1, HES4, DTX1, PTPRC, CR2, LZTFL1, TASP1 and RHOU. This confirms that the mutations manifest functionally at the protein level in patient samples. We identified ten patients that lacked NOTCH1 and/or FBXW7 mutations that either expressed high levels of ICN or that expressed NOTCH1 target genes. As we did not find chromosomal translocations or other types of rearrangements involving the NOTCH1 locus, this implies that additional mutation mechanisms in NOTCH1 or directly downstream regulatory genes must exist, that so far been left unnoticed in T-ALL. Although cMYC was identified as a prominent NOTCH1 target in T-ALL cell lines, it was not identified as target gene in primary samples. However, two cases expressed ectopic cMYC levels due to a t(8;14)(q24;q11) translocation which were both wild-type for NOTCH and FBXW7, supporting a role for MYC as NOTCH1 target. Further research will be required to establish whether cMYC is generally upregulated by means of other oncogenic mechanisms in addition to activated NOTCH1 in primary samples and therefore left undetected, or that the expression of cMYC is rapidly lost upon isolation of primary leukemic cells.

NOTCH1/FBXW7 mutations were identified at a lower frequency in T-ALL cases with a mature immunophenotype. This may explain the low incidence of NOTCH1/FBXW7 mutations in the TAL/LMO subgroup because TAL1 rearrangements, which are the most recurrent abnormality in this subgroup, are associated with a mature T-cell development arrest. This is an interesting finding and suggests that the oncogenic role of NOTCH1 is less prominent in T-ALL cases arrested at a relative mature T-cell developmental stage. Interestingly, NOTCH1/FBXW7 mutations were identified at a higher frequency in TLX3-rearranged T-ALL. The oncogenic activation of NOTCH1 thus far has been regarded as one of the earliest acquired abnormalities in a pre-leukemic progenitor cell that therefore becomes committed to the T-ALL. In this perspective, our data indicate that the importance of deregulated NOTCH1 as initiating event during T-cell oncogenesis depends on additional collaborating events like TLX3 or TAL1 rearrangements. It also suggests that the oncogenic program that is followed by T-ALL cases that eventually arrest at the mature development stage may be less dependent on NOTCH1. Whether NOTCH1-activating mutations represent truly initiating leukemic events or not needs to be established, as evidence is emerging that NOTCH1 activation in some T-ALL cases may have occurred as a secondary event which may be acquired or lost at relapse.
In the study of Breit et al (2006), NOTCH1 mutations were associated with a good initial prednisone response and a significantly lower minimal residual disease (MRD) content at day 78. Our study supports this association with initial prednisone response for NOTCH1/FBXW7 mutant patients. This association is also validated for patients of the EORTC-CLG study. In that study, NOTCH1-activating mutations were also associated with reduced minimal residual disease during therapy. The association for NOTCH1-activating mutations with initial good prednisone response seems to be in contrast with the finding that gamma-secretase inhibitors (GSIs) can sensitize for glucocorticoids in glucocorticoid resistant cells. It may be that the NOTCH pathway has opposing effects in the glucocorticoid response in responsive versus resistance patients, but it now seems clear that activation of NOTCH1 by mutations does not drive glucocorticoid resistance. Further research will be required to clarify this seeming contradiction.

NOTCH1 mutations are not associated with a superior outcome for patients treated on the BFM-like DCOG protocols or the COALL-97 protocol. The survival of NOTCH1-activated patients was actually lower than for wild-type patients. Separating patients carrying strong NOTCH1-activating mutations from those with weak NOTCH1-activating mutations or patients that were wild-type, demonstrated a significant poor outcome for patients having strong NOTCH1-activating mutations in the DCOG cohort. This could not be reproduced for T-ALL patients treated on the German COALL-97 protocol. In the accompanying paper of Clappier and coworkers, NOTCH1-activating mutations did not predict improved outcome for patients treated on the BFM-derived EORTC-CLG protocols either. These observations are in contrast to the findings by the BFM study group. In the accompanying paper of Kox et al (2009), this finding is now validated in an extended series comprising 301 pediatric T-ALL patients treated on the ALL-BFM 2000 protocol. A favorable prognostic effect of NOTCH1 and/or FBXW7 mutations was also identified in a recent study by Park et al (2009), although the overall incidence of identified NOTCH1 mutations was only 31%. No favorable outcome of NOTCH1 and/or FBXW7-mutated cases has been observed for adult T-ALL patients treated on GMALL 05/93 and 06/99 multicenter protocols, nor for patients treated on the MRC UKALLXII/ECOG E2993 or LALA-94 protocols. A significant association with improved outcome for NOTCH1-activating mutations has only been observed for adult T-ALL patients treated on the GRAALL-2003 multicenter protocol. These results indicate that the prognostic effect of NOTCH1/FBXW7 mutations may strongly depend on the treatment protocol given.

Compared to the ALL-BFM-2000 protocol, the DCOG ALL-7/8 protocol in general showed an inferior outcome. Although both protocols are highly related, part of the patients treated on the DCOG ALL-7/8 cohort received less chemotherapy and none of them received prophylactic cranial irradiation, except for patients with initial central-nervous system involvement. NOTCH1-activating mutations may provoke CNS relapse due to the activation of the CCR7 chemokine. This study therefore predicts that NOTCH1-activating mutations would result in increased risk for CNS relapse via the CCL19-CCR7 axis in the absence of cranial irradiation. However, the numbers of CNS relapses in our cohorts were too low to substantiate this notion. Besides, neither the CCR7 gene nor its ligand CCL19 were identified as significantly differentially expressed.
genes that were activated in NOTCH1/FBXW7-mutated T-ALL patients based on our microarray expression dataset (data not shown). As our patient biopsies were all obtained from peripheral blood or bone marrow samples, we cannot exclude that these genes are only upregulated in malignant blasts in the context of a neuronal environment. Cranial radiation may contribute to the differences in prognostic value for NOTCH1-activating mutations between the DCOG and ALL-BFM-2000 cohorts, but this does not apply for the COALL-97 cohort that includes cranial irradiation. Therefore, other differences among treatment protocols seem important.

In conclusion, NOTCH1/FBXW7 mutations that activate the NOTCH1 pathway are identified in more than 60 percent of pediatric T-ALL patients and result in elevated ICN levels and activation of NOTCH1 target genes. Mutations were more often found in association with TLX3-rearranged T-ALL, but were less frequently identified in TAL/LMO T-ALL patients and T-ALL patients with a mature T-cell phenotype. NOTCH1/FBXW7 mutations predict for an initial good prednisone response, which does not translate into a superior outcome of T-ALL on DCOG ALL-7/8, ALL-9 or COALL-97 protocols.

AUTHORSHIPS AND DISCLOSURES

L.Z. designed experiments, performed research and wrote manuscript, I.H. performed research and wrote manuscript, V.C. performed RPMA analysis, M.L.W. performed research, J.B.-G. performed NOTCH1 and FBXW7 mutation analysis, C.K. performed western blot analysis, W.S. prepared samples for RPMA analysis, E.S., A.J.P.V, W.K. and M.H. provided patient samples and clinical and immunophenotypic data, E.P. supervised study, and wrote manuscript, R.P. designed and supervised study and wrote manuscript, J.P.P.M. was principal investigator, designed and supervised the study, and wrote manuscript.

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NOTCH1 and FBXW7 mutations in T-ALL

SUPPLEMENTARY DATA

Figure S1 | NOTCH1 mutations in pediatric T-ALL patients. Amino acid changes in the HD, JM and PEST domains of NOTCH1, as a result of NOTCH1 mutations, are listed for each patient. New mutations and the reference of each known mutation are indicated.
NOTCH1 and FBXW7 mutations in T-ALL

Figure S2 | FBXW7 mutations in pediatric T-ALL patients. Amino acid changes in the WD40-repeats, as a result of FBXW7 mutations, are listed for each patient. New mutations and the reference of each known mutation are indicated.

Figure S3 | ICN levels in wild-type, weak NOTCH1-activated (single HD, single PEST, single FBXW7 mutation) and strong NOTCH1-activated (HD+PEST, HD+FBXW7, JM mutation) T-ALL patients analyzed with reverse-phase protein microarray.
Figure S4 | Heatmap showing the TOP50 most differentially expressed genes between patients with strong NOTCH1-activating mutations versus wild-type patients. NOTCH1 JM mutations or combinations of NOTCH1 HD mutations with PEST mutations or FBXW7 mutations were considered as strong NOTCH1-activating mutations. Annotations indicated are genetic rearrangements, gender and NOTCH1/FBXW7 mutation status. Genetic rearrangements indicated are: T, TAL1 or ST-TAL1; L, LMO1 or LMO2 (includes del(11)(p12p13)); A, HOXA-activated (includes cases with SET-NUP214; CALM-AF10 or Inv(7)(p15q34)); 1, TLX1; 2, TLX2; 3, TLX3; O, Other; U, Aberration unknown. Gender is indicated F, Female or M, Male. NOTCH1/FBXW7 mutation status is indicated “1” in blue box for wild-type, “2” in yellow box for patients with weak NOTCH1-activating mutations and “3” in red box for patients having strong NOTCH1-activating mutations.

Figure S5 | The prognostic effect of NOTCH1/FBXW7 mutations. Weak NOTCH1-activating mutations were considered as NOTCH1 HD, PEST or FBXW7 mutations, whereas strong NOTCH1-activating mutations were considered as NOTCH1 JM mutations or NOTCH1 HD mutations in combination with PEST or FBXW7 mutations. A. For DCOG T-ALL patients, strong NOTCH1-activating mutations are significantly associated with poor outcome, with p-values of $p=0.012$ and $p=0.048$, compared to patients without NOTCH1/FBXW7 mutations (wild-type) or patients with weak NOTCH1-activating mutations, respectively. B. No significant association with poor outcome was observed for weak or strong-activated NOTCH1 T-ALL patients treated according to the COALL-97 protocol.
The significance of PTEN and AKT aberrations in pediatric T-cell acute lymphoblastic leukemia

Linda Zuurbier¹, Maartje J. Vuerhard¹, Valerie Calvert², Clarissa Kooi², Jessica G.C.A.M. Buijs-Gladdines¹, Willem K. Smits¹, Edwin Sonneveld¹, Anjo J.P. Veerman¹⁺, Willem A. Kamps¹⁺, Martin Horstmann⁶⁺, Emanuel F. Petricoin III²⁺, Rob Pieters¹ and Jules P.P. Meijerink¹

From the ¹Department of Pediatric Oncology/Hematology, Erasmus MC Rotterdam-Sophia Children’s Hospital, Rotterdam, the Netherlands; ²Center for Applied Proteomics and Molecular Medicine, George Mason University, Manassas, VA, USA; the ³Dutch Childhood Oncology Group (DCOG), the Hague, the Netherlands; the ⁴Department of Pediatric Oncology/Hematology, VU University Medical Center, Amsterdam, ⁵Department of Pediatric Oncology, University of Groningen-Beatrix Children’s Hospital, Groningen, the Netherlands; the ⁶German Cooperative Study Group for Childhood Acute Lymphoblastic Leukemia (COALL), Hamburg, Germany; ⁷the Research Institute Children’s Cancer Center Hamburg, Clinic of Pediatric Hematology and Oncology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; ⁸NCI-FDA Clinical Proteomics Program, Food and Drug Administration, Bethesda, MD, USA.

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ABSTRACT

PI3K/AKT pathway mutations are found in T-cell acute lymphoblastic leukemia, but impact and associations with other genetic aberrations is unknown. PTEN mutations have been proposed as secondary mutations that follow NOTCH1-activating mutations and that cause cellular resistance to gamma-secretase inhibitors. The impact of PTEN, PI3K and AKT aberrations was studied in genetically well-characterized pediatric T-cell leukemia patient cohort (n=146) treated on DCOG or COALL protocols. PTEN and AKT E17K aberrations were detected in 13% and 2% of patients, respectively. Defective PTEN-splicing was identified in incidental cases. Patients without PTEN protein but lacking exon-, splice-, promoter mutations or promoter hypermethylation were present. PTEN/AKT mutations were especially abundant in TAL- or LMO-rearranged leukemia but nearly absent in TLX3-rearranged patients (p=0.03), an association pattern that seems reciprocal to NOTCH1-activating mutations. Most PTEN/AKT mutant patients either lacked NOTCH1-activating mutations (p=0.006) or had weak NOTCH1-activating mutations (p=0.011), and consequently expressed low intracellular NOTCH1, cMYC and MUSASHI levels. T-cell leukemia patients without PTEN/AKT and NOTCH1-activating mutations fared well, with a cumulative incidence of relapse of only 8% versus 35% for PTEN/AKT and/or NOTCH1-activated patients (p=0.005). In conclusion, PI3K/AKT pathway aberrations are present in 18% of pediatric T-cell acute lymphoblastic leukemia patients. Absence of strong NOTCH1-activating mutations in these cases may explain cellular insensitivity to γ-secretase inhibitors.
INTRODUCTION

Despite improved treatment outcome, children with T-cell Acute Lymphoblastic Leukemia (T-ALL) have a higher relapse risk than children with B-lineage ALL. T-ALL is characterized by mutually exclusive abnormalities in TAL1, LMO2, TLX3/HOX11L2, TLX1/HOX11 or HOXA oncogenes. Gene expression analyses supported the view that the aberrations delineate specific T-ALL subgroups, in which cases with TAL1 or LMO2 aberrations share an identical expression profile and may be considered as a single TALLMO subgroup. These abnormalities are accompanied by other genetic aberrations, denoted as so-called type B mutations, that are found in nearly all subgroups. These latter mutations include NOTCH1-activating mutations affecting the NOTCH1 gene itself and/or inactivating mutations in the F-Box WD40 domain containing protein FBXW7 gene, which is a ubiquitin ligase that apart of NOTCH1 can also target various other molecules. NOTCH1-activating mutations have been observed in more than 60% of T-ALL pediatric patients. We recently observed that the incidence of NOTCH1-activating mutations is higher for TLX3-rearranged patients while lower for TAL- or LMO-rearranged patients. NOTCH1 is a transmembrane receptor that is activated upon ligand binding, and these mutations result in ligand independent activation. The prognostic consequences of NOTCH1-activating mutations are different in various studies.

Recurrent mutations in the phosphatase and tensin homolog (PTEN) gene were discovered in T-ALL patient samples following common PTEN deletions in the triple knockout mouse model (Terc, Atm and Trp53) that developed T-cell lymphomas as well as by a genome-wide copy number analysis in ALL samples. PTEN mutations were also observed in T-ALL cell lines, and analyses of T-ALL patient samples revealed PTEN mutations and deletions in 5% and 15%, respectively. Palomero and coworkers found absence of PTEN expression in T-ALL cell lines that were resistant to γ-secretase inhibitors (GSI). Sequence analysis revealed PTEN mutations in 9 out of 111 primary T-ALL samples, suggesting that PTEN mutations that follow NOTCH1 mutations may provoke GSI resistance. Various other studies showed a variable incidence of PTEN mutations and/or deletions in T-ALL patients (range 18-63%).

PTEN acts downstream of the T-cell receptor and various other pathways. It controls the PI3K/AKT pathway by dephosphorylating PtdIns(3,4,5)P3 into PtdIns(3,4)P2. PI3-kinase (PI3K) has an opposite function and phosphorylates PIP2 into PIP3, which allows activation of AKT via PDK1. PTEN-inactivating mutations result in an overactive PI3K/AKT pathway. Few mutations are found in PI3K and AKT1 genes itself as alternative mechanisms to activate AKT. Activated AKT can act on multiple downstream targets that are involved in proliferation, cell metabolism and apoptosis. One major downstream target is TSC2, which is repressed by AKT that therefore facilitates protein synthesis through activation of mTOR.

The prognostic significance of an aberrantly activated PI3K/AKT pathway by mutations in pediatric T-ALL is fairly unknown. Also, PTEN, PI3K or AKT1 aberrations in relation to NOTCH1-activating mutations are unclear. We therefore investigated the incidence of mono-allelic or bi-allelic PTEN-inactivating events and PI3K or AKT1 aberrations in genetic subtypes of
MATERIALS AND METHODS

Patient samples
A total of 146 primary pediatric T-ALL patients were included in this study: 72 enrolled on the Dutch Childhood Oncology Group (DCOG) protocols ALL-7/8 (n=30) or ALL-9 (n=42), and 74 patients enrolled on the German Co-Operative Study Group for Childhood Acute Lymphoblastic Leukemia study (COALL-97, n=74) with median follow-up of 67 and 52 months, respectively. The patients' parents or legal guardians provided informed consent to use diagnostic patient biopsies for research in accordance with the Institutional Review Board of the ErasmusMC Rotterdam and the declaration of Helsinki. Isolation of leukemia cells was described before, with all samples containing >90% of leukemic blasts. Clinical and immunophenotypic data were supplied by the study centers. Patients were classified into T-cell development stages based on EGIL criteria, i.e. the pro-/pre-T-cell subgroup (CD7+, CD2+ and/or CD5+ and/or CD8+, but CD1- and sCD3-), the cortical T-cell (CD1+) or the mature T-cell subgroup (sCD3+/CD1). Patients were indicated positive for an immunophenotypic marker when ≥25% of leukemic blast stained positive for this marker.

Cell culture, γ-secretase inhibitor treatment and cell cycle analysis
T-ALL cell lines (DSMZ, Braunschweig, Germany) were cultured in RMPI-1640 supplemented with 10–20% fetal calf serum (Integro, Zaandam, the Netherlands), 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.125 μg/ml fungizone (Invitrogen, Life Technologies, Breda, the Netherlands) at 37°C under 5% CO₂. T-ALL cell lines (JURKAT, CEM, LOUCY, SKW3, ALL SIL, HPBALL, PF382, HSB2, PEER, MOLT3, MOLT16, P12 ICHIKAWA, KARPAS45, RPMI8402, BE13, TALL1, SUPT1, KE37 and DND41) were grown under 1μM Compound E (Enzo Life sciences (Alexis), Lausen, Switzerland) or 0.002% DMSO for 4 days, and 1*10⁶ cells were harvested. Cell were fixed with 70% cold ethanol and stained with propidium iodide (Invitrogen), after trypsin (Gibco BRL, Life Technologies, Breda, the Netherlands) and RNase A (Sigma, Zwijndrecht, the Netherlands) treatment. DNA content was measured and analyzed by flow cytometry (FACSCalibur, Becton Dickson, San Jose, CA, USA).

Genomic DNA and RNA extraction
Genomic DNA and RNA were isolated from at least 5*10⁶ leukemic cells using the Trizol reagent (Invitrogen) according to the manufacturer with minor modifications. Copy-DNA synthesis of 1μg of total RNA was performed as described before. DNA was stored at 4°C, whereas RNA and cDNA were stored at -80°C.

Detection of mutations and splice variants
The phosphatase domain and C2-domain of PTEN (exons 1-9), the pleckstrin homology (PH)
domain of AKT1 (exon 4), the SH2-domain of PIK3CA (p85, exon 12 and 13) and the accessory domain of PIK3RI (p110, exon 10) were amplified and sequenced. Primers used are described in Table S1. PCR reactions were performed on 50ng of DNA, 300nM of primers, 200μM of dNTPs, 4mM MgCl₂, 1.25U of ampliTaq gold (Applied Biosystems (AB), Foster City, CA, USA) in 1x PCR buffer II (Applied Biosystems) in a volume of 50μl. After denaturation at 94°C for 5', PCR was performed for 40 cycles at 94°C for 15'' and 60°C for 1'. Due to the GC-rich content, PCR of PTEN exon 1 was followed by a second asymmetric PCR for 10 cycles, using the forward or reverse primer. PCR products were purified with the Millipore Vacuum Manifold filter system (Millipore, Billerica, MA, USA) and sequenced (BigDye Terminator v3.1 Cycle sequencing Kit, AB) on an ABI PRISM 3130 DNA Analyzer (AB). Amplicons of patients that demonstrated two mutations were cloned using the TOPO-TA cloning kit (Invitrogen) to determine whether mutations occurred in cis or trans.

To examine promoter mutations, one primer set was used to amplify the promoter area. PCR-reactions were carried out as described above in the presence of 2mM MgCl₂ and 5% DMSO. Annealing temperature started at 63°C, and was lowered by 0.5°C each cycle till a final annealing temperature of 58°C. To investigate alternative PTEN-splicing, two primer pairs were used to amplify the complete PTEN transcript, using PCR conditions as described above in the presence of 2mM MgCl₂. NOTCH1 mutations were identified as described in our previous study14.

Methylation specific PCR (MSP)
For methylation specific PCR (MSP), sodium bisulfite conversion was done using the EZ DNA methylation kit (Zymo research, Orange, CA, USA). Primers used are listed in Table S1. PCR was performed using 0.6U Hotstar Taq plus DNA polymerase (Qiagen, Venlo, the Netherlands), 1x PCR buffer, 200μM dNTPs, 300nM primers, 1x Q-solution, 3.5mM MgCl₂ and 100ng converted DNA in a total volume of 50μL. Taq polymerase was activated by 5’ at 95°C, followed by 35 PCR cycles at 95°C for 30”, 59°C for 30” and 72°C for 1’ and a final elongation step of 10’ at 72°C. In-vitro methylated DNA with CpG methyltransferase Sss1 and co-substrate S-adenosylmethionine (SAM, New England Biolabs, Ipswich, MA, USA) served as positive control, untreated genomic DNA served as negative control.

Fluorescence in-situ hybridization analysis (FISH) and RQ-PCR
Rearrangements of the TLX1, TLX3, TAL1, LMO2 and MLL loci were determined with fluorescence in-situ hybridization analysis (FISH) as described before4,32,33. SET-NUP214 or CALM-AF10 fusion products or expression levels of SIL-TAL1, TLX1 or TLX3 were detected by an RQ-PCR strategy as described4,32,33. BAC clones and RQ-PCR primers/probes are summarized in Table S2. To identify PTEN deletions by FISH, bacterial artificial chromosomes (BAC) clones RP11-846G17 and/or RP11-124B18 were used. Probe RP11-265H15 covering the X-chromosomal BEX1 gene was used as control. BACs were obtained from BAC/PAC Resource Center (Children’s Hospital, Oakland, CA).
Microarray-based comparative genome hybridization (array-CGH)
Array-CGH analysis was performed on the human genome CGH Microarray 44A (n=33), 105K (n=2), and 400K (n=78) (Agilent Technologies, Santa-Clara, CA, USA), which consists of 60-mer oligo-nucleotide probes, that span both coding and non-coding sequences. The procedure was done as described before.

Western Blot procedure
Western blot was performed as described previously. Antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA) for PTEN (Cat#9552), phosphorylated (S380) PTEN (Cat#9551), phosphorylated (Thr308 and S473) AKT (Cat#9275 and 9271), phosphorylated (S2448 and S2481) mTOR (Cat#2971 and 2974), phosphorylated (Thr389) p70 S6 kinase (Cat#9205), phosphorylated (S65 and T70) 4E-BP1 (Cat#9451 and 9455), phosphorylated (Y1571) TSC2 (Cat#9514), phosphorylated (S256) FOXO1 (Cat#9461), intracellular NOTCH1 (ICN) Val1744 (Cat#2421), cMYC (Cat#9402) and MUSASHI1/2 (Cat#2154). To detect phosphorylated (S246) PRAS40, Cat#44-1100 from Invitrogen/Biosource was used. Total protein load was determined by staining for actin (Sigma, Cat#2547).

Reverse-phase protein microarray analysis (RPMA)
Reverse-phase protein microarray construction and analysis was performed essentially as previously described.

Statistics
Statistics was done in SPSS 15.0 software. Pearson’s Chi-square or the Fisher’s exact tests were performed to test significance levels for nominal data distributions, whereas the Mann-Whitney-U test was used for continuous data. Differences in cumulative incidence of relapse (CIR), relapse-free survival (RFS) and event-free survival (EFS) were tested by the log-rank test. An EFS event is defined as relapse, non-response to induction therapy, toxicity related death or development of a secondary malignancy. Data were considered significant when $p \leq 0.05$ (two-sided).

RESULTS
Inactivating PTEN aberrations in pediatric T-ALL patients
To determine the prevalence of PTEN mutations, all 9 coding exons were amplified and sequenced, providing data for 142 out of 146 pediatric T-ALL patients. Twenty-seven mutations were identified in 16 patients (11%), mostly representing heterozygous nonsense mutations that truncate the PTEN protein (Figure 1A; Table S3). Ten patients had two mutations in single exons or distributed over different exons. Re-sequencing of cloned PCR products revealed compound heterozygous insertion mutations in 8 out of 9 patients. One patient (#1959) comprised two
mutations that occurred in cis. Most deletion/insertion mutations occurred in exon 7, truncating PTEN in the C2-domain. Other mutations were detected in exon 5, 6 and 8. Two patients (#335 and #9963) had missense mutations, of which the R129G mutation was proven to inactivate phosphatase activity before 36.

We also detected four patients that had intron mutations located at the 3’-end in introns 1-2, 2-3 or 4-5, but amplification and sequencing of PTEN transcripts in these patients revealed no alternative PTEN splice isoforms.

High resolution array-CGH was performed on 113 out of 146 pediatric T-ALL patients, and heterozygous PTEN deletions were observed in three patients (#531, #8815 and #321, Table S3; Figure S1A). Loss of one PTEN allele in patient #531 explained its homozygous mutation pattern. Patient #2486 had a homozygous deletion of both PTEN alleles and since the deleted areas were identical, homozygosity may be due to uniparental disomy in this patient. Deletions could be validated by FISH in 3 patients except for patient #2486 due to the relatively small size of that deletion (Figure S1B). Array-CGH analysis also revealed subclonal deletions in two PTEN-mutated patients (#344 and #1959) that both carried two nonsense mutations affecting exons 5 and 7 (Table S3; Figure S1C). Validation by FISH demonstrated copy loss in 40% of the leukemic
blasts for patient #344, for which material was available (Figure S1D). Taken together, 19 of the 142 pediatric T-ALL patients (13%) harbored inactivating \( \text{PTEN} \) aberrations including missense and nonsense mutations as well as deletions of the entire \( \text{PTEN} \) locus. Bi-allelic \( \text{PTEN} \) inactivation was evident for 12 out of 19 patients.

**PTEN protein levels in relation to the \( \text{PTEN} \) mutation status**

Nonsense \( \text{PTEN} \) mutations result in loss of \( \text{PTEN} \) protein levels in T-ALL cell lines\(^{19,24} \). Using reverse-phase protein microarray (RPMA), total \( \text{PTEN} \) protein levels as well as phosphorylated (inactivated) \( \text{PTEN} \) protein levels (S380) were quantified. \( \text{PTEN} \) mutant T-ALL cell lines had significantly lower \( \text{PTEN} \) and phosphorylated \( \text{PTEN} \) levels than wild-type cell lines, and validated this technique (Figure 1B-C \( p=0.004 \) and \( p=0.004 \), respectively). Material for RPMA analysis was available for 66 out of 146 T-ALL patient samples. Total \( \text{PTEN} \) (Figure 1B \( p<0.0001 \)) as well as phosphorylated \( \text{PTEN} \) protein levels (Figure 1C \( p<0.0001 \)) were significantly lower for patients bearing inactivating \( \text{PTEN} \) mutations. One \( \text{PTEN} \)-mutated patient (#335) expressed \( \text{PTEN} \) protein (Figure 1B-C) from the mutant allele carrying the missense R129G mutation while the second allele was lost due to a frameshift insertion in exon 5. \( \text{PTEN} \) protein levels were absent or low in all other \( \text{PTEN} \)-mutated patients. \( \text{PTEN} \) levels were significantly lower for bi-allelic affected patients compared to mono-allelic affected patients (Figure S2 \( p=0.04 \)). Some mono-allelic-mutated/deleted patients had expression levels that were comparable to bi-allelic patients, indicating that the remaining wild-type allele in these patients may be silenced through yet unknown mechanisms. In addition, three seemingly \( \text{PTEN} \) wild-type patient samples (#769, #8629 and #9243) and the \( \text{PTEN} \) wild-type cell line HPBALL lacked \( \text{PTEN} \) protein (Figure 1B-C black squares). Array-CGH data were not available for these patients, but large \( \text{PTEN} \) deletions were excluded by FISH analysis in 2 out of these 3 patients and HPBALL (data not shown).

**Defective PTEN-splicing in pediatric T-ALL patients**

We then investigated whether absence of \( \text{PTEN} \) protein in these three patients and HPBALL was due to splice defects, mutations or hypermethylation of the \( \text{PTEN} \) promoter region. In addition, we further investigated the seven T-ALL patients that seemed to comprise mono-allelic mutations or deletions (#9160, #9919, #9963, #2759, #2852, #321 and #8815). One out of the three seemingly \( \text{PTEN} \) wild-type patients (#9243) and 2 out of 7 patients with mono-allelic \( \text{PTEN} \) mutations (#2852 and #8815) demonstrated aberrant \( \text{PTEN} \)-splicing and lacked expression of the full-length \( \text{PTEN} \) isoform (Figure 2; Table S3). PCR-sequence analysis for patient #9243 confirmed defective splicing of exon 3 to exon 6, whereas intron 1-2 was defectively spliced to exon 4 in patient #2852. Patient #8815 demonstrated defective \( \text{PTEN} \) exon 4 to exon 6 splicing that eliminates the phosphatase domain. Miss-splicing therefore provides an additional mechanism to eliminate wild-type \( \text{PTEN} \) expression. So far, no explanation was found for defective \( \text{PTEN} \)-splicing as no mutations were identified in the first 20-30 intronic bases flanking acceptor/donor splice sites of affected exons. Defective splicing in the absence of full-length \( \text{PTEN} \) transcript was also observed in the mono-allelic \( \text{PTEN} \)-deleted cell line LOUCY. All 11 control T-ALL patient
samples expressed the PTEN wild-type isoform only (Figure 2, 7 controls are shown).

Figure 2 | Defective splicing of PTEN transcripts. Analysis of alternative PTEN splicing in two wild-type PTEN patients and PTEN expression (#419 and #914), seven PTEN wild-type patients and cell lines without PTEN expression (#768, #8628, #9243, HPBALL, LOUCY, HS62 and KE37), patients with silent or intronic mutations (#2720, #2698, #2845, #2790 and #540) and seven patients with mono-allelic PTEN mutations or deletions (#9160, #9919, #9963, #2759, #2852, #321 and #8815). RT-PCR I covers wild-type and alternative PTEN transcripts from exon 1 through exon 6, whereas RT-PCR II covers wild-type and alternative PTEN transcripts from exon 6 through 9. Patients and cell lines expressing aberrant transcripts while lacking the full-length PTEN transcript are marked with an asterisk.

These three PTEN wild-type patients with reduced PTEN expression as well as these seven PTEN mono-allelic-mutated patients were also investigated for PTEN promoter hypermethylation as potential mechanism to silence wild-type PTEN alleles. For this, methylation specific PCR (MSP) was performed for the -1223 to -1032 region upstream relative to the transcriptional start site of PTEN (Figure S3A), in which hypermethylation has been described before in solid tumors and T-ALL.37-39 However, we found no evidence for PTEN promoter hypermethylation (Figures S3B-C). We also did not find evidence for deletions or mutations in the PTEN promoter region (-1414 to -613bp) in any of these T-ALL patients.

PI3K/AKT pathway mutations in pediatric T-ALL patients
PTEN regulates the PI3K/AKT pathway, and inactivation of PTEN may result in constitutive activation of the AKT pathway. Rare activating mutations in PI3K and AKT have been described in T-ALL patient samples. To screen for such mutations, exons 12 and 13 of PIK3RI (p85 regulatory subunit) and exon 10 of PIK3CA (p110 catalytic subunit class IA) and exon 4 of AKT1 were amplified and sequenced, and results were obtained for 135 out of the 146 T-ALL patients. No mutations were identified in PIK3RI or PIK3CA. Three patients (2%) had a mutation in AKT1 changing glutamic acid into lysine at position 17 (E17K) (Table S3). This mutation has been reported before in a single T-ALL patient, and constitutively activates AKT1.

All AKT1-mutated patients lacked PTEN aberrations. Overall, PTEN mutations or AKT1 mutations were identified in 25 out of 142 pediatric T-ALL patients (18%), and were denoted as the PTEN/AKT mutant patient group. This group comprised both PTEN wild-type patients that lacked PTEN protein expression (#769 and #8629). Based on our findings and literature, more than half of T-ALL cell lines have inactivated PTEN (summarized in Table S4).

Comparing the activation status of AKT and potential downstream signaling molecules in
PTEN/AKT-mutated versus wild-type patients using RPMA, we did not observe differences in phosphorylated AKT (Ser473 and Thr308) levels, nor in the phosphorylated status of downstream AKT targets including mTOR, p70 S6 kinase, 4E-BP1, TSC2, PRAS40 and FOXO1 (Figure S4A-B). As AKT activation has also been described to occur downstream of the NOTCH1 pathway24,41, we distinguished between PTEN/AKT-mutant patients, NOTCH1-activated and patients lacking PTEN/AKT or NOTCH1/FBXW7 mutations but did not identify significant differences in phosphorylated AKT levels nor downstream AKT targets (not shown).

PTEN/AKT aberrations in relation to biological, clinical and molecular-cytogenetic parameters

PTEN/AKT mutations were not associated with gender (p=0.97) or white blood cell counts (p=0.61), but seemed associated with younger age (Table 1 p=0.05).

Eight out of 25 PTEN/AKT patients had TAL1 rearrangements (Table 1 and Table S5, p=0.05), whereas only 3 out of 25 PTEN/AKT patients had TLX1 or TLX3 rearrangements (1 TLX3- and 2 TLX1-rearranged patients; p=0.003). Similar associations were observed for PTEN-mutated patients only. For T-ALL clusters based on unsupervised gene expression profiling6, we noticed that PTEN/AKT mutations were predominantly present in TAL/LMO cluster patients albeit not significant, while the incidence of these mutations was significantly lower for the TLX cluster that comprise most TLX3- and HOXA-rearranged cases (p=0.002). No associations were observed with PHF6 or WT1 mutations nor with CDKN2A/B deletions, in line with previous findings as reported by Gutierrez et al (2009)26.

Initially, PTEN mutations have been suggested as secondary mutations following NOTCH1-activating mutations, rendering cells insensitive to γ-secretase inhibitors24. We therefore compared the distribution of PTEN/AKT mutations with that of NOTCH1-activating mutations. NOTCH1-activating mutations (in NOTCH1 and/or FBXW7) were present in 63% of the patients14. Strikingly, patients carrying NOTCH1-activating mutations seemed to have a lower incidence of PTEN/AKT aberrations as only 10 out of 90 NOTCH1/FBXW7-mutated patients carried PTEN/AKT aberrations, in contrast to 15 out of 51 NOTCH1/FBXW7 wild-type patients (Table 1, p=0.006). Remarkably, PTEN/AKT-mutated patients that had NOTCH1/FBXW7 mutations especially harbored weak NOTCH1-activating mutations only (9 out of 10 cases, p=0.011)14,42.

So NOTCH1-activating mutations and PTEN/AKT mutations seem hits that are associated with different molecular cytogenetic T-ALL subgroups (14, and this study). These observations were further strengthened by our RPMA analyses that showed that PTEN/AKT mutant patients have low expression of intracellular NOTCH1 (ICN, p=0.003), cMYC, as a prime NOTCH1-target gene24,43 (p=0.01) and MUSASHI1/2 (MSI1/2, Figure S4C p=0.002), which is a repressor of the NOTCH1 negative regulator NUMB44. This is different in T-ALL cell lines, as 10 out of 13 PTEN/AKT-mutated cell lines also harbor NOTCH1-activating mutations (Table S4).

We then investigated whether PTEN/AKT mutations are associated with resistance to γ-secretase inhibitors such as previously suggested24. For this purpose, we measured the G1-arrest in a large panel of T-ALL cell lines following γ-secretase inhibitor treatment. Various cell lines (JURKAT, P12Ichikawa, PF382, MOLT16 and KARPAS45) that had PTEN-inactivating
mutations (Table S4) were resistant to γ-secretase inhibitor treatment (Figure S5A)\textsuperscript{24}, but four lines with PTEN-inactivating aberrations (SKW3, SUPT1, LOUCY, KE37) rapidly underwent G1-arrest following treatment. So, PTEN loss-of-function mutations are not necessarily associated with resistance towards γ-secretase inhibitors. All PTEN mutant lines lacked PTEN protein expression regardless of their γ-secretase inhibitor response, with the exception of SUPT1 and RPMI8402 that had PTEN missense mutations (Figure S5B).

<table>
<thead>
<tr>
<th>Clinical (n=442)</th>
<th>PTEN mutation/deletion</th>
<th>PTEN or AKT1 mutation/deletion + patients with a low PTEN protein expression (PTEN/AKT)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>WT n(%) Mut n(%) p\textsuperscript{=}</td>
<td>WT n(%) Mut n(%) p\textsuperscript{=}</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>85 (12) 27 (96) 1 (4) 0.002\textsuperscript{‡}</td>
<td>7 (1.1-17.8) 1 (2.2-15.9) 0.071</td>
</tr>
<tr>
<td>Female</td>
<td>38 (83) 7 (17) 0.02\textsuperscript{‡}</td>
<td>13 (68) 6 (32) 0.11</td>
</tr>
<tr>
<td>Median age (range)</td>
<td>7.8 (1.1-17.8) 4.3 (2.2-15.9) 0.071</td>
<td>7.1 (1.1-17.8) 4.3 (2.2-15.9) 0.052</td>
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<tr>
<td>Median WBC (range)</td>
<td>120 (2.0-900.0) 136 (3.0-600.0) 0.331</td>
<td>120 (2.0-900.0) 136 (3.0-600.0) 0.611</td>
</tr>
<tr>
<td>Phenytoin status (n=99)</td>
<td></td>
<td></td>
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<tr>
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<td>WT n(%) Mut n(%) p\textsuperscript{=}</td>
<td></td>
</tr>
<tr>
<td>TAL1 + (n=24)</td>
<td>20 (77) 6 (23) 0.11</td>
<td>18 (80) 8 (40) 0.05</td>
</tr>
<tr>
<td>LMO2 + (n=13)</td>
<td>13 (93) 1 (7) 0.0001</td>
<td>13 (93) 1 (7) 0.462</td>
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<tr>
<td>TLX3 + (n=18)</td>
<td>28 (100) 0 (0) 0.002\textsuperscript{‡}</td>
<td>27 (96) 1 (4) 0.031</td>
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<tr>
<td>HOXA + (n=13)</td>
<td>6 (88) 1 (14) 0.021</td>
<td>5 (71) 2 (29) 0.611</td>
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<tr>
<td>JAGGED1 + (n=6)</td>
<td>13 (100) 0 (0) 0.024</td>
<td>13 (100) 0 (0) 0.133</td>
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<td>LMO2 + (n=14)</td>
<td>5 (83) 1 (17) 0.001</td>
<td>5 (83) 1 (17) 0.11</td>
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<td>UHRF1 (n=2)</td>
<td>1 (100) 0 (0) 0.22</td>
<td>1 (100) 0 (0) 0.133</td>
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<td>Gene expression clusters (n=113)</td>
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<tr>
<td>WT n(%) Mut n(%) p\textsuperscript{=}</td>
<td>WT n(%) Mut n(%) p\textsuperscript{=}</td>
<td></td>
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<tr>
<td>TAL/LMO + (n=62)</td>
<td>41 (80) 10 (20) 0.07</td>
<td>38 (75) 12 (25) 0.09</td>
</tr>
<tr>
<td>TLX + (n=28)</td>
<td>28 (100) 0 (0) 0.022\textsuperscript{‡}</td>
<td>27 (96) 1 (4) 0.031</td>
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<tr>
<td>ProB/mye + (n=19)</td>
<td>16 (84) 3 (16) 0.021</td>
<td>13 (68) 6 (32) 0.11</td>
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<td>immatureB/TP/ALL + (n=15)</td>
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<tr>
<td>TAL/LMO + (n=62)</td>
<td>13 (87) 2 (13) 0.07</td>
<td>13 (87) 2 (13) 0.732</td>
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<table>
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<tr>
<th>NOTCH1/FBXW7 status (n=142)</th>
<th>WT n(%) Mut n(%) p\textsuperscript{=}</th>
<th>WT n(%) Mut n(%) p\textsuperscript{=}</th>
<th>WT n(%) Mut n(%) p\textsuperscript{=}</th>
<th>WT n(%) Mut n(%) p\textsuperscript{=}</th>
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<tbody>
<tr>
<td>Wild-type</td>
<td>39 (76) 12 (24) 0.008 \textsuperscript{‡}</td>
<td>36 (71) 15 (29) 0.006 \textsuperscript{‡}</td>
<td>33 (76) 12 (24) 0.008 \textsuperscript{‡}</td>
<td>30 (70) 14 (30) 0.008 \textsuperscript{‡}</td>
</tr>
<tr>
<td>Mutant</td>
<td>3 (8) 7 (18) 0.0001 \textsuperscript{‡}</td>
<td>5 (81) 3 (19) 0.001 \textsuperscript{‡}</td>
<td>2 (5) 8 (18) 0.0001 \textsuperscript{‡}</td>
<td>2 (5) 8 (18) 0.0001 \textsuperscript{‡}</td>
</tr>
<tr>
<td># weak mutant (n=62)</td>
<td>36 (71) 15 (29) 0.006 \textsuperscript{‡}</td>
<td>33 (76) 12 (24) 0.008 \textsuperscript{‡}</td>
<td>33 (76) 12 (24) 0.008 \textsuperscript{‡}</td>
<td>30 (70) 14 (30) 0.008 \textsuperscript{‡}</td>
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<tr>
<td># strong mutant (n=76)</td>
<td>26 (86) 7 (14) 0.0001 \textsuperscript{‡}</td>
<td>24 (80) 6 (20) 0.001 \textsuperscript{‡}</td>
<td>23 (88) 5 (12) 0.0001 \textsuperscript{‡}</td>
<td>20 (77) 6 (23) 0.0001 \textsuperscript{‡}</td>
</tr>
<tr>
<td>PhF6 status (n=62)</td>
<td>11 (100) 0 (0) 0.33 \textsuperscript{‡}</td>
<td>10 (91) 1 (9) 0.674 \textsuperscript{‡}</td>
<td>10 (91) 1 (9) 0.674 \textsuperscript{‡}</td>
<td>10 (91) 1 (9) 0.674 \textsuperscript{‡}</td>
</tr>
<tr>
<td>Wild-type</td>
<td>44 (86) 7 (14) 0.031 \textsuperscript{‡}</td>
<td>42 (82) 1 (8) 0.74 \textsuperscript{‡}</td>
<td>41 (82) 1 (8) 0.74 \textsuperscript{‡}</td>
<td>41 (82) 1 (8) 0.74 \textsuperscript{‡}</td>
</tr>
<tr>
<td>Mutant</td>
<td>17 (85) 19 (15) 0.021 \textsuperscript{‡}</td>
<td>16 (88) 2 (12) 0.74 \textsuperscript{‡}</td>
<td>16 (88) 2 (12) 0.74 \textsuperscript{‡}</td>
<td>16 (88) 2 (12) 0.74 \textsuperscript{‡}</td>
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<tr>
<td>Wild-type</td>
<td>73 (84) 14 (16) 0.521 \textsuperscript{‡}</td>
<td>72 (83) 15 (17) 0.76 \textsuperscript{‡}</td>
<td>72 (83) 15 (17) 0.76 \textsuperscript{‡}</td>
<td>72 (83) 15 (17) 0.76 \textsuperscript{‡}</td>
</tr>
<tr>
<td>Mutant</td>
<td>23 (92) 2 (8) 0.22 \textsuperscript{‡}</td>
<td>22 (88) 3 (12) 0.63 \textsuperscript{‡}</td>
<td>22 (88) 3 (12) 0.63 \textsuperscript{‡}</td>
<td>22 (88) 3 (12) 0.63 \textsuperscript{‡}</td>
</tr>
</tbody>
</table>

Table 1 | Overall clinical, immunophenotypic and molecular cytogenetic characteristics of PTEN or PTEN/AKT-mutated patients versus wild-type patients. Significant P values are indicated in bold; all P values were calculated by using Pearson’s χ2 test, unless indicated otherwise; WT: wild-type; Mut: mutant; P: P value; \textsuperscript{‡} statistical analysis of the frequency of PTEN or PTEN/AKT aberrations for specific genetic T-ALL subgroups indicated compared to all other T-ALL subgroups combined; Median age indicated in years; WBC: white blood cell count; white blood cell counts are indicated as number of blasts (x109/L); *Mann-Whitney-U test; \#Fisher’s exact test; \$Different genetic aberrations have been identified that all result in the activation of the MEF2C or NXX2-1/NXX2-2 oncogenes that define novel genetic T-ALL subtypes;\$] out of 117 T-ALL patients included in the gene expression profiling study\$5 had a known PTEN and AKT mutation status. T-ALL patients were assigned to clusters based on unsupervised gene expression cluster analysis.\$5 The TAL/LMO group is based on the presence of TAL1 or LMO2 rearrangements or by having a TAL1/LMO2 expression signature;\$5 \#Weak NOTCH1 activating mutations are considered as mutations in the NOTCH1 heterodimerization (HD) domain or NOTCH1 PEST domain or in FBKW7;\$9 \#Strong NOTCH1 activating mutations are considered as mutations in the juxtamembrane (JM) or mutations in the NOTCH1 HD domain in combination with mutations in the NOTCH1 PEST domain or FBKW7.
Good outcome for T-ALL patients lacking PTEN/AKT and/or NOTCH1/FBXW7 aberrations

In relation to outcome, relapse-free survival (RFS) and event-free survival (EFS) rates for PTEN/AKT mutant patients did not differ compared to wild-type patients (Figures S6A-B). In contrast to previous observations, no differences in outcome for PTEN-deleted patients versus other patients were observed nor for patients having mono-allelic versus bi-allelic PTEN mutations (data not shown). As the PTEN/AKT wild-type patient group is enriched for patients that harbor NOTCH1-activating mutations, which were previously associated with a trend towards poor outcome, we compared CIR and EFS rates for patients with PTEN/AKT aberrations and/or NOTCH1-activating mutations versus patients lacking these mutations (wild-type patients). Wild-type patients had a significantly lower 5-yr CIR rate (8%) compared to PTEN/AKT and/or NOTCH1-activated patients (35%) in a stratified analysis in our cohorts (Figure 3A and S6B, p=0.005). Only 2 out of 36 wild-type patients relapsed versus 33 out of 105 patients that had NOTCH1-activating and/or PTEN/AKT mutations (Table S6, p=0.002). The 5-yr EFS rate for wild-type patients was 75±7.7% versus 60±5.0% for NOTCH1/FBXW7 and/or PTEN/AKT mutant patients (Figure 3B, p=0.15), due to a relatively high number of toxic deaths or secondary malignancies in the wild-type patient group (Table S6, p=0.03; Figure S6B). We further investigated clinical and molecular-genetic parameters with 5-years relapse-free survival (RFS) rates (Table S7). We found improved 5-yr RFS rates for male patients (p=0.01), but inferior RFS rates for TLX3-rearranged T-ALL (p=0.04) as well as for patients having PTEN/AKT and/or NOTCH1 activating mutations (p=0.005). Multivariate analysis demonstrated that male gender and PTEN/AKT/NOTCH1/FBXW7 mutations remained independent predictors for improved or worse outcome, respectively (Table S8).

Figure 3 | T-ALL patients without PTEN/AKT and/or NOTCH1-activating mutations have a good outcome. Cumulative incidence of relapse (CIR) (A) and event-free survival (EFS) for DCOG and COALL pediatric T-ALL patients. Different patients groups are indicated in the legend. Log-rank p-values in a stratified analysis for different DCOG and COALL mutation groups relative to PTEN/AKT and NOTCH1/FBXW7 non-mutated patients (i.e. wild-type patients) have been indicated.

DISCUSSION

Eighteen percent of the patients have aberrations that affect the PI3K/AKT pathway in our pediatric T-ALL patients cohort (n=146). PTEN aberrations were identified in ~16%, whereas
AKT mutations were observed in ~2% of T-ALL patients. In other studies, PTEN mutations were identified in 5-27% of patients. Gutierrez et al (2009) identified PTEN/AKT mutations in ~48% of the patients, which is considerably higher compared to our study.

Major PTEN inactivation mechanisms are nonsense mutations and deletions. We identified defective splicing as an alternative mechanism to reduce PTEN expression in 2% of T-ALL patients. No mutations in donor/acceptor sites or closely flanking intronic sequences of the exons involved were identified, but we cannot exclude that intronic mutations at greater distance from these donor/acceptor sites are present that affect PTEN-splicing. Splice-defective patients did not express full-length PTEN transcript, indicating that both PTEN alleles were inactivated. Alternative splice isoforms of PTEN have been described before in Cowden Syndrome (CS), sporadic breast cancer or Bannayan-Riley-Ruvalcaba syndrome (BRRS), and were shown to alter full-length PTEN expression levels. Two PTEN wild-type T-ALL patients completely lacked PTEN protein. Although PTEN promoter mutations have been described for patients with CS and autism spectrum disorders and PTEN promoter hypermethylation was described for endometrial cancer, sporadic breast cancer and T-ALL, evidence for promoter mutations or promoter hypermethylation was not identified in our T-ALL patient series, so additional mechanisms to inactivate PTEN may exist in T-ALL. We cannot exclude that these mechanisms involve microRNAs, including miR-19b or miR-20a. Also, a regulatory role for the PTEN pseudogene PTENP1 on PTEN expression have been identified before with PTENP1 acting as a decoy transcript that bind miR-19b and miR-20a, resulting in elevated PTEN levels. Other miRNAs have been identified that regulate PTEN expression.

Mono-allelic inactivation of PTEN in cancer led to the hypothesis that PTEN is a haploinsufficient tumor suppressor gene. We have identified PTEN aberrations in a single allele in approximately one third of PTEN-mutated T-ALL patients, and these patients expressed lower PTEN protein levels compared to wild-type patients, in concordance with previous findings. These expression levels were still significantly higher compared to patients with bi-allelic PTEN mutations/deletions. Thus, mono-allelic loss of PTEN may be sufficient to provide a proliferation advantage in T-ALL, but an oncogenic pressure remains ongoing to inactivate the second functional PTEN allele. This notion is further substantiated by subclonal PTEN deletions in two T-ALL patients that already had one dysfunctional PTEN allele.

Inactivation of PTEN results in ectopic activation of AKT. However, using RPMA, we did not find different phospho-AKT levels or phosphorylation of downstream AKT targets in PTEN/AKT patients compared to patients lacking PTEN/AKT aberrations, and differences were also not observed between PTEN/AKT mutant cell lines and wild-type lines. It may be that differences in phosphorylation levels for AKT and downstream targets between PTEN/AKT mutant patients and wild-type patients are subtle and difficult to be accessed on primary patient material by RPMA or that AKT may also be regulated through other oncogenic pathways. In this respect, activation of the AKT pathway has been identified in over 75 percent of T-ALL cases, which is well above the incidence of PTEN/AKT aberrations (this study). In T-ALL, activation of AKT has been described downstream of NOTCH1, and AKT may be activated upon transcriptional
repression of PTEN by the NOTCH1-activated transcriptional repressor HES1\textsuperscript{24}. So AKT activation as consequence of PTEN/AKT mutations or through NOTCH1-activating mechanisms could explain the lower frequency of PTEN/AKT mutations in patients that have NOTCH1-activating mutations in our cohort. Furthermore, the 9 out of 10 PTEN/AKT-mutated patients that had NOTCH1-activating mutations only had weakly NOTCH1-activating mutations (i.e. NOTCH1-HD or PEST domain mutations or FBXW7 mutations)\textsuperscript{43}, pointing to a common downstream target and therefore eliminating the necessity to accumulate both PTEN/AKT and strong NOTCH1-activating mutations. Also in the study of Medyoyf et al (2010), 4 of the 6 primary T-ALL samples with PTEN inactivating mutation were also NOTCH1 mutated and only carried weakly activating PEST domain mutations\textsuperscript{41}. Consequently, ICN, MYC (another NOTCH1 target gene) and the indirectly NOTCH1 activator MUSASHI1/2 are expressed at lower levels in PTEN/AKT-mutated patients. Although PTEN/AKT mutations and NOTCH1-activating mutations may both converge on the activation of AKT, the association of PTEN/AKT mutations with TAL/LMO-rearranged patients (this study) and NOTCH1-activating mutations that are especially predominant in TLX3-rearranged patients\textsuperscript{42} also imply that both oncogenic pathways may activate different routes within different T-ALL subtypes.

The lower frequency of combined PTEN/AKT and NOTCH1-activating mutations may further explain cellular insensitivity of PTEN/AKT mutant cases towards γ-secretase inhibitors (GSI)\textsuperscript{24}. Our data show that most PTEN-inactivating mutations occur independently from NOTCH1-activating mutations, implying that PTEN/AKT-mutant leukemic cells are not sensitive towards GSIs rather than PTEN aberrations would provoke γ-secretase resistant\textsuperscript{24}. In this respect, we demonstrated that various T-ALL cell lines that have PTEN mutations (SKW3, SUPT1, LOUCY and KE37) respond to γ-secretase inhibitors. Our findings are in concordance to one previous study which showed that PTEN negative primary T-ALL cells or NOTCH1-induced T-ALL cells in mice on a Pten null background are equally sensitivity to γ-secretase inhibitors than primary T-ALL cells or NOTCH-induced tumors with unaffected PTEN loci, respectively\textsuperscript{51}. In the study of Gutierrez et al (2009), PTEN/AKT patients did not predict for event-free survival, but PTEN deletions seemed to be associated with early treatment failure\textsuperscript{26}. Jotta et al (2010) demonstrated poor overall survival rates for PTEN-mutated high-risk patients\textsuperscript{25}. In this last study, a trend towards poor outcome was related to the presence of mono-allelic or bi-allelic PTEN mutations/deletions. We could not confirm this, as most of our patients demonstrated bi-allelic inactivation of PTEN through additional mechanisms, such as alternative splicing that have thus far not been investigated in T-ALL. Distinguishing patients groups in our cohort based on the presence or absence of PTEN/AKT and NOTCH1/FBXW7 mutations revealed that patients with PTEN/AKT mutations fared as poorly as patients with NOTCH1/FBXW7 mutations or both. The patients without PTEN/AKT and NOTCH1/FBXW7 mutations had a good outcome, and almost no relapses were observed for these patients.

In conclusion, missense or nonsense mutations or deletions affecting the PTEN gene occur in 13 percent of pediatric T-ALL patients, and may result in the activation of the AKT pathway. The AKT E17K activating mutation was observed in ~2% of T-ALL patients. Defective PTEN splicing is an
additional PTEN-inactivating event, but so far the underlying mechanism is poorly understood. PTEN/AKT mutations are predominantly associated with TAL/LMO-rearranged T-ALL, with most PTEN/AKT-mutated patients lacking NOTCH1-activating mutations. T-ALL patients that lack PTEN/AKT and NOTCH1/FBXW7 mutations demonstrated a good overall outcome.

AUTHORSHIPS AND DISCLOSURES

L.Z. designed experiments, performed research and wrote manuscript, M.J.V. performed research and wrote manuscript, V.C. performed RPMA analysis, J.B.-G. performed PTEN mutation analysis and FISH, C.K. performed MSP, PCRs, cell cycle analyses and FISH, W.K.S. performed PTEN mutation analysis and prepared samples for RPMA analysis, E.S., A.J.P., W.K. and M.H. provided patient samples and clinical and immunophenotypic data, E.P. supervised study, and wrote manuscript, R.P. designed and supervised study and wrote manuscript, J.P.P.M. was principal investigator, designed and supervised the study, and wrote manuscript. None of the authors had competing financial interest.

ACKNOWLEDGEMENTS

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REFERENCES

SUPPLEMENTARY DATA

Figure S1 | PTEN deletions in T-ALL patients detected by array CGH and FISH analysis. (A) Array CGH and (B) FISH results of T-ALL patients with a clonal PTEN deletion. (C) Array CGH and (D) FISH results of patients with a subclonal PTEN deletion.

PTEN/AKT ABBREVIATIONS IN T-ALL
Chapter 4

Figure S2 | PTEN protein expression levels in PTEN wild-type patients, PTEN monoallelic and biallelic-mutated T-ALL patients.

Figure S3 | Methylation-specific PCR of the PTEN promoter region in T-ALL patients. (A) Schematic overview of PTEN promoter area depicting overlapping sequences between the PTEN gene and the PTENP1 pseudogene in gray and the unique PTEN sequence in black. Primers used for methylation-specific PCR are indicated by arrows where M indicates primers used for the amplification of methylated DNA and U indicates primers used for the amplification of unmethylated DNA. -1223 and -1032 indicate the numbers of base pairs before the PTEN start site. (B) PCR results of methylation-specific PCR in mono-allelic and bi-allelic mutated/deleted patients and (C) in PTEN wild-type patients and cell lines with and without PTEN expression. + indicates the positive control. U shows the PCR result for unmethylated DNA (124bp) and M for methylated.
Figure S4 | Total and phosphorylated levels of PTEN/AKT and NOTCH pathways mediators in T-ALL. (A) Schematic overview of AKT and its potential downstream signaling partners. (B) The expression of phosphorylated AKT (T308 and S473) levels as well as the activation status of potential downstream signaling components in PTEN/AKT mutant versus PTEN/AKT non-mutated (wild-type) patients, analyzed by reverse-phase protein microarray. Potential downstream targets include mTOR (S2448 and S2481), p70 S6 kinase (T389), 4EBP1 (S65 and T70), TSC2 (Y1571), PRAS40 (S246), and FOXO1 (S256). The p-value for each comparison is indicated. (C) The expression of intracellular NOTCH1 (ICN), the NOTCH1 target molecule cMYC, and the indirect NOTCH1 activator MUSASHI1/2 (MSI1/2), in PTEN/AKT mutant and PTEN/AKT non-mutated T-ALL patient samples. The p-value for each comparison is indicated.
Figure S5 | PTEN mutations are not necessarily related with resistance towards the γ-secretase inhibitor compound E. (A) Response of indicated T-ALL cell lines towards the γ-secretase inhibitor compound E, measured by G0/G1-arrest following 96 h of γ-secretase inhibitor treatment relative to DMSO-treated control cells. Cell lines that do not undergo G0/G1-arrest are indicated as resistant cell lines, whereas cell lines that do undergo G0/G1-arrest following incubation with compound E are indicated as sensitive. *Cell lines with reduced PTEN expression through mutations, deletions or aberrant splicing. PTEN and NOTCH1/FBXW7 mutational status. Genetic aberrations in the HPBALL cell line that result in low or loss of PTEN protein levels have not been identified. (B) Western blot analysis of PTEN protein levels. Cell lines have been ordered based on their compound E resistance with most resistant cell lines at the upper left corner to most sensitive cell lines in the lower right corner. β-actin is used as loading control.
Figure S6 | Survival curves of T-ALL patients in both cohorts separately. (A) Relapse free survival and event free survival of PTEN/AKT wild-type (gray line) and mutant patients (black line) in DCOG and COALL cohorts. (B) Relapse free survival and event free survival of PTEN/AKT and NOTCH1/FBXW7 wild-type patients (dark gray line), PTEN/AKT mutant (black line), NOTCH1/FBXW7 mutant (light gray line) and of patients with PTEN/AKT as well as NOTCH1/FBXW7 mutations (dotted black line) in DCOG and COALL cohorts.
**Table S1 | Primers used for PCR amplification of PTEN, AKT, and PI3K.**

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<th>Exon</th>
<th>Primer Sequence Forward</th>
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<td>5′-TTTGCACGTCCTACTC-3′</td>
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<td>5′-GTTGAAGTCTTGATGAT-3′</td>
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<tr>
<td>PTEN exon 3</td>
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<td>PTEN exon 4</td>
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<tr>
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**Table S2 | RQ-PCR primer and probes and FISH BAC-clones.**

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<th>BAC clones</th>
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**Table S3 | RQ-PCR primer and probes for identification of PTEN, AKT, and PI3K.**

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<th>Exon</th>
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<th>Primer Sequence Reverse</th>
<th>Product (Bp)</th>
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<td>5′-AGCTTCTGCCATCTCCTC-3′</td>
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<tr>
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<td>exon 2</td>
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<tr>
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<tr>
<td>PTEN</td>
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**Table S4 | FISH BAC-clones.**

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### Table S3 | PTEN and AKT aberrations in pediatric T-ALL.

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### Table S4 | PTEN genetics of T-ALL cell lines.

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<td>WT/FBXW7</td>
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<td>WT/WT</td>
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<td>HD/FBXW7</td>
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### Table S5 | Patients' characteristics and mutation overview for 146 pediatric T-ALL patients. Available on request.
### Chapter 4

#### Table S6 | Clinical and immunophenotypic data of PTEN/AKT and NOTCH1/FBXW7-mutated versus wild-type patients.

Significant P values are indicated in bold. All P values were calculated using Pearson’s chi2 test, unless indicated; WT: wild-type; Mut, mutant; P: P value; SD: standard deviation; WBC: white blood cell count; white blood cell counts are indicated as number of blasts (x10⁹/L); ‡Fisher’s exact test.

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<th>DCOG</th>
<th>COALL</th>
<th>Overall stratified analysis</th>
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<td>5-yr RFS (% ± SD)</td>
<td>P</td>
<td>5-yr RFS (% ± SD)</td>
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<td>Male (n=73) vs female (n=73)</td>
<td>84 ± 6 vs 64 ± 7</td>
<td>0.18</td>
<td>62 ± 8 vs 63 ± 8</td>
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<tr>
<td>age &lt;50 (n=114) vs ≥50 yrs (n=32)</td>
<td>82 ± 13 vs 74 ± 6</td>
<td>0.52</td>
<td>62 ± 8 vs 68 ± 8</td>
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<tr>
<td>WBC =&lt;10 (n=56) vs &gt;10 (n=89)</td>
<td>89 ± 6 vs 82 ± 13</td>
<td>0.4</td>
<td>73 ± 8 vs 61 ±10</td>
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</table>

**Immunophenotypic (n=138)***

<table>
<thead>
<tr>
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<th>WT n(%)</th>
<th>Mut n(%)</th>
<th>P</th>
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<tbody>
<tr>
<td>Pre-T/Pro-T +</td>
<td>9 (23%)</td>
<td>30 (77%)</td>
<td>0.61</td>
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<td>Cortical T +</td>
<td>12 (21%)</td>
<td>46 (79%)</td>
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<tr>
<td>Mature T +</td>
<td>15 (37%)</td>
<td>26 (63%)</td>
<td>0.06</td>
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</table>

**Table S7**

Significant log rank P values for DCOG or COALL cohort analyses are indicated in bold; RFS: relapse free survival; SD: standard deviation; P: P value; WBC: white blood cell count; □Different genetic aberrations have been identified that all result in the activation of the MEF2C or NXX2-1/NXX2-2 oncogenes that define novel genetic T-ALL subtypes; †All patients who have one of the above described cytogenetic aberrations (known) versus all patients without any of these above described aberrations (unknown); 1113 out of 117 T-ALL patients included in the gene expression profiling study had a known PTEN and AKT mutation status. T-ALL patients were assigned to the TAL/LMO group based on the presence of TAL1 or LMO2 rearrangements or by having a TAL/LMO expression signature.
PTEN/AKT aberrations in T-ALL

Table S8 | NOTCH1-activating and PTEN/AKT mutations predict for poor outcome in pediatric T-ALL. Univariate and multivariate Cox's regression analyzes using relapse free survival for various parameters that were significantly associated with good or poor relapse free survival.

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<thead>
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<th>95% confidence interval</th>
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<td>0.122 - 0.820</td>
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<td>1.030 - 4.339</td>
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<td><strong>Multivariate analyses using Cox regression model</strong></td>
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<tr>
<td>male vs female</td>
<td>146</td>
<td>0.37</td>
<td>0.141 - 0.959</td>
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CHAPTER 4
PTEN micro-deletions in T-cell acute lymphoblastic leukemia are caused by illegitimate RAG-mediated recombination events

Rui D. Mendes1,9, Leonor M. Sarmento2,9, Kirsten Canté-Barrett1, Linda Zuurbier1, Jessica G.C.A.M. Buijs-Gladdines1, Vanda Póvoa2, Willem K. Smits1, Miguel Abecasis1, J. Andres Yunes4, Edwin Sonneveld1, Martin A. Horstmann6,7, Rob Pieters1,8, João T. Barata2,10 and Jules P.P. Meijerink1,10

1Department of Pediatric Oncology/Hematology, Erasmus MC Rotterdam-Sophia Children’s Hospital, Rotterdam, the Netherlands; 2Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Lisboa, Portugal; 3Cardiologia Pediatrica Medico Cirúrgica, Hospital Sta. Cruz, Lisboa, Portugal; 4Centro Infantil Boldrini, Campinas, SP, Brazil; 5Dutch Childhood Oncology Group (DCOG), the Hague, the Netherlands; 6German Cooperative Study Group for Childhood Acute Lymphoblastic Leukemia (COALL), Hamburg, Germany; 7Research Institute Children’s Cancer Center Hamburg, Clinic of Pediatric Hematology and Oncology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; 8Princess Maxima Center for Pediatric Oncology, Utrecht, Netherlands

9These authors are co-first authors; 10These authors contributed equally to this work

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ABSTRACT

PTEN inactivating mutations and/or deletions is an independent risk factor for relapse just like NOTCH-activating mutations or male gender for T-cell acute lymphoblastic leukemia (T-ALL) patients treated on DCOG or COALL protocols. Some monoallelic mutated or PTEN wild-type patients lack PTEN protein, implying that additional PTEN inactivation mechanisms exist. We show that PTEN is inactivated by small deletions affecting a few exons only in 8% of pediatric T-ALL patients. These micro-deletions were clonal in 3% and sub-clonal in 5% of patients. Conserved deletion breakpoints are flanked by cryptic RAG-recombination signal sequences (cRSS) and frequently have non-template derived nucleotides inserted in between breakpoints, implying that micro-deletions are the result of illegitimate RAG recombination activity. Identified cRSSs drive RAG-dependent recombination in a reporter system as efficiently as bona fide RSSs that flank gene segments of the T-cell receptor locus. Remarkably, equivalent micro-deletions were also detected in thymocytes of healthy individuals. Similar to other PTEN aberrations, micro-deletions strongly associate with the TALLMO-subtype characterized by TAL1 or LMO2 rearrangements. Primary and secondary xenotransplantation of TAL1-rearranged leukemia allowed development of leukemic subclones with newly acquired PTEN micro-deletions. Ongoing RAG activity may therefore actively contribute to the acquisition of pre-leukemic hits, clonal diversification and disease progression.
INTRODUCTION

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive leukemia caused by the malignant transformation of T-cell precursors in the thymus. T-ALL represents 10-15% of pediatric acute leukemias. Despite major therapeutic improvements due to treatment intensification and refined risk-adapted stratification during the past decade, ~30% of T-ALL cases relapse with very poor prognosis.1

T-cell transformation is characterized by aberrant expression of oncogenic transcription factors combined with inactivation of tumor suppressor genes (e.g. PTEN, CDKN2A) and/or activation of the NOTCH1 pathway.2 The ectopic expression of oncogenes is typically caused by chromosomal rearrangements—the so-called type A hits—that place T-ALL oncogenes under the control of strong T-cell specific promoters or enhancer elements.3,4 In many cases, the analysis of translocation breakpoint regions revealed illegitimate V(D)J recombination to be involved in this process, through the binding of the recombination activating gene (RAG)-1/2 proteins to conserved DNA sequences that resemble authentic recombination signal sequences (RSS).5 These recurrent chromosomal rearrangements activate several oncogenes, such as TAL1, LMO2, TLX3, TLX1 or NKX2-1/NKX2-2, which are believed to represent the clonal drivers that occur in a nearly mutually exclusive pattern.2,6

Besides the type A mutations, recurrent genetic aberrations that affect cell viability and/or proliferation—the so-called type B hits—are found in nearly all T-ALL genetic subgroups. Type B mutations include NOTCH1-activating mutations affecting NOTCH1 and FBXW7 that are found in over 60% of pediatric T-ALL patients (11; reviewed in 12), as well as less frequent events such as IL7R mutations in around 10% of T-ALL cases.13,14 In addition, mutations in the phosphatase and tensin homolog (PTEN) tumor suppressor gene have been associated with poor prognosis,15-18 resulting in overactive PI3K–AKT signaling that drives enhanced cell proliferation and cell metabolism, and impairs apoptosis.16,19,20 PTEN is considered to be a haploinsufficient tumor suppressor gene, because PTEN dose determines cancer susceptibility.21-22 The majority of PTEN aberrations in T-ALL are deletions affecting the entire PTEN locus or mutations that truncate the membrane binding C2-domain.15,18

In our previous studies, we detected PTEN aberrations in 13-20% of T-ALL patients18,24 and revealed that those mutations are especially associated with TAL or LMO rearrangements and nearly absent in TLX3-rearranged T-ALL.18 In general, PTEN mutated T-ALL appears to be devoid of NOTCH1-activating mutations.18 Interestingly, we did not observe differential AKT activation when comparing PTEN mutant/deleted with wild-type patient samples, indicating that other mechanisms may influence the PI3K-AKT pathway. In this respect, non-deletional posttranslational inactivation of PTEN,24 rare mutations in PIK3CA (encoding PI3K) and AKT themselves,16 or PI3K-AKT pathway activation downstream of activated NOTCH1 have been described.15 However, none of these mechanisms explain the absence of PTEN protein in some T-ALL patient samples that have retained at least one PTEN wild-type allele.18

In this study, we have used multiplex ligation-dependent probe amplification (MLPA) to
investigate copy-number variations among PTEN exons to detect potential additional PTEN deletions. We identified PTEN micro-deletions in T-ALL patient samples and we provide evidence that these are driven by illegitimate RAG-mediated recombination events.

MATERIALS AND METHODS

Samples
A total of 146 primary pediatric T-ALL patient samples were enrolled in the Dutch Childhood Oncology Group (DCOG) protocols (n=72) or the German Co-Operative Study Group for Childhood Acute Lymphoblastic Leukemia study (COALL-97) (n=74) were included in this study. The patients’ parents or legal guardians provided informed consent to use leftover diagnostic material for research in accordance with the Institutional Review Board of the ErasmusMC (Rotterdam, the Netherlands), the Ethics Committee of the City of Hamburg, Germany, and the Declaration of Helsinki. Leukemia cells were harvested and enriched from blood or bone marrow samples as described previously, all containing >90% of leukemic cells.

Normal thymocytes were isolated from thymic tissue obtained from children undergoing cardiac surgery at the Hospital Sta. Cruz, Centro Hospitalar de Lisboa Ocidental (Lisboa, Portugal) as described. Informed consent and approval by the local ethical committee were obtained in accordance with the Declaration of Helsinki.

Multiplex ligation-dependent probe amplification (MLPA) reaction
For MLPA analysis, the SALSA MLPA kit (MRC-Holland, Amsterdam, the Netherlands) was used in combination with the P200-A1 Human DNA reference-1 probemix kit (MRC-Holland). Two synthetic left and right hybridization probe oligonucleotide (LPO and RPO) pairs were designed per exon for all 9 exons of PTEN. Probe pairs were designed according to the manufacturer guidelines, and obtained from IDT (Coralville, USA). Probe pairs were combined into a probemix with a concentration of 4nM per oligonucleotide (Table S1). Reactions were carried out in a model 2320 thermocycler (Applied Biosystems, Bleiswijk, the Netherlands). MLPA fragment analysis was performed using GeneMarker V1.85 (Softgenetics, State College, PA, USA).

Breakpoint Mapping and PCR-based screening
To map breakpoints for PTEN exons 2-3 and 4-5 deletions, multiple forward or reverse primers were designed for PTEN introns 1, 3 and 5 (Table S2 and S3). Specific primers (Table S4) were selected for PTEN exon 2-3 and 4-5 breakpoint screening. Positive reactions were directly sequenced for both strands or following cloning into the TOPO TA vector (Life Technologies). Sequence reactions were run on the 3130x capillary sequencer (Applied Biosystems) and analyzed using CLC Main Workbench software (Aarhus, Denmark).

Computational detection of putative RAG recombination signal sequences
The human PTEN gene (ENSG00000171862) was screened for the presence of cryptic RAG recombination signal sequences (cRSS) using the PERL software algorithms developed by Cowell et al.\textsuperscript{31} Mouse 12- and 23-RSSs (n=356) from all TCR and Ig loci were used for modeling. Both the program and 12-/23-RSSs were available at http://www.duke.edu/~lgcowell/. The program computes RSS information content (RIC) scores for 12-spacer RSSs (i.e. 28-bp sequences comprising the heptamer, a 12-bp spacer and the nonamer sequences: RIC12) or 23-spacer RSSs (i.e. 39-bp sequences comprising the heptamer, a 23-bp spacer and the nonamer sequences: RIC23) to predict cryptic RSSs (cRSS) based on a comparison to the RIC score for actual immunoglobulin and T-cell receptor RSS. RSSs that potentially function within the range of actual antigen receptor loci RSSs are predicted to score above the -38,81 as RIC12 and -58,45 as RIC23 thresholds. RSSs that potentially function within the range predicted for degenerated RSSs, i.e the so-called cryptic RSSs (cRSS), score below these thresholds but above the background (e.g. the mean of RIC scores determined for non-RSS DNA sequences: -60,07 for RIC12 and -77,76 for RIC23) as previously described.\textsuperscript{31}

**Generation of GFPi-PTEN cRSS reporter constructs and recombination assay**

To measure efficiencies of predicted cRSSs in mediating recombination of GFPi-mRFP reporter constructs, PCR amplified PTEN cRSS1-4 or defined RSS control sequences were cloned into this reporter construct as previously described.\textsuperscript{32} Predicted PTEN cRSS1-4 or control RSSs were tested in combination with a consensus 12 or 23 RSS (Figure 3B). All RSS or cRSS used in the GFPi-mRFP constructs are summarized in Table S5.

Recombination assays were carried out as described, with minor adaptations. Briefly, HEK293T cells grown in standard culture conditions were seeded at 2x10\textsuperscript{5} cells per well in 12-well plates. After 24h, cells were transfected with a transfection mix that contains 5 μL of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), 300 μL of Optimem (Gibco, Life Technologies, Paisley, UK), 0.8 μg of CMV-RAG1, 0.7 μg of CMV-RAG2, and 2.5 μg of a GFPi-mRFP construct variant. Medium was replaced 16h after transfection. Cells were harvested at 48h following transfection, and analyzed by flow cytometry for GFP and RFP fluorescence using a FACSAria III (BD Bioscience, Madrid, Spain). Data analysis was performed using the FlowJo software (TreeStar Inc., Ashland, OR).

**Xenotransplantation**

NOD-SCID-IL2Rγnull (NSG) mice were bred and housed under specific pathogen free (SPF) conditions at the Experimental Animal Facility of ErasmusMC. All experiments have been approved by the committee on animal welfare of ErasmusMC and are in compliance with Dutch legislation. Sets of 6–10 week old mice were intravenously injected with 1-5x10\textsuperscript{6} human bone marrow leukemic cells. Upon signs of illness, mice were euthanized and leukemic cells were collected from the different organs. Likewise, primary transplanted leukemic cells from bone marrow, thymus or spleen were retransplanted.
Statistics
Statistics was performed using IBM SPSS Statistics 21 software (IBM, Armonk, NY, USA). The Pearson's Chi-square was used to test statistical significant differences for nominal distributed data, the Fisher's exact test in tests where the number of patients tested in individual groups was lower than five and the Mann-Whitney-U test for continuously distributed data. Data were considered significant when $p \leq 0.05$ (two-sided). Differences in relapse-free survival were tested by using the log-rank test. Proportional risk for relapse was done by univariate and multivariate Cox regression analysis. The recombination efficiencies of cRSSs were compared using a one-way ANOVA with the Bonferroni’s multiple comparison post-test and significant differences were considered when $p \leq 0.05$.

RESULTS

Identification of PTEN micro-deletions
In our previous study, we identified various T-ALL primary patient samples that lack PTEN protein expression and seemed PTEN wild-type or that contained an inactivating mutation or PTEN deletion in only one allele (summarized in Table 1). To identify additional PTEN inactivating mechanisms, we performed MLPA analysis to screen for potential micro-deletions affecting single or a few PTEN exons that had been missed by array-CGH and FISH analyses. We analyzed 146 T-ALL patient samples for copy-number alterations in any of all 9 coding exons. Heterozygous micro-deletions were detected in 3 T-ALL patients (Figure 1A), encompassing exons 2 and 3 in 2 patients (#21, #11) and exons 4 and 5 in another patient (#20). Accordingly, 2 out of the 3 patients (#11, #20) demonstrated defective PTEN-splicing with previously unknown underlying genetic aberrancies. A fourth patient (#12) was identified with a homozygous deletion of exons 2 and 3 that was confirmed by high-resolution array-CGH analysis (Figure 1A, B).

In order to clone the breakpoint regions of these micro-deletions, a PCR-based strategy was designed for introns 1 and 3 (patients #21, #11 and #12) and intron 3 and 5 (patient #20) (Figure 2A, B), and resulting positive reactions were cloned and sequenced. These analyses predicted micro-deletions of ~65Kb that encompassed exons 2-3 and of ~11Kb that encompassed exons 4-5 (Figure 2B). The homozygously deleted patient (#12) revealed different breakpoints that point to independent deletion events for each allele, with insertion of random bases in between breakpoints for one allele (Figure 2B). Breakpoints for the exon 2-3 micro-deletion in patient #21 were identical to breakpoints of one allele of patient #12 and also lacked insertion of random bases. T-ALL patient #11 had a similar exon 2-3 deletion that shared the identical breakpoint in intron 3 but had an alternate breakpoint in intron 1 (Figure 2A-C). Breakpoints for the fourth T-ALL patient #20 with a micro-deletion that affected exons 4 and 5 were located in introns 3 and 5 (Figure 2B). All three types of micro-deletions result in out-of-frame PTEN transcripts (Figure 2C).
Table 1 | **PTEN genetics of T-ALL patients.** *PTEN* frameshift mutations are indicated with the number of encoded amino acids in the alternative reading frame. *PTEN* deletion status was determined by fluorescent in situ hybridization (FISH), array-comparative genomic hybridization (array-CGH) and/or multiplex ligation-dependent probe amplification (MLPA). Introns harboring the genomic breakpoints of *PTEN* deletions and/or micro-deletions have been indicated. Exons for alternative spliced *PTEN* transcripts are indicated. Abbreviations: Del, deletion; FS, frameshift; HD, heterodimerization domain mutation; Het, heterozygous; Hom, homozygous; PEST, glutamine, serine and threonine-rich C-terminal region; Subcl, subclonal.

### Clonal iactivaton of 1 allele

<table>
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<th>Patient</th>
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<th>PTEN Deletion</th>
<th>PTEN Deletion</th>
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<th>PTEN protein</th>
<th>Cytogenic aberration</th>
<th>NOTCH1/FBXW7</th>
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<tr>
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<td>Abole B</td>
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### Multivariate analyses using Cox regression model

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Table 2 | **NOTCH1-activating and PTEN/AKT mutations predict for poor outcome in pediatric T-ALL treated on DCOG ALL7/8/9 or COALL-97/03 protocols.** Univariate and multivariate Cox regression analyses stratified for DCOG or COALL treatment protocols using relapse-free survival for various parameters that were significantly associated with poor relapse-free survival (see supplementary table S7). §Includes T-ALL patients that do not express PTEN protein while lacking *PTEN* aberrations, but does not include patient samples with *PTEN* aberrations only on the subclonal level.
CHAPTER 5

Figure 1 | Identification of PTEN micro-deletions in T-ALL patients. (A) MLPA electropherograms of normal reference DNA and representative examples of T-ALL patients with heterozygous or homozygous PTEN micro-deletions affecting exons 2-3 or a heterozygous deletion of exons 4-5. Fluorescence intensities of amplified PCR products for specific PTEN exons are shown. PCR product sizes are shown at the top. Each arrow points to a homo- or heterozygously deleted exon. (B) Array-CGH plot exhibiting the homozygous PTEN exon 2-3 micro-deletion in one T-ALL patient sample.
Microdeletions in PTEN in T-ALL

Type-I DNA breakpoints

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Phosphatase domain

C2 domain
Figure 2 | Breakpoints of PTEN micro-deletions. (A) Schematic representation of the PTEN gene. Missense mutations are represented by open triangles above the exons, whereas a silent mutation is presented as a filled grey triangle as shown before 18. Nonsense insertion/deletion mutations are indicated by a filled black triangle. Left or right-pointing open triangles in introns 1, 3 and 5 represent cRSSs. (B) Sequences of cloned intron 1-3 type I and type II breakpoints and the intron 3-5 type III breakpoint for T-ALL patients with PTEN micro-deletions. Cryptic recombination signal sequences (cRSS) are indicated by a box with the canonic CAC trinucleotide sequences or the corresponding GTG nucleotides in heptamer sequences indicated in bold and underlined. Insertion of non-template, random nucleotides are shown in bold. (C) Examples of sequence traces of cDNA resulting from type I, II and III micro-deletions. (D) Involvement of specific cRSSs in illegitimate RAG-mediated recombination events resulting in types I and II micro-deletions (signal joint) and aberrant PTEN splice variant or the type III with the aberrant exon 4-5 micro-deletion PTEN transcript (coding joint).
As MLPA does not allow for sensitive detection of subclones with micro-deletions, we performed PCR analysis to screen the T-ALL cohort for similar micro-deletions. Seven additional patients were identified with deletions affecting exons 2-3 that were similar to the breakpoints as observed in patients #21 and #12 (Figure 2B). Based on the conservation of breakpoints, this micro-deletion was denoted as a type I micro-deletion. One additional patient was identified with breakpoints similar to patient #11, therefore this deletion was denoted as a type II micro-deletion. The deletion affecting exons 4 and 5 as identified in patient #20 was accordingly denoted as a type III micro-deletion. These deletions had not been detected before by array-CGH, FISH or MLPA analyses. One patient sample (#20) had already been identified by MLPA as having a clonal micro-deletion affecting exons 4 and 5, but also contained a subclonal type I micro-deletion in exons 2 and 3 as detected by PCR (Figure 2B and Table 1). In another case (#19), array-CGH had revealed a heterozygous PTEN deletion of exons 3-9, but now PCR also revealed a subclonal type I exon 2-3 micro-deletion (Table 1). Sequencing of the breakpoints in these additional T-ALL cases revealed that five out of the seven type I deletions and the type II deletion involved the insertion of unique, random nucleotide sequences, thereby excluding false positives due to PCR contamination. Notably, the PCR product for these patient samples as visualized by gel electrophoresis was much weaker than those for the 4 patient samples with clonal micro-deletions (data not shown). This strongly indicates that these deletions must be present on the subclonal level and therefore only detectable by specific PCRs. Overall, we have identified PTEN micro-deletions in 11 out of 146 T-ALL patients (8%), comprising a total of 13 deletional events. Only 4 patients presented these mutations at the clonal level.

The PTEN breakpoints are flanked by cryptic RAG recombination signal sequences
The conservation of breakpoints among patient samples and the inclusion of non-template derived nucleotides by terminal deoxynucleotidyltransferase (TdT) in most breakpoint regions pointed to a RAG-mediated deletion mechanism. We then searched for the presence of cryptic RAG recombination signal sequences (cRSS) that could function as putative RAG-mediated recombination signals, such as the RSS involved in T- or B-cell receptor gene segment rearrangements. Analysis of sequences directly flanking the breakpoints immediately revealed typical CAC canonnic trinucleotides (Figure 2B and Table S5), which is a hallmark of the heptamer sequences of RSSs. The search for nearby nonamer sequences with A-nucleotide enrichment revealed a putative 12-spacer cRSS in intron 3 with a 5' to 3' orientation (cRSS1). A 23-spacer RSS was identified that directly flanks the breakpoint in intron 5 (cRSS2), and two others were identified flanking both breakpoints in intron 1 (cRSS3 and cRSS4; Figure 2A, B and Table S5). All 23-spacer cRSSs (cRSS2, cRSS3 and cRSS4) are present in a 3' to 5' orientation with respect to the PTEN reading frame orientation, and are therefore correctly positioned to allow illegitimate RAG-mediated recombinations with cRSS1 (Figure 2A, C). In this scenario, RAG1/2 molecules bind a pair of 12- and 23-RSSs resulting in two DNA double-strand breaks adjacent to each heptamer. Most micro-deletion breakpoints are the consequence of heptamer-to-heptamer sequence fusions resembling signal joints of excision circles that are generated during normal T-
or B-cell receptor gene segment rearrangements (Figure 2D, top): Types I and II micro-deletions result from cleaved DNA sequences 3-prime of cRSS3 or cRSS4, respectively, that are fused to sequences 5-prime of cRSS1. This retains both cRSSs in the genomic sequences that flank the deletion breakpoints as depicted in Figure 2D. The type III deletion resembles a typical coding joint that results from cleaved DNA sequences 5-prime of cRSS1 that are fused to sequences 3-prime of cRSS2 resulting in the loss of cRSSs from the genomic sequence (Figure 2D, bottom). T- or B-cell receptor coding joints give rise to fused gene segments with potential exonuclease processing of both ends and incorporation of random nucleotides whereby directly flanking RSSs and intervening DNA sequences are lost as excision circles. For the type III deletion of patient #20 (Figure 2B), this led to the fusion of sequences 5-prime of cRSS1 to sequences of 3-prime of cRSS2 with loss of 14 nucleotides and incorporation of 17 GC-rich N-nucleotides.

Cryptic RSS prediction using the Positional Information Algorithm

To further characterize these cRSSs and estimate their recombination potential, we scanned the human PTEN locus using the Positional Information Algorithm designed by Cowell and colleagues that calculates RSS Information Content (RIC) score. Cryptic RSSs with RIC scores close to RIC score threshold levels that discriminate bona-fide functional RSSs flanking gene segments of antigen receptor loci from cRSSs (i.e. -38.81 for 12-spacer RSSs and -58.45 for 23-RSSs) were further investigated. This search in the PTEN locus predicted a 12-spacer cRSS1 with a strong RIC score of -34.23 as well as 23-spacer cRSS2 (-55.59) and cRSS3 (-59.78) with RIC scores that were close to the threshold levels separating RSSs from cRSSs. A 23-spacer cRSS4 was predicted with a RIC score of -75.59 that is barely above the mean background RIC score value for 39-nucleotide non-RSS DNA sequences (-77.76). Thus, the obtained RIC scores for cRSS1, cRSS2 and cRSS3 strongly support PTEN micro-deletions as RAG-mediated recombination events with similar recombination potential to that of bona-fide RSSs flanking immunoglobulin V(D)J gene segments.

Cryptic RSS1-4 support RAG-mediated recombination in vitro

We then tested whether the predicted cRSS1-4 could functionally mediate RAG recombination events. We used the GFPi-mRFP RAG reporter construct, in which the 12-spacer cRSS1 was cloned in combination with a consensus 23-spacer RSS. Also, the 23-spacer RSSs (cRSS2, cRSS3 and cRSS4) were cloned in combination with a consensus 12-spacer RSS. RAG-mediated recombination activities were tested in HEK293T cells. Recombination efficiency of each variant GFPi-cRSS-mRFP construct was measured by flow cytometry as the frequency of GFP-positive (recombination-positive) cells within the population of RFP-positive (transfected) cells (Figure 3A). Indeed, all four PTEN cRSSs were able to mediate RAG-dependent recombination of the GFPi substrate (Figure 3B). Recombination efficiencies were 7.5±0.19% for cRSS1 (Figure 2B, left panel), 2.2±0.15% for cRSS2, 4.1±0.21% for cRSS3 and 2.1±0.16% for cRSS4 (right panel). For comparison, the putative 12-spacer cRSS SCL(12) or the 23-spacer cRSS SCL(23) from the human SCL gene yielded 1.3±0.09% and 1.1±0.13% of GFP-positive cells, respectively. Both of
Microdeletions in PTEN in T-ALL

A

GFP (MSCV-GFP\textsuperscript{inverted-RES}-RFP)

12-RSS 23-RSS

RAG1/2

GFP (Con\textsuperscript{12/23} - RAG1/2)

GFP (Con\textsuperscript{12/23} + RAG1/2)

B

12-RSS\textsubscript{s} / Con\textsuperscript{23}

- RAG1/2

+ RAG1/2

C

Recombinant index

GFP, cTos3, SCL\textsuperscript{(12)}, cTos3, cTos5, cTos6, SCL\textsuperscript{(23)}

*** *
Figure 3 | Intronic PTEN cryptic RSS mediate RAG recombination events. (A) Upper panel: Linear representation of the GFPI reporter construct that results in the inversion of GFP coding sequence during RAG-mediated recombination, and consequent GFP expression. The inverted GFP sequence (light green box) is flanked by a proximal 12-spacer RSS (light grey triangle) and a distal 23-spacer RSS (dark grey triangle) followed by the IRES-RFP as transfection control reporter (red box). GFP positivity is a measure for recombination potential. Lower panel: Control *in vitro* RAG recombination assay; flow cytometry analysis of HEK293T cells transiently transfected with either an irrelevant, mock vector (in the absence of RAG1/2 expression vectors; negative control) or the GFPI-reporter construct containing the consensus 12- and 23-RSS in the presence of RAG1/2 expression vectors. The flow cytometry plots show the expression of GFP and RFP within gated live cells defined by FSC and SSC parameters (not shown) and the values represent the percentage of each cell population in the quadrants. The gate used to discriminate RFP-positive from RFP-negative cells is depicted by a red square and used for the contour plot analysis. The efficiency of recombination is indicated as the percentage of GFP-positive (recombination positive) cells within the RFP-positive (transfected) population. (B) Flow cytometry analysis of HEK293T cells transiently transfected with the GFPI variant constructs containing specific 12-spacer cRSS (LMO2, SCL/TAL1, PTEN-cRSS1 or the Jβ2-2-RSS) site combined with the consensus 23-spacer RSS (left panel). The GFPI variant constructs containing the consensus 12-RSS was combined with 23-spacer PTEN cRSS2, cRSS3, cRSS4 or the control SCL/TAL1 23-spacer cRSS version (right panel). The human LMO2 12-spacer cRSS and the mouse Jβ2-2 *bona fide* RSS were used to establish the range of recombination activities for low-efficiency RSSs as measured by the GFPI reporter assay. The 12- and 23-spacer versions of the human SCL/TAL1 cRSS were used to define the lower limit of detection of cRSS function in this reporter assay. Average percentage ±SD of GFP+ cells in the RFP+ population are derived from 4-5 independent experiments. (C) Recombination index was determined by normalizing the recombination efficiencies of each indicated reporter to that of GFPI Con12/23 and recombination efficiencies were calculated subtracting the GFP background of each respective unrecombined control. Values represent the mean±SEM of 3 independent experiments with 3 replicates per condition; * p < 0.05; ** p < 0.01 and *** p < 0.0001.

these SCL cRSSs were used as references for the lower limit of detection in the GFPI-mRFP RAG reporter assay, as these do not give rise to distinct GFP-positive cell populations in the reporter assay. In contrast, the 12-spacer RSS that flanks the Jβ2-2 gene segment of the mouse TCRβ locus yielded 8.0±0.31% of GFP-positive cells. Also, the 12-spacer cRSS that is involved in recurrent LMO2 translocations in T-ALL yielded 11.4±0.30% of GFP-positive cells. These reporters highlight the capability of the recombination assay to measure low-efficiency RSS and cRSS activities. Despite the low frequencies of recombination, cRSS2-4 reporters give rise to distinct populations of GFP-positive cells (Figure 3B), in contrast to SCL(12) and SCL(23) cRSSs. Moreover, the efficiencies of recombination of PTEN cRSS1-4 differed significantly from those of SCL(12) or SCL(23) cRSSs (Figure 3C). These results strongly support the involvement of predicted cRSS1-4 in PTEN micro-deletions in illegitimate RAG-mediated recombination events. Additionally, the recombination potential of these cRSSs are in line with the observed frequencies of type I micro-deletions (cRSS3-cRSS1) versus type II (cRSS4-cRSS1) and type III (cRSS1-cRSS2) micro-deletions in T-ALL patients (Figure 2B).

RAG-mediated PTEN deletions in xenografted human T-ALL cells

Subclonal micro-deletions in PTEN, even in patients that already had undergone clonal inactivating events affecting one allele, strongly imply that acquisition of micro-deletions is an ongoing phenomenon in T-ALL leading to clonal diversity. To test this, we performed primary and secondary xenotransplantation experiments into NSG mice (Figure 4A) using TAL1-rearranged T-ALL blasts from patient (#24) at diagnosis that had a subclonal micro-deletion (Figure 4B). Several months post-transplantation, mice developed overt leukemia. Primary (X1)
and secondary (X2) xenotransplanted material was then analyzed for the presence of PTEN micro-deletions in bone marrow, thymus, spleen and liver biopsies. Using MLPA analysis, no PTEN micro-deletions were detected (data not shown), indicating that the subclonal PTEN micro-deletion in the diagnostic patient material had not been clonally selected following xenotransplantation. Three distinct, subclonal PTEN micro-deletions were detected by PCR in thymocyte and liver biopsies: One (X1-24 thymus-1) was identical to the micro-deletion as originally identified in this patient (Figure 4B), whereas 2 novel micro-deletions were detected suggesting that these had occurred upon serial retransplantation.

Figure 4 | PTEN micro-deletions in xenotransplants of a T-ALL primary patient sample and in human thymocytes from healthy individuals. (A) Schematic representation of the xenotransplantation strategy. Several months post-transplant of the patient’s (X24) leukemic cells into immunodeficient NSG mice (n=9), we collected cells from bone marrow (BM), thymus (Thy), spleen (Spl) and liver. Primary (X1) and secondary (X2) xenotransplanted material was then analyzed for the presence of any of the three different PTEN micro-deletions. (B) Breakpoint sequences of PTEN micro-deletions as detected in samples from primary (X1) and secondary (X2) xenotransplanted mice. Canonic CAC trinucleotide sequences or the corresponding GTG nucleotides in heptamer sequences are indicated in bold and underlined. (C) Sequences of the breakpoints for PTEN type I micro-deletions as identified in thymocytes of healthy individuals (H-Thy1 – H-Thy3).
PTEN deletions are associated with TALLMO T-ALL patients

*PTEN* aberrations have been associated with a low incidence of NOTCH1-activating mutations, but with a high incidence of rearrangements in TAL1- and/or LMO2-related oncogenes. We now extend these findings, totaling 26 out of 146 T-ALL patients (18%) that have *PTEN* aberrations including point, missense or nonsense mutations, entire locus deletions and/or micro-deletions at the clonal or subclonal level as summarized in Table 1. Twelve patients had clonally inactivated *PTEN* on both alleles and 10 patients on one allele. Evidence for subclonal *PTEN* aberrations was found in 11 patients, seven of whom also had clonally inactivated *PTEN* at least in one allele. The other four patients had either a subclonal missense mutation (patient #23) or subclonal micro-deletions (3 patients) only. Still, for 8 T-ALL patients for whom protein data were available, absence of PTEN protein could not be solely explained by the genetic aberrations found, suggesting that additional *PTEN* inactivating mechanisms await identification. Overall, our previously observed association with TAL or LMO-rearranged leukemia became considerably more significant (p=0.003; Table S6). Also, the significance levels for absence of these mutations in TLX3-rearranged T-ALL (p=0.002; Table S6) and reduced overlap with NOTCH1-activating mutations were further strengthened (p=0.001, Table S6).

PTEN aberrations and outcome

We then investigated the relationship of *PTEN/AKT* aberrations with outcome. Our results do not support observations that *PTEN*-inactivated T-ALL subclones become selected during disease progression giving rise to relapse. Therefore, we regarded T-ALL patients with subclonal *PTEN* aberrations as wild type patients in outcome analyses. *PTEN/AKT* aberrant T-ALL patients, including those patients without *PTEN* protein expression, were not significantly associated with poor outcome in both treatment cohorts (5yrs RFS for *PTEN/AKT* mutant patients is 64±15% versus 70±5% for wild type patients on DCOG protocols and 57±15% versus 76±6% for patients on COALL protocols). This is due to the fact that *PTEN/AKT* mutations and NOTCH-activating mutations predominantly behave as mutually exclusive mutations. In addition, NOTCH-activating mutations have a strong trend towards poor outcome (5yrs RFS for NOTCH-activated patients is 62±8% versus 82±8% for wild type patients on DCOG protocols and 68±8% versus 80±9% for patients on COALL protocols; stratified p=0.06 (for protocol); and supplementary Table S7). However, if NOTCH-activated and *PTEN/AKT* mutated T-ALL patients are being compared to wild type patients, wild type patients demonstrate significantly fewer relapses (stratified p=0.04; Figure 5), albeit having more frequent events including toxic deaths and secondary malignancies. Using the Cox regression proportional hazard method, NOTCH-activating and *PTEN/AKT* mutations were investigated along with male gender and the presence of TLX3 rearrangements that are negatively related with poor outcome (Supplementary Table S7 and Table 2). Even though both NOTCH1-activating mutations and *PTEN/AKT* mutations did not significantly predict for increased risk for relapse in univariate analyses, both were identified as strong, independent risk factors along with male gender in a multivariate analysis (Table 2).
Figure 5 | T-ALL patients lacking PTEN/AKT mutations and NOTCH-activating mutations have a good outcome. Relapse free survival curves (RFS) for T-ALL patients treated on (A) Dutch DCOG ALL7/8 or 9 protocols or (B) German COALL-97/03 protocols. Green line; NOTCH-activating mutations including mutations in NOTCH and FBXW7, red line; PTEN-inactivating or AKT-activating mutations, blue line; NOTCH-activating mutations and PTEN-inactivating or AKT-activating mutations combined, black line; wild type for NOTCH/FBXW7 and PTEN/AKT. Tick-marks in figures refer to patients that are lost from further follow-up. The numbers of patients included at various time points in these studies have been shown.

Illegitimate RAG-mediated PTEN micro-deletions in healthy human thymocytes

The presence of recombination-prone cRSSs in PTEN intron sequences led us to speculate that micro-deletions may occur in healthy thymocytes. Screening DNAs that were isolated from thymocytes of non-leukemic children for the presence of PTEN micro-deletions by PCR revealed evidence for subclonal type I deletions in 3 out of 11 (27%) thymocyte biopsies. Two of those micro-deletions had unique random nucleotide sequences inserted in between the breakpoints (Figure 4C), ruling out false PCR positivity due to contamination. Thus, RAG-mediated PTEN micro-deletions are not exclusive to T-ALL, but also occur during normal T-cell development.
PTEN has been identified as a haploinsufficient tumor suppressor gene,\textsuperscript{21-23} for which gene mutations and/or deletions have been associated with poor outcome in T-ALL in various\textsuperscript{17,18,38,39} but not all studies.\textsuperscript{16,40} For T-ALL patients treated on DCOG ALL-7/8/9 or COALL97/03 treatment protocols, we demonstrate that clonal PTEN inactivating aberrations or loss of PTEN protein is an independent factor that predicts for relapse just like NOTCH-activating mutations and male gender. About half of the T-ALL patients that retained one wild type allele do not express PTEN protein. This indicates that additional genetic, epigenetic or post-translational inactivating events are expected. In this study, we have identified recurrent inactivating PTEN micro-deletions in T-ALL patients due to illegitimate RAG-mediated recombination events, mediated through cryptic RSSs (cRSSs). Taking into account all point, missense or nonsense mutations as well as deletions including micro-deletions, 18\% of T-ALL patients in our patient cohort harbor PTEN inactivating aberrations.

Increasing evidence suggests that cRSSs can participate in oncogenic mechanisms, including chromosomal translocations in lymphoma, B-ALL\textsuperscript{41,42} and T-ALL\textsuperscript{4,43,44}, such as S1L-TAL1 gene fusions and HPRT deletions. Different chromosomal translocation mechanisms have been described as a consequence of erroneous rearrangements between cRSS that flank oncogenes with RSS-sequences of T-cell receptor gene segments. Alternatively, broken DNA strands near oncogenes become mistakenly fused through a non-cRSS mechanism to T-cell receptor gene during V(D)J-assembly (reviewed in \textsuperscript{45}). Other illegitimate, cRSS-driven coding-joint recombination events may cause intrachromosomal deletions such as described for IKZF1,\textsuperscript{46,47} ERG1,\textsuperscript{48} BTG1,\textsuperscript{49} and CDKN2A/B\textsuperscript{50-52} in humans and Notch1\textsuperscript{53,54} and Bcl11b\textsuperscript{55} in mice.

PTEN micro-deletions occur as a consequence of aberrant RAG-mediated recombination events. This is supported by the fact that the breakpoints are flanked by cRSSs, which contain heptamer and nonamer sequences that are separated by 12 or 23 nucleotide spacers. Three out of the four identified cRSSs have high RIC scores.\textsuperscript{56} In recombination assays, 12- or 23-nucleotide spacers cRSS1-4 facilitate recombinations when combined with 23- or 12-nucleotide spacers consensus RSSs, respectively.\textsuperscript{57} Efficiencies for those recombinations equals those of functional RSSs that flank gene segments of immunoglobulin or TCR genes when tested in this system.\textsuperscript{32} The frequencies of different types of micro-deletions matches with the efficiency levels of cRSSs in the recombination assay with cRSS1 and cRSS3 being responsible for the type I micro-deletion in 10 out of 13 micro-deletion events.

Four out of 12 (33\%) signal joint-related type I and II micro-deletions have perfect heptamer-to-heptamer fusions that lack incorporation of random nucleotides just like signal-joints of excision circles as generated during IgH or TCR assembly processes. The other 8 signal junctions (67\%) represented atypical joints that incorporated 3 to 13 random GC-rich N-nucleotides. Twenty to thirty percent of V(D)J-associated signal junctions in mouse lymphocytes represent atypical joints,\textsuperscript{58} and the T-cell receptor-mediated translocation breakpoint as observed in the SUP-T1 T-ALL cell line is a comparable atypical signal junction.\textsuperscript{59} Three out of these 8 atypical
signal joints (patient #12, #26 and #11) also demonstrated evidence for exonuclease processing of signal ends (Figure 2B). Arnal and co-workers proposed that non-canonic heptamer sequence variations may destabilize the RAG post-cleavage complex that make coding-ends and signal-ends available for alternative joining mechanisms. Such a mechanism could also result in a RAG-mediated open-and-shut joint recombination, which includes RAG cleavage adjacent to a cRSS heptamer, processing of the open coding-end by exonuclease activity and insertion of random nucleotides by TdT that is followed by re-ligation of the previously adjacent coding and signal ends. This may explain at least one rare T-ALL case with a mutation affecting the start codon of PTEN. This mutation is flanked by a 12-spacer cRSS with a strong RIC score of -45.48 (Sarmento et al, unpublished results). It resulted in a deletion of 13 nucleotides including the ATG start codon with the insertion of 15 random nucleotides. This cRSS allowed RAG-mediated recombinations equal to the efficiency (2.5±0.13%) of the mouse IgH locus VH/87 RSS that rarely undergoes recombination in-vitro. However, no other T-ALL patient in our current series was identified with an equivalent mutation at this position, indicating that aberrant open-and-shut recombinations at this position are rare. For missense in/del mutations in exon 7 of PTEN, no cRSS sequences could be identified by the positional information algorithm indicating that these mutations are generated by means of other mechanisms.

The identification of subclonal PTEN micro-deletions—as well as entire PTEN locus deletions—indicates that RAG activity may be ongoing in (at least part of) the leukemic cell population. This may explain clonal diversity and selection that results in disease progression and relapse. This latter is also supported by in-vitro recombination assays using T-ALL cell lines, and demonstrate that about one percent of leukemic cells or less will undergo recombinations of the reporter construct within a one week time-frame. Since intraclonal heterogeneity at diagnosis and clonal evolution at relapse are known to occur in ALL, we checked whether PTEN micro-deletions present in a minor leukemic clone at presentation of disease could become clonally selected following xenograft transplantation such as previously demonstrated for PTEN-inactivated T-ALL blasts by means of lentiviral shRNA transfer or gain of BTG1 micro-deletions at relapse of B-ALL patients. In contrast to the study of Clappier et al., we did not observe preferential selection of leukemic cells with PTEN micro-deletions to near clonal levels following xenotransplantation. This could be explained by the preferential outgrowth of other leukemic subclones with certain mutations that are specifically advantageous for engraftment in mice over leukemic subclones having PTEN micro-deletions. Furthermore, additional and new illegitimate RAG-mediated PTEN micro-deletions were detected that had not been found in the primary leukemic patient cells possibly as consequence of ongoing RAG activity. However, at present we cannot formally rule out that subclonal selection of a leukemic subpopulation with a novel PTEN micro-deletion occurred from a PCR-undetectable subclone that was already present at diagnosis. In addition, one patient had a subclonal missense mutation, indicating that there is an ongoing pressure on TALLMO-dysregulated leukemic cells to inactivate remaining wild-type PTEN alleles. RAG activity also results in PTEN micro-deletions in developing thymocytes of healthy individuals. These rearrangements may facilitate a pre-malignant condition from which
leukemia can develop. Likewise, Marlunescu et al. described two mechanisms of illegitimate V(D)J chromosomal rearrangement that were found in healthy children, i.e. the Dδ2/LMO2 recombination in the t(11;14)(p13;q11) and the TAL2/TCRβ translocation t(7;9)(q34;q32) that are known to represent driving oncogenic lesions in T-ALL.

Overall, our discovery of PTEN micro-deletions has reinforced the fact that PTEN aberrations are especially abundant in TAL- or LMO-rearranged leukemia but not in TLX3-rearranged patients, as previously observed for adult T-ALL patient series. PTEN abnormalities seem to be associated with a reduced incidence of NOTCH1-activating mutations. The TALLMO subtype represents an immunophenotypically mature subtype of arrested leukemic cells in T-ALL, in which ongoing RAG-activity creates an opportunistic and extended time window for cRSS-mediated illegitimate recombination events. These may provoke disease progression and relapse in leukemia patients, adding a new level of complexity that should be addressed in the development of future antileukemic strategies for ALL. Taking into account all currently known PTEN inactivation mechanisms (PTEN mutations, entire locus deletions and PTEN micro-deletions), some seemingly wild type T-ALL patients still lack PTEN protein expression indicating that other PTEN inactivation mechanisms await identification.

AUTHORSHIPS AND DISCLOSURES

Contribution: R.D.M., L.M.S., J.T.B. and J.P.P.M. designed the study and wrote the manuscript; R.D.M., W.K.S., L.Z. and J.G.C.A.M.B.-G. performed the MLPA analyses, breakpoint mapping and PCR-based screening of PTEN micro-deletions; J.A.Y. performed the computational detection of putative RAG recombination signal sequences; L.M.S. and V.P. were responsible for generation of GFPi-PTEN cRSS reporter constructs and recombination assays; K.C.-B. and W.K.S. performed the xenotransplantation experiments; J.P.P.M. performed the statistic analyses of the data; M.A., E.S., M.H. collected and provided patient samples and their characteristics; R.D.M., L.M.S., K.C.-B., R.P., J.T.B., J.P.P.M. analyzed and interpreted data; and all authors read, revised, and approved the paper. Conflict-of-interest disclosure: no competing financial interests.

ACKNOWLEDGEMENTS

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40. Larson Gedman A, Chen Q, Kugel Desmoulin S, et al. The impact of NOTCH1, FBXW7 and PTEN mutations on prognosis and downstream signaling in pediatric T-cell acute lymphoblastic leukemia: a report from
59. Baer R, Forster A, Rabbitts TH. The mechanism of chromosome 14 inversion in a human T cell
## SUPPLEMENTARY DATA

### Table S1 | Design of LPO and RPO PTEN hybridization probes for MLPA amplification.

<table>
<thead>
<tr>
<th>Primer sequence</th>
<th>Forward/Reverse</th>
<th>Primers position (GRCh37)</th>
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<tbody>
<tr>
<td>FW: S'-CGACGCCCTCAGCCTGTA-3'</td>
<td>Chr:10 89,524,110-89,624,125</td>
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<tr>
<td>FW: S'-TCTGCGAGACGAGATTTCCTTTC-3'</td>
<td>Chr:10 89,526,319-89,626,336</td>
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<tr>
<td>FW: S'-TGGGTAATCCCATCTCAGC-3'</td>
<td>Chr:10 89,528,98-89,628,216</td>
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<tr>
<td>FW: S'-TGCCAGATTGCTGCATATTTCAGATATTTCTTTCCTTAACTAAAGTACTCATGATATTTATCCAAACATTATTGCTATGGGATTTCCTGCAGAAAGACTTGTTTATTATATCTAGATTGGATCTTGCTGGCAC-3'</td>
<td>Chr:10 89,530,012-89,630,029</td>
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<tr>
<td>FW: S'-TGCTGTAGATGTGGAAGTGCT-3'</td>
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<tr>
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<tr>
<td>FW: S'-AGCTTGGACGAGTGATGAGAT-3'</td>
<td>Chr:10 89,538,040-89,638,051</td>
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<tr>
<td>FW: S'-GCTGTGATATTTTCATCTGCA-3'</td>
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<td>FW: S'-GCCAGTCCAGTACTGCTGC-3'</td>
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<td>FW: S'-TGGGATTGGGATTGAATGACG-3'</td>
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<td>Chr:10 89,590,830-89,690,812</td>
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### Table S2 | Primers used for PTEN exon 2_3 breakpoint mapping.

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<th>Primer sequence</th>
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<td>S'-GGTCTAAGGGACCTGGAC-3'</td>
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<td>S'-GGTCTAAGGGACCTGGAC-3'</td>
<td>Chr:10 89,539,751-89,639,771</td>
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---

**Microdeletions in PTEN in T-ALL**
Table S3 | Primers used for PTEN exon 4_5 breakpoint mapping.

Table S4 | Specific primers to screen for PTEN exon 2_3 and 4_5 breakpoints.

<table>
<thead>
<tr>
<th>Screening breakpoint</th>
<th>PTEN deletion</th>
<th>Primer sequence Forward/Reverse</th>
<th>Primers position (GRCh37)</th>
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<td>Exon 2_3: DNA</td>
<td>FW: 5'-GCTGTCCTCTTACCTTT-3’ or FW: 5'-GGCTGTCCTCTTACCTTT-3’</td>
<td>Chr:10 89.624.121-89.624.340</td>
<td>Chr:10 89.624.320-89.624.338</td>
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<tr>
<td>Exon 2_3: cDNA</td>
<td>FW: 5'-CGAGGGGATCGACTGTA-3’ or FW: 5'-CCGGTTTATGTTCTACGGTC-3’</td>
<td>Chr:10 89.624.110-89.624.125</td>
<td>Chr:10 89.625.124-89.625.267</td>
</tr>
<tr>
<td>Exon 4_5: DNA</td>
<td>FW: 5'-GAAAATGTTACGTGTTATT-3’ or RV: 5’-TCCGACTGCACTGTAATTATC-3’</td>
<td>Chr:10 89.685.486-89.685.509</td>
<td>Chr:10 89.701.203-89.701.182</td>
</tr>
<tr>
<td>Exon 4_5: cDNA</td>
<td>FW: 5'-AAGCTGAACATACATACATAC-3’ or RV: 5’-CAGTGACACTGTCTATAC-3’</td>
<td>Chr:10 89.685.283-89.685.305</td>
<td>Chr:10 89.711.961-89.711.941</td>
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### Table S5 | RSSs tested with the GFPi reporter construct.

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<tr>
<th>12-spacer RSS</th>
<th>heptamer</th>
<th>12-spacer</th>
<th>nonamer</th>
<th>RIC*</th>
</tr>
</thead>
<tbody>
<tr>
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<td>CACAGTG</td>
<td>CTACAGACCTGGA</td>
<td>ACAAAAACCC</td>
<td></td>
</tr>
<tr>
<td>Human PTEN cRSS1</td>
<td>CACAGAT</td>
<td>AAATATACCTTTT</td>
<td>ACATAAAACA</td>
<td>-34.2</td>
</tr>
<tr>
<td>Human PTEN exon 1</td>
<td>CAAAGAG</td>
<td>ATCGTGTAGCAGA</td>
<td>AACAACAGG</td>
<td>-45.48</td>
</tr>
<tr>
<td>Human SCL12</td>
<td>CACAGCC</td>
<td>TCGCGCATTTCCT</td>
<td>GTATATTGC</td>
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<tr>
<td>Human LMO2</td>
<td>CACAGTA</td>
<td>TTTGCTTACCACA</td>
<td>GCAAATATT</td>
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<tr>
<td>Mouse jβ2-1</td>
<td>CACAGTC</td>
<td>GTCAAAATGCCTG</td>
<td>GCACAAACC</td>
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<tr>
<td>Mouse VH/87 IgH</td>
<td>CACTATT</td>
<td>AGGATCAATCCT</td>
<td>TCAATCCCC</td>
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</table>

<table>
<thead>
<tr>
<th>23-spacer RSS</th>
<th>heptamer</th>
<th>23-spacer</th>
<th>nonamer</th>
<th>RIC*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Consensus 23-sp</td>
<td>CACAGTG</td>
<td>CTACAGCTCCACTGTCTACTGGA</td>
<td>ACAAAAAACCC</td>
<td></td>
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<tr>
<td>Human PTEN cRSS2</td>
<td>CACAGTA</td>
<td>TTTTCACTTCTATGAAACTAATTA</td>
<td>TTGAGAACCA</td>
<td>-55.59</td>
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<tr>
<td>Human PTEN cRSS3</td>
<td>CACCCCTA</td>
<td>GGTGGAATACACAGAAAGGAAAC</td>
<td>ACAAATATT</td>
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<tr>
<td>Human PTEN cRSS4</td>
<td>CACTGTA</td>
<td>TGAAAAAGCTAACATACCTACA</td>
<td>ATCTAAAGC</td>
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<tr>
<td>Human SCL 23</td>
<td>CACAGCC</td>
<td>TCGCGCATTTCGTATATTGCCTG</td>
<td>AAGGAAAAAG</td>
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*RIC scores are only stated for newly identified cRSS in PTEN.*

References:

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<tr>
<th>Clinical (n=142)</th>
<th>WT</th>
<th>Mut</th>
<th>p-value</th>
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<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td>0.91</td>
</tr>
<tr>
<td>Male (n=97)</td>
<td>79</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Female (n=45)</td>
<td>37</td>
<td>8</td>
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<tr>
<td>Median age (range)</td>
<td>7.9 (1.3-17.8)</td>
<td>4.7 (1.1-15.9)</td>
<td>0.03*</td>
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<tr>
<td>Median WBC (range)</td>
<td>119.2 (2.0-900.0)</td>
<td>134 (5.0-600.0)</td>
<td>0.14*</td>
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</table>

<table>
<thead>
<tr>
<th>Cytogenetics (n=142)</th>
<th>WT n(%)</th>
<th>Mut n(%)</th>
<th>p-value^</th>
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<tr>
<td>TAL-related§ (n=29)</td>
<td>18 (60%)</td>
<td>11 (40%)</td>
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<tr>
<td>LMO-related§ (n=19)</td>
<td>16 (84%)</td>
<td>3 (16%)</td>
<td>1#</td>
</tr>
<tr>
<td>TLX3+ (n=28)</td>
<td>28 (100%)</td>
<td>0 (0%)</td>
<td>0.002#</td>
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<td>TLX1+ (n=7)</td>
<td>6 (86%)</td>
<td>1 (14%)</td>
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<tr>
<td>HOXA+ (n=13)</td>
<td>13 (100%)</td>
<td>0 (0%)</td>
<td>0.13#</td>
</tr>
<tr>
<td>Unknown (n=48)</td>
<td>37 (77%)</td>
<td>11 (23%)</td>
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<table>
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<th>Unsupervised clusters (n=113)</th>
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<th>Mut n(%)</th>
<th>p-value^</th>
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<td>TAL/LMO+ (n=51)</td>
<td>35 (69%)</td>
<td>16 (31%)</td>
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<tr>
<td>Proliferative (n=19)</td>
<td>16 (84%)</td>
<td>3 (16%)</td>
<td>1#</td>
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<tr>
<td>Immature (n=15)</td>
<td>13 (87%)</td>
<td>2 (13%)</td>
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<tr>
<td>TLX (n=28)</td>
<td>28 (100%)</td>
<td>0 (0%)</td>
<td>0.002#</td>
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<table>
<thead>
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<th>NOTCH1/FBXW7 status (n=141)</th>
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<th>p-value^</th>
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<td>wild-type (n=51)</td>
<td>34 (66%)</td>
<td>17 (34%)</td>
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<tr>
<td>mutant (n=90)</td>
<td>81 (90%)</td>
<td>9 (10%)</td>
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Table S6 Overall clinical and molecular cytogenetics of PTEN-mutated patients versus wild-type patients. ^, statistical analysis of the prevalence of PTEN aberrations within specific genetic T-ALL subgroups, as indicated, compared to other all other T-ALL subgroups combined. Abbreviations: Mut, mutant; WT, wild-type; WBC, white blood cell count. Significant p-values are indicated in bold. All p-values were calculated by using Pearson's Chi-square test, unless indicated; *Mann-Whitney U test, #Fisher's exact test. §Two T-ALL cases have TAL2 and LMO1 or TAL1 and LMO2 rearrangements; The TAL/LMO group consists of TAL1 and/or LMO2 rearranged patients and patients lacking these aberrations but that have an identical or TAL/LMO-like gene expression profile.
Table S7 | 5-years relapse free survival of patient groups based on clinical, (cyto)genetic or biological characteristics. Significant log-rank p-values for DCOG or COALL cohort analyses are indicated in bold; RFS, Relapse free survival; SD, standard deviation; P, p-value; WBC, white blood cell count; †All patients having one of the specified cytogenetic aberrations are being compared to patients lacking those specific aberrations (indicated as “other”); §Different genetic aberrations have been identified that activate HOXA or MEF2C oncogenes in specific genetic subtypes of T-ALL patients (Meijerink JP. Best Pract Res Clin Haematol, 2010; Homminga I. et al., Cancer Cell 2011); ‡Unsupervised clusters as defined in Homminga I. et al., Cancer Cell 2011. ∫The del9p21 status was determined by multiplex ligation-dependent probe amplification (MLPA) analysis for p15/CDKN2B and p16/CDKN2A loci as designed by MRC-Holland, Amsterdam, the Netherlands. ¶PTEN status does not include patient samples having PTEN mutations or deletions on the subclonal level.
Mutually Exclusive Mutations in the IL7-Receptor Signaling Pathway in T-Cell Acute Lymphoblastic Leukemia Respond to Combined Inhibition of MEK/ERK and PI3K/AKT/mTOR Pathways

Kirsten Canté-Barrett1, Linda Zuurbier1, Jessica G.C.A.M. Buijs-Gladdines1, Willem K. Smits1, Maartje J. Vuerhard1, Clarissa Kooi1, Mahban Irandoust1, Dirk Geerts1, Edwin Sonneveld2, Martin Horstmann3,4, Rob Pieters1,5, and Jules P.P. Meijerink1

1Department of Pediatric Oncology/Hematology, Erasmus MC Rotterdam-Sophia Children’s Hospital, Rotterdam, the Netherlands; 2Dutch Childhood Oncology Group (DCOG), the Hague, the Netherlands; 3German Cooperative Study Group for Childhood Acute Lymphoblastic Leukemia (COALL), Hamburg, Germany; 4Research Institute Children’s Cancer Center Hamburg, Clinic of Pediatric Hematology and Oncology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; 5Princess Máxima Center of Pediatric Oncology, Utrecht, the Netherlands.
ABSTRACT

The JAK-STAT, RAS-MEK-ERK and PI3K-AKT-mTOR pathways are important for growth and survival signaling pathways in many cell types, and are frequently mutated in cancer. In leukemic cells, recurrent somatic mutations in JAK, RAS and AKT genes result in uncontrolled proliferation. In the present study of 146 sequenced pediatric T-cell acute lymphoblastic leukemia (T-ALL) patients, 64 harbor mutations in JAK1, JAK3, N-RAS, K-RAS, NF1, AKT, PTEN or in IL7Ra, activating all three pathways. No mutations were identified in JAK2 and TYK2. Strikingly, these mutations occur in a predominantly mutually exclusive fashion, suggesting they share aberrant activation of similar downstream targets. We adapted the Ba/F3 IL3-dependent cell line to express many of these mutated genes in a doxycycline-inducible manner and found that expression of these mutations—in contrast to expression of the wild type genes—render Ba/F3 cells IL3-independent. Additionally, the IL7Ra- and JAK-mutated Ba/F3 cells are sensitive to JAK inhibition by Ruxolitinib or JAK inhibitor I, but relatively resistant to downstream RAS-MEK-ERK or PI3K-AKT-mTOR inhibition, indicating that inhibition of one of these downstream pathways is insufficient. This result can be explained by our observation that inhibition of just one pathway triggers enhanced signaling of the other pathway. We therefore tested combinations of inhibitors for the ability to completely block the IL7R signaling pathway irrespective of the level of activation by specific mutations. The combination of the MEK inhibitor CI-1040 with the PI3K inhibitor Ly294002 proved most efficient in blocking signaling in IL7Ra- or JAK1-mutated Ba/F3 lines as exemplified by the complete absence of p70-S6Kinase phosphorylation that is downstream of both pathways. Since most leukemias depend on one or both pathways, combined MEK and PI3K inhibition should be investigated further and considered a therapeutic option in addition to current treatment protocols.
INTRODUCTION

During the last two decades, T-ALL has been extensively typed for recurrent chromosomal rearrangements and mutations\(^1\). Distinct T-ALL subtypes have been identified that are characterized by specific driving oncogenic lesions—i.e. the so-called type A mutations—that facilitate differentiation arrests at specific T-cell developmental stages\(^4\). Type A mutations are accompanied by type B mutations\(^5,6\) that disturb a multitude of cellular processes such as cell cycle, apoptosis, ubiquitin-mediated proteolysis, or kinase activity by activating pathways including NOTCH1\(^9,10\), N/K-RAS\(^11-14\), JAK-STAT\(^3,15\), PTEN-PI3K-AKT\(^10,16\).

Mutations in interleukin-7 receptor alpha chain (IL7Ra) have recently been identified as an additional type B mutation in approximately 9% of pediatric T-ALL patients\(^17,18\). Activating IL7Ra mutations generate a cysteine residue in the juxta-membrane-transmembrane domain of the receptor that facilitates formation of intermolecular disulfide bonds, homodimerization, and IL7-independent signaling of the mutant IL7Ra\(^17,18\). IL7R signaling results in the phosphorylation and activation of STAT5, JAK1 and PI3K-AKT and provides a proliferation and survival advantage in T-cell development and T-ALL\(^18-20\). Survival of normal developing thymocytes depends on the IL7R-mediated induction of anti-apoptotic BCL2\(^21-23\). Signaling through the intact heterodimeric IL7 receptor α- with common γ-chain is required for normal thymocytes development beyond the early double-negative 2 (DN2) stage\(^24,25\). The IL7Ra gene is one of many transcriptional targets of NOTCH1; its expression can be regulated by interaction of NOTCH1 with a distal IL7Ra enhancer\(^26-28\). When expressed, the IL7R is activated by interaction with its ligand IL7 and recruits Janus kinases (JAK1 and JAK3) and STATS\(^29,30\). In malignant T-cells, IL7-dependent survival and cell cycle progression primarily act via the downstream PI3K-AKT pathway that not only induces BCL2 expression, but also down-regulates the cell cycle inhibitor p27\(^KIP1\)\(^31,32\). Additionally, IL7-induced lymphomas in mice depend on STATS\(^33\) and ectopic STAT5 expression in transgenic mice or in bone marrow transplantation assays is sufficient to induce thymic T-cell lymphomas\(^34,35\). Recently, activating STAT5B mutations have been identified in human T-ALL\(^36-38\).

Mutations in JAK1 have been found in 4-27% of T-ALL patients\(^3,15,39,40\). Even though they have been detected in AML, pre-B-ALL and solid tumors, JAK1 mutations are most frequent in T-ALL\(^15,39-42\). Activating JAK1 mutations induce IL3-independent growth of Ba/F3 cells and activate downstream AKT and ERK\(^35,42\). Similar to the JAK2\(_{V617F}\) mutation in myeloid disorders\(^43-48\), JAK1 mutant molecules need the FERM domain to interact with interleukin homodimers or heterodimers to initiate ligand-independent activation of STAT proteins\(^49,50\). The gene encoding the non-receptor type protein tyrosine phosphatase 2 (PTPN2), an important negative regulator of JAK1, is deleted in 6% of (predominantly TLX1-rearranged) T-ALL patients\(^51,52\). PTPN2 deletion is associated with activation of both wild type and mutant JAK1 and decreases the sensitivity of mutant JAK1-transformed Ba/F3 cells to the JAK inhibitor\(^i\)\(^52\).

In contrast to other hematological malignancies\(^41,53\), JAK2 mutations have not been found in T-ALL. Several JAK2 fusion products exist in a variety of hematological disorders (reviewed in\(^53\)). Of these fusions, the ETV6-JAK2 fusion product is associated with T-ALL\(^54\). This raises the
possibility that JAK2 fusion products can act as oncogenes and contribute to the pathogenesis of T-ALL, as was demonstrated in ETV6-JAK2 transgenic mice.53

JAK3 is activated by interacting with the common γ-chain of several interleukin heterodimeric receptors, has an important role in lymphoid development, and was found mutated in adult T-cell leukemia/lymphoma and acute megakaryoblastic leukemia (AMKL) (reviewed in 53,57). Even though it was discovered in AMKL, the JAK3_A572V pseudo-kinase domain mutant causes lymphoproliferative disease in mice, activates STAT5, AKT and ERK and induces ligand-independent growth when transfected in Ba/F3 cells.58,59 Interestingly, both JAK3_A572V and JAK3_A573V mutations were found in natural killer/T-cell lymphoma, while the JAK3_A573V and two other JAK3 mutations (JAK3_R657Q and JAK3_M511I) have recently also been found in T-ALL patients.5,61

In addition to IL7Ra and JAK mutations, previous studies reported other mutated signaling molecules in T-ALL that normally act in response to IL7 signaling including N/K-RAS, NF1, PTEN, PI3K, AKT, N/K-RAS, NF1, PTEN, PI3K, AKT, and PI3K-AKT-mTOR. Here, we set out to uncover the degree to which all of these mutations of IL7Ra and its downstream signaling molecules co-occur in T-ALL and the degree to which they can activate (independent of ligand stimulation) the different signal-transduction pathways JAK-STAT, RAS-MEK-ERK and PI3K-AKT-mTOR. We tested clinically relevant inhibitors for their ability to block activation of these pathways and found that combined inhibition of MEK and PI3K/AKT abolishes all activation of downstream signaling molecules.

MATERIALS AND METHODS

Patient samples
In this study, 146 primary pediatric T-ALL patient samples were included. The patients’ parents or legal guardians provided informed consent to use leftover diagnostic material for research in accordance with the Institutional Review Board of the Erasmus MC Rotterdam and the Declaration of Helsinki. Leukemia cells were harvested from blood or bone marrow samples and enriched to a purity of at least 90%.

Detection of mutations
Hotspot areas of N-RAS and K-RAS (exons 2 and 3), the FERM domain (exons 3-9), pseudokinase domain (exons 12-18) and kinase domain (exons 19-25) of JAK1, the pseudokinase (exons 12-19) and kinase domain (exons 20-25) of JAK2, the SH2 domain (exon 11), pseudokinase (exons 12-17) and kinase domain (exons 18-24) of JAK3, and the complete TYK2 gene (exon 1-23) were sequenced. PCR reactions were performed using 25-50ng genomic DNA, 300nM primers, 200μM dNTPs, 2mM MgCl₂, 1.25 units of ampliTaq gold (Applied Biosystems) in 1x PCR buffer II (Applied Biosystems) in a volume of 50μl. PCR products were purified with the Millipore Vacuum Manifold filter system (Millipore) and sequenced (BigDye Terminator v3.1 Cycle sequencing Kit, Applied Biosystems) on the ABI PRISM 3130 DNA Analyzer (Applied Biosystems).
Ba/F3 transfectants
Gateway multi-site recombination (Invitrogen) was used to simultaneously clone multiple DNA fragments (Supplementary Figure S1A) into our Gateway-adapted pcDNA3.1 destination vector that contains either a SV40-driven neomycin or puromycin cassette. Ba/F3 cells were transfected by electroporation and transfected cells were enriched to >95% purity using the CD271 (LNGFR) MicroBead kit and magnetic separation (MiltenyiBiotec). Single-cell clones were grown after plating the enriched cells at 0.5 cells/well in 96-well plates.

Doxycycline-dependent induction of mutant genes in Ba/F3 cells
We developed a doxycycline-inducible system in the murine Ba/F3 cell line that normally depends on IL3 for survival and proliferation (Supplementary Figure S1). IL3 withdrawal was done regularly to ensure that all selected Ba/F3 clones remain IL3-dependent. Doxycycline-induced expression of the mutant gene was done for 24 hours after which IL3-independent proliferation and activation of signaling molecules was tested for each of the lines.

Inhibitors
IC50 values for each of the inhibitors were determined using the following concentration ranges: 0.5 nM-2 µM for JAK inh 1 (Merck #420099), 0.5 nM-5 µM for Ruxolitinib (SelleckChem#S1378), 0.2-50 µM for Pimozide (Sigma-Aldrich #P1793), 0.2-16.7 µM for Ly294002 (Cell Signaling #9901), 0.06-5 µM for MK-2206 (SelleckChem#S1078), 0.4 nM-4 µM for Rapamycin (SelleckChem#S1039), and 0.5-125 µM for CI-1040 (Axon Medchem #Axon 1368).

Antibodies
Antibodies used for western blots: phospho-AKT S473 (#9271), phospho-ERK1/2 (#4370), phospho-JAK1 (#3331), phospho-MEK1/2 (#9154), phospho-mTOR(#2971), phospho-p70S6Kinase (#9204), phospho-STAT1 (#9167), phospho-STAT3 (#9145), phospho-STAT5 (#9351), phospho-TYK2 (#9321), DYLDDDDK (#2368) (Cell Signaling Technology), CD127(IL7Ra) (R&D systems #MBAb036), RAS (Millipore #05-516) and β-actin (Sigma #2547). CD127-FITC (#130-094-888) and CD271(LNGFR)-APC (#130-091-884, both from Miltenyi Biotec) were used for flow cytometry.

Statistics
Statistics were performed using SPSS 15.0 software. Pearson’s Chi-square test was performed to test statistical significant differences in the distribution of nominal data. If the number of patients tested in individual groups was lower than five, Fisher’s exact test was used instead. Statistical significance for continuous distributed data was tested using the Mann-Whitney-U test. Data were considered significant when p<0.05 (two-sided).
RESULTS AND DISCUSSION

Identification of JAK1, JAK3, N-RAS and K-RAS mutations in T-ALL

IL7R mutations were previously identified in 9% of pediatric T-ALL cases. In addition, mutations affecting all three of the following pathways occur in T-ALL: JAK-STAT, RAS-MEK-ERK and PI3K-AKT-mTOR. Since these pathways are downstream of IL7R signaling, we questioned whether these mutations occur in a mutually exclusive manner in T-ALL. We screened 146 pediatric T-ALL patient samples for mutations in the Janus kinase gene family, as well as for mutations in N-RAS and K-RAS. While we did not detect any mutations in JAK2 or TYK2, several T-ALL patient samples revealed JAK1, JAK3, N-RAS or K-RAS mutations (Table 1). Out of 146 samples, 10 harbor JAK1 and/or JAK3 mutations, 2 of which have both a JAK1 and a JAK3 mutation. Out of 146 samples, 15 have N-RAS or K-RAS mutations. NF1 negatively regulates RAS signaling and is deleted in 3 instances. From the 18 cases in which RAS signaling is enhanced by an N/K-RAS mutation or NF1 deletion, only 3 co-occur with JAK mutations, and one with an IL7Ra mutation. Except for this co-occurrence with one N-RAS mutation, IL7Ra mutations do not co-occur with JAK, RAS, PTEN and AKT mutations. Previously described inactivating PTEN and activating AKT mutations also occur in a mutually exclusive manner with IL7Ra and JAK mutations, and rarely occur together with N-RAS or K-RAS mutations (Table 1). Together, the results summarized in Table 1 reveal that activating mutations in JAK-STAT, RAS-MEK-ERK and PI3K-AKT-mTOR pathways downstream of IL7R signaling are essentially mutually exclusive in T-ALL. Furthermore, IL7Ra mutations predominantly fall in the TLX subgroup; one has a gene expression profile (GEP) of the TALLMO subgroup whereas no IL7Ra mutations were detected in the proliferative or ETP-ALL subgroups (Supplementary Table S1). JAK mutations are associated with a GEP cluster other than the TALLMO group and RAS or NF1 mutations frequently occur in ETP-ALL (Supplementary Table S1). Combined, the IL7R pathway mutations IL7Ra, JAK and RAS tend to correlate with the TLX and ETP-ALL GEP clusters, which consist of TCRgd lineage (TLX3) and immature (ETP-ALL) arrested T-ALL cells. The IL7Ra, JAK and RAS mutated cases frequently also contain NOTCH1/FBXW7 mutations (Table 1). Indeed, RAS-activating and (weak) NOTCH-activating mutations cooperate in T-ALL; Notch1-activating mutations are acquired in a majority of oncogenic K-RAS or RASGRP1-induced mouse models of T-ALL. In contrast, PTEN/AKT mutated cases do not frequently contain NOTCH1/FBXW7 mutations (Table 1). This concurs with our previous findings that PTEN/AKT mutations predominantly associate with the TALLMO subgroup and are mostly mutually exclusive with NOTCH-activating mutations.

Transforming capacities of JAK1 and JAK3 mutants

IL7 signaling is important for survival and growth of developing T-cells and activates the JAK-STAT and PI3K-AKT-mTOR pathways. Because we determined that both IL7Ra and mutations in JAK1/3, N/K-RAS, PTEN and AKT that are in pathways downstream of IL7R signaling occur in a mutually exclusive manner, we next explored whether these mutations could function in a similar way. In order to test this, we cloned and transfected several constructs containing the
From a cohort of 146 screened pediatric T-ALL patients, 64 are listed with mutations in at least one of the following genes: IL7Ra, JAK1, JAK3, N-RAS, K-RAS, NF1, PTEN and AKT. The NOTCH/FBXW7 mutational status is listed in the last column. The column 'oncogene' lists the driving oncogene or translocation based on the known genetic aberration. GEP cluster: unsupervised clustering groups the patients based on gene expression profile similarity. ND: not done; -: tested negative.

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Table 1
truncated NGFR reporter (LNGFR) and (wild-type or mutant) gene with a C-terminal DDK (Flag) tag (JAK and AKT constructs) or without a tag (IL7Ra and N-RAS constructs) into Ba/F3 cells (Supplementary Figure S1A, B). All Ba/F3 lines express LNGFR (Supplementary Figure S1C). Upon addition of doxycycline, the wild type and mutant genes are induced as measured by DDK expression (western blot, Supplementary Figure S1D) and IL7Ra/CD127 or RAS expression (flow cytometry; Supplementary Figure S1C, E). We tested whether each of these stable Ba/F3 lines could support cell proliferation in the absence of IL3 when induced with doxycycline. All JAK mutants support IL3-independent proliferation (Figure 1A) and activation of certain signal transduction pathways, whereas expression of wild-type JAK1 or JAK3 does not (Figure 1B-G and Supplementary Figure S2). Activation of doxycycline-induced signaling pathways was usually 2-4 hours delayed when compared to IL3 induction (after which signaling is detectable virtually immediately). This delay is due to the time needed to express the mutant protein after addition of doxycycline (Figure 1B-G and Supplementary Figure S2).
Mutations in the IL7-R pathway in T-ALL

Figure 1 | JAK1 and JAK3 mutations promote IL3-independent proliferation and JAK1 mutants activate downstream signaling. A. Survival curves of Ba/F3 lines with different JAK1 or JAK3 constructs after induction by doxycycline (top) or in the presence of IL3 (control; bottom). The cultures were each initiated with 2*10^5 cells at day 0 after extensive washing. B-G. Western blot analyses of DDK and multiple phospho-proteins of lysates from a time-series after stimulation with IL3 or induction of the mutant construct by doxycycline (DOX) of Ba/F3 lines containing JAK1_wt (B), JAK1_L624YPILKV (C), JAK1_E668Q (D), JAK1_R724H (E), JAK1_P815S (F), and JAK1_T901G (G). Levels of b-actin were used as total protein loading controls.

The cysteine-mutated IL7Ra line results in ligand-independent downstream signaling and cell proliferation

IL7R mutations were previously identified in 9% of pediatric T-ALL cases, most of which result in the introduction of a cysteine amino acid that facilitates receptor homodimerization and ligand-independent activation\(^1\). We explored activation of JAK-STAT, RAS-MEK-ERK, and PI3K-AKT-mTOR signal transduction pathways upon expression of IL7Ra_wt, a non-cysteine mutant IL7Ra_V253GPSL (also designated as IL7Ra_GPSL) and a cysteine mutant IL7Ra_PILLT240-244RFCPH (also designated as IL7Ra_RFCPH). The IL7Ra_RFCPH mutant, but not the IL7Ra_wt and GPSL mutant line, supports IL3-independent proliferation (Supplementary Figure S3A). When IL7 was added instead of IL3, the IL7Ra_wt and IL7Ra_GPSL lines also supported cell proliferation, indicating that the IL7R pathway is functionally intact and actively signaling when stimulated (Supplementary Figure S3A). The IL7L-dependent proliferation of all three IL7Ra lines and the IL3- and IL7-independent proliferation of only the IL7Ra_RFCPH cysteine mutant line is supported by activated downstream signaling as evident from the expression of key phosphorylated molecules in the JAK-STAT, RAS-MEK-ERK and PI3K-AKT-mTOR pathways (Supplementary Figure S3B-D). In addition, these data clearly demonstrate that apart from the JAK-STAT and PI3K-AKT-mTOR pathways, IL7R signaling also results in the activation of the RAS-MEK-ERK pathway.

While the cysteine mutant IL7Ra_RFCPH supports IL7-independent proliferation and activation of downstream signaling molecules (Supplementary Figure S3A, D), there is currently no evidence from these in vitro studies that the non-cysteine mutant (IL7Ra_GPSL) has any transforming capacities. Because IL7Ra_GPSL supports Ba/F3 growth in the presence of IL7, it is possible that this mutation leads to a selective advantage in the context of IL7 signaling in leukemia patients. The cysteine mutation in the transmembrane region of the IL7 receptor—that is lacking in the IL7Ra_GPSL mutant but present in most other IL7Ra mutations—allows
cysteine-cysteine bonds and homodimerization of the receptor (Supplementary Figure S4), which results in IL7-independent signaling\textsuperscript{17,18}.

Transforming capacities of AKT and N-RAS mutants
Similar to the IL7Ra and JAK mutant lines, both AKT\textsubscript{E17K} and N-RAS\textsubscript{G12D} mutations (but not the wild-type molecules) result in IL3-independent growth and activation of downstream signal transduction (Supplementary Figure S5). The AKT\textsubscript{E17K} mutant induces proliferation, but at a lower rate than the JAK and IL7Ra mutants. The N-RAS\textsubscript{G12D} mutant does not provide a growth advantage right after doxycycline induction, but starts proliferating after approximately 7 days. The proliferation rate after 7 days, however, is comparable to that of the JAK and IL7Ra mutants (Supplementary Figure S5A).

Different mutants result in different activation of signal-transduction pathways
After 24 hours of doxycycline induction the signaling pathways seem maximally active and thus this time-point was taken to compare signaling between the different mutant lines. Even though the JAK1, JAK3 and IL7Ra mutants all give rise to a growth advantage, not all downstream signaling molecules are activated to the same extent (Figure 2). For example, the JAK1\textsubscript{R724H} and JAK1\textsubscript{T901G} mutant lines display stronger signaling than the JAK3 mutants as determined by the expression of phospho-STAT proteins, p-MEK, p-AKT and p-S6Kinase (Figure 2).

Figure 2 | Differential activation of downstream signaling by IL7Ra, JAK1, and JAK3 mutants. Western blot analysis of several phospho-proteins to indicate the relative signal transduction strength between different wild type and mutant Ba/F3 lines after 24 hours of induction with doxycycline.
Remarkably, these JAK1 mutants also cause TYK2 phosphorylation. The AKT, JAK1 and IL7Ra mutants have comparable p-S6Kinase activation as a final read-out of pathway activation, but the IL7Ra lines do not yield similarly high levels of p-STAT, p-MEK and p-AKT compared to the JAK1 mutants. Furthermore, the IL7Ra cysteine mutant RFCPH signals somewhat stronger than the non-cysteine mutant IL7Ra_GPSL (Figure 2). IL7Ra mutations that result in IL7Ra homodimers due to cysteine bonds enhance JAK1 activation but not JAK3\(^{18}\). A possible explanation is that mutant IL7Ra homodimerization occurs at the expense of heterodimerization with the common γ-chain that is normally required for binding and activation of JAK3\(^{56,68}\). While the JAK3 mutant lines result in very robust IL3-independent growth (Figure 1A), the phosphorylated signaling molecules are very weak in comparison to the JAK1 and IL7Ra mutants (Figure 2). We speculate that activation of the RAS-MEK-ERK pathway by JAK3 remains dependent on the presence of the IL7R when JAK3 is mutated, whereas signaling of mutant JAK1 could have become independent of receptor binding.

IL7R pathway inhibitors affect the transforming capacities of the different mutants

Because the growth curves and downstream signaling of the different mutant lines are not identical, it is plausible that responses to the inhibitors of different downstream signaling molecules in the IL7R pathway also differ. Multiple Ba/F3 lines were subjected to a concentration range of several inhibitors (Figure 3A) and cell growth was tested in an MTT assay to address differences in sensitivity. Ba/F3 lines without doxycycline-induction of the transgene and in the presence of IL3 (as indicated by the solid black symbols (Figure 3) behave like the non-manipulated parental Ba/F3 cell line and are resistant to all inhibitors except Ruxolitinib (Figure 3C) and the RAS inhibitor Tipifarnib (not shown). Despite the fact that Ba/F3 cells are somewhat sensitive to the JAK inhibitor Ruxolitinib in the presence of IL3 signaling, Ba/F3 lines that are induced to express IL7Ra, JAK1 and JAK3 mutant versions (that grow independently of IL3) are extremely sensitive to Ruxolitinib (red symbols, Figure 3C). On the other hand, Ba/F3 lines that depend only on active AKT or N-RAS signaling are completely unaffected by JAK inhibition (Figure 3C). Ruxolitinib inhibits constitutively active JAK1 and JAK3 signaling as well as signaling from the upstream IL7Ra mutants, but not the downstream AKT or RAS signaling, indicating the specificity of this JAK inhibitor.

Experiments with the JAK inhibitor 1 show a similar pattern (Figure 3B), although not all lines are sensitized to the same extent: IL7Ra_RFCPH and JAK3_{R657Q} seem less sensitive to JAK inhibitor 1 when compared to the non-induced (+IL3) controls. Except for a moderate effect on JAK1 mutants, the STAT5 inhibitor Pimozide has no effect on the mutant lines (Figure 3D), suggesting additional pathways activated by IL7R signaling are involved. Indeed, the PI3K inhibitor Ly294002 results in less growth of the JAK1, JAK3 and AKT mutant lines, but not of the IL7Ra lines (Figure 3E). The AKT and mTOR inhibitors affect only the mutant AKT lines effectively (Figure 3F-G). MEK inhibition slows growth of the mutant N-RAS lines, and the JAK lines to varying degrees, but has no effect on the IL7Ra and mutant AKT lines (Figure 3H).

Combined, these data indicate that inhibiting JAK can be effective in targeting leukemic cells...
harboring activating mutations in IL7Ra, JAK1 and JAK3. However, leukemic cells with mutations in any molecules downstream of or parallel to this pathway (PI3K, PTEN, AKT, N-RAS, K-RAS, NF1) will grow independent of inhibition of upstream signaling.

Figure 3 | Sensitivity levels of IL7Ra, JAK, AKT, and RAS mutant lines to various JAK, PI3K-AKT, and RAS-MEK pathway inhibitors. A. Inhibitors that were tested in MTT assays. B-H. Summary of the IC50 values obtained for each of the indicated Ba/F3 lines in the presence of IL3 (control, indicated by the average of all Ba/F3 lines; solid black circles) or after doxycycline-induction of the (mutant) protein in the absence of IL3 (open red triangles). The maximum used concentrations of the inhibitors were 2 µM for JAK inh 1 (B), 5 µM for Ruxolitinib (C), 50 µM for Pimozide (D), 16.7 µM for Ly294002 (E), 5 µM for MK-2206 (F), 4 µM for Rapamycin (G), and 125 µM for CI-1040 (H); points that reach the maximum value represent a completely resistant condition for which an IC50 could not be determined. Varying sensitivity of control Ba/F3 cells to JAK inh 1, Ruxolitinib, and Ly294002 is indicated by the darker shaded area between dashed lines (B, C, E).
Inhibiting PI3K-AKT or RAS-MEK-ERK signaling cross-activates the other pathway
In addition to reducing the cell proliferation (Figure 3), the use of single JAK inhibitors JAK inh
I or Ruxolitinib effectively prevents phosphorylation of downstream molecules in IL7Ra mutant
and JAK mutant Ba/F3 lines (Figure 4A). In line with inhibition of IL7Ra proximal signaling,
downstream mutant RAS and AKT are insensitive to these JAK inhibitors, but do respond to their
specific inhibitors. For example, Tipifarnib and CI-1040 (inhibition of RAS and MEK, respectively)
block signaling by the N-RAS_G12D mutant as demonstrated by the lack of phosphorylated
p70S6K (Figure 4A). Inhibitors of PI3K pathway molecules Ly294002, MK-2206 and Rapamycin
effectively inhibit growth (Figure 3) and p-p70 S6K (Figure 4A) of the AKT_E17K, but not the
N-RAS_G12D mutant. Exposure of IL7Ra or JAK mutants to any of the single agents Ly294002,
MK-2206, Rapamycin or CI-1040 only has a partial effect (JAK mutants) or no effect (IL7Ra lines)
on reducing proliferation (Figure 3E-H) and a partial effect on reduction of p-p70 S6K (Figure 4A).
The data suggest that IL7R signals through an alternate pathway than RAS-MEK-ERK and PI3K-AKT-mTOR, or that the IL7Ra lines enhance activation of one pathway when the other pathway is blocked. Some evidence points to the latter mechanism. In the JAK1 and IL7Ra lines, the p-AKT levels in the presence of the MEK inhibitor CI-1040 are as high as or higher than in control cells without any inhibitor (Figure 4A), suggesting the AKT pathway is preferred when the MEK-ERK pathway is blocked. RAS can signal to PI3K, and may do so in an enhanced manner in tumors that depend on this signaling for survival and in which the MEK-ERK pathway is blocked or in which a potential negative feedback loop is circumvented.

Combined inhibition of PI3K and MEK results in complete loss of downstream signaling

To test whether the RAS-MEK-ERK and PI3K-AKT-mTOR pathways are both important for the signaling capabilities of the Ba/F3 mutant lines, we analyzed the effect of an AKT- or PI3K inhibitor in combination with a MEK inhibitor in comparison to the effect of the single agents. Many cancers depend on these important pathways and combining MEK and PI3K inhibitors has been suggested to be effective in several cancer types. We tested activation of signaling...
Mutations in the IL7-R pathway in T-ALL

molecules in the presence of the following combinations of inhibitors: CI-1040 (MEK) with Ly294002 (PI3K), CI-1040 (MEK) with MK-2206 (AKT), and Rapamycin (mTOR) with Ly294002 (PI3K) (Figure 4B). All three combinations of inhibitors lead to significant reduction of p-p70S6K in the JAK1_T901G as well as the IL7Ra_RFCPH mutant line as compared to the inhibition by each of the individual agents (Figure 4B). The most potent combination tested here is the CI-1040 and Ly294002, resulting in complete absence of p-p70 S6K (Figure 4B) and suggesting a synergistic mechanism. In addition to inhibiting both arms of signal transduction at the levels of MEK and PI3K simultaneously (Figure 4C), this combination of inhibitors also prevents any potential activation by cross-signaling between RAS and PI3Kinase⁶⁹, by the PI3K-dependent feedback loop from mTORC1 to ERK activation⁷³, or potential other cross-signaling events between these pathways⁷⁴.

Many leukemias depend on one or more of the central JAK-STAT, RAS-MEK-ERK and PI3K-AKT-mTOR growth and survival signaling pathways. In T-ALL, somatic mutations in genes of these pathways are acquired across different oncogene-driven subtypes, can be subclonal and/or arise at relapse, and are mostly mutually exclusive. Multiple inhibitors of the MEK and PI3K pathways are available, some of which are used clinically. Inhibiting both pathways simultaneously has a synergistic effect on blocking downstream signal transduction and also targets potential subclones. Therefore, combined MEK and PI3K pathway inhibition should be considered as a therapeutic option in addition to current treatment protocols.
REFERENCES

64. Kindler T, Cornejo MG, Scholl C, et al. K-RasG12D-induced T-cell lymphoblastic lymphoma/leukemias harbor Notch1 mutations and are sensitive to gamma-secretase inhibitors. Blood. 2008;112:3373-
Chapter 6

SUPPLEMENTARY DATA

A
Untagged IL7Rα and N-RAS wt and mutant versions:

DDK-tagged JAK1, JAK3 and AKT wt and mutant versions

B
No DOX

With DOX (●)

No oncogene expression

Oncogene expression

C

D

E

Figure S1 | Schematic representation of the mutant constructs and doxycycline-inducible Ba/F3 clones.
Mutations in the IL7-R pathway in T-ALL

Figure S2 | JAK3 mutations activate downstream signaling.

A  JAK3_wt

B  JAK3_M511I

C  JAK3_R657Q

Figure S2 | JAK3 mutations activate downstream signaling.
Figure S3 | The IL7Ra cysteine-mutant harbors autonomous transforming capacity and activates downstream signaling.
Figure S4 | The IL7R a cysteine-mutant results in higher-order multimer formation.
Figure S5 | N-RAS and AKT mutations activate downstream signaling.
Table S1 | Co-occurrence of IL7Ra, JAK1/3 and NF1, N/K-RAS mutations with unsupervised gene expression profile (GEP) clusters.

<table>
<thead>
<tr>
<th>Unsupervised GEP clusters</th>
<th>IL7Ra mutations (n=7)</th>
<th>p-value</th>
<th>JAK1/3 mutations (n=9)</th>
<th>p-value</th>
<th>NF1, N/K-RAS mutations (n=14)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TALLMO (n=53)</td>
<td>1.0%</td>
<td>0.13</td>
<td>1.0%</td>
<td>0.039*</td>
<td>6.3%</td>
<td>0.08</td>
</tr>
<tr>
<td>TLX (n=30)</td>
<td>20%</td>
<td>0.001*</td>
<td>13.3%</td>
<td>0.23</td>
<td>18.5%</td>
<td>0.35</td>
</tr>
<tr>
<td>Proliferative (n=19)</td>
<td>0%</td>
<td>0.60</td>
<td>5.3%</td>
<td>1</td>
<td>0%</td>
<td>0.12</td>
</tr>
<tr>
<td>ETP-ALL/immature (n=15)</td>
<td>0%</td>
<td>0.59</td>
<td>20%</td>
<td>0.09</td>
<td>46.2%</td>
<td>0.002*</td>
</tr>
<tr>
<td>Total (n=117)</td>
<td>6.0%</td>
<td>7.7%</td>
<td>12.0%</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 51 | Co-occurrence of IL7Ra, JAK1/3 and NF1, N/K-RAS mutations with unsupervised gene expression profile (GEP) clusters.
Immature MEF2C-dysregulated T-cell leukemia patients have an early T-cell precursor acute lymphoblastic leukemia gene signature and typically have non-rearranged T-cell receptors

Linda Zuurbier1, Alejandro Gutierrez2,3, Charles G. Mulighan4, Kirsten Canté-Barrett1, A. Olivier Gevaert5, Johan de Rooi6,7, Yunlei Li1, Willem K. Smits1, Jessica G.C.A.M. Buijs-Gladdines6, Edwin Sonneveld6, A. Thomas Look2,3, Martin Horstmann9,10, Rob Pieters1,8, and Jules P.P. Meijerink1

1Department of Pediatric Oncology/Hematology, Erasmus MC Rotterdam-Sophia Children’s Hospital, Rotterdam, the Netherlands; 2Department of Pediatric Oncology, Dana-Farber Cancer Institute, Boston, MA, USA; 3Division of Hematology/Oncology, Children’s Hospital, Boston, MA, USA; 4Department of Pathology, St Jude Children’s Research Hospital, Memphis, TN, USA; 5Center for Cancer Systems Biology (CCSB) & Department of Radiology, Stanford University School of Medicine, Stanford, CA, USA; 6Department of Biostatistics, Erasmus MC Rotterdam, Rotterdam, the Netherlands; 7Department of Bioinformatics, Erasmus MC Rotterdam, Rotterdam, the Netherlands; 8Dutch Childhood Oncology Group (DCOG), the Hague, the Netherlands; 9German Cooperative Study Group for Childhood Acute Lymphoblastic Leukemia (COALL), Hamburg, Germany; 10Research Institute Children’s Cancer Center Hamburg, Clinic of Pediatric Hematology and Oncology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

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ABSTRACT

Three distinct immature T-cell acute lymphoblastic leukemia entities have been described including cases that express an early T-cell precursor immunophenotype or expression profile, immature *MEF2C*-dysregulated T-cell acute lymphoblastic leukemia cluster cases based on gene expression analysis (immature cluster) and cases that retain non-rearranged *TRG@* loci. Early T-cell precursor acute lymphoblastic leukemia cases exclusively overlap with immature cluster samples based on the expression of early T-cell precursor acute lymphoblastic leukemia signature genes, indicating that both are featuring a single disease entity. Patients lacking *TRG@* rearrangements represent only 40% of immature cluster cases, but no further evidence was found to suggest that cases with absence of bi-allelic *TRG@* deletions reflect a distinct and even more immature disease entity. Immature cluster/early T-cell precursor acute lymphoblastic leukemia cases are strongly enriched for genes expressed in hematopoietic stem cells as well as genes expressed in normal early thymocyte progenitor or double negative-2A T-cell subsets. Identification of early T-cell precursor acute lymphoblastic leukemia cases solely by defined immunophenotypic criteria strongly underestimates the number of cases that have a corresponding gene signature. However, early T-cell precursor acute lymphoblastic leukemia samples correlate best with a CD1 negative, CD4 and CD8 double negative immunophenotype with expression of CD34 and/or myeloid markers CD13 or CD33. Unlike various other studies, immature cluster/early T-cell precursor acute lymphoblastic leukemia patients treated on the COALL-97 protocol did not have an overall inferior outcome, and demonstrated equal sensitivity levels to most conventional therapeutic drugs compared to other pediatric T-cell acute lymphoblastic leukemia patients.
INTRODUCTION

During normal T-cell development, early T-cell precursors (ETPs) migrate to the thymus to differentiate into mature T-cells.\(^1,2\) T-cell acute lymphoblastic leukemias (T-ALL) represent malignant counterparts of thymocytes that have arrested at specific developmental stages that are coupled to specific patterns of T-cell receptor rearrangements.\(^3\) Developmental arrest seems dependent on the presence of so-called “type A mutations”, which activate either T-ALL oncogenes such as \(TAL1, LMO2, TLX3, TLX1, NXX2-1/NXX2-2\) or fusion proteins that activate \(HOXA\) genes.\(^4-6\) For TLX oncoproteins, it has recently been found that these can directly interfere with \(TRA\@\) rearrangements by binding to \(ETS1\) on the \(Ea\) enhancer resulting in a block of active transcription, histone modification-dependent chromatin opening and rearrangements resulting in a developmental arrest.\(^7\)

Various studies have identified T-ALL entities that arrest at an extremely immature developmental stage. Using transcriptome analysis, it was first described as the \(LYL1\) subgroup based on the appreciation of high \(LYL1\) expression.\(^8\) Three years later, the immature subgroup was also identified by unsupervised cluster analysis and that expressed a early thymocyte profile.\(^9\) Coustan-Smith and co-workers identified the ETP-ALL subtype that is characterized by a distinct ETP gene-expression profile and immunophenotype.\(^10,11\) Using unsupervised transcriptome analysis, we described in 2011 that immature T-ALL cluster patients are characterized by rearrangements of either \(MEF2\) or \(MEF2\)-regulating transcription factors.\(^6\) Another immature T-ALL entity was described in 2010 that is characterized by absence of bi-allelic deletions of the T cell receptor gamma gene locus (\(TRG\@\))—and denoted as ABD cases—possibly representing early maturation arrest before the onset of T-cell receptor rearrangements.\(^12\)

ETP-ALL as first described by Coustan-Smith and colleagues predicts poor outcome for patients treated on St. Jude (XIII, XIV and XV) or AIEOP ALL-2000 protocols.\(^10\) Although immature T-cell development arrest was identified as a poor prognostic factor for T-ALL before,\(^13-18\) that study identified a uniform entity that expresses a gene signature alike early thymic progenitor cells.\(^10,11\) In children, the incidence of ETP-ALL is approximately 13 percent of all T-ALL cases, but varies among different cohorts: St Jude Children’s research Hospital study (17 ETP-ALL out of 135 T-ALL patients), AIEOP ALL-2000 study (13/100 cases), COGP9404 and DFCI00-01 (14/40 cases), the Tokyo Children’s Cancer Study group L99-15 (5/91 cases) and Shanghai Children’s Medical Center study (12/72 cases).\(^10,12,19,20\) ETP-ALL patients—as predicted by the mouse ETP gene signature\(^10\)—that were treated on Children’s Oncology Group Study (COG) P9404 or Dana-Farber Cancer Institute (DFCI) 00-01 protocols or ETP-ALL patients based on the ETP-ALL immunophenotype in the Japanese L99-15 study or the Shanghai study were also related with poor outcome.\(^12,19,20\) For adult T-ALL patients, the incidence of immunophenotypic ETP-ALL was 7.4% and was also associated with poor outcome for patients treated on German ALL multicenter study group (GMALL) protocols alike the inferior outcome for early T-ALL.\(^21\) Molecular analysis by whole-genome sequencing revealed that most ETP-ALL cases harbor loss-of-function alterations in regulators of hematopoietic and lymphoid development (\(RUNX1, IKZF1, ETV6, GATA3\) and
EP300) or in components of the polycomb repressor complex 2 (PRC2). The gene-expression profile includes many genes that are expressed in both normal and malignant hematopoietic stem cells, suggesting that ETP-ALL represents an immature leukemia with stem cell and myeloid features. Accordingly, recurrent mutations in myeloid-specific oncogenes (e.g., IDH1, IDH2, DNMT3A, FLT3, NRAS), were identified in immature or ETP-ALL T-ALL patients while having a low incidence of NOTCH1-activating mutations. At this stage, it remains unknown whether ETP-ALL cases are related to the AML entity that has C/EBPA hypermethylation with expression of T-lineage markers and/or T-ALL mutations like NOTCH1.

In the study of Gutierrez and co-workers, ABD T-ALL was associated with a poor response to induction chemotherapy, 5-year event-free survival and overall survival in pediatric T-ALL patients who were treated using the COG P9404 or DFCI 00-01 protocol. Similar results were described for ABD T-ALL in children treated on Taiwanese TPOG-ALL-97/2002 protocols, as well as for pediatric T-cell lymphoblastic lymphoma patients.

In the present study, we investigated the extent to which ETP-ALL, immature cluster T-ALL and ABD overlap by comparing gene expression and immunophenotypic profiles of the ETP-ALL and immature cluster cases and determining the ABD status of these cases. Our findings strongly suggest that—based on gene expression—ETP-ALL and immature cluster T-ALL represent a single entity in which ABD is a subgroup. Furthermore, classifying ETP-ALL cases purely based on the previously proposed ETP immunophenotype significantly underestimates the number of actual patients with an immature cluster/ETP-ALL gene expression profile.

**MATERIALS AND METHODS**

**Patient samples**

For this study, we used diagnostic samples from 117 patients for which gene expression data were available. These patients had enrolled in the Dutch Childhood Oncology Group (DCOG) ALL-7/8 (n=19) and ALL-9 (n=26) protocols, together with 72 patients who were enrolled in the German Co-Operative Study Group for Childhood Acute Lymphoblastic Leukemia study (COALL-97). Seventeen COALL patients underwent bone marrow transplantation due to non-response to therapy at day 29. T-ALL was defined as being positive for TdT, CD2, cytoplasmic CD3 (CyCD3) and/or CD7. The median follow-up times for the DCOG and COALL patients were 63 and 52 months, respectively. Each patient’s parents or legal guardian provided informed consent to use excess diagnostic material for research purposes as approved by the Institutional Review Board/Ethics committee of the Erasmus MC Rotterdam and in accordance to the Declaration of Helsinki. Leukemic cells were harvested from blood or bone marrow samples and enriched as described previously. Enriched samples contained >90% leukemic cells.

**Fluorescent in-situ hybridization (FISH).**

FISH analysis was performed on thawed cytospin
slide as described before. To identify rearrangements of the LYL1 locus, we used homelabeled BAC clones RP11-352L7 and RP11-356L15 (BAC/PAC Resource Center, Children’s Hospital, Oakland, USA).

**Genomic DNA and RNA extraction and ABD status detection by quantitative PCR**

Genomic DNA and RNA were isolated from ≥5x10⁶ leukemic cells using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol, with minor modifications. DNA was stored at 4°C. For RNA isolation, an additional phenol-chloroform extraction was performed as a minor modification of the protocol, and RNA was precipitated with isopropanol together with 1 μl (20 μg/ml) glycogen (Roche, Almere, the Netherlands). After precipitation, the RNA pellets were dissolved in 20 μl RNAse-free TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and the concentration was measured spectrophotometrically. The presence of absence of bi-allelic TRG deletions (ABD) was determined using quantitative PCR, targeting the intron between TRGV11 and TRGJP1 as described previously.

**In vitro cytotoxicity assay**

In vitro cytotoxicities for leukemic cells towards serial dilutions of L-Asparaginase (0.003-10 IU/mL, Paronal, Christiaens, Breda, The Netherlands), prednisolone (0.08-250 μg/mL, BUFA BV, Uitgeest, the Netherlands), vincristine (0.05-50 μg/mL, TEVA Pharma BV, Mijdrecht, The Netherlands), daunorubicin hydrochloride (0.002-2.0 μg/mL, Ceubidine, Rhône-Poulenc Rorer, Amstelveen, The Netherlands) or cytarabine (0.002-2.5 μg/mL, Cytosar, Pharmacia & Upjohn, Woerden, the Netherlands) were determined using the MTT assay. Briefly, 1.6x10⁵ leukemic cells (>90% purity as determined by morphological examination of May-Grünwald-Giemsa (Merck, Darmstadt, Germany) stained cytospin slides) were exposed to serial dilutions of chemotherapeutic drugs in duplicate in a final volume of 100 μL culture media (RPMI 1640 (Life Technologies, Breda, The Netherlands) supplemented with 10% fetal calfs serum (Integro, Zaandam, The Netherlands), 5 μg/mL transferrin, 5 ng/mL sodium selenite (ITS media supplement, Sigma-Aldrich, Zwijndrecht, The Netherlands), 100 IU/mL penicillin, 100 μg/mL streptomycin, 0.125 μg/mL fungizone (Life Technologies)). Following 4 days of incubation in a humified incubator at 37°C with 5% CO2, 5 mg/mL MTT (3-[4,5- dimethylthiazol-2-yl]-2,5- diphenyltetrazoliumbromide, Sigma-Aldrich) was added and incubated for an additional 6 hrs at 37°C to facilitate reduction of MTT tetrazolium salt into formazan crystals by viable cells. Total formazan production was measured spectrophotometrically at 562 nm. For each patient sample, leukemic cells incubated in the absence of drugs was used as control, whereas the assay performed on pure media containing wells were used for background correction (blank values). Leukemic cells survival following exposure to each drug concentration was calculated for background corrected OD values using the formula: ((OD drug incubated wells / OD control wells) x 100%), and the LC50 drug concentrations, at which 50% of the leukemic cells die, were calculated.
Microarray expression analyses
RNA integrity testing, copy-DNA and copy-copy RNA (ccRNA) syntheses, washing, hybridization to Human Genome U133 plus 2.0 microarrays (Affymetrix, Santa Clara, CA), extraction of probe set intensities from CEL-files and normalization with RMA or VSN methods were performed as previously described.\(^5\) Geneset enrichment analysis (GSEA; \(^34\)) was performed on our Affymetrix U133 plus 2.0 microarray expression dataset for 117 T-ALL cases\(^6\) using 1000 random permutations. Enrichment scores and nominal p-values were obtained for up- or down-regulated probe sets among the TOP100, 200 or 500 most significantly, differentially expressed probesets for human ETP-ALL compared to other T-ALL cases.\(^11\) Also up- or down-regulated genes or probesets for C/EBPA-mutated AML\(^25\) and early T cell (MPP-ETP-DN2A) and committed T cell (DN2B-and later) subsets\(^35\) were tested. ETP-ALL patients were identified by Prediction Analysis of Microarrays (PAM)\(^36\) implemented in \(R\) using the human ETP-ALL probe set signature.\(^11\) The classifier was built on the 100, 200 or 500 most significant probe sets from this ETP-ALL gene signature (Supplementary Table S1)\(^11\) using class labels immature cluster \((n=15)\) and non-immature cluster \((n=102)\) in our gene expression cohort comprising 117 T-ALL patient samples. Patients with a cross-validated probability greater than 0.6 for being classified as immature were considered to be ETP-ALL patients. Microarray data are available at the gene expression omnibus (http://www.ncbi.nlm.nih.gov/geo/), accession GSE10609 and GSE26713. Differentially expressed genes between ETP-ALL cases with or without bi-allelic \(TRG\) rearrangements (ABD versus non-ABD ETP-ALL) were obtained by regression analysis using the \textit{limma} package in the \(R\) statistical software package. VSN- or RMA-normalized expression values for \textit{MEF2C} (probe set 239966\_at), \textit{LMO1} (probe set 206718\_at), \textit{LMO2} (probe set 204249\_s\_at), \textit{LYL1} (probe set 210044\_s\_at), \textit{ERG1} (probeset 1563392\_at) and \textit{BAALC} (probe set 222780\_s\_at) were used to test for differential expression between the T-ALL groups (representative probe sets were used). Human hematopoietic progenitor signature genes (probesets) as established by Novershtern and coworkers\(^37\) within the immature cluster gene signature were enriched using the Fisher’s exact test. Differentially expressed genes between the immature cluster \((n=15)\)\(^6\) versus other T-ALL cases \((n=102)\) in our T-ALL microarray expression set were analyzed using the Wilcoxon statistical test and corrected for multiple testing error using the false discovery rate procedure as previously described\(^38\) using the Bioconductor package Multitest. A total of 784 probe sets with an FDR p-value less than \(p=0.01\) was selected as the Immature cluster expression signature gene set (Supplementary Table S5).

Statistics
Statistics were performed using the PASW Statistics 1 software program. The Pearson’s Chi-square test was performed to test for statistically significant differences in the distribution of nominal data; if fewer than five patients were tested in the individual groups the Fisher’s exact test was used instead (as indicated in the corresponding tables). Statistical significance for continuous distributed data was tested using the Mann-Whitney \(U\) test. Differences between patient populations with respect to relapse-free survival (RFS) and event-free survival (EFS)
were tested using the log-rank test. An event for EFS is defined as relapse, lack of response to induction therapy, death in remission due to toxicity, or the development of a secondary malignancy. Differences were considered to be significant when \( p < 0.05 \) (two-sided).

**RESULTS**

**ETP-ALL, immature cluster (MEF2C), and ABD T-ALL patients**

We investigated whether immature T-ALL cluster cases (15 cases) as previously identified using our unsupervised clustering approach\(^6\) displayed ETP-ALL or ABD immature features consistent with two previous studies.\(^{10,12}\) For this gene expression cohort comprised of 117 pediatric T-ALL patient samples, prediction analysis of microarrays (PAM) predicted immature cluster cases as ETP-ALL based on the 100, 200 or 500 most significant probe sets from the human ETP-ALL gene signature\(^{11}\) (Supplementary Table S1). Most significant up- and down-regulated probe sets from the ETP-ALL gene signature are strongly enriched as assessed by GSEA analysis in immature cluster and non-immature cluster cases, respectively (Supplementary Figure S1). By using PAM analysis, 13 out of 15 immature cluster cases were consistently predicted as ETP-ALL based on these ETP-ALL probe sets, while none of the remaining 102 non-immature cluster cases were predicted (\( p < 0.001 \); Supplementary Figure S2). This implies that ETP-ALL and the immature cluster represent single or strongly overlapping T-ALL entities (Table 1 and Supplementary Table S2). Two out of 15 immature cluster cases were not predicted by the ETP-ALL gene signature. Seven patients (6%) were identified as ABD cases based on the preservation of non-rearranged TRG\(@\) loci as detected by RQ-PCR\(^{12}\) (Table 1). Six of the seven ABD cases were immature cluster cases,\(^6\) and five out of these six also had an ETP signature (Supplementary Figure S3). Thus, approximately 40% (5/13 or 6/15) of immature cluster/ETP-ALL cases have retained non-rearranged TRG\(@\) loci.

**Relation to immunophenotype**

ETP-ALL cases were originally defined by the absence of both CD1 and CD8 (present in fewer than 5% of leukemic cells), absent or weak expression of CD5 (in \( \leq 75\% \) of total cells or \( \geq 10\)-fold lower than in normal T-cells), or the expression of one or more of the markers CD117, CD34, HLA-DR, CD13, CD33, CD11b and CD65 (in \( \geq 25\% \) of total cells).\(^{10}\) We next defined whether ETP-ALL gene signature positive cases met with the immunophenotypic criteria as originally proposed for ETP-ALL.\(^{10}\) In the present study, historic flow cytometry data were not obtained on the gated leukemic cell population and various markers were missing, so we used a simplified ETP-ALL immunophenotype as being CD1 negative, CD8 negative, weak CD5 \( (\leq 75\%) \) or CD5 negative with positive expression of CD34 or CD13/33.\(^{10}\) We identified only five samples out of the 111 patients for whom both immunophenotype and gene-expression data were available that had such an immunophenotype, and only three of these five cases expressed an ETP-ALL signature (\( p = 0.01 \), Table 2). Leaving out the CD5 marker led to the identification of 10 out of 13 ETP-
information and significance levels have been indicated. Log-rank p-value.

Expression of (combinations of) immunophenotypic markers for predicted ETP-ALL cases versus other T-ALL cases

Table 2 | Characteristics of immature T-ALL, ETP-ALL (predicted ETP-ALL), ABD patients and patients that have an ETP-ALL immunophenotype. Immature, ETP-ALL, and ABD patients are depicted in bold. ETP-ALL, early T-cell precursor ALL; ABD, absence of bi-allelic deletions; For the chromosomal rearrangement analysis, one case has TAL1 and LMO2 translocations and one case has TAL2 and LMO2 translocations. Unsupervised subgroups have been published before; ETP-ALL immunophenotypes for DCOG and COALL cohort was based on <25% expression of CD1 and CD8, ≥25% expression of CD13, CD33 or CD34 with ≤75% CD5 expression.

<table>
<thead>
<tr>
<th>Immunophenotypic markers</th>
<th>ETP-ALL GEP</th>
<th>p-value</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34+ (n=111)</td>
<td>yes</td>
<td>0.021</td>
<td>65%</td>
<td>73%</td>
</tr>
<tr>
<td>CD33/CD13+ (n=111)</td>
<td>yes</td>
<td>0.0076</td>
<td>96%</td>
<td>88%</td>
</tr>
<tr>
<td>CD54/CD33/CD13+ (n=110)</td>
<td>yes</td>
<td>0.007</td>
<td>77%</td>
<td>64%</td>
</tr>
<tr>
<td>CD2- (n=114)</td>
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<td>0.27</td>
<td>69%</td>
<td>18%</td>
</tr>
<tr>
<td>CD5-CD5weak (n=115)</td>
<td>yes</td>
<td>0.26</td>
<td>71%</td>
<td>87%</td>
</tr>
<tr>
<td>CD25- (n=110)</td>
<td>yes</td>
<td>0.011</td>
<td>92%</td>
<td>49%</td>
</tr>
<tr>
<td>CD4- (n=110)</td>
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<td>100%</td>
<td>72%</td>
</tr>
<tr>
<td>CD8- (n=110)</td>
<td>yes</td>
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<td>100%</td>
<td>71%</td>
</tr>
<tr>
<td>CD4/CD8 DN (n=115)</td>
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<td>100%</td>
<td>86%</td>
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<td>CD1+, CD8+, CD34/CD33/CD13+, CD6 (n=111)</td>
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<td>0.01</td>
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<td>98%</td>
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<td>89%</td>
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<tr>
<td>CD1+, CD4/CD8+DN, CD34/CD33/CD13+ (n=111)</td>
<td>yes</td>
<td>0.001</td>
<td>77%</td>
<td>94%</td>
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</table>

Table 2 | Immunophenotypic markers predicting for immature T-ALL cases with an ETP-ALL gene expression profile. Expression of (combinations of) immunophenotypic markers for predicted ETP-ALL cases versus other T-ALL cases (all categories are also CD7+). GEP: gene expression profile. The number of patient samples with available marker information and significance levels have been indicated. Log-rank p-value.
ALL but also identified 11 out of 98 T-ALL cases that lack an ETP-ALL gene expression signature ($p<0.001$, Table 2).

We then investigated which other (combinations of) immunophenotypic markers could be defined that most strongly associates with cases expressing the ETP-ALL gene signature (Table 2). This analysis showed that these ETP-ALL cases strongly associated with absence of CD4 expression in addition to absence of CD8. Inclusion of absence of CD4 expression in addition to absence of expression of CD1 and CD8 but presence of expression of CD34 and/or CD13/33 markers (i.e. CD1-, CD4/CD8 DN, CD34/CD33/CD13+) predicted 10 out of 13 cases with an ETP-ALL gene signature compared to only 6 out of 98 T-ALL cases that lacked an ETP-ALL gene signature, resulting in 77% sensitivity and 94% specificity levels (Table 2). This immunophenotype also strongly associated with the immature cluster cases; 10 of the 15 immature cluster cases had this immunophenotype compared to only 6 of the 96 non-immature cluster cases ($p<0.001$, sensitivity 67%, specificity 94%; Supplementary Table S3). Also, four out of seven ABD cases had such an immunophenotype compared to 12 out of 104 non-ABD patients ($p=0.008$, Supplementary Table S4).

**Clinical and molecular-genetic features**

Predicted ETP-ALL patients did not associate with gender or age, but white blood cell (WBC) counts tended to be lower for both ETP-ALL cases and immature cluster cases, and were significantly lower for ABD cases ($15\times10^9$ vs. $129\times10^9$ cells/L; $p=0.002$; Table 3, Supplementary Tables S3 and S4). Predicted ETP-ALL patients lacked rearrangements in TAL1, LMO2, TLX3, TLX1, MYB or NKX2-1/NKX2-2 genes (Tables 1 and 3). However, three of the 13 ETP-ALL cases had HOXA-dysregulating events.\(^9,39\) These same three cases also represent immature cluster cases and have an immature immunophenotype with expression of the myeloid marker CD33.\(^4\) MEF2C-dysregulating mechanisms—including ETV6-NCOA2 (1 case), RUNX1-AFF3 (1 case), NKX2-5 (1 case) and PU.1/SPI1 (1 case) translocations—or a MEF2C rearrangement (2 cases) were originally identified in six out of 15 immature cluster cases.\(^4\) Five of these six cases expressed the human ETP-ALL gene signature (Tables 1 and 3; $p<0.001$). The PU.1/SPI1 immature cluster case was the only MEF2C-dysregulated case that was not predicted by the human ETP-ALL gene signature. No genetic aberrations have been identified for 5 ETP-ALL cases yet. With respect to other recurrent T-ALL mutations, the incidences of NOTCH1-activating mutations (NOTCH1/FBXW7) and PTEN/AKT mutations in the immature cluster, ETP-ALL and ABD cases did not differ significantly from other T-ALL patients (Table 3, Supplementary Tables S3 and S4). Similar results were obtained with respect to PHF6 and WT1 mutations. ETV6 and RUNX1 mutations, as previously associated with early T-ALL\(^2,3\) or immunophenotypic ETP-ALL,\(^11\) were only identified in two and one cases out of 71 COALL-97 T-ALL patients, respectively (data not shown), but none of these mutant cases expressed an ETP-ALL gene signature.

The ETP-ALL cases in our cohort were associated with high MEF2C, LMO2 and LYL1 expression levels (Figure 1 (panels A, C and D); $p<0.0001$, $p<0.0001$ and $p=0.0002$ respectively). LMO2 and LYL1 were previously identified as direct target genes for MEF2C.\(^5\) MEF2C-dysregulating events...
Table 3 | Overall clinical, immunophenotypic and molecular cytogenetic properties of predicted ETP-ALL and non-ETP-ALL patients. Unsupervised clusters were based on gene-expression signatures mainly representing known cytogenetic subgroups, with the TALLMO cluster consisting of patients with a TALLMO gene signature, of which the various patients have a TAL1, TAL2, LYL1 or LMO1, LMO2 or LMO3 rearrangement. Patients from the proliferative cluster are primarily characterized by an NKX2-1/NKX2-2 or TLX1 rearrangement. The immature cluster includes patients with high MEF2C expression and MEF2C dysregulating aberrations. The TLX cluster is comprised of patients who are predominantly characterized by having HOXA or TLX3 rearrangements. Significant p-values are indicated in bold. The p-values were calculated using the Fisher’s exact test except as follows: *Mann-Whitney U test; #Pearson’s Chi-square test. ^The significance levels of ETP-ALL frequencies within the indicated cytogenetic subtypes were compared to all other subtypes. ETP, early T-cell precursor; WBC, white blood cell count; the Bonferroni-Holm significance level for multiple testing correction is indicated.

<table>
<thead>
<tr>
<th>Variable</th>
<th>ETP-ALL prediction</th>
<th>p-value</th>
<th>Bonferroni-Holm alpha</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>No (%) or range</td>
<td>Yes (%)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
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</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>76 (75%)</td>
<td>2 (2%)</td>
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<tr>
<td>Female</td>
<td>28 (25%)</td>
<td>6 (46%)</td>
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<tr>
<td>Median age yrs (range)</td>
<td>8 (1.5-17.8)</td>
<td>10.8 (3.7-16.4)</td>
<td>0.19*</td>
</tr>
<tr>
<td>Median WBC x10e9 cells/liter (range)</td>
<td>119 (2-900)</td>
<td>94 (2-435)</td>
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<td>0.36</td>
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<td>0 (0%)</td>
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<td>MEF2C (n=6)</td>
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<td>38 (38%)</td>
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<tr>
<td>mutant (n=14)</td>
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<td>0 (0%)</td>
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Figure 1 | ETP-ALL patients express high MEF2C, LMO2 and LYL1 levels but not ERG or BAALC. The expression of (A) MEF2C (probe set 239966_at), (B) LMO2 (probe set 206718_at), (C) LMO2 (probe set 204249_s_at), (D) LYL1 (probe set 210044_s_at), (E) ERG (probe set 1563392_at), and (F) BAALC (probe set 222780_s_at) was based on VSN normalized microarray gene expression data for ETP-ALL (grey squares) and non-ETP-ALL cases (white squares). For the ETP-ALL subgroup, the ABD and non-ABD cases are indicated separately.
were identified in both ETP-ALL cases with and without ABD characteristics: three ABD ETP-ALL cases had $MEF2C$-dysregulating events compared to two non-ABD ETP-ALL cases (Table 1). Cytogenetic defects underlying the other two ETP-ALL cases with ABD-characteristics include a $HOXA$ case due to an inversion on chromosome 7 and an unknown case. Two other ABD cases did not have an ETP-ALL profile: one had a MYB translocation and was identified as immature cluster case before whereas the other belonged to the TALLMO cluster for which the underlying genetic defect remains unknown (Table 1 and Supplementary Table S4). LIMMA (Linear Models for MicroArray data) analysis revealed that no genes were differentially expressed between the ABD and non-ABD ETP-ALL cases, suggesting that these cases likely represent a single disease entity. Although the expression of $LYL1$ was higher in the ABD cases than in the non-ABD ETP-ALL cases, significance was lost following correction for multiple testing. $ERG1$ or $BAALC$ levels, previously linked to adult immature T-ALL/ETP-ALL and poor outcome in some studies, were not significantly elevated in our pediatric ETP-ALL series.

Both pediatric and adult ETP-ALL cases are characterized by the expression of hematopoietic stem cell signature genes. ETP-ALL may therefore resemble a stem cell-like leukemia with myeloid and lymphoid features. Consistent with this hypothesis, the gene signature of our immature cluster (Supplementary Table S5) was significantly enriched for genes (probe sets) that are expressed in sorted hematopoietic stem cells, early erythroid precursor cells, and B-cell fractions as established by Novershtern and co-workers (Supplementary Table S6). Remarkably, genes that are typically down-regulated during normal T-cell development were enriched in cases with the immature cluster signature. Also B-cell genes were significantly enriched possibly reflecting the early status of ETP-ALL as an entity that has not yet committed to T-cell development. This is further strengthened by strong enrichment of genes that are expressed in normal MMP-ETP-DN2A immature stages rather than genes from later T-cell development stages beyond DN2B (Supplementary Figure S4A-B). It was also reported that ETP-ALL cases express myeloid signature genes. We therefore tested whether ETP-ALL cases would be enriched for differentially expressed genes of AML cases with hypermethylation or mutation of $C/EBPA$. Although gene set enrichment (GSEA) results overall were not significant possibly due to the limited number of probe sets, most of these up- or down-regulated signature genes were strongly enriched in immature cluster/ETP-ALL cases (Supplementary Figure S4C-D). These data confirm that immature cluster/ETP-ALL is a stem cell-like leukemia that arises at the decision point between early myeloid and lymphoid development.

Relation to outcome
ETP-ALL has been associated with extremely poor outcome in several studies. Therefore, we investigated the outcome of immature cluster/ETP-ALL patients in the COALL-97 protocol: 72 out of the 117 patients for which gene expression signatures were available had been enrolled in the COALL-97 protocol, and these 72 included 11 of the 15 immature cluster patients and 10 of the 13 predicted ETP-ALL cases. Surprisingly, ETP-ALL patients were not significantly different than the other T-ALL patients with respect to relapse-free survival (5-year RFS was...
89±11 vs. 71±7%, respectively; \( p=0.31 \)) or event-free survival (5-year EFS was 70±15 vs. 61±7%, respectively; \( p=0.66 \)) (Figure 2). Moreover, no differences were detected in the 5-year overall survival (OS) curves (not shown). Also with respect to the immature cluster cases, the ABD cases and the cases with an ETP-ALL immunophenotype as defined by our criteria, no differences in the RFS or EFS curves were identified (Supplementary Figure SSA-C). For ETP-ALL patients, high-dose cytarabine has been suggested to improve outcome of ETP-ALL patients.\(^1\) As the COALL-97 protocol is a high-dose cytarabine-containing treatment, this may be one of the reasons for the relative good outcome of ETP-ALL and ABD patients in this study compared to other studies.\(^{10,12,19,26}\) However, we could not demonstrate differential sensitivity levels to various drugs including cytarabine for ETP-ALL patients in our \textit{in vitro} cytotoxicity assay (Supplementary Figure S6).

![Figure 2](image-url) | ETP-ALL patients who were treated using the COALL-97 protocol were not associated with poor outcome. (A) Relapse-free survival (RFS) and (B) event-free survival (EFS) curves were generated for the COALL pediatric T-ALL patients. Shown are the RFS and EFS curves for ETP-ALL cases (black line) versus non ETP-ALL cases (grey line).\(^4\) Vertical tick marks represent individual cases for which no further follow-up data is available.

**DISCUSSION**

In this study, we found that 13 of our 15 previously reported immature cluster cases\(^6\) are consistently predicted by PAM based on the human ETP-ALL expression signature,\(^{10,11}\) strongly suggesting that ETP-ALL and immature cluster cases represent a single entity. Consistent with
previous observations, we found that ABD T-ALL patients represent a subset of the immature cluster/ETP-ALL cases. Six out of 7 ABD patients had an ETP-ALL signature. So, approximately 40% of immature cluster cases retain non-rearranged TRG loci, and perhaps represents an even more immature entity among immature cluster/ETP-ALL cases. This entity may closely overlap with the FLT3 mutant adult ETP-ALL cases that fail to demonstrate monoclonal TCR rearrangements in line with expected results for ABD ETP-ALL patients. Although we identified some genes using our microarray analysis that were differentially expressed between the ABD and non-ABD immature cluster/ETP-ALL cases (including higher levels of LYL1 in ABD cases), none of these differences remained significant after correcting for multiple testing. The total number of ABD and non-ABD immature cluster/ETP-ALL patients in our cohort may have been insufficient to reveal subtle differences in gene expression levels. On the other hand, we did not detect differences in oncogenic rearrangement types between the ABD and non-ABD immature cluster/ETP-ALL cases. We also found that both ABD-ETP-ALL as well as non-ABD ETP-ALL entities are associated with MEF2C-dysregulating mechanisms and both entities lack TAL1, TLX1 and NKX2-1/NKX2-2 rearrangements that were previously associated with other T-ALL subgroups. A low white blood cell count was significantly associated with both the ETP-ALL and ABD cases. Taken together, based on expression profiles, genetic data and clinical findings, ABD and non-ABD ETP-ALL cases seem to reflect a single ETP-ALL entity. In line with others, immature cluster/ETP-ALL cases are enriched for stem-cell gene signatures. ETP-ALL cases are also strongly enriched for genes that are normally up-regulated in MMP-ETP-DN2A immature T-cell subsets, but do not express genes that are normally expressed in T-cell subsets beyond the DN2B stage. Although overall enrichment results for AML with inactivated C/EBPA expression were not significant, most of the up- or down-regulated signature genes for that AML cluster were concordantly up- or down-regulated in immature cluster/ETP-ALL cases. Lack of significance may be due to the limited size of the gene signatures. Alternatively, it may be due to the fact that this cluster contains 2 AML entities of which only C/EBPA-hypermethylated AML cases have T-ALL characteristics. These data imply that immature cluster/ETP-ALL and C/EBPA-hypermethylated AML cases may be closely-related disease entities that needs further investigation. To date, exome sequencing of ETP-ALL patient samples has identified mutations in genes that regulate hematopoietic and lymphoid development, cytokine and/or Ras signaling pathways and the polycomb repressor complex 2 (PRC2) reflecting important chromatin-modifying enzymes and myeloid-leukemia associated oncogenes. In addition, the prevalence of NOTCH1-activating mutations is reduced in ETP-ALL patient samples.

Our immature cluster/ETP-ALL cases lack PHF6 and WT1 mutations, and no differences were observed with respect to the frequency of NOTCH1-activating mutations or PI3K/AKT-activating events between the immature cluster/ETP-ALL cases and the other T-ALL cases. The low incidence of ETV6 and RUNX1 mutations may reflect the lower incidence of mutations in children as compared to adult leukemia patients. Although none of these mutations have previously been explicitly associated with outcome in our relatively limited number of patient
samples, additional mutation screens using an expanded series of patient samples are required to determine whether our immature cluster/ETP-ALL samples differ from the spectrum of mutations identified in the St. Jude's and COG patient samples.\textsuperscript{11,22} In contrast to adult T-ALL studies,\textsuperscript{40-42} we did not observe low \textit{ERG/BAALC} expression in our immature cluster/ETP-ALL samples in line with results for adult T-ALL patients that enrolled on GRAALL protocols.\textsuperscript{42}

With respect to our immature T-ALL cluster, we previously identified a variety of distinct genetic rearrangements that result in the activation of oncogenes (e.g., \textit{NKX2-5}, \textit{PU.1}, and \textit{MEF2C}), \textit{RUNX1}, or \textit{ETV6} fusion products, all of which converge on the activation of \textit{MEF2C}.\textsuperscript{6} Although \textit{MEF2C} seems to be activated in multiple immature cluster/ETP-ALL samples for which the underlying genetic defect has not yet been identified, some cases had low levels of \textit{MEF2C} expression, suggesting that alternate pathogenic pathways may also be affected in ETP-ALL. The expression of \textit{LMO2} and \textit{LYL1} was significantly elevated in immature cluster/ETP-ALL samples, and both of these genes were previously identified as direct target genes for \textit{MEF2C}.\textsuperscript{6} Unlike previous findings,\textsuperscript{8} immature cluster/ETP-ALL patients lack \textit{LYL1} rearrangements as assessed by FISH. The only \textit{LYL1}-rearranged case in our cohort has a \textit{TALLMO} gene signature, consistent with the high homology between TAL1 and \textit{LYL1} oncoproteins.\textsuperscript{45}

Coustan-Smith and colleagues originally described an ETP-ALL immunophenotype that was associated with T-ALL cases that were predicted by a mouse ETP-like signature.\textsuperscript{10} This immunophenotype includes multiple markers and may therefore be less useful for identifying ETP-ALL cases in retrospective studies for which in general relatively fewer parameters are available. With respect to our cohort, immunophenotypic parameters were measured on the bulk of mononuclear cells following Ficoll gradient centrifugation. The leukemic population may therefore be contaminated with low numbers of normal cells. We then set the positivity threshold for various markers to \(\geq 25\%\). Using the immunophenotypic data, we characterized ETP-ALL cases by a simplified ETP-ALL immunophenotype that apart from expressing CD7 also expressed CD34 and/or myeloid markers CD13 and/or CD33, no or weak (<75\%) expression of CD5 in the absence of CD1 and CD8. Only five out of 117 patients were identified in our study that met this criterion and only 3 of these samples had an ETP-ALL gene signature. In the study of Gutierrez \textit{et al.},\textsuperscript{12} only one out of 14 cases as identified by the mouse ETP-like gene signature has such an ETP-ALL immunophenotype.\textsuperscript{10} Also in the original ETP-ALL study of Coustan-Smith and coworkers,\textsuperscript{10} only nine of the 14 initial cases that were identified using the mouse ETP gene signature had a bona fide ETP-ALL immunophenotype. In all of these instances the current ETP-ALL immunophenotype may severely underestimate the actual number of ETP-ALL cases that express an ETP-ALL gene signature. For our cohort, the CD34+ and/or CD13/33+, CD1- CD4- and CD8- immunophenotype in addition to CD7 positivity most closely associated with cases that expressed the human ETP-ALL gene signature, with a sensitivity level of 77\% and a specificity level of 94\%.

Predicted ETP-ALL patients based on the human ETP-ALL gene signature that were treated on the COALL-97 protocol did not show a worse outcome in comparison to non ETP-ALL patients in contrast to some other studies.\textsuperscript{10,19-21} We also did not observe a worse outcome for immature
cluster cases, ABD patients and immunophenotypic ETP-ALL patients (regardless of the inclusion of CD5 data). As ETP-ALL cases express human hematopoietic stem cell gene signatures and early myeloid-associated gene signatures,\textsuperscript{11,22} treatment with high-dose cytarabine as included in AML treatment protocols has been suggested to improve the outcome or to increase the cure rate of ETP-ALL patients.\textsuperscript{11} High-dose cytarabine has been incorporated into the COALL-97 treatment protocol, and this may explain the relatively good outcome in contrast to various other studies.\textsuperscript{10,19-21} So far, in vitro cytotoxicity data for various conventional therapeutic drugs including cytarabine failed to reveal differences in sensitivity levels for immature cluster/ETP-ALL compared to other T-ALL patient samples.

In conclusion, the expression of the ETP-ALL gene signature and clustering in the immature T-ALL cluster (following unsupervised cluster analysis) are highly overlapping and point to a single ETP-ALL entity with respect to biology and genetics. We found no evidence to suggest that ABD and non-ABD cases reflect distinct entities among ETP-ALL cases. Different ETP-ALL patient populations may be identified based on immunophenotypic or gene expression data (ETP-ALL gene signature) and could explain differences in outcome between our and other studies. In this study, samples with an ETP-ALL gene signature correlated best with a CD34/13/33+, CD1- and CD4/CD8 double negative immunophenotype. ETP-ALL cases in the COALL-97 study did not associate with a poor outcome compared to other T-ALL cases. High-dose cytarabine as incorporated in the COALL-97 protocol may have improved the outcome for ETP-ALL patients. Limited numbers of ETP-ALL cases have been investigated in various studies so far. Better definitions for ETP-ALL based on immunophenotypic and gene expression data and clinical outcome require a systematic review as part of a large international meta-analysis.

**AUTHORSHIPS AND DISCLOSURES**

JM and RP designed the study, analyzed data and wrote manuscript. RP, ES and MH provided study center data and patient materials. LZ, KC-B, OG, JdR, YL, WKS, and JB-G performed research, analyzed data and wrote manuscript. AG, CGM, ES, MLB, ATL, MH analyzed data and wrote manuscript. ATL is consultant, advisor and stock owner of Oncomed.

**ACKNOWLEDGEMENTS**

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REFERENCES


SUPPLEMENTARY DATA

Figure S1 | ETP-ALL signatures genes are enriched in the immature T-ALL cluster. Gene set enrichment analysis (GSEA) for (A) up- and (B) down-regulated probesets from the top100, 200 or 500 most significantly differentially expressed probesets from the human ETP-ALL gene signature (Zhang et al., nature 2012) for immature cluster versus other T-ALL patients. Significance levels for each analysis has been indicated. Heatmaps for (C) up- and (D) down-regulated probesets for immature cluster patients versus other T-ALL patients.
Figure S2 | PAM prediction of immature cluster cases versus non-immature cluster T-ALL patients based on the TOP100, 200 or 500 most significant probe sets from the human ETP-ALL gene signature (Zhang et al., Nature 2012). Only results for the TOP100 most significant ETP-ALL gene signature probe sets are shown predicting 13 out of 15 immature cluster cases while none of the 102 non-immature cluster cases on this probe selection (p<0.001).

Figure S3 | Overlap between the immature cluster, ETP-ALL and ABD characteristics in our pediatric T-ALL cohort. The number of ETP-ALL positive patients in this Venn diagram is depicted by the gray sphere, the number of ABD patients is depicted by blue sphere, and the number of immature cluster patients is depicted by the dark blue sphere. This diagram is based on 117 T-ALL patients for whom gene expression array data were available.

Figure S4 | Immature cluster/ETP-ALL cases are enriched for early T-cell development signatures. Enrichment of (A) MPP-ETP-DN2A or (B) post-DN2A T-cell signatures genes in immature cluster/ETP-ALL T-ALL cases. GSEA for (C) up- or (D) down-regulated genes of CEBPA-inactivated AML patient samples in immature cluster/ETP-ALL T-ALL cases.
Immature T-ALL/ETP-ALL and outcome

Figure S5 | Immature cluster T-ALL, ABD and immunophenotypic ETP-ALL patients treated on the COALL-97 protocol do not predict for poor outcome. Relapse free survival (RFS) and event free survival (EFS) curves for COALL-97 pediatric T-ALL patients. RFS and EFS curves (A) for immature cluster T-ALL cases upon unsupervised cluster (green line) versus other T-ALL cases (blue line), (B) for ABD T-ALL cases (green line) versus non-ABD cases (blue line), and (C) for ETP-ALL cases as identified upon immunophenotypic parameters (green line) compared to other T-ALL cases (blue line). Cases that are lost from further follow-up are represented by vertical tick marks.

Figure S6 | In vitro sensitivity of Immature cluster/ETP-ALL leukemic cells towards chemotherapeutic agents. In vitro drug sensitivity levels towards prednisolone (squares), vincristine (downside-facing triangles), daunorubicin (circles), L-asparaginase (upside-facing triangles) and cytarabine (diamonds) were measured for leukemic cells of Immature cluster/ETP-ALL patients (open symbols) versus other T-ALL cases (closed gray symbols). The sensitivity level for each sample is indicated as the LC50 drug concentration that is lethal for 50 percent of the leukemic cells. Significance levels have been indicated. NS, not significant.
### Table S2

Available on request.

### Table 51 | Immunophenotype of the immature cluster, ETP-ALL, and ABD T-ALL patients and patients with an ETP immunophenotype.

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</table>

### Table S3 | Overall clinical, immunophenotypic and molecular cytogenetic characteristics of the immature cluster (MEF2C) versus non-immature cluster T-ALL patients.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Wild-Type MEF2C</th>
<th>Mutant MEF2C</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>12.6 (4.5-17.8)</td>
<td>10.1 (3.1-16.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gender</td>
<td>50% Male, 50% Female</td>
<td>50% Male, 50% Female</td>
<td>0.69</td>
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<tr>
<td>ETP-Immunophenotype</td>
<td>Yes</td>
<td>No</td>
<td>&lt;0.001</td>
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<tr>
<td>CD56 Expression</td>
<td>90% Normal</td>
<td>60% Normal</td>
<td>0.025</td>
</tr>
<tr>
<td>NOTCH1/FBXW7 Status</td>
<td>Wild-Type</td>
<td>Mutant</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PIK3C2G Status</td>
<td>Wild-Type</td>
<td>Mutant</td>
<td>0.017</td>
</tr>
<tr>
<td>WTI Status</td>
<td>Wild-Type</td>
<td>Mutant</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

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## Immature T-ALL/ETP-ALL and outcome

### Table S4 | Overall clinical, immunophenotypic and molecular cytogenetic characteristics of ABD versus non-ABD T-ALL patients.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>ABD (n=117)</th>
<th>p-value</th>
<th>Bonferroni-Holm alpha</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>110 (89%)</td>
<td>7 (11%)</td>
<td></td>
</tr>
<tr>
<td>Clinical (n=117)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
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</tr>
<tr>
<td>Male</td>
<td>81 (73%)</td>
<td>3 (41%)</td>
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</tr>
<tr>
<td>Female</td>
<td>29 (27%)</td>
<td>4 (59%)</td>
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<tr>
<td>Median age yrs (range)</td>
<td>7.7 (1.5-17.8)</td>
<td>10.8 (5.4-16.1)</td>
<td>0.13*</td>
</tr>
<tr>
<td>Median WBC x10^9 cells/liter (range)</td>
<td>120 (2900)</td>
<td>15 (2248)</td>
<td>0.002* 0.003</td>
</tr>
<tr>
<td><strong>ETP-immunophenotype: CD5 ≤75%</strong></td>
<td></td>
<td></td>
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<tr>
<td>ETP immunophenotype</td>
<td>3 (3%)</td>
<td>1 (14%)</td>
<td></td>
</tr>
<tr>
<td>non ETP immunophenotype</td>
<td>101 (97%)</td>
<td>6 (86%)</td>
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<tr>
<td><strong>CD1-, CD4/CD8 DN, CD34/CD33/CD13+</strong></td>
<td></td>
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</tr>
<tr>
<td>yes</td>
<td>12 (12%)</td>
<td>4 (57%)</td>
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<tr>
<td>no</td>
<td>92 (88%)</td>
<td>3 (43%)</td>
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<tr>
<td><strong>Chromosomal rearrangements (n=17)</strong></td>
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<tr>
<td>TAL1/2/3LYL1 (n=7)</td>
<td>27 (24%)</td>
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<tr>
<td>LMO1/2/3 (n=14)</td>
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<td>0 (11%)</td>
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<tr>
<td>TLX3 (n=22)</td>
<td>22 (20%)</td>
<td>0 (0%)</td>
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<tr>
<td>TLX1 (n=7)</td>
<td>7 (6%)</td>
<td>0 (0%)</td>
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<tr>
<td>HOXA (n=10)</td>
<td>9 (8%)</td>
<td>1 (22%)</td>
<td></td>
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<tr>
<td>MEF2C (n=6)</td>
<td>3 (2%)</td>
<td>3 (33%)</td>
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<tr>
<td>NKX2-1/NKX2-2 (n=6)</td>
<td>7 (6%)</td>
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<tr>
<td>Unknown (n=26)</td>
<td>23 (21%)</td>
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<td><strong>Unsupervised T-ALL clusters (n=116)</strong></td>
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<tr>
<td>TAL/LMO (n=53)</td>
<td>52 (48%)</td>
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<tr>
<td>Proliferative (n=19)</td>
<td>19 (17%)</td>
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<tr>
<td>Immature (n=15)</td>
<td>9 (8%)</td>
<td>6 (86%)</td>
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<tr>
<td>TLX (n=29)</td>
<td>29 (27%)</td>
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<td><strong>NOTCH1/FSXW7 status (n=112)</strong></td>
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<td>wild-type (n=42)</td>
<td>39 (37%)</td>
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<td>mutant (n=70)</td>
<td>66 (63%)</td>
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<td><strong>PTEN/AKT status (n=113)</strong></td>
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<td>wild-type (n=95)</td>
<td>85 (80%)</td>
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<td>mutant (n=18)</td>
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<td>wild-type (n=32)</td>
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<td>mutant (n=9)</td>
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<tr>
<td>wild-type (n=100)</td>
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<td>7 (100%)</td>
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<tr>
<td>mutant (n=15)</td>
<td>15 (13%)</td>
<td>0 (0%)</td>
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### Table S5 | Gene signature of the immature cluster. Available on request.

- Available on request.

### Table S6 | Genes (probe sets) expressed in sorted hematopoietic cells, early erythroid precursor cells, and B-cell fractions established by Novershtern and co-workers. Available on request.
CHAPTER 7
CHAPTER 8

Differential activation of pathways in genetic subgroups of T-cell acute lymphoblastic leukemia

Linda Zuurbier1, Emanuel F. Petricoin III2,3, Valerie Calvert2, Jessica G.C.A.M. Buijs-Giaddines1, Willem K. Smits1, Edwin Sonneveld3, Rob Pieters1,4 and Jules P.P. Meijerink1

From the 1Department of Pediatric Oncology/Hematology, Erasmus MC Rotterdam-Sophia Children’s Hospital, Rotterdam, The Netherlands; 2Center for Applied Proteomics and Molecular Medicine, George Mason University, Manassas, VA, USA; 3NCI-FDA Clinical Proteomics Program, Food and Drug Administration, Bethesda, MD, USA; 4Princess Maxima Center for pediatric Oncology, Utrecht, The Netherlands.
ABSTRACT

Using an approach combining transcriptome data with proteome data that includes the activation state of molecules, we could identify pathways that seem inactive or active in T-ALL subgroups. Based on these data, TALLMO cluster patients seem to express molecules involved in T-cell receptor (TCR) signaling and metabolic glycolysis and lipid signaling. In TLX cluster patients a NOTCH1 pathway signature was observed independent of NOTCH genetic status. Proliferative cluster patients showed an active cell cycle signature, combined with a high expression of DNA repair genes but low p53 activity and circadian rhythm gene expression. In contrast, immature patients seem to express molecules involved in cell proliferation and migration at low levels. Furthermore, they had a low expression of TCR and high expression of B-cell receptor signaling genes. The alternative TGF-beta signaling via p38 MAPkinases may be important for MEF2C activation in immature patients. Regarding type B T-ALL mutation groups, NOTCH1-activated patients seem to have low and/or inactive PKCθ and p27 levels. This is in contrast to PTEN/AKT-mutated patients, whom seem to have high PKCθ levels. Moreover high expression of cell cycle and glycogenolysis genes were measured in these patients. WT1-inactivated patients had increased expression of gene involved in tRNA thiolation, which possibly reflects high levels of dominant negative WT1 transcripts. Also, WT1-inactivated patients seem to normally repress growth factor pathways, including TCR, cKIT and EGFR and JAK/STAT, AKT and mTOR.
INTRODUCTION

Pediatric T-cell acute lymphoblastic leukemia (T-ALL) accounts for 15% of all acute lymphocytic leukemia diagnosed in children. T-ALL is a high-risk disease, and 20 percent of T-ALL patients relapse. Patients receive intensive chemotherapy which impedes further intensification of therapy. Once relapsed, curative treatment options are limited and most relapsed patients ultimately die. To improve cure rates and to reduce treatment related toxicities, it is of utmost importance to understand pathogenic mechanisms of the disease and to develop new therapies for personalized medicine.

T-ALL is characterized by specific genetic alterations that have been categorized into type A and type B mutations. Type A mutations define at least 4 genetic subtypes that comprise TAL1, LMO2, TLX3, TLX1 or NKX2-1/NKX2-2 rearrangements or HOXA- or MEF2C-activating chromosomal and mutual exclusive rearrangements that drive specific gene expression signatures. Type B mutations are distributed across all T-ALL subgroups, including NOTCH1-activating mutations, mutations that drive the PTEN/AKT pathway, RAS mutations and others as reviewed elsewhere. Some aberrations have been linked with poor or good prognosis, but studies so far revealed that the prognostic significance of many markers were not consistent among studies impeding implementation for treatment stratification in current patient care.

Gene expression profiling (GEP) combined with detailed molecular-cytogenetic analyses has improved understanding disease biology and discovering new patient groups. This identified various TALLMO cases with unknown abnormalities that had activating LMO2 deletions, TAL2, LYL1 or LMO3-rearrangements and new genetic subtypes that comprise MEF2C or NKX2-1/NKX2-2-activating rearrangements. Unsupervised cluster analysis helped to distinguish 4 major T-ALL clusters, i.e. the TALLMO, TLX, proliferative and immature clusters. Most TAL- or LMO-rearranged cases fell into the TALLMO cluster, whereas the proliferative cluster comprised mostly NKX2-1 or TLX1-rearranged cases that all expressed CD1 and highly expressed cell-cycle regulating genes. The TLX cluster almost exclusively comprised TLX3 or HOXA-rearranged cases whereas the immature cluster comprised all MEF2C-activated cases as consequence a variety of different oncogenic rearrangements. This immature cluster was recently proven to overlap with the early T-cell progenitor ALL (ETP-ALL), an immature T-ALL entity that was associated with poor outcome in various studies.

Oncogenic transcription factors transform cells by regulating the expression levels of downstream genes. As mRNA expression levels do not strictly recapitulate the proteomic fingerprint due to post-transcriptional and -translational mechanisms, we combined gene expression analyses with oncogene-related proteome signatures based on the activation status of various signal transduction pathway proteins, to study pathway analysis in T-ALL. This may help to distinguish drugable targets for future tailored treatment strategies, as well as to discover new diagnostic and prognostic biomarkers.

Using the reverse-phase protein array technique (RPMA) we previously showed that primary patient samples with NOTCH1 and/or FBXW7 mutations (NOTCH1-activated patients) have...
increased cleaved (activated) intracellular NOTCH1 protein (ICN) expression compared to wild-type NOTCH1/FBXW7 patients, as was only known for T-ALL cell lines16-19. Chan et al (2007) demonstrated that NOTCH1 was an important regulator of multiple mTOR signaling proteins in T-ALL cell lines, mediated by cMYC20. Furthermore, using RPMA we also demonstrated that PTEN-mutated patient samples that lack PTEN protein expression were surprisingly not associated with increased AKT signaling activity as suggested in other studies21-24. This indicated that, apart from PTEN mutations/deletions, other aberrations exist that also drive activation of AKT, including NOTCH1-activating mutations24. PTEN-mutated patients expressed low levels of ICN, MYC and Musashi proteins that are indicative of decreased NOTCH1 signaling, in line with the reduced incidence of NOTCH-activating mutations in PTEN-mutated patient samples23.

In the present study, we integrated transcriptome and proteome data to study pathway activation in T-ALL groups. We used global test analysis of gene expression data combined with RPMA-generated protein data describing the activation status of a selected panel of signal transduction molecules that may be dysregulated in pediatric T-ALL patients. To the best of our knowledge, broad screens on the activation status for such a panel of signal transduction components simultaneously and in combination with transcriptional analyses, have not been done before on primary T-ALL patient samples. We investigated differential expression of pathway and protein activation among T-ALL clusters, as well as among patient samples that presented with different type B mutations.

MATERIALS AND METHODS

Patient samples
A total of 117 primary pediatric T-ALL patients were included in this study. The patients’ parents or legal guardians provided informed consent to use leftover diagnostic material for research in accordance with the Institutional Review Board of the Erasmus MC Rotterdam and the Declaration of Helsinki. Leukemia cells were harvested and enriched of blood or bone marrow samples as described before, with all samples containing >90% of leukemic cells25. Clinical data were supplied by both study group centers.

Reverse Phase Protein Microarray analysis (RPMA)
Reverse phase protein microarray construction and analysis was performed essentially as previously described26-27. To isolate proteins from $10^6$ leukemic cells, lysis was performed in 20µL Tissue Protein Extraction Reagent (TPER, Pierce Biotechnology, Rockford, IL, USA) with 300 nM NaCl, 1 mM orthovanadate and protease inhibitors. Cells were incubated at 4°C for 20’ and subsequently centrifuged at 10,000 rpm for 5’ in an Eppendorf centrifuge. Supernatants were stored at -80°C prior to printing on the microarrays. Lysates were diluted to 1.0 mg/ml protein concentration and mixed 1:1 with 2x SDS Tris-glycine buffer (Invitrogen) containing 5% 2-mercaptoethanol (Sigma, Zwijndrecht, the Netherlands) (FC = 0.5 mg/ml). Lysates were
Differentially expressed pathways in T-ALL

spotted at a concentration of 0.5 µg/µl (neat spot) and 0.125 µg/µl in duplicate with 350 micron pins on glass-backed nitrocellulose coated array slides (FAST slides, Whatman plc, Kent, UK) using an Aushon Biosystems 2470 (Aushon Biosystems, Billerica, MA, USA). To prevent bias through the composition of the array, patient samples of different clusters were randomly distributed over the RPMA array. Printed slides were stored at -20°C or directly used. The first of each 25 slides printed were subjected to Sypro Ruby Protein Biot staining (Invitrogen) to determine total protein amount. These slides were visualized on a NovaRay CCD fluorescent scanner (Alpha Innotech. San Leandro, CA, USA). The remaining slides were used for staining with a specific antibody. Prior to this, slides were incubated with 1x Reblot (Chemicon, Temecula, CA, USA) for 15' and subsequently washed with PBS twice. This was continued with a blocking procedure for 5 hrs using 1gr I-block (Applied Biosystems) diluted in 500mL PBS with 0.5% Tween-20. Slides were stained with an automated slide stainer (Dako) according to manufacturer’s instructions using the Autostainer catalyzed signal amplification (CSA) kit (Dako). In each staining run, a negative control slide was stained with the secondary antibody only for background substraction. Briefly, endogenous biotin was blocked for 10 minutes with the biotin blocking kit (Dako), followed by application of protein block for 5 minutes; primary antibodies were diluted in antibody diluent and incubated on slides for 30 minutes and biotinylated secondary antibodies were incubated for 15 minutes. Signal amplification involved incubation with a streptavidin-biotin-peroxidase complex provided in the CSA kit for 15 minutes, and amplification reagent (biotinyl-tyramide/hydrogen peroxide, streptavidin-peroxidase) for 15 minutes each. A signal is generated using streptavidin-conjugated IRDye680 (LI-COR Biosciences, Lincoln, NE, USA). Slides were allowed to air dry following development. Stained slides were scanned individually on the NovaRay scanner (Alpha Innotech) and files were saved in TIF format in Photoshop 7.0. All slides were subsequently analyzed with the MicroVigene v2.8.1.0 program (VigeneTech, Carlisle, MA, USA).

Genomic DNA extraction, PCR and sequencing
Genomic DNA isolation, PCR and sequencing analyses of the NOTCH, FBXW7, PTEN, AKT, PIK3CA, PIK3RI, and WT1 genes were previously described

Fluorescence in-situ hybridization analysis (FISH) and RQ-PCR
Rearrangements of the TLX1, TLX3, TAL1, LMO2 and MLL loci were determined using fluorescence in-situ hybridization analysis (FISH) or CALM-AF10 fusion products or expression levels of SIL-TAL1, TLX1 or TLX3 were detected by an RQ-PCR strategy.

Gene expression arrays
Gene expression arrays and analyses were done using Humane Genome U133 plus2.0 oligonucleotide microarrays (Affymetrix, Santa Clara, CA, USA), according to the procedure described before, and have been deposited at the GEO database (http://www.ncbi.nlm.nih.gov/geo/), accession GSE10609 and GSE26713).
**Microarray-based comparative genome hybridization (array-CGH)**

Array-CGH analysis was performed on the human genome CGH Microarray 44A \((n=4)\), 105K \((n=2)\), and 400K \((n=54)\) (Agilent Technologies, Santa-Clara, CA, USA). These arrays harbor 60-mer oligo-nucleotide probes, spanning both coding and non-coding sequences. The procedure was done as described before\(^8\). Analyses were done using Agilent Genomic Workbench software v3.1.28.

**Statistics**

Statistics were performed using SPSS 18.0 software. Statistical significance for continuous distributed data was tested using the Mann-Whitney-U test. Data were considered significant when \(p \leq 0.05\) (two-sided). The Bioconductor package Global test\(^9\) on gene expression data was performed in R for pathway analysis and is based on the empirical Bayesian generalized linear model and determines whether a pre-specified group of genes is differentially expressed in relation to sample parameters. It does, however, not take into account whether genes are up- or downregulated. Correction for multiple testing (Benjamini and Hochberg’s method) was applied and sampling analyses revealed a comparative P-value (<0.05) and was done to exclude pathways which are coincidently detected as being significantly differentially expressed. Using the Bioconductor package Pathview\(^{31}\) expression changes were imaged in KEGG images. When multiple genes were involved at one pathway node, the sum of all expression levels was taken. Probe sets linked to multiple genes were excluded. The probe set with the highest variance was used when multiple probe sets represented one gene, using the Bioconductor Genefilter package.

**RESULTS**

In this study, we investigated the activation status of cellular signaling pathways at transcriptional and protein level in specific T-ALL subgroups. We examined differential expression of pathways in various unsupervised T-ALL clusters as previously described\(^6\), i.e. the TALLMO, TLX, proliferative and immature/ETP-ALL cluster\(^12\) (herein called immature). These clusters are almost exclusively associated with the presence of specific driving oncogenic transcription factor rearrangements\(^6\). Also, patients with various type B aberrations were investigated for differentially expressed pathways, including NOTCH1-activated patients (having NOTCH1 and/or FBXW7 mutations), PTEN/AKT-mutated patients (having silenced PTEN and/or AKT mutations) and WT1-inactivated patients (having WT1 mutations or deletions)\(^{19,23,28}\).

For analyses of gene expression data by microarray analysis, the global test analysis was performed in R, using KEGG pathway information including 229 KEGG cellular signaling pathway maps. Due to extensive crosstalk between pathways, many KEGG pathway maps contain overlapping molecules. In this study, 117 T-ALL patient samples were used of which 53 belongs to TALLMO, 30 to TLX, 19 to the proliferative and 15 to the immature cluster. Sixty-eight
patients have NOTCH1-activating mutations, whereas 20 are mutated in PTEN or AKT and 14 are WT1-inactivated. Significantly differentially expressed pathway maps identified in each group were categorized by body task according to supplied KEGG information; organismal system, environmental information processing, cellular processes and metabolism. The expression direction of each pathway was determined by imaging expression levels in KEGG maps using Pathview in R, which visualizes significant up-or downregulation of particular genes within a pathway. Only pathway maps showing a clear expression direction were further investigated and described (Table 1 and 2; marked in gray).

To determine total and phosphorylated protein levels of 91 selected signal transduction molecules, we used the reverse-phase protein microarray (RPMA) technique (see Table S1). This technique was applied on protein lysates of 66 primary T-ALL patients. Differential protein expression was examined using Mann-whitney U statistics between one and all other subgroups (p≤0.05, Table 1 and 2). Sixty-one out of the 66 patients had corresponding gene expression data. These included 25 TALLMO cluster patients, 14 TLX and 10 proliferative cluster and 12 immature cluster patients. It also included 38 NOTCH1-activated patients, 17 PTEN/AKT-mutated patients and 8 WT1-inactivated patients. Clinical and genetic patient data were available for all these patients (Table S2).

**TALLMO cluster**
First, we examined transcriptionally differentially expressed KEGG pathways in TALLMO cluster patients (n=53) when compared to all other T-ALL patients (Table 1). We observe a clear differential expression pattern of the phosphatidylinositol signaling system, the inositol phosphate metabolism, glycerolipid metabolism, starch and sucrose metabolism, gap junctions and the T-cell receptor (TCR) signaling pathway in these patients (Figure 1A and Figure S1A-E). TALLMO patients seem to actively alter their phosphorylated phosphatidylinositols (PtdIns/PIP) status as well as these of the phosphorylated inositol (IP) derivates (FDR p=5.5*10^{-11}, respectively FDR p=5.3*10^{-11}), which is mediated by various differentially expressed phosphatases and kinases including PTEN and PI3K isoforms. Both processes are important for membrane-trafficking and lipid signaling (second messengers) leading to cellular growth, migration, survival and differentiation. Downstream signaling can occur through the AKT/mTOR pathway, among others. Within the glycerolipid metabolism (FDR p=<1*10^{-12}), we observe conversion of molecules mainly towards diacylglycerols (DAG) or its phosphorylated form phosphatidic acid (diacylglycerophospholipid). Both can function as second messengers within the PtdIns and IP metabolisms. Gene expression within the starch and sucrose metabolism points towards active glycolysis in TALLMO patients, meaning the generation of ATP molecules. Furthermore, the formation of gap junctions seems inactive in TALLMO patients (FDR p=2.9*10^{-10}) indicating that intercellular channels essential for transendothelial migration of lymphocytes may not be actively formed. Interestingly, TALLMO cells appear to have a differential expression of genes encoding TCR signaling drivers (FDR p=6.0*10^{-11}), which is indicated by high expression of e.g. multiple TRA (TRCα) and TRB (TCRβ) variants, LCK, CD40LG (CD40 ligand), LAT, CD28, NFATC1,
182

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Folate biosynthesis

Metabolic pathways

00790

01100

Global test results
KEGG map
pathway
TALLMO cluster (n =53)
Organismal system
04660
T cell receptor signaling pathway
04270
Vascular smooth muscle contraction
04970
Salivary secretion
04972
Pancreatic secretion
04974
Protein digestion and absorption
04971
Gastric acid secretion
04720
Long-term potentiation
Environmental Information Processing
04070
Phosphatidylinositol signaling system
Cellular processes
04540
Gap junction
Metabolism
00561
Glycerolipid metabolism
00590
Arachidonic acid metabolism
00500
Starch and sucrose metabolism
00562
Inositol phosphate metabolism
00512
Mucin type O-Glycan biosynthesis
00830
Retinol metabolism
00750
Vitamin B6 metabolism
TLX cluster (n=30)
Organismal system
04670
Leukocyte transendothelial migration
04270
Vascular smooth muscle contraction
04614
Renin-angiotensin system
Environmental Information Processing
04070
Phosphatidylinositol signaling system
04060
Cytokine-cytokine receptor interaction
04630
Jak-STAT signaling pathway
Cellular processes
04115
p53 signaling pathway
04540
Gap junction
04330
NOTCH signaling pathway
Genetic information processing
04122
Sulfur relay system
Metabolism
00920
Sulfur metabolism
00750
Vitamin B6 metabolism
00532
Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate
00562
Inositol phosphate metabolism
00830
Retinol metabolism
Proliferative cluster (n =19)
Organismal system
04610
Complement and coagulation cascades
04914
Progesterone-mediated oocyte maturation
04910
Insulin signaling pathway
04640
Hematopoietic cell lineage
04710
Circadian rhythm
04380
Osteoclast differentiation
Environmental Information Processing
04310
Wnt signaling pathway
04514
Cell adhesion molecules (CAMs)
04010
MAPK signaling pathway
Cellular processes
04520
Adherens junction
04115
p53 signaling pathway
04110
Cell cycle
04114
Oocyte meiosis
Genetic information processing
03440
Homologous recombination
03030
DNA replication
03420
Nucleotide excision repair
03430
Mismatch repair
03410
Base excision repair
03060
Protein export
Metabolism
00062
Fatty acid elongation
00330
Arginine and proline metabolism
00670
One carbon pool by folate
00230
Purine metabolism
00240
Pyrimidine metabolism
00531
Glycosaminoglycan degradation
1,84E-09
3,97E-09
5,24E-09
1,54E-11
1,95E-10
5,93E-10
2,83E-09
6,23E-09
8,52E-09
3,91E-09
5,72E-11
5,89E-11
1,02E-10
1,60E-10
1,83E-09

6,36E-08
9,68E-08
1,27E-07
1,77E-07
2,79E-07
6,67E-07
3,48E-08
7,09E-08
6,33E-07
3,52E-10
3,70E-09
2,21E-08
1,06E-07

Immnune system
Circulatory system
Endocrine system
Sign. Transduction
Sign. molecules + interaction
Sign. Transduction
Cell growth and death
Cell cummunication
Sign. Transduction
Folding, sorting, degradation
Energy metabolism
Metab. cofactors + vitamins
Glycan biosynth.+ metab.
Carbohydrate metabolism
Metab. cofactors + vitamins

Immune system
Endocrine system
Endocrine system
Immune system
Environmental adaptation
Development
Sign. Transduction
Sign. molecules + interaction
Sign. Transduction
Cell cummunication
Cell growth and death
Cell growth and death
Cell growth and death

Metab. cofactors + vitamins

1,06E-06

4,52E-07

8,79E-08
1,08E-07
1,14E-07
1,48E-07
1,99E-07
3,50E-07

<1E-12
<1E-12
1,19E-12
1,97E-12
3,51E-12
6,14E-11
2,61E-08

Lipid metabolism
Lipid metabolism
Carbohydrate metabolism
Carbohydrate metabolism
Glycan biosynth.+ metab.
Metab. cofactors + vitamins
Metab. cofactors + vitamins

Lipid metabolism
Amino acid metabolism
Metab. cofactors + vitamins
Nucelotide metabolism
Nucelotide metabolism
Glycan biosynth.+ metab.

2,49E-11

Cell cummunication

7,41E-08
5,28E-07
6,50E-07
6,56E-07
7,05E-07
1,31E-06

3,20E-12

Sign. Transduction

Replication and repair
Replication and repair
Replication and repair
Replication and repair
Replication and repair
Folding, sorting, degradation

4,25E-12
2,78E-12
<1E-12
<1E-12
2,54E-12
2,10E-11
1,48E-11

P

Sign. Transduction
Circulatory system
Digestive system
Digestive system
Digestive system
Digestive system
Nervous system

Class

5,37E-06

3,57E-06

1,87E-06
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2,12E-06
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4,13E-06

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1,94E-06
2,39E-06
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4,13E-06

3,37E-09
3,37E-09
4,60E-09
5,22E-09
3,84E-08

6,50E-08

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1,08E-07

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7,50E-08

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5,62E-10
8,29E-08

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5,45E-11

6,02E-11
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5,31E-11
2,53E-10
1,88E-10

Adjusted P

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0,012

0,010
0,049
0,008
0,002
0,005
0,026

0,003
0,005
0,010
0,008
0,012
0,029

0
0
0
0,005

0,008
0,025
0,030

0,012
0,009
0,017
0,003
0,009
0,041

0.009
0.009
0.012
0.030
0.006

0.033

0.031
0.032
0.055

0.004
0.023
0.032

0.034
0.046
0.022

0,036
0,049
0,045
0,045
0,037
0,015
0,039

0,039

0,031

0,032
0,032
0,039
0,032
0,045
0,037
0,025

Comparative P

0.002
0.011
0.020
0.021
0.021
0.028
0.039
0.050
0.001
0.044
0.044

<0.001
<0.001
0.003
0.011
0.020
0.032
0.038
0.008
0.016
0.031

TLX cluster (n=14)
Musashi
pSMAD158
S/S, S/S, S/S
pp70 S6
T389
caspase 3
cleaved Asp175
MYC
β-catenin
pPKCαβ
T638/41
pLCK
Thr 505
p27
pSHIP1
Y1020

Proliferative cluster (n=10)
pABL
T735
pc-KIT
Y719
pIGF-1R/IRb
Y1135/36, Y1150/51
pLCK
Thr505
PI3K
pPYK2
Y402
pPKCαβ
T638/41
MYC
caspase 3
caspase 3
cleaved Asp175
pBRAF
S445

high
high
high
high
low
low
low
low
low
low
low
low
low
low
low

high
high
high
high
high
high
high
high
low
low
low

high
high
high
high
high
high
high
low
low
low

expression

P
0.004
0.016
0.019
0.039
0.003
0.019
0.030
0.030
0.031
0.039
0.039
0.041
0.043
0.044
0.048

RPMA results
Protein
specific residue
TALLMO cluster (n=25)
pGSK3αβ
S21/9
p27
GSK3β
pPKCθ
T538
pABL
T735
PI3K
pMEK1/2
S217/221
pp27
T187
cHER2
CD44
pMTOR
S2481
pc-KIT
Y719
pTSC2
Y1571
pp70 S6
T389
MYC

Chapter 8


### Differentially expressed pathways (global test) and proteins (RPMA) in unsupervised T-ALL clusters.

<table>
<thead>
<tr>
<th>Pathway/Protein</th>
<th>Global Test Adjusted P</th>
<th>RPMA P</th>
<th>Gene/Protein</th>
<th>Description</th>
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<tr>
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<td>0.039</td>
<td>pPDK1 S241</td>
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<tr>
<td>Butanoate metabolism</td>
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<td>9.16E-07</td>
<td>0.045</td>
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<tr>
<td>Purine metabolism</td>
<td>1.48E-07</td>
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<td>Pyrimidine metabolism</td>
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<tr>
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<tr>
<td>RNA degradation</td>
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<td>0.023</td>
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<td>Metabolic pathways</td>
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<td>Oocyte meiosis</td>
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<td>6.50E-08</td>
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<td>5.53E-07</td>
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</tr>
</tbody>
</table>
Chapter 8
Differentially expressed pathways in T-ALL

C

D
**Differentially expressed pathways in T-ALL**

Figure 1 | A selection of differentially expressed KEGG pathway maps. Red indicates higher, green indicates lower expression of a particular gene compared to all other subgroups. (A) phosphatidylinositol signaling system in TALLMO patients, (B) NOTCH signaling in TLX patients, (C) circadian rhythm in proliferative patients, (D) MAPK signaling pathway in immature patients, (E) calcium signaling pathway in NOTCH1-activated patients, (F) cell cycle in PTEN/akt-mutated patients and (G) histidine metabolism in WT1-inactivated patients.

ZAP70, VAV1, CD3D (CD3δ), CD247 (CD3ζ), CD3G (CD3γ) and FYN genes and a low expression of TCRA/D (αδ) loci (data not shown).

Proteome analyses revealed 15 proteins that were differentially expressed in TALLMO patients \((n=25, \text{Table 1})\). The transcriptional activities regarding active glycolysis seem to be reflected by abundant expression of total GSK3β \((p=0.02)\) and inactive phosphorylated GSK3β proteins in TALLMO samples compared to all other clusters \((p=0.004, \text{Figure 2})\). GSK3β is inactive when phosphorylated at Ser9, prevents glycogen storage and has a function within Wnt signaling. GSK3β/phosphorylated-GSK3αβ ratios are similar in TALLMO and all other patients, suggesting that effectively more non-phosphorylated/active GSK3 is present in TALLMO patients. In support of this notion, based on two GSK3β gene expression array probe sets that had above-background levels, GSK3β RNA levels are slightly increased in TALLMO patients (data not shown). In addition, a strong correlation is observed between total GSK3β protein level and phosphorylated GSK3αβ \((R^2=0.804)\). This indicates that GSK3β transcription is relatively equal to phosphorylation in these patients and implies GSK3αβ phosphorylation is not enhanced in these patients, but more likely, GSK3β transcription is enhanced. Whereas high numbers of various (other) PI3K subunits were just described to be observed at transcriptional level, protein levels of the regulatory PI3K...
class I α-subunit (p85 class I regulatory subunit) appear low in this T-ALL subgroup (Figure 2, p=0.019). Also PI3K-downstream effectors like AKT-mTOR, seem inactive (Figure 2) as observed by low levels of phospho-TSC2 (p=0.04), phospho-mTOR (p=0.04) and phospho-p70 S6 kinase (RPS6KB1; p=0.04). Levels of the cell cycle inhibitor protein p27 (CDKN1B; p=0.02) are high but predominantly present in their non-phosphorylated, active form (Figure 2, p=0.03). Among others, transcription of p27 can be initiated by Forkhead transcription factors (downstream AKT) but also HES1 (downstream NOTCH1) and kinases that can phosphorylate p27, including AKT. The cHER2 receptor (p=0.03), that partially fulfills an mTOR-like function upon HER2 expression, is also expressed at low levels in TALLMO cluster patients. Furthermore, the novel isoform PKCθ that is important for TCR signaling is highly expressed (p=0.04) and corresponds to a high expression of TCR signaling genes in this group. Also, phospho-ABL (p=0.003), phospho-MEK1/2 (p=0.03), receptor tyrosine kinase phospho-c-KIT (p=0.04), cell migratory protein CD44 (p=0.04) and MYC (Figure 2, p=0.05) are expressed at significantly lower levels in this cluster.

**TLX cluster**

Differential transcriptional expression analysis of KEGG pathways within the TLX T-ALL cluster patients (n=30) compared to all other T-ALL patients (Table 1) points towards a high gene expression signature of the NOTCH1 pathway (Figure 1B, FDR p=1.1*10^-7). As this cluster predominantly consists of TLX3-rearranged patients, this validates our previous finding of a higher incidence of NOTCH1-activating mutations within TLX3-rearranged patients\(^{19}\). However interestingly, NOTCH1 signature genes seem also highly expressed in NOTCH1 wild-type TLX cluster patients (Figure 3). Furthermore, in contrast to TALLMO cluster patients TLX cluster patients may have an active PtdIns signaling system and IP metabolism (Figure S2A,B, FDR p=1.8*10^-8 and FDR p=5.2*10^-8 respectively). Signaling for leukocyte transendothelial migration seems shut-off in TLX cluster patients (Figure S2A, C, FDR p=3.8*10^-4) and many cytokine receptors are significantly differently expressed (Figure S2D, FDR p=5.6*10^-9). Among these are reduced expression levels of IL4 and IL4R genes and a high expression of PDGF family members and ligands.

Ten proteins were differentially expressed in the TLX cluster (n=14) compared to all other T-ALL patients. The TLX cluster shows significant enrichment of NOTCH1 signaling proteins Musashi (MSI1/2; p<0.001) as well as the downstream target MYC (p=0.02) (Table 1, Figure 2) which validates our global test result. TLX cluster patients do not seem to express the cell cycle inhibitor p27 of which transcription can be regulated by NOTCH1 target HES1 as well as by Forkhead transcription factors (Figure 2, CDKN1B; p=0.016). The phosphatase phospho-SHIP1 seems not expressed (INPP5D; p=0.031). SHIP1 is involved in PIP and IP signaling which appear not to be active in TLX patients. Furthermore, this cluster highly expresses the calcium-sensitive kinase phospho-PKCa/β (Figure 2, p=0.038), β-catenin that is important for migration but also for transcriptional activation of the Wnt signaling (Figure 2, p=0.03), the mTOR target phospho-p70 S6 kinase (Figure 2, RPS6KB1; p=0.003), phospho-SMAD1/5/8 (Figure 2, p<0.001), and cleaved pro-apoptotic caspase 3 (Figure 2, p=0.011) but low levels of the TCR protein phospho-LCK (p=0.008).
Figure 2 | Protein expression levels of proteins that are differentially expressed in TALLMO, TLX, proliferative or immature patient clusters. Differential expression of a selection of proteins in TALLMO cluster patients, TLX cluster patients, proliferative and TLX or immature cluster patients and immature cluster patients. P-value is calculated by Mann-whitney U, in which expression levels of specific molecules in one cluster are compared to these of all other subtypes together. Expression levels are depicted by boxplots showing the distribution of values with standard deviations. Median expression is shown by a bar. Bold boxplots represent the distribution of expression values that are significantly different compared to all others together.
Proliferative cluster

We described before that the proliferative cluster (n=19) is associated with a high expression of genes involved in the cell cycle and spindle assembly. Indeed, our global test result shows cell cycle and DNA replication (Table 1 and Figure S3A,B, FDR p=1.7*10^{-6} and FDR p=4.0*10^{-6} respectively) as one of the top pathways differentially and highly expressed in this cluster compared to all other clusters. This was supported by an increased gene expression of these involved in the production of the one carbon pool by folate and folate biosynthesis, as well as of purine and pyrimidine metabolism (Figure S3C-F, FDR p=1.9*10^{-6}, FDR p=3.6*10^{-6}, FDR p=2.1*10^{-6} and FDR p=2.5*10^{-6} respectively). Differentially expressed pathways also include those that involve the same genes, like spindle assembly processes (Figure S3G,H, progesterone-mediated oocyte maturation, FDR p=1.9*10^{-6} and oocyte meiosis, FDR p=1.9*10^{-6}). Furthermore, there seem to be active amino acid conversions along with decreased breakdown of amino acids through the urea cycle (arginine and proline metabolism, FDR p=1.9*10^{-6}), possibly to save nitrogen groups for the synthesis of purines. A high expression of cell cycle genes seems to be accompanied by an increase in DNA repair genes which is reflected by the differential high expression of genes involved in homologous recombination, nucleotide excision repair, mismatch repair, base excision repair and the p53 pathway (Figure S3K-N, FDR p=1.9*10^{-6}, FDR p=4.1*10^{-6}, FDR p=4.1*10^{-6}, FDR p=4.1*10^{-6} and FDR p=4.2*10^{-7} respectively). CHEK2 and the TP53 gene itself are highly expressed, but most of p53’s target genes are expressed on a low level. In addition, CDK4 and CDK2 genes that are normally suppressed by p53 effectors are elevated in this cluster and support the high expression cell cycle genes. Our data points towards inactivation of the biological rhythm that coordinates cell cycle activity (Figure 1C, circadian rhythm, FDR p=3.0*10^{-4}). Generation of ATP seems to
be reflected by high expression of genes that are involved in the intra-mitochondrial export of freshly produced proteins but not of these produced in the endoplasmatic reticulum (protein export Figure S3O, FDR $p=6.2\times10^{-6}$). Mitochondrial genes mainly encode proteins involved in the aerobic mitochondrial energy metabolism. Also genes resulting in fatty acid degradation from ketone bodies that can serve as fuel supply are highly expressed in this cluster (Figure S3P, FDR $p=9.8\times10^{-4}$). Regarding signal transduction pathways, there is a very low expression of MAPK pathway genes involved in classical or p38/Jnk branches (Figure S3Q, FDR $p=4.1\times10^{-4}$), normally resulting in proliferation, differentiation or apoptosis of cells. Also the canonical Wnt signaling pathway responsible for cell cycle activity seems inactive. In contrast, the Wnt/Ca$^{2+}$ pathway that can regulate transcription through the NFAT transcription factor and thereby cellular activation seems active (Figure S3R, FDR $p=1.9\times10^{-4}$). Surprisingly, genes of the AKT/mTOR pathway are low expressed in this cluster (Insulin signaling pathway, Figure S3S, FDR $p=1.9\times10^{-6}$), which is one of the major routes of protein translation. Expression of genes involved in cell adhesions are low expressed (adherens junction Figure S3T, FDR $p=8.1\times10^{-8}$), except for ACTN1 which is essential for cytokinesis. Moreover membrane-bound adhesion molecules that can regulate immune responses are low expressed including ICOS, HLA-A, CD34, F11R, CD6, PVRL2 and ITGB7 (cell adhesion molecules Figure S3U, FDR $p=1.9\times10^{-6}$). This did not include the T-cell marker and TCR co-receptor CD8, which can bind major histocompatibility complex I (MHC-I) and is highly expressed. Furthermore, genes regulating osteoclast differentiation are expressed at low levels (Figure S3V, FDR $p=4.1\times10^{-4}$).

On the protein level, 11 proteins were differentially expressed in these patients ($n=10$). A high expression of cell cycle players in these patients is also observed on protein level as patients demonstrate higher levels of various kinases including the cell cycle protein phospho-ABL ($p=0.002$), phospho-cKIT ($p=0.01$), phospho-LCK ($p=0.02$), PI3K (Figure 2, $p=0.02$), phospho-PYK2 ($p=0.03$) and phospho-PKCab (Figure 2, $p=0.04$) (Table 1). Also MYC levels are high in this cluster (Figure 2, $p=0.05$). The membrane receptor phospho-IGF-1R/Rb ($p=0.02$) is also highly expressed, and low levels of pro-apoptotic proteins caspase 3 ($p=0.001$) and cleaved caspase 3 are observed ($p=0.04$) (Figure 2). Caspase 3 can be activated by p53, among others, upon irreparable DNA damage. This is in line with our global test results that points towards a low p53 activity. Phosphorylated B-RAF, a member of the MAPK pathway, is low expressed in this cluster ($p=0.04$), which correlates with the low expression of MAPK genes that we observe in these patients.

**Immature cluster**

Immature cluster patients ($n=15$) express high levels of genes involved in the B-cell receptor (BCR) signaling pathway (Table 1 and Figure S4A,B, BCR signaling, FDR $p=8.9\times10^{-8}$ and FcεRI signaling pathway, FDR $p=4.1\times10^{-4}$). In contrast, genes encoding key proteins of the TCR signaling are not expressed in these patients (Figure S4C, FDR $p=2.5\times10^{-7}$). This is in line with the immature early T-cell progenitor state of this T-ALL subtype$^{12}$. This is also reflected by the low expression of T-cell markers but expression of myeloid markers (Figure S4D, hematopoietic cell lineage,
FDR $p=5.5 \times 10^{-7}$). Whereas proliferative patients seem to have an elevated expression of cell cycle genes, immature patients do not (Figure S4E-G, cell cycle, FDR $p=1.3 \times 10^{-6}$, progesterone-mediated oocyte maturation, FDR $p=6.0 \times 10^{-7}$ and oocyte meiosis, FDR $p=4.0 \times 10^{-7}$). This inactive proliferative stage is further supported by a low expression of some genes of the RNA degradation machinery and various metabolisms including ketone bodies synthesis and degradation, N-glycan biosynthesis for protein folding, butanoate and purine metabolisms (Figure S4G-L, FDR $p=2.6 \times 10^{-6}$, FDR $p=6.6 \times 10^{-6}$, FDR $p=8.8 \times 10^{-6}$, FDR $p=9.2 \times 10^{-7}$ and FDR $p=9.3 \times 10^{-7}$ respectively). Interestingly, tight and adherens junctions seem not expressed (Figure S4M,N, FDR $p=3.6 \times 10^{-9}$ and FDR $p=6.6 \times 10^{-9}$ respectively). These are normally formed during cell migration. In contrast, the map of actin cytoskeleton regulation illustrates that focal adhesions seem to be formed in immature cluster patients (regulation of actin cytoskeleton, Figure S4O, FDR $p=1.9 \times 10^{-7}$). These normally link lymphocytes to the extracellular matrix to transmit signals. In addition to this notion, we observe a low expression of enzymes involved in dephosphorylation and thereby inactivation of sphingolipids (Figure S4P, FDR $p=3.3 \times 10^{-7}$). Like focal adhesions, these are important for outside-inwards signaling and consequent regulation of intracellular cascades. Differential expression of genes involved in TGF-beta (TGFbeta) signaling (Figure S4Q, FDR $p=2.1 \times 10^{-4}$) point to inactivity of canonical cascades despite the fact that TGFβ and TGFβ receptors are actively transcribed. The MAPK signaling pathway is an alternative potential TGFβ downstream pathway involving MAPK12 (p38, Figure 1D, FDR $p=1.9 \times 10^{-7}$) and seems active, which may explain MEF2C activation in this cluster. Furthermore genes involved in the JNK-axis including MAPK9 (JNK2) and downstream Jun transcription factor seem to have a low transcriptional expression. Jun is an anti-apoptotic and cell cycle promoting protein.

At the protein level, 17 proteins were differential expressed in immature cluster patients ($n=12$); inactivity of the canonical TGFβ/BMP signaling observed at the transcriptional level is confirmed by low expression of phosphorylated SMAD1/5/8 proteins ($p<0.001$, Figure 2). Similar, the low adhesion gene expression signature is supported by low phosphorylated adducin levels (Figure 2, ADD1-3; $p=0.01$) and β-catenin (Figure 2, CTNNB1; $p=0.005$). Interestingly, the presence of pro-apoptotic molecules caspase 3 and BAX (Figure 2, $p=0.005$ and $p=0.04$ respectively) and the low levels of the cell cycle proteins CDK2 and MYC (Figure 2, $p=0.03$ and $p=0.04$ respectively) confirm the low proliferative activity of T-ALL cells in this immature cluster. This is also supported by a low expression on the cell cycle inhibitor p27 (Figure 2, CDKN1B; $p=0.02$). Phospho-SAPK/JNK ($p=0.008$) proteins are observed at low levels, which coincide with the global test result showing low activity of JNK2 and subsequent Jun and suggesting a low anti-apoptotic and low cell cycle activities. Furthermore, many other proteins are low expressed which may support the low proliferative activity, including phospho-PKCζ/λ (Figure 2, PRKCZ/I $p=0.024$) and phospho-PKCαβ (Figure 2, PRKCA/PRKCB), calcium-dependent phospholipid binding protein annexin A1 (ANXA1; $p=0.03$) and various AKT pathway molecules including phospho-SHIP1 (INPP5D; $p=0.02$), phospho-PDK1 ($p=0.04$), phospho-4EBP1 (EIF4EBP1; $p=0.04$) and transcription factors phospho-FOXO3 ($p=0.04$) and phospho-CREB (Figure 2, $p=0.01$) of which the latter can also signal downstream of the p38 MAPK.
Differentially expressed pathways in T-ALL

**NOTCH1-activating mutations**

As expected, global test analysis of NOTCH1-activated patients \( (n=68) \) compared to NOTCH1/FBXW7 wild-type patients demonstrated that the NOTCH1 pathway is markedly induced in patients with NOTCH1-activating mutations \( (FDR \ p=7.2\times10^{-4}, \text{ Table 2 and Figure S5A}) \). High differential expression of genes that are involved in the calcium signaling pathway \( (\text{Figure 1E, FDR } p=2.3\times10^{-2}) \) suggests active release of calcium through ERBB4, PLCB4 and ITPR2 and subsequent PKC alpha activation. This activation pattern was also visible in the phosphatidylinositol signaling system KEGG map \( (\text{Figure S5B, FDR } p=1.3\times10^{-2}) \), however this map also depicts a low expression of genes of the connected PIP/IP3 lipid signaling cascades. Interestingly, NOTCH1-activated patients have a very low expression of cell membrane proteins that are involved in immunity, including HLA proteins (cell adhesion molecules map \( \text{Figure S5C, FDR } p=1.6\times10^{-2} \)). Also molecules involved in phagosome and endocytosis processes seem to be low expressed \( (\text{Figure S5D-F, FDR } p=2.0\times10^{-2} \text{ and FDR } p=3.1\times10^{-2} \text{ respectively and natural killer cell-mediated cytotoxicity map FDR } p=1.6\times10^{-2}) \). These processes are normally involved in antigen presentation on the cell surface. Furthermore, myeloid/platelet-T-cell membrane markers are expressed at low levels (hematopoietic cell lineage \( \text{Figure S5G, FDR } p=1.3\times10^{-2} \)) whereas T-cell maturity markers CD4 and CD8 are abundantly expressed. Also, genes involved in cell migration are expressed at low levels (leukocyte transendothelial migration \( \text{Figure S5H, FDR } p=2.3\times10^{-2} \)).

In addition to protein expression data we described before\(^9\), we observe high levels of activated PKCα/β \( (\text{Figure 4A, PRKCA/B; } p=0.001) \) and the calcium-dependent phospholipid-binding protein annexin A1 \( (\text{ANXA1; } p=0.04; \text{ Figure 4A}) \) in NOTCH1-activated patients \( (n=38) \) compared to NOTCH1/FBXW7 wild-types patients \( (\text{Table 2}) \). This supports our global test results that indicate a high expression of calcium signaling genes. In contrast, low activity of the calcium-independent PKCθ isoform was measured \( (\text{Figure 4A, PRKCQ; } p=0.04) \). We also observe a low expression of the cell cycle inhibitor p27 \( (\text{Figure 4A, CDKN1B; } p=0.03) \), and residual p27 seems to be predominantly present in its inactive, phosphorylated state in these patients \( (\text{Figure 4A, } p=0.03) \).

**PTEN/AKT mutations**

Regarding PTEN/AKT-mutated patients \( (n=20) \), global testing supports low NOTCH1 pathway activation in these patients as we observed before at protein level \( (FDR \ p=4.8\times10^{-2}, \text{ Table 2 and Figure S6A}) \). This finding nicely matches our previous observations in which we noticed that NOTCH1-activating mutations and PTEN/AKT mutations hardly co-express\(^{23} \). Data support a high expression of genes of the TCR signaling but a low expression of these of the B-cell receptor signaling in PTEN/AKT-mutated patients \( (\text{Figure S6B,C, FDR } p=7.2\times10^{-2} \text{ and FDR } p=4.8\times10^{-2} \text{ respectively}) \). Many genes encoding differentiation markers of all blood lineages and many cytokine and cytokine-receptors are differentially expressed \( (\text{Figure S6D,E}, \text{ hematopoietic cell lineage map FDR } p=7.2\times10^{-2} \text{ and cytokine-cytokine receptor interaction FDR } p=5.7\times10^{-2} \text{ respectively}) \). These include high expression of pro-inflammatory IL2, IL4, IL9 and IL17 receptors and CXCR4 but low expression of B-cell-specific cytokines IL6 and CXCR5. Data indicates an active...
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Differentially expressed pathways (global test) and proteins (RPMA) in T-ALL type B mutation groups. NOTCH1-activations, PTEN/AKT mutations and WT1-inactivating mutations.
cell proliferation signature (Figure 1F and S6F-J, cell cycle FDR \(p=5.3\times10^{-2}\), p53 signaling pathway FDR \(p=4.8\times10^{-2}\), oocyte meiosis FDR \(p=4.8\times10^{-2}\), progesterone-mediated oocyte maturation, FDR \(p=4.8\times10^{-2}\), pyrimidine metabolism FDR \(p=8.4\times10^{-2}\) and base excision repair FDR \(p=7.2\times10^{-2}\)). Genes of the fructose and mannose metabolism point towards glycogen breakdown for energy supply (Figure S6I, FDR \(p=4.8\times10^{-2}\)). Genes of the bone morphogenic pathway (BMP) including receptor-regulated SMAD1, SMAD5 or SMAD8 and consequent ID1 transcription are not expressed whereas these of the ACVR2B and TGFBR downstream routes including receptor-regulated SMAD2 or SMAD3 are expressed (Figure S6L, FDR \(p=4.8\times10^{-2}\)).

Figure 4 | Significant differentially expressed proteins in type B T-ALL subgroups. P-value is calculated by Mann-Whitney U. Expression levels are depicted by box plots showing the distribution of values with standard deviations. Median expression is shown by a bar. (A) NOTCH1-activated (NOTCH1 and/or FBXW7-mutated), (B) PTEN/AKT-mutated, (C) WT1-inactivated patients.
Members and transcriptional targets of the JAK/STAT pathway PIM1, CISH, MYC, CCND1 and SPRED1 are not actively transcribed in PTEN/AKT-mutated patients (Figure S6M, FDR \( p = 4.8 \times 10^{-2} \)). Expression levels of TGFB and JAK/STAT pathway molecules may explain low MYC levels in these patients.\textsuperscript{23}

Regarding expression levels of proteins in PTEN/AKT-mutated patients (\( n = 17 \)), 10 were differentially expressed of which 5 were described by us before.\textsuperscript{23} Due to the near mutual exclusiveness of NOTCH1-activating and PTEN/AKT mutations in our cohort\textsuperscript{23} and only 19 out of the 66 patients were genetically wild-type for both abnormalities, various proteins were expressed in PTEN/AKT-mutated and NOTCH1-activating patients in this manner. This includes calcium-dependent annexin A1 (ANXA1; \( p = 0.03 \)) and PKCa/β (PRKCA; \( p = 0.006 \)) that are expressed at low levels in PTEN/AKT-mutated patients. In contrast, they have high levels of phospho-PKCθ (PRKCQ; \( p = 0.01 \)) (Figure 4B and Table 2). In line of an active cell cycle gene signature, we measured low p27 levels and its phosphorylated inactive form (CDKN1B; \( p = 0.04 \)), which coincides with low p27 gene expression levels (data not shown). Also, BMP-regulated SMAD1/5/8 proteins are expressed at low levels, as observed on transcript level.

**WT1-inactivating mutations**

Differential expression of genes in WT1-inactivated patients (\( n = 14 \)) compared to all other T-ALL patients (Table 2) points to calcium release through phospholipase C and ITPR1, which correlates with a high expression of downstream PRKCA (protein kinase C alpha) (Figure S7A, FDR \( p = 2.0 \times 10^{-2} \)). In line with this, there is a high expression of genes involved in IP4 to IP3 to IP2 and IP conversions which are important in calcium signaling, but not of those conversing from IP to IP4. Also genes regulating PIP conversions seem to be expressed at low levels (inositol metabolism Figure S7B,C, FDR \( p = 1.7 \times 10^{-2} \) and phosphatidylinositol signaling system, FDR \( p = 1.5 \times 10^{-2} \)). Surprisingly, genes involved in the formation of stress fibers (leukocyte transendothelial migration Figure S7D,E, FDR \( p = 2.0 \times 10^{-2} \) and vascular smooth muscle contraction, FDR \( p = 1.8 \times 10^{-2} \)) are abundantly found leukemic cells of WT1-mutated patients, although these processes normally occur in endothelial cells. Expression of genes involved in the histidine metabolism support degradation of L-histidine and histamine in these patients (Figure 1G, FDR \( p = 2.0 \times 10^{-2} \)). Histidine can be converted to aspartate, which is a precursor of lysine. During this reaction glutamate is released. Genes of the sulfur relay system are highly expressed (Figure S7F, FDR \( p = 2.0 \times 10^{-2} \)) and may indicate high levels of thiolation of tRNAs which is important for efficient translation of genes enriched for lysine, glutamine and glutamate codons.

Out of the 91 measured proteins, 32 were differentially expressed in WT1-mutated T-ALL patients compared to all other patients (\( n = 8 \), Table 2). These patients have high levels of membrane receptors phospho-cKIT (\( p = 0.005 \)) and phospho-EGFR (Figure 4C, \( p = 0.02 \)) as well as further downstream effectors including phospho-JAK1 (\( p = 0.03 \)), phospho-STAT3 (\( p = 0.05 \)), phospho-STAT5 (\( p = 0.005 \)) and phospho-STAT6 (Figure 4C, \( p = 0.02 \)). Also the AKT/MTOR pathway seems activated which is indicated by high levels of inactive phospho-TSC2 (\( p = 0.04 \)), phospho-mTOR on residues S2448 (Figure 4C, \( p = 0.006 \)) and S2481 (\( p = 0.02 \)) and high expression of the
protein translation downstream target phospho-4EBP1 \((p=0.03)\), as well as phospho-FOXO3 \((\text{Figure 4C}, \ p=0.006)\) and phospho-FOXO1/FOXO3 \((p=0.009)\) suppression of their downstream target p27 \((\text{Figure 4C}, \ p=0.002)\). We also observe abundant expression of various T-cell receptor (TCR) signaling components including CD3ζ \((\text{Figure 4C}, \ p=0.002)\) and its interacting partner phospho-ZAP70/phospho SYK \((p=0.01)\), phospho-SYK \((p=0.005)\), phospho-PKCa/β \((p=0.007)\), phospho-PYK2 \((p=0.01)\), phospho-SHC \((p=0.02)\), phospho-PKC6 \((\text{PRKCD}, \ p=0.04)\) and phospho-FYN \((p=0.05)\), as well as high expression of PI3K \((p=0.02)\) and RAS \((p=0.05)\) \((\text{Table 2})\).

DISCUSSION

By combining gene expression and protein data, we analyzed differentially expressed pathways in T-ALL pediatric patient subgroups at the transcriptome and proteome level. The main results are shown in \textbf{table 3}. In TALLMO cluster patients, we measured high transcript levels of genes and proteins involved in metabolic processes. First, this includes those involved in the prevention of glucose to glycogen conversion \((\text{GSK3})\) and those involved in active glycolysis in which ATP molecules are produced. A second glycolysis-connected metabolism seems to be relatively active in these patients; \textit{de novo} fatty acid synthesis \((\text{lipogenesis})\). During aerobic glycolysis acetyl-CoA can be provided for the \textit{de novo} lipogenesis when glucose levels are excessive. It is known that many cancer cells are highly dependent on \textit{de novo} lipogenesis for their proliferation and survival and many enzymes regulating these mechanisms are upregulated in many cancers\(^{32,33}\). Increased lipogenesis in TALLMO patients is indicated by a high expression of genes encoding enzymes that can convert phosphorylated phosphatidylinositols \((\text{PtdIns/PIP})\) and inositol \((\text{IP})\) lipids. Signaling through PtdIns and IP are important for membrane-trafficking and cell and lipid signaling \((\text{second messengers, eg. diacylglycerol and calcium})\) leading to cellular growth, migration, survival and differentiation. Although one downstream signaling cascade is the AKT/mTOR pathway, we did not observe AKT/mTOR pathway activation in TALLMO patients. Lipogenic and glycolytic enzymes are promising targets for therapy as they can be remarkably active in cancer cells in contrast to normal cells. Inhibitory substances are currently under extensive research as anti-cancer agents in general\(^{34}\). This is similar for GSK3 inhibitors which, based on this data, might be very effective in TALLMO cells. Remarkably, in our cohort this cluster contains the most loss of function mutations in \textit{PTEN}\(^{23}\). \textit{PTEN} is a phosphatase also involved in these metabolic systems and can confer PtdIns(3,4,5)P\(_3\) \((\text{PIP}_3)\) into PtdIns(4,5)P\(_2\) \((\text{PIP}_2)\) as well as PtdIns(3,4) P\(_2\) into PtdIns(4)P \((\text{PIP})\) and PtdIns(3)P into PIP. Regarding \textit{PTEN} mutations in T-ALL, literature predominantly focusses on PIP\(_2\) and subsequent downstream AKT/mTOR activation\(^{24}\) which we did also not observe for these patients in our cohorts\(^{25}\). However, based on these data one might suggest that one of the mechanisms that contribute to the oncogenic effects of \textit{PTEN} mutations is through other then PIP\(_2\), PtdIns and IP lipids, which seem to be particularly important in TALLMO patients. Alternatively, high expression of enzymes involved in the IP metabolism can be a bystander result of active T-cell receptor (TCR) signaling in TALLMO cluster patients, e.g. via the high expression of TCR-downstream Ca\(^{2+}\)-independent PKCβ proteins which can activate...
the PLCγ/Ca2+/IP route. Active TCR signaling may reflect the maturation status of the TALLMO subtype as immunophenotypically being the most mature T-ALL entity\textsuperscript{25}. These data suggest that neither TALLMO nor PTEN/AKT-mutated patients will benefit from PI3K/mTOR inhibitors.

Based on these data, it seems that NOTCH1 signaling is important in TLX patients. Whereas the frequency of NOTCH1-activating mutations is enriched in TLX patients, a NOTCH1 signature was observed in all TLX patients independent of NOTCH genetic status. This suggests addiction to NOTCH signaling in TLX cluster patients. The TLX cluster comprised predominantly TLX3- and HOXA-rearranged patients. Direct relations between HOX family members and NOTCH1 signaling are described and may be partially responsible for the increased NOTCH activity in this cluster. First, this concerns a relation between HOXA and MSI (Musashi; an indirect NOTCH activator) expression. A putative HOXA9 transcription factor binding site is known for the MSI2 gene and the fusion protein NUP98-HOXA9 is able to induce MSI2 expression\textsuperscript{36}. On the other hand, MSI is able to induce HOXA9 and HOXA10 expression\textsuperscript{37}. These data suggest a possible interplay between HOXA transcription factors and MSI. Second, the NOTCH1 downstream gene taspase 1\textsuperscript{38} is essential for MLL-cleavage that can regulate HOX gene expression\textsuperscript{38}. Based on this data, this second largest pediatric T-ALL cluster might be very well treatable with gamma-secretase inhibitors (GSI), independently of NOTCH1 genetic status. GSIs are currently being intensively investigated as therapeutic approach in T-ALL\textsuperscript{39,40}, and high MSI2 expression has already been identified by Palomero et al., to be related to GSI sensitivity in cell lines\textsuperscript{24}.

The proliferative cluster comprises patients that are predominantly characterized by NKX2-1, NKX2-2 or TLX1 translocations and has been associated with expression of genes involved in cell cycle regulation\textsuperscript{6}. We now confirmed this by the global test analysis that showed a high expression of genes regulating the cell cycle, DNA repair and replication and ATP-generating metabolisms. In addition, this was indicated by a high expression of active (cell cycle) kinases and MYC as shown on protein level. Although high p53 transcript levels were observed, downstream pathway molecules were expressed at low levels and suggest a low p53 activity. p53 becomes activated upon cellular stress. Its activity can change rapidly and largely depends on alterations in the p53 protein and to a minor extend, relieve of translational repression. For example, upon stress signals p53 stability, change of p53 cellular localization, blockage of the p53 transactivation domain or enhancement of p53 DNA-binding capacity can occur\textsuperscript{41}. A possible low functional activity of p53 can be due to interference with these mechanisms. For example, inactivating p53 or ATM kinase mutations (both found in ~5% of pediatric T-ALL patients) as well as PTEN/AKT mutations (~5-63% of pediatric T-ALL patients) can reduce p53 activity through different mechanisms and might have an important function in proliferative cluster patients\textsuperscript{23,42,43}. Interestingly, we observed that T-cells of the proliferative cluster expressed genes involved in the circadian rhythm at very low levels. This is an important regulator of the cell cycle, DNA damage responses, senescence and metabolism and dysregulation is linked with cancer. Restoring it would be a potential for therapy, though the exact regulatory mechanisms are still unclear\textsuperscript{44}.

The early T-cell maturity state of immature patients that resemble early T-cell precursor (ETP)
ALL patients, was confirmed by a significantly low expression of key T-cell receptor pathway genes along with a high expression of B-cell receptor (BCR) pathway genes as well as the expression of myeloid surface markers. This is in line with our previous report in which we recently showed that our ETP-ALL cohort is enriched for genes expressed in hematopoietic stem cells, early erythroid precursor cells, T-cell and B-cell sorted fractions (established by Novershtern and co-workers) and also enrichment of B-cell-related genes, using gene set enrichment analysis\(^\text{12}\). We now also show that this immature state seems to be reflected by an inactive proliferative and migratory behavior as samples expressed cell cycle genes and various proteins at very low levels -as reported before\(^\text{6}\), as well as these of genes involved in spindle assembly and RNA degradation and junction formation. Pro-apoptotic proteins BAX and caspase 3 were highly expressed compared to all other clusters indicating that apoptotic thresholds may be relatively high in these patients and may be exploited in treatment strategy for this high-risk cluster. Interestingly, the canonical TGF-beta signaling pathways seem inactive in these patients, despite a high expression of TGF-beta ligands and TGF-beta receptor. We suggest alternative TGF-beta signaling is very important in immature patients through downstream MAPKs (p38 MAPKs) that may result in ectopic MEF2C levels. MEF2C is a well known transcription factor downstream of p38 MAPkinases which can also be activated through the BCR signaling, also active in immature patients\(^\text{45}\).

NOTCH1-activated patients were characterized by differential expression of hematopoietic cell lineage markers, indicated by a high expression of both CD4/CD8 T-cell maturity markers. This probably reflects the intermediate/cortical T-cell differentiation stage which was immunophenotypically shown by us before\(^\text{19}\). Remarkably, NOTCH1-activated patients had a very low expression of genes encoding major histocompatibility complexes (MHC) and other genes involved in phagosome and endocytosis processes, indicating a possible mechanism of the cancerous cells to evade the immune system. Furthermore, NOTCH1-activated patient samples seem to carry low levels of p27 proteins, which is in line with previous studies that reported cell cycle inhibitor p27 as being a target of the HES-1 repressor\(^\text{46,47}\), one of the most important NOTCH1 downstream target genes. Second, HES1 can transcriptionally alter PTEN levels. PTEN regulates AKT activity that can in turn, repress p27 activity either by direct phosphorylation or transcription via Forkhead transcription factors\(^\text{48}\). Third, residual p27 levels are almost entirely phosphorylated and therefore inactive in NOTCH1-activated patients. NOTCH1 also upregulates SKP2 directly\(^\text{46}\), that is responsible for the recognition of phosphorylated p27 at Thr187, and thereby targets p27 for ubiquitination-dependent protein degradation. It is also shown that p27 gene expression is increased after GSI treatment that inhibits NOTCH signaling\(^\text{20}\). Furthermore, we suggest a role for calcium and calcium-dependent processes in NOTCH1-activated patients, involving PKCab and annexin A1. Interestingly, NOTCH1-activated patients had low levels of PKCθ, a protein which is very recently described as being indirectly downregulated by NOTCH1; PKCθ expression is correlated with high levels of reactive oxygen species (ROS) and thereby decreases activity of leukemia-initiating cells (LIC’s)\(^\text{49}\). PKCθ is induced by RUNX1 that is repressed by RUNX3, which is a direct NOTCH1 target. It is suggested that patients with low PKCθ activity will
have a poor outcome\(^9\). In previous studies we showed a poor outcome for NOTCH1-activated patients\(^9\) so it should be investigated whether direct targeting of PKCθ may be a therapeutic option for T-ALL.

High levels of PKCθ are detected in PTEN/AKT-mutated patients. In addition to low ICN (activated-NOTCH) levels in PTEN/AKT-mutated patients, high PKCθ levels can be as a consequence of an increased expression of TCR signaling genes. In addition to decreased LIC activity as a result of high PKCθ levels, Silva et al. described that high ROS levels abolish PTEN function as alternative mechanism for genetic inactivation\(^2\). Interestingly, PTEN/AKT-mutated patients also have a very high expression of genes involved in cell cycle and spindle formation, which is accompanied by a high expression of genes involved in active glycogenolysis to supply ATP (glycogen degradation). Like TALLMO cluster patients that are enriched for PTEN/AKT mutations in our cohorts, it might be worthwhile to verify whether these patients are sensitive for inhibitors of glycolysis.

Concerning pathway activation in WT1-mutated patients we observed expression of genes involved in calcium release and subsequent activated pathways. Also glutamate production seemed to be increased in WT1-mutated patients as well as thiolation of tRNA’s which is important for efficient translation of genes enriched for glutamate, among others. Interestingly, WT1 is one of such glutamate-rich genes implying the translation of truncated WT1 proteins may be very active and might act in a dominant negative fashion. Protein data showed that WT1-mutated patients have a very active protein signature compared to all other T-ALL samples. WT1 is suggested to participate in the RNA splicing process of genes and is a transcription factor acting as a haploinsufficient tumor suppressor gene in T-ALL, though the transcriptional targets of WT1 are largely unknown. Literature suggest its role in growth factor receptor inhibition by transcriptional repression\(^50,51\). Nonsense and missense mutations or deletions in the WT1 gene effect the DNA-binding domain of WT1, and are expected to thereby disable its DNA binding activity and thereby disability transcriptional repression\(^56,54-59\). It should be investigated whether this may explain high expression of activated receptors cKIT and EGFR and subsequent active AKT and JAK/STAT signaling pathways. In addition, downstream TCR proteins were highly expressed in their active form. This supports the suggestion for a role of WT1 in growth factor inhibition, which function is diminished upon WT1 loss. Kinase inhibitors are under extensive development and might be very well useful in proliferative patients, WT1-mutated patients or patients with high TCR signaling, e.g. sunitinib which targets c-kit among other kinases, bosutinib which can inhibit src kinases or dasatinib which targets the TCR and downstream src kinases.

Taken together, these data confirm and suggest specific pathway activation in T-ALL in various genetic subgroups, possibly as a direct or indirect result of genetic changes. Therefore, these data provides targets of therapeutic approach in specific T-ALL subgroups which should be further investigated.
Table 3 | Main results of this study. Deregulated pathways and therapeutic possibilities that should be further investigated in TALLMO, TLX, Proliferative and immature cluster patients, NOTCH1-activated, PTEN/AKT-mutated and WT1-inactivated patients.

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<td>chemotherapy, CDK inhibitors</td>
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AUTHORSHIPS AND DISCLOSURES

L.Z. designed experiments, performed research and wrote manuscript, E.P. supervised study, and wrote manuscript, V.C. performed RPMA analysis, J.B.-G. prepared samples, W.K.S prepared samples, E.S provided patient samples and clinical and immunophenotypic data, R.P. designed and supervised study and wrote manuscript, J.P.P.M. was principal investigator, designed and supervised the study, and wrote manuscript. None of the authors had competing financial interest.

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SUPPLEMENTARY DATA

A

B
Differentially expressed pathways in T-ALL
Figure S1 | Differentially expressed KEGG pathway maps of TALLMO cluster patients. (A) inositol phosphate metabolism, (B) glycerolipid metabolism, (C) starch and sucrose metabolism, (D) gap junction and (E) T-cell receptor signaling pathway.
Differentially expressed pathways in T-ALL

A

B

Data on KEGG graph
Rendered by Pathview
Figure S2 | Differentially expressed KEGG pathway maps of TLX cluster patients. (A) phosphatidylinositol signaling system, (B) inositol phosphate metabolism, (C) leukocyte transendothelial migration, (D) cytokine-cytokine receptor interaction.
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Figure S3 | Differentially expressed KEGG pathway maps of proliferative cluster patients. (A) cell cycle, (B) DNA replication, (C) one carbon pool by folate, (D) folate biosynthesis, (E) purine metabolism, (F) pyrimidine metabolism, (G) progesterone-mediated oocyte maturation, (H) oocyte meiosis, (K) nucleotide excision repair, (L) mismatch repair, (M) base excision repair, (N) p53 signaling pathway, (O) protein export, (P) synthesis and degradation of ketone bodies, (Q) MAPK signaling pathway, (R) Wnt signaling pathway, (S) insulin signaling pathway, (T) adherens junction, (U) cell adhesion molecules, (V) osteoclast differentiation.
Differentially expressed pathways in T-ALL
Differentially expressed pathways in T-ALL
Differentially expressed pathways in T-ALL
Differentially expressed pathways in T-ALL

[Diagram of regulative pathways in actin cytoskeleton and sphingolipid metabolism]

Data on KEGG graph
Rendered by Pathview
Figure S4 | Differentially expressed KEGG pathway map of immature cluster patients. (A) B-cell receptor signaling pathway, (B) FcεRI signaling pathway, (C) T-cell receptor signaling pathway, (D) hematopoietic cell lineage, (E) cell cycle, (F) progesterone-mediated oocyte maturation, (G) oocyte meiosis, (G) RNA degradation, (I) synthesis and degradation of ketone bodies, (J) N-glycan biosynthesis, (K) butanoate metabolism, (L) purine metabolism, (M) adherens junction, (N) tight junction, (O) regulation of actin cytoskeleton, (P) sphingolipid metabolism, (Q) TGF beta signaling pathway.
Differentially expressed pathways in T-ALL

A

NOTCH SIGNALING PATHWAY

B

PRAGMATIC/PROVOCAL SIGNALING SYSTEM

Data on KEGG graph
Rendered by Pathview.
Differentially expressed pathways in T-ALL

Diagram E

Diagram F
Differentially expressed pathways in T-ALL

Figure S5 | Differentially expressed KEGG pathway map of NOTCH1-activated patients. (A) NOTCH signaling pathway, (B) phosphatidylinositol signaling system, (C) cell adhesion molecules, (D) phagosome, (E) endocytosis, (F) natural killer cell mediated cytotoxicity, (G) hematopoietic cell lineage, (H) leukocyte transendothelial migration.
Differentially expressed pathways in T-ALL

[Diagram of Differentially Expressed Pathways]

[Diagram of Progesterone-Mediated Oocyte Maturation]
Differentially expressed pathways in T-ALL
Figure S6 | Differentially expressed KEGG pathway maps of PTEN/AKT-mutated patients. (A) NOTCH signaling, (B) T-cell receptor signaling, (C) B-cell receptor signaling, (D) hematopoietic cell lineage, (E) cytokine cytokine receptor interaction, (F) p53 signaling pathway, (G) oocyte meiosis, (H) progesterone-mediated oocyte maturation, (I) pyrimidine metabolism, (J) base excision repair, (K) fructose and mannose metabolism, (L) TGF beta signaling pathway, (M) JAK-STAT signaling pathway.
Differentially expressed pathways in T-ALL

A

B

Data as KEGG graph
Rendered by Pathview

Data as KEGG graph
Rendered by Pathview
Figure S7 | Differentially expressed KEGG pathway maps of WT1-mutated patients. (A) calcium signaling pathway, (B) inositol phosphate metabolism, (C) phosphatidylinositol system, (D) leukocyte transendothelial migration, (E) vascular smooth muscle contraction, (F) sulfur relay system.
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Table S2 | Characteristics of patients used for gene expression arrays and reverse phase microarrays.
Summary

Future prospective
Nederlandse samenvatting
SUMMARY

T cell acute lymphoblastic leukemia (T-ALL) is a very heterogeneous disease involving a spectrum of genetic events resulting in the uncontrolled expansion of immature T-lymphocytes (blasts). This multicombination of genetic events seems different in each patient. Two types of mutations occur in T-ALL which we categorized as type A mutations and type B mutations. Type A mutations are characterized as clonal chromosomal rearrangements that activate oncogenes mainly involved in cell differentiation. Ectopic expression of the oncogenes provoke a specific T cell developmental block and accompanying gene expression profile. Four expression clusters have been identified so far, i.e. the TALLMO, TLX, proliferative and immature/ETP-ALL (early thymic progenitor ALL) clusters. In general, type B mutations occur in any of the identified T-ALL clusters, although some may have preferences for particular subtypes. Type B mutations affect molecules that participate in classical signal transduction pathways like NOTCH or PI3K/AKT signaling, cell cycle of cytokine receptors.

Associations of particular mutations with T cell phenotype or with additional mutations have been noticed, however, the biological rationale behind this is still not clear. An explanation for the association of mutations with a T cell phenotype is that the activity of a gene can vary between T cell development stages under normal circumstances. It may therefore be more prone to mutate during the stage in which it is active (open chromatin). Alternatively, mutations (or their counterpart) are potential oncogenic at one particular development stage only. An explanation for the co-expression of particular mutations may be the additive/synergistic oncogenic potential of some combinations. Another reason may be that a cell needs guaranty that a particular mechanism is dysregulated, in terms of oncogenic addiction, and therefore multiple hits interfering with one mechanism may be present. Gaining knowledge regarding these topics is hampered by the fact that T-ALL involves many genetic alterations and many are still undiscovered. This comes along with the drawback that a leukemic cell can have many mutations but only a few have the potential to alter cell proliferation or self-renewal activity (driver mutations) and others are present but lack oncogenic capacity (passenger mutations).

Currently, the survival rate of children with T-ALL is around 80% for patients treated on one of the latest treatment protocols, e.g. the ALL10 protocol. Twenty percent of patients relapse. Outcome for relapsed patients is extremely poor due to acquired therapy resistance, resulting in death for most relapsed patients. T-ALL treatment is based on combinational chemotherapy. As children are still in development late side-effects can have a major impact. For this reason, the reduction of side-effects in children is very important which can be achieved by treatment stratification. Based on current protocols many patients may receive super-or suboptimal therapy. High doses of drugs are already given to high-risk patients and improvement of treatment now requires targeted therapy. This therapy should, in most optimal situation, be directed to driving oncogenic aberrations.
To answer questions above and to improve treatment stratification, we aimed to map the most frequent aberrations in a cohort of 146 pediatric T-ALL patients treated according to DCOG ALL7/8/9 or COALL-97 protocols to determine the heterogeneous landscape of alterations in children with T-ALL. Furthermore, we have correlated specific features with survival of patients. In addition, we extended our genetic analyses by examining pathway expression at transcriptional and translational level that are changed as a consequence of different genetic aberrations that occur in T-ALL patients.

**Chapter 2** provides an overview of our current understanding of genetic aberrations in T-ALL. This includes type A aberrations, but focuses more on the different pathways that are most likely to be dysregulated as a consequence of type B aberrations.

In our study NOTCH1-activating type B mutations were fist mapped (chapter 3), which activate the NOTCH pathway and is the most recurrently mutated pathway in T-ALL found so far. Sixty-three percent of patients in our cohorts have NOTCH1-activating mutations. Most other studies have screened for NOTCH1-activating mutations in mutational hotspots. We screened almost the entire gene except for the EGF-repeats and the LNR domain of the \( \text{NOTCH1} \) gene, as well as a large part of the \( \text{FBXW7} \) gene. FBXW7 can target NOTCH1 for proteosomal degradation. Our study confirmed that most \( \text{NOTCH1} \) mutations are present in the heterodimerization and juxtamembrane domain as well as in the \( \text{FBXW7} \)-targeted PEST domain of NOTCH1. Mutations are rare in the ANK, TAD and RAM domains\(^7,8\), and none were found in our cohort. New mutations in the \( \text{FBXW7} \) gene were revealed, of which most were at the far C-terminal end of the gene. We observed a negative association of NOTCH1-activating mutations and \( \text{TAL1} \) or \( \text{LMO2} \) family members. As most of these patients are arrested at a late T cell developmental stage, NOTCH1-activating mutations were also negatively associated with the presence of mature T cell markers. Mutations were also positively associated with \( \text{TLX3} \) rearrangements. Patients with NOTCH1-activating mutations expressed a clear distinct NOTCH1-activated RNA signature, that included some known NOTCH1-regulated genes. This signature can contribute to the biological understanding of NOTCH1 activity which is important for specific therapeutic development. For the first time in patient samples we revealed that mutated patients have, as was already hypothesized based on cell line data, increased activated NOTCH1 (ICN) protein levels. Interestingly, when patients were subdivided based on weak or strong NOTCH1-activating mutations, as previously documented in \textit{in vitro} and \textit{in vivo} models\(^9\), ICN was indeed expressed at lower levels in patients presenting with weak NOTCH1-activating mutations compared to patients with strong NOTCH1-activating mutations. This pattern was similar at gene expression level. An additional ten patients showed either high ICN levels or its target genes and seem to lack NOTCH1-activating mutations or NOTCH1 translocations. This indicates the presence of additional, yet unidentified genomic alterations may be present in a minor part of patients presumably in the \( \text{NOTCH1} \) EGF or LNR repeats, remaining sequences of the \( \text{FBXW7} \) gene or in other NOTCH1-regulating genes. This means not all NOTCH1-activated patients are identified
yet. Patients with NOTCH1-activating mutations have poor relapse-free survival rates in our cohorts, but strikingly, have a better initial prednisone response \textit{in vitro}. The clinical impact of NOTCH1 mutations and prognosis is inconsistent among study cohorts. These discrepancies might be created by several factors; the existence of different treatment protocols and outcome parameters used, yet unidentified NOTCH1-activating mutations and differences in distribution of weak and strong NOTCH1-activating mutations which may be different entities with a distinct treatment response. Indeed, we noticed different survival rates between these (weak and strong) patient groups in DCOG cohort patients, however this could not be confirmed in COALL cohort patients.

A relation between NOTCH1 and PTEN expression was described by Palomero et al.\textsuperscript{10}, who described PTEN as being a downstream NOTCH1 target via HES1 and leading to AKT activation. As a proper GSI response required low or normal AKT levels, loss of the AKT inhibitor PTEN was believed to result in GSI resistance. To investigate the role of PTEN in T-ALL and its relation to NOTCH1 and potential therapeutic inhibition, our cohort was screened for \textit{PTEN}, \textit{PIK3CA (PI3K isoform)}, \textit{PIK3RI (PI3K isoform)} and \textit{AKT1} type B genetic aberrations (chapter 4). During this screen, we identified a new mechanism for PTEN-silencing in pediatric T-ALL in a minor part of patients, which involved aberrant \textit{PTEN}-splicing that resulted in absence of PTEN protein. Taken all patients with identified aberrations (\textit{PTEN} mutations/deletions, \textit{AKT1} mutations and loss of PTEN protein due to incorrect splicing or yet unidentified mechanisms) together, 13% of patients have \textit{PTEN/AKT} mutations. All patients with nonsense mutations or deletions of \textit{PTEN} did have low or no PTEN protein expression, depending on the mono-allelic or bi-allelic mutation status. Total absence of PTEN in some mono-allelic mutated patients suggest for alternative yet unidentified inactivation of PTEN. No differences in the levels of activated downstream proteins involved in AKT-mTOR pathway, or other AKT downstream proteins (PRAS40 en forkhead transcription factors) were measured in \textit{PTEN/AKT}-mutated patients compared to wild-type patients. In contrast to what we expected based on the data from the Palomero study\textsuperscript{10}, \textit{PTEN/AKT} mutations were negatively associated with NOTCH1-activating mutations. Some \textit{PTEN/AKT}-mutated patients had only weak NOTCH1-activating mutations. Patients with \textit{PTEN/AKT} mutations had significantly low levels of NOTCH1 pathway proteins MYC and musashi. \textit{PTEN/AKT} mutations were predominantly present in TALLMO patients and were associated with a mature T cell development stage. TLX patients were remarkably devoid of \textit{PTEN/AKT} mutations, a pattern that seemed nearly reciprocal to NOTCH1-activating mutations. \textit{PTEN/AKT} mutations were not associated with outcome per se. Nevertheless, taken poor prognostic NOTCH1-activating mutations into account as their presence negatively correlates with \textit{PTEN/AKT} mutations, we observed good survival rates for \textit{NOTCH1/FBXW7/PTEN/AKT} wild-type patients and poor survival rates for NOTCH1 activating and/or \textit{PTEN/AKT}-mutated patients.

We further investigated \textit{PTEN} splice defects in chapter 5. There we found evidence that these splice variants were due to micro-deletions affecting one or a few exons. Deletions were flanked by conserved immunoglobulin recombination signals and contained insertion of random nucleotides. This suggested that these deletions were a consequence of aberrant RAG-mediated
recombination events.

Screening of type B mutations was extended by the examination of IL7Ra, JAK and RAS mutations in chapter 6. We did not detect any JAK2 or TYK2 mutations. IL7Ra mutations in our cohort have been reported before and were detected in 8% of the patients. N-RAS/K-RAS mutations were detected in 10% and JAK1 and/or JAK3 mutations in 7% of patients. Strikingly, except for one case, IL7Ra mutations were devoid of any other of these mutations. Also PTEN/AKT mutations did not co-occur together with JAK mutations and seldomly with RAS mutations. All JAK mutants that we tested had transformation capacity, as they showed IL3-independent growth in Ba/F3 cell lines. Wild-type JAK1 or JAK3 did not. Also AKT and N-RAS mutations induced IL3-independent growth. Protein analysis revealed increased expression of phosphorylated JAK-STAT, RAS-MEK-ERK and PTEN/AKT pathway proteins in IL7Ra-mutated patients. Interestingly, different IL7Ra and JAK1 mutants did not activate similar downstream molecules to the same extent, however they all resulted in comparable elevated p70 S6 kinase activity. Also, JAK3 mutants resulted in very robust IL3-independent growth compared to IL7Ra and JAK1 mutants, although downstream phosphorylated signaling molecules were relatively weakly expressed. IL7Ra and JAK1/3 but not N-RAS and AKT mutant cell lines were sensitive to the JAK inhibitor Ruxolitinib, which was accompanied by reduced downstream signaling molecules in IL7Ra and JAK1/3-mutated lines but not in N-RAS and AKT-mutated lines. Downstream STAT5 inhibitor did only moderately reduce growth of JAK1 mutant lines but not in IL7Ra mutant lines, suggesting that IL7Ra mutants can signal through an alternative pathway, eg. RAS-ERK-MEK or PI3K/AKT. JAK1/3 mutants but not IL7Ra mutants were sensitive to the PI3K/AKT-blocking agent LY294002. Combined RAS-MEK-ERK and PI3K/AKT pathway inhibitors in a JAK1 and IL7Ra mutant line, significantly reduced phosphorylated signaling compared to single agent treatment. The insensitivity for single LY294002 treatment but sensitivity for LY294002 treatment combined with RAS-ERK-MEK inhibition suggests that the IL7R can signal through both pathways, but activates the RAS-MEK-ERK pathway for sustained survival when the PI3K/AKT pathway is blocked and vice versa.

Our group recently described a new cluster of patients based on unsupervised gene expression cluster analysis which have been associated with genetic aberrations that activate MEF2C. This group of patients is a very immature T-ALL phenotype with frequent co-expression of myeloid markers and herein called immature cluster. Two studies have been published that described a comparable group of patients; one T-ALL entity denoted as early T cell precursor (ETP) ALL which was identified using cluster analysis based on a previously established early T cell precursor (ETP) mouse RNA expression profile. ETP-ALL is described as being an immature leukemia with stem cell and myeloid features with mutations in genes involved in hematopoietic and lymphoid development as well as in myeloid-specific oncogenes. Another early T-ALL entity was identified by lack of the TCRyδ (TRG@) gene and were denoted as ABD patients. In chapter 7, we investigated the overlap of these different features in our T-ALL cohort. Our described immature cluster patients strongly resemble ETP-ALL patients based on the conservation of a human ETP-ALL gene expression profile (13 of our 15 immature patients were also ETP-ALL).
Chapter 9

About half of our immature T-ALL cluster patients had no TCRγδ deletions, indicating that ABD may represent a subtype within immature/ETP-ALL patients. We further observed that our immature cluster patients could not be predicted by the originally proposed immunophenotype for ETP-ALL patients. We proposed an alternative immunophenotype that correlated with our ETP-ALL patients. Our patients were associated with absence of CD1 and CD8 expression but also absence of CD4 expression, but with frequent expression of CD34 and/or CD13 or CD33. The stem cell-like phenotype of immature/ETP-ALL patients based on gene set enrichment analyses of gene expression microarray data was confirmed. In contrast to what has been described for ETP-ALL patients, immature/ETP-ALL patients treated on the COALL97/03 protocol did not have very poor survival rates and had equal sensitivity in vitro to various chemotherapeutic agents compared to other T-ALL patients. Based on the first described ETP-ALL study and two others that followed, it had been proposed to intensify treatment for these patients in the clinic as soon as possible. Our data point to more careful further evaluation of the prognostic significance of immature/ETP-ALL patients first. A trend for lower responsiveness of immature T-ALL/ETP-ALL patients to steroid treatment was observed. Our patients had received higher doses of cytarabine compared to other studies. We therefore suggest to evaluate the cytarabine dose for these patients that might explain the higher survival rates we observed for immature/ETP-ALL patients compared to other studies.

The consequences of all different genetic hits on pathway level are largely unknown. Therefore, in chapter 8 we investigated differential pathway expression in T-ALL patient groups that reflect type A mutations or T-ALL patient groups based on type B mutations, using whole genomic transcriptome data and proteome data. In TALLMO patients, we observed a high expression of genes and proteins of T cell receptor (TCR) signaling and of metabolic glycolysis and lipid signaling, including GSK3. This suggest that these processes are important for cell function in these patients and therefore, it would be worthwhile to test the effect of kinase inhibitors, glycolysis and lipid inhibitors or specific GSK3 inhibitors in TALLMO patient samples. TLX cluster patient samples were clearly associated with NOTCH1 signaling. This extended our previous observations from chapter 3 that pointed to a very high NOTCH1-activating mutation rate which predominantly involved strong NOTCH1-activating mutations in TLX3 patients. Besides this notion, NOTCH signaling was also observed in some NOTCH1 wild-type patients. This supports the idea of existing yet unidentified genetic aberrations that activate the NOTCH1 pathway (chapter 3) or suggest addiction to NOTCH1 signaling within this patient group, which is mediated by HOX-regulated NOTCH signaling. It would be very interesting to consider the TLX3 T-ALL group for therapeutic NOTCH1 inhibition. Proliferative cluster patients had a high expression of genes involved in circadian rhythm. Restoring it would be a potential for therapy. The very high proliferative activity and DNA damage response in this cluster was confirmed in this study. We measured high levels of p53 gene transcripts but a low expression of downstream genes/proteins, what might suggest low function activity of p53 through interference with p53 stability, localization, transactivation or DNA-binding capacity in this group. In addition to conventional chemotherapy, kinase inhibitors should be carefully studied as a potential therapeutic option for
these patients. This analysis showed that immature cases have a very low TCR signaling but very high B cell receptor signaling. Expression of cell adherence molecules is low. These observations may reflect the very early T cell arrest of this leukemia. Interestingly, these cells seem relatively proliferative and migratory inactive. We observed an interesting link between MEF2C and the p38 MAPK which seems very promising for further investigation. According to type B mutations, we confirmed the importance of the tumor suppressor gene p27 downstream of NOTCH1 and observed low levels of PKCθ. PKCθ is a protein very recently described as being indirectly downregulated through NOTCH1 via RUNX3 and RUNX1. In contrast, PKCθ levels were high in PTEN/AKT-mutated patients that are mostly devoid of NOTCH1-activating mutations or bear only weakly-activating mutations (chapter 4). These patients also had high expression levels of TCR genes and a very active proliferative signature. This makes also this group attractive for kinase inhibitors. WT1-inactivated patients showed very high levels of growth factor receptors and downstream signaling. As the functional role of WT1 in T-ALL is unknown, the role of WT1 in translational repression of growth factors is an interesting mechanism to investigate, as well as the sensitivity of WT1-inactivated patient cells to well-known growth factor inhibitors.
FUTURE PROSPECTIVE

T-ALL is characterized by a variety of rearrangements and gene mutations, making a uniform targeted therapeutic approach difficult for this disease. Each patient displays a different mutation pattern and may therefore have a different response to therapy. To improve the survival rates of children with T-ALL and to decrease the severity of (long-term) side-effects, we aim for a better stratification of patients based on molecular-cytogenetic data and the development of targeted therapy.

Progression of T-ALL research with new tools

Major steps have been made due to new analytical techniques that continue to be developed in the near future, and that may help us to clarify disease biology and develop better treatment strategies. For example, next generation sequencing (NGS) in which whole patient genomes are screened is more and more being applied, enabling investigators to obtain a full spectrum of genetic aberrations that are present in each patient. Furthermore, data on relatively newly discovered transcriptional mechanisms including epigenetic modifications like DNA methylation and gene expression regulation by miRNA’s, will further complete and complicate the picture. Bioinformatic tools will become more applicable and understandable, making it easier to integrate and interpret all data. These new techniques and data analyses tools enable researchers to obtain a more complete, and profound overview of oncogenic mechanisms. For example, novel NOTCH1-activating mutations may be identified with these tools in the future, that may clarify observed differences in the prognostic significance of NOTCH1-activating mutations among study cohorts. Also, other new genetic aberrations can now be easily identified and help to improve patient stratification. Information about downstream effects at the protein level is needed to provide more knowledge about interactions of activated pathways, which may reveal potential druggable targets or disease biomarkers. New techniques like the reverse-phase protein microarray (RPMA) provide accessibility to measure and understand these data as they include less experimental bias and more endpoints in one assay compared to conventional techniques like western blotting. Our studies already showed the advance of proteomic studies by identifying an additional mechanism of PTEN-silencing as well as by revealing the possibility for presence of novel NOTCH1-activating mutations. Techniques like NGS and RPMA can also be used when rather limited amounts of patient materials are available which is often a weakness of older techniques. High-throughput screens to identify druggable targets have also become a useful tool, for example siRNA libraries can identify expression of pivotal genes for tumorigenesis or disease resistance by silencing.

NOTCH1 and PTEN in T-ALL

An interesting topic in pediatric T-ALL is the relation between aberrant NOTCH1 and PTEN signaling. In 2007, Palomero et al. reported a direct mechanistic association between NOTCH1 and PTEN in T-ALL[10]. They suggested that PTEN is a downstream target of NOTCH1 via HES1,
resulting in AKT upregulation. As a consequence, \textit{PTEN/AKT} mutations would provoke resistance to gamma-secretase inhibitors (GSIs) as these mutations result in continuously NOTCH1 downstream signaling independent of NOTCH1 activity. We doubt the clinical relevance of this proposed mechanism as we showed these two classes of mutations are almost never co-expressed in the same leukemic clone. In fact, NOTCH1-activating mutations and \textit{PTEN/AKT} mutations seem to have a nearly reciprocal pattern. As a consequence, GSI resistance in \textit{PTEN/AKT}-mutated patients may be due to the wild-type \textit{NOTCH1/FBXW7} genotype of these cells which are less sensitive for GSIs, rather than due to interference with downstream NOTCH1 signaling. The inverse correlation of both mutations in one leukemic clone can be due to their association with different T cell development stages or type A mutations or due to the participation of both proteins in the same signaling pathway, as proposed\textsuperscript{10}. In the latter situation, mutations in both genes might not have an additive effect concerning strong NOTCH1-activating mutations. However, weak NOTCH1-activating mutations may not be potent enough for proper AKT activation. Therefore, additional \textit{PTEN/AKT} mutations may have benefits in weak NOTCH1-activated patients by provoking adequate AKT activation. If NOTCH1-activating mutations indeed result in PTEN inhibition and subsequent AKT activation, this may explain the equal levels of AKT and downstream AKT proteins between \textit{PTEN/AKT}-mutated and \textit{PAKT} wild-type (but predominantly NOTCH1-activated) patients. Our studies focuses on differences between groups and might therefore not identify important mechanisms in groups with similar pathway activation. Instead, more attention should be drawn to the similarities between the NOTCH1 and \textit{PTEN/AKT} pathway in future studies.

\textbf{NOTCH1 and patient outcome}

Various studies have investigated the role of the NOTCH pathway for prognosis prediction of pediatric T-ALL patients, but the overall conclusion is unclear. Whereas some studies reported good survival rates for \textit{NOTCH1}-mutated patients, other studies, among ours, reported similar or poor survival rates between mutated and \textit{NOTCH1} wild-type patients\textsuperscript{3,7,18-24}. This variation between studies is probably due to the widespread role of the NOTCH pathway, the high rate of different and probably yet undiscovered mutations and the variety in treatment protocols around the world. In addition, interpretation is hampered as different studies use different outcome parameters, like minimal residual disease, \textit{in vitro} therapy response, relapse-free, event-free or overall survival rates. Moreover, mutational screening is often incomplete thereby introducing extra bias. In this light, is of utmost important that clinical and research-based studies become more generalized. Furthermore, it is important to take additional aberrations into account when performing analyses as T-ALL turns out to be more a heterogeneous disease than originally thought.

\textbf{Future therapy}

The NOTCH and \textit{PTEN/AKT} pathway would be attractive therapeutic targets, although more pathways as described above should be considered for therapeutic targeting. So far, none of
the targeted agents reached the clinic for T-ALL patients. A major drawback so far is that these agents are not effective enough as they block only one pathway and a cancer cell may simply escape by gaining additional mutations in different pathways to sustain its oncogenic potential. To overcome this problem, research is more focused now on combinational therapies targeting several key nodes of one or multiple pathways. GSIs are promising agents to treat patients that have active NOTCH signaling. Unfortunately, a first clinical trial with GSIs was hampered by extensive gastro-intestinal toxicity provoked by this compound and had to be retracted. Still, GSIs are of clinical interest as it may exert synergistic cytotoxic effects in lower doses when combined with other drugs. For example, GSIs were able to restore glucocorticoid-sensitivity in a glucocorticoid resistant T-ALL mouse model, while glucocorticoids prevent the GSI induced gastro-intestinal toxicity. GSIs have further been demonstrated to enhance the cytotoxic effects of dexamethasone in vitro. Furthermore, various GSI compounds are now under extensive development to overcome drug toxicity. Since the PI3K/AKT and NOTCH1 pathway might intertwine it is postulated that a combination of inhibitors targeting both pathways would be effective. Indeed, Rapamycin is shown to work additionally with NOTCH inhibitors in mice and also Chan et al and Sanda et al observed synergism between NOTCH1 and mTOR inhibitors or PI3K inhibitors in T-ALL cells in vitro. This indicates combinational therapies have significant potential for future treatment of T-ALL.
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**NEDERLANDSE SAMENVATTING**

T cel acute lymfatische leukemie (T-ALL) wordt gekarakteriseerd door veel verschillende genetische afwijkingen. Deze afwijkingen leiden tot ongecontroleerde celdeling van onrijpe lymfocyten (blasten). Een blast cel van een patiënt met T-ALL heeft meerdere genetische afwijkingen. Iedere patiënt heeft een andere combinatie van genetische afwijkingen. Bij T-ALL komen twee verschillende mutaties voor, welke wij type A en type B mutaties hebben genoemd. Type A mutaties zijn chromosomale herschikkingen welke oncogenen activeren die voornamelijk betrokken zijn cel differentiatie. Abnormale activatie van deze oncogenen zorgt voor een cel differentiatie blokkade en wordt gekenmerkt door een specifiek genexpressie profiel. Tot nu toe zijn vier gen expressie clusters geïdentificeerd; TALLMO, TLX, proliferatief en immatuur/ETP ALL cluster. Over het algemeen komen type B mutaties in ieder cluster voor, hoewel sommige wel voorkeur hebben voor een specifiek cluster. Type B mutaties komen voor in genen die betrokken zijn bij de signalering binnen en buiten een cel, zoals bijvoorbeeld de PI3K/AKT, NOTCH signalering of de cel cyclus.

Er zijn associaties gezien tussen mutaties en het T cel fenotype en tussen mutaties onderling. Een echte verklaring hiervoor is nog niet ontdekt.

Een verklaring voor de associatie van mutaties met T cel fenotype zou kunnen zijn dat onder normale omstandigheden, de activiteit van een gen verschilt tussen de verschillende T cel ontwikkeling stadia. Een gen is veel vatbaarder voor mutaties wanneer het actief is en het chromatine open gevouwen is. Een alternatief is dat een eiwit (of het eiwit waarmee het samenwerkt) alleen oncogene potentie heeft in een bepaald T cel ontwikkeling stadium.

Een verklaring voor het feit dat er combinaties van mutaties zijn die vaak binnen een patiënt voorkomen, zou kunnen zijn omdat bepaalde mutaties samen additief of synergistisch werken. Dit zou ook kunnen zijn omdat een cel zogenoemde “oncogene verslaving” heeft. Dit houdt in dat de cel meerdere punten van een signalerings route muteert, om er zeker van te zijn dat deze aangedaan is. Daardoor zijn er meerdere mutaties die interfereren met een mechanisme. Omdat T-ALL veroorzaakt wordt door veel genetische mutaties en doordat er nog steeds velen niet zijn ontdekt, is het moeilijk om een goede verklaring te vinden. Daar komt bij dat een cel ook veel mutaties heeft die geen invloed hebben op het gedrag en overleving van de cel (passerende mutaties) en dat er slechts een paar oncogeen zijn (driver mutaties).

Met een van de recentste behandelprotocollen (ALL-10) is de overleving van kinderen met T-ALL 80%. Twintig procent van de kinderen krijgt een terugval. Overlevingscijfers van deze kinderen zijn erg laag, omdat ze vaak resistent zijn geworden voor de behandeling en daardoor niet goed zijn te behandelen en overlijden. Voor deze patiënten is het cruciaal effectievere behandeling te ontwikkelen. De behandeling van T-ALL is gebaseerd op om een combinatie van chemotherapie. Omdat kinderen nog volop in ontwikkeling en groei zijn, kunnen late bijwerkingen van medicatie grote invloed hebben. Daarom is het bij kinderen heel belangrijk om deze bijwerkingen te verminderen.
verminderen. Het is daarvoor van belang dat patiënten, therapie “op maat” krijgen. Daarvoor is het identificeren van patiëntengroepen met overeenkomstige eigenschappen belangrijk. Met het huidige behandelprotocol krijgen patiënten suboptimale of superoptimale behandeling. Hoog-risico patiënten krijgen al een hoge dosis chemotherapie en verbetering van behandeling moet bereikt worden door kinderen een specifieker behandeling te geven. Het meest ideale zou zijn wanneer deze behandeling specifiek gericht is tegen oncogenen.

Om bovenstaande vragen te beantwoorden, hebben wij van iedere patiënt in kaart gebracht welke combinaties van mutaties zij hebben. We hebben dit gedaan in een cohort van 146 patiënten welke behandelt zijn met het DCOG ALL7/8/9 protocol of COALL-97 protocol. Deze mutaties en andere patiënten eigenschappen hebben we gecorreleerd aan overlevingscijfers. Daarnaast hebben we op transcriptie- en translatie-niveau gekeken, welke veranderingen er zijn opgetreden in T-ALL patiënten met bepaalde mutaties.

In hoofdstuk 2 is een overzicht van mutaties beschreven, die tot dusver bekend zijn in T-ALL. Dit bevat type A mutaties, maar is meer gespitst op type B mutaties en het (mogelijk) signaleringspad dat als gevolg van deze mutatie aangetast is in activiteit.

We zijn begonnen met het identificeren van NOTCH1-activerende mutaties binnen het cohort (hoofdstuk 3). Deze mutaties activeerden de NOTCH1 signalering en is een van de meest voorkomende mutaties in kinderen met T-ALL. Drieënzestig procent van de kinderen heeft een NOTCH1-activerende mutatie. De meest beschreven studies dusver, hebben alleen gekeken naar mutaties in genetische hotspots. Wij hebben behalve het EGF- en LNR-domein van NOTCH1, bijna het hele NOTCH1 gen en FBXL7 gen gescreefd. FBXL7 is een eiwit dat er voor kan zorgen dat NOTCH1 wordt afgebroken in het proteosoom. Onze studie bevestigt dat NOTCH1 mutaties voornamelijk voorkomen in het heterodimerisatie domein, juxtamembraan domein en het PEST domein waaraan FBXL7 kan binden, zoals eerder in de literatuur beschreven. Mutaties zijn zeldzaam in het ANK, TAD en RAM domein van NOTCH1 en ook wij vonden geen mutaties hierin. Wij hebben nieuwe mutaties in FBXL7 ontdekt en de meeste mutaties waren aanwezig in het C-terminale einde van het gen. Wij zagen een negatieve associatie tussen NOTCH1-activerende mutaties en afwijkingen in het TAL1 en/of LMO2 gen. TAL1/LMO2 patiënten hebben vaak een matuur T cel fenotype waardoor NOTCH1-activerende mutaties ook negatief geassocieerd waren met de aanwezigheid van mature T cel markers. NOTCH1-activerende mutaties waren positief geassocieerd met TLX3 herschikkingen. Patiënten met NOTCH1-activerende mutaties hadden een specifiek RNA expressie signatuur welke bekende genen bevat die door NOTCH1 worden gereguleerd. Dit signatuur kan bijdragen aan de begrijpbaarheid van de werking van NOTCH1, wat belangrijk is voor het ontwikkelen van geneesmiddelen die zich specifiek richten op NOTCH1. Wij hebben voor het eerst in patiënten samples aangetoond dat ook de actieve NOTCH1 eiwitvorm verhoogd is in gemuteerde patiënten. Dit was al eerder gehypothetiseerd op basis van data welke waren gegenereerd in cellijnen. Wanneer we patiënten indeelden op zwakke en sterke NOTCH1-activerende mutaties, gebaseerd op een eerdere publikatie
waarin in vitro en in vivo experimenten werden beschreven, zagen we dat patiënten met een zwakke NOTCH1-activerende mutatie verhoogd actief NOTCH1 eiwit hadden ten opzichte van NOTCH1/FBXW7 wild-type patiënten, en dat deze nog hoger was in patiënten met sterke NOTCH1-activerende mutaties. Gelijke resultaten zagen we op RNA expressie niveau. Ook tien wild-type patiënten lieten een hoge expressie van het actieve NOTCH1 eiwit zien, dan wel zijn target genen op RNA niveau. Echter deze patiënten hadden geen mutaties in de gescreende NOTCH1/FBXW7 domeinen of NOTCH1 translocaties. Dit suggereert dat deze patiënten mutaties hebben in het EGF- of LNR-domein van NOTCH1, in de delen van FBXW7 die niet gescreend zijn of in andere genen die NOTCH1 reguleren. Dit betekent dat niet alle NOTCH1-geactiveerde patiënten nog geïdentificeerd zijn. In deze cohorten hebben NOTCH1-geactiveerde patiënten vaak een terugval, maar reageren ze goed op prednisone behandeling in een in vitro setting. De relatie tussen NOTCH1-activerende mutaties en de prognose van patiënten is vaak inconsistent tussen verschillende beschreven studiecohorts. Dit kan verklaard worden door verschillende factoren; er zijn verschillende behandelprotocolen en parameters voor overleving gebruikt in de verschillende studies, nog niet alle NOTCH1-activerende mutaties zijn ontdekt wat de screening onvolledig maakt en de distributie van zwakke en sterke NOTCH1-activerende mutaties kan verschillen tussen studiecohorts. In onze studie hebben wij aangetoond dat beide type mutaties geassocieerd zijn met andere overlevingscijfers binnen het DCOG cohort, maar niet het COALL cohort.

Palomero en collega’s beschreef dat er een correlatie bestaat tussen NOTCH1 en PTEN expressie, waarbij PTEN een target van NOTCH1 zou zijn via HES1. Een goede response van de NOTCH1 γ-secretase inhibitor zou alleen mogelijk zijn wanneer er lage of normale AKT concentraties aanwezig zouden zijn. Een mutatie in PTEN zou leiden tot hoge AKT concentraties wat daardoor zou leiden tot resistentie voor γ-secretase inhibitors. Om de rol van PTEN in T-ALL en zijn relatie met NOTCH γ-secretase inhibitors te onderzoeken, hebben wij onze cohorten gescreend op de aanwezigheid van PTEN, PIK3CA (PI3K isoform), PIK3RI (PI3K isoform) and AKT1 mutaties (hoofdstuk 4). Hierbij hebben wij bij een klein aantal patiënten een nieuw mechanisme van PTEN inactivatie als gevolg van defecte PTEN splicing ontdekt, wat resulteert in de afwezigheid van PTEN eiwit. Dertien procent van de kinderen heeft een afwijking in PTEN, PIK3RI of AKT1 (PTEN/AKT mutaties). Alle patiënten met een nonsense mutatie of deletie hadden geen of laag PTEN eiwit expressie, wat gecorreleerd was aan een mono-alleelische of bi-alleelische PTEN mutatie status. Afwezige PTEN eiwitexpressie in enkele mono-allelisch gemuteerde patiënten suggereert dat er meer PTEN-inactiverend mutaties zijn. We zagen geen verschil in eiwit expressie van de AKT-mTOR of andere downstream AKT eiwitten (zoals PRAS40 of forkhead transcriptiefactoren) tussen wild-type en PTEN/AKT gemuteerde patiënten. In tegenstelling tot onze verwachtingen gebaseerd op de Palomero studie, waren PTEN/AKT mutaties negatief geassocieerd met NOTCH1-activerende mutaties. Wanneer patiënten met een PTEN/AKT mutatie ook een NOTCH1-activerende mutatie hadden, was dit een zwakke NOTCH1-activerend mutatie. PTEN/AKT gemuteerde patiënten hadden een lage expressie van MYC en musashi eiwitten, welke betrokken zijn bij de NOTCH1 signalering. PTEN/AKT mutaties waren voornamelijk aanwezig in
patiënten met een TALLMO herschikking en hadden vaak expressie van mature T cel markers. Patiënten met een TLX herschikking hadden nagenoeg geen additionele PTEN/AKT mutatie. Dit patroon lijkt tegenovergesteld aan dat van NOTCH1-activerende mutaties. Op zichzelf zijn PTEN/AKT mutaties niet geassocieerd met overleving in dit cohort. Echter, wanneer we rekening houden met de negatieve associatie van PTEN/AKT mutaties en NOTCH1-activerende mutaties welke juist gecorreleerd zijn aan overleving, zien we dat NOTCH1/FBXW7/PTEN/AKT wild-type patiënten een goede overleving hebben en NOTCH1 activerend en/of PTEN/AKT-gemuteerde patiënten een slechte.

PTEN splice-defecten hebben we verder onderzocht in hoofdstuk 5. Hierbij hebben we ontdekt dat deze splice-defecten het gevolg waren van microdeleties in een of meerdere exonen. Deleties waren omgeven door immunoglobuline recombinatie signalen en bevatte inserties van random nucleotiden. Dit suggereert dat deze deleties als gevolg van abnormale RAG combinatie events zijn ontstaan.

In hoofdstuk 6 werd het screenen van type B mutaties verder uitgebreid, met het identificeren van mutaties in de IL7R (IL7 receptor), JAK en RAS genen. We vonden geen mutaties in JAK2 en TYK2. De mutaties die in het IL7Ra gen waren gedetecteerd, zijn al eerder gerapporteerd en kwamen voor in 8% van de kinderen met T-ALL. N-RAS/K-RAS mutaties werden gevonden in 10% van de patiënten en JAK1 en/of JAK3 mutaties in 7%. Opvallend was dat IL7Ra mutaties, met uitzondering van 1 patiënt, nooit samen voorkwamen met een van de andere mutaties. Ook PTEN/AKT mutaties kwamen nooit tegelijk voor met JAK of RAS mutaties. Alle JAK mutaties die we hebben getest, waren in staat om een cel te transformeren. Hiervoor werden IL3-afhankelijke Ba/F3 cellijnen gebruikt. Wildtype JAK1 en JAK3 cellijnen konden dit niet. Ook AKT en N-RAS mutaties zorgden voor IL3-onafhankelijke groei. In IL7Ra-gemuteerde patiënten zagen we op eiwitniveau een verhoogde fosforylatie van eiwitten die een rol spelen in de JAK-STAT, RAS-MEK-ERK of PTEN/AKT signaalpaden. Echter activeerden de verschillende IL7Ra en JAK1 mutanten niet dezelfde downstream eiwitten en in gelijke mate, maar hadden ze wel allemaal evenveel downstream p70 S6 kinase activiteit. Wat we ook zagen, was dat JAK3 mutanten een erg robuuste versnelling in IL3-onafhankelijk groei lieten zien, vergeleken met IL7Ra en JAK1 mutanten, maar dat ze in vergelijking weinig verhoging van downstream gefosforyleerde eiwitten hadden. IL7Ra en JAK1/3, maar niet N-RAS en AKT mutanten, waren gevoelig voor de JAK inhibitor Ruxolitinib. Ook hadden de gevoelige lijnen na behandeling een verlaagde expressie van downstream signaleringseiwitten, maar de ongevoelige lijnen niet. Inhibitie van STAT5, een downstream eiwit, zorgde voor matige reductie van groei in JAK1 mutante lijnen maar niet in IL7Ra mutanten lijnen. Dit suggereert dat IL7Ra mutanten ook via andere downstream paden signaleren dan STAT5, zoals bijvoorbeeld het RAS-ERK-MEK of PI3K/AKT signaalpad. JAK1/3 mutanten waren in tegenstelling tot IL7Ra mutanten, gevoelig voor de PI3K/AKT inhibitor LY294002. Wanneer inhibitie van het PI3K/AKT pad in een JAK1 of IL7Ra mutante cellijn gecombineerd werd met inhibitie van het RAS-MEK-ERK pad, observeerden we een lagere expressie van downstream signaleringseiwitten dan wanneer de lijnen met 1 inhibitor werden behandeld. De ongevoeligheid voor de LY294002 inhibitor in combinatie met de extreme gevoeligheid voor een tweetal van PI3K/AKT en RAS-
MEK-ERK inhibitors suggereert dan de IL7R via beide signaalpaden kan signaleren, maar dat deze signaleert via het RAS-MEK-ERK pad wanneer het PI3K/AKT pad wordt geblokkeerd en andersom.

Onze onderzoeksgroep heeft recentelijk een nieuw patiëntencollectie in T-ALL beschreven, welke gebaseerd is op genexpressie data en geassocieerd is met genetische afwijkingen die \textit{MEF2C} activeren. Deze groep patiënten heeft een zeer immatuur T cel fenotype en brengt vaak myeloïde markers tot expressie. Daarom wordt deze groep het immature cluster genoemd. Twee andere studies beschreven een soortgelijke patiëntengroep: vroege voorloper T-ALL welke geïdentificeerd is op basis van het genexpressie profiel dat overeenkomt met het genexpressie profiel van muizen met vroege voorloper T-ALL. Vroege voorloper T-ALL is beschreven als een immature leukemie, welke eigenschappen heeft die overeenkomstig zijn met stamcellen en myeloïde cellen. Deze groep is geassocieerd met mutaties die de hematopoïetische, lymfatische alsmede myeloïde celrijping verstoren. Een andere groep patiënten werd gekarakteriseerd door een deletie in het TCRγδ (TRG@) gen. Deze patiënten worden ABD patiënten genoemd en worden ook geassocieerd met immature T-ALL. We hebben de overlap van deze drie immature patiëntengroepen binnen ons T-ALL cohort bestudeerd in hoofdstuk 7. Hierbij zagen we dat het genexpressie profiel van onze immature cluster patiënten sterk overeen kwam met dit van de vroege voorloper T-ALL patiënten (13 van de 15 immature patiënten werden ingedeeld als vroege voorloper T-ALL patiënten). Ongeveer de helft van de immature patiënten had geen TCRγδ deletie, wat suggereert dat ABD een subtype binnen de immature/vroege voorloper T-ALL patiënten is. We merkten ook dat onze immature cluster patiënten niet konden worden geïdentificeerd op basis van het voorgestelde gepubliceerde immunofenotype dat bij vroege voorloper T-ALL patiënten hoort. Daarom hebben we een andere immunofenotype voorgesteld, dat wel karakteristiek is voor onze immature cluster patiënten; onze patiënten waren geassocieerd met de afwezigheid van CD1 en CD8 expressie, maar ook van CD4, en hadden frequent expressie van CD34 en/of CD13 of CD33. Met behulp van GSEA (Gene Set Enrichment Analysis; verrijking voor bekende genensets) op onze genexpressie data, konden we bevestigen dat deze patiënten een stamcel-achtig fenotype hebben. In tegenstelling tot wat eerder beschreven was, waren COALL97/03 patiënten niet gecorreleerd met een slechte overleving en hebben deze \textit{in vitro} eenzelfde gevoeligheid voor chemotherapeutische middelen, dan andere T-ALL patiënten. In de eerdere publicatie over vroege voorloper T-ALL, alsmede in twee andere publicaties, was voorgesteld om vroege voorloper T-ALL patiënten zo snel mogelijk te behandelen volgens een intensief behandelprotocol. Onze data geeft aan om hier beter naar te kijken. We zagen wel een trend voor lagere gevoeligheid voor steroïden in immature patiënten. Vergeleken met andere behandelprotocollen, bevat het COALL97/03 protocol een hoge concentratie cytarabine. Deze hogere dosis verklaart mogelijk de betere overlevingscijfers van immature patiënten in ons cohort. Daarom stellen we voor om goed te kijken naar het effect van cytarabine in immature patiënten.

De gevolgen op eiwitniveau van genetische afwijkingen in T-ALL is grotendeels onbekend. Om dit te onderzoeken hebben we in hoofdstuk 8 de differentiële expressie van signaleringspaden
op genexpressie- en eiwitniveau bestudeert in patiëntengroepen die type A mutaties reflecteren en in patientengroepen gebaseerd op type B mutaties. TALLMO patiënten hadden een hoge expressie van genen en eiwitten die betrokken zijn bij de T cel receptor (TCR) signalering, metabole glycolyse en vetzuur signaleringspaden waarbij onder andere GSK3 betrokken is. Dit geeft aan dat deze processen mogelijk belangrijk zijn voor celoverleving van deze patiënten. Daarom is het de moeite waard om het effect van remmers van deze processen, in deze patiënten te testen. Dit zijn bijvoorbeeld kinase inhibitors, glycolyse of vetzuur inhibitors of specifieke GSK3 inhibitors. TLX patiënten waren duidelijk geassocieerd met een actieve NOTCH signalering. Dit versterkt onze eerdere observatie in hoofdstuk 3, waarin we een positieve associatie van (voornamelijk sterke) NOTCH1-activerende mutaties met TLX3 hergeschikte patiënten zagen. Activatie van NOTCH signalering zagen we ook in enkele wild-type patiënten. Dit kan komen door, zoals eerder gesuggereerd in hoofdstuk 3, de aanwezigheid van nog onbekende NOTCH1-activerende mutaties of komt door een verslaving aan actieve NOTCH signalering, mogelijk gedreven door HOX. Het is zeer interessant om te kijken of de TLX groep ook zeer gevoelig is voor NOTCH inhiberende y-secretase remmers, welke gebruikt kunnen worden in therapeutische setting. Proliferatieve patiënten hadden een hoge expressie van genen die betrokken zijn bij de regulatie van de biologische klok. Herstel van deze verstooring zou een goede therapeutische mogelijkheid zijn voor deze patiënten. De hele hoge proliferatieve activiteit en DNA schade response die eerder geobserveerd zijn in deze patiënten, zagen we ook in deze studie. Ook zagen we een hoge expressie van p53 transcripten maar een lage expressie van downstream p53 genen en eiwitten. Dit suggereert dat de activiteit van p53 heel laag is doordat er bijvoorbeeld verstoring is in de stabiliteit, localisatie, transactivatie of DNA bindingscapaciteit van p53 in deze groep patiënten. Naast conventionele chemotherapie, zou er gekeken moeten worden naar de effectiviteit van kinase remmers als mogelijke behandelmethode in proliferatieve patiënten. Immature cluster patiënten lijken een erg lage activiteit van T cel signalering en een erg hoge activiteit van B cel signalering te hebben. Expressie van cel adhesie moleculen in deze groep is laag. Deze observaties zijn mogelijk een weerspiegeling van de erg vroege T cel differentiatie stop van deze leukemie. Opvallend is dat deze cellen weinig proliferatie en migratie lijken te vertonen. We vonden een erg interessante link tussen MEF2C en p38 MAPkinase, dat waardevol kan zijn voor verder onderzoek. Wat betreft type B mutaties, bevestigden we dat p27 belangrijk is downstream van NOTCH en dat deze patiënten een lage expressie hebben van PKCβ. Recentelijk is beschreven dat PKCβ via RUNX3 en RUNX1 een indirect target van NOTCH1 is. In tegenstelling tot lage PKCβ concentraties in NOTCH1-geactiveerde patiënten, zagen we een hoge expressie van dit eiwit in PTEN/ACT gemuteerde patiënten. Deze patiënten hebben nauwelijks NOTCH1-activerende mutaties en als ze mutaties hebben, dan zijn deze zwak (hoofdstuk 4). PTEN/ACT gemuteerde patiënten lijken ook een actieve TCR signalering te hebben en erg proliferatief te zijn. Dit maakt deze groep daarom ook aantrekkelijk voor kinase inhibitors. Ingeactiveerde WT1 patiënten hadden een hoge concentratie van groeireceptoren en downstream signalering. De rol van WT1 in translationele inhibitie van groeireceptoren en de gevoeligheid van therapeutische remmers van groeifactoren in deze patiënten, is erg interessant om te onderzoeken omdat de
functie van WT1 in T-ALL nog grotendeels onbekend is.
CHAPTER 9
CHAPTER

10

About the author
LIST OF PUBLICATIONS


* shared first authorship


CURRICULUM VITAE


Direct na het behalen van haar bachelor diploma, is zij de Master Oncology gestart aan het VUmc. Tijdens deze Master heeft zij twee stages gedaan. Een was aan het Nederlands Kanker Instituut (afdeling Tumor Biologie), waar zij heeft gekeken naar het antisense RNA van de p53 en cycline D1 genen. De andere stage was in de UK bij het Wellcome Trust Centre for Cell Biology te Edinburgh, waar zij eiwitten onderzocht die mogelijk aan condensin, een mitose-eiwit, binden.

In 2006 behaalde Linda haar Master of Science. Voordat zij aan haar PhD begon, heeft zij om werkvaring op te doen, zes maanden als analist binnen de diagnostiek van de klinische genetica aan het VUmc gewerkt. Haar promotieonderzoek is gestart in 2007 aan het Erasmus Medisch Centrum/Sophia kinderziekenhuis te Rotterdam, op de afdeling Kinderoncologie/hematologie, onder leiding van Prof. Rob Pieters en Dr. Jules Meijerink. Gedurende deze promotietijd heeft zij onderzoek gedaan naar genetische afwijkingen bij kinderen met T cel acute lymfatische leukemie (T-ALL), zoals beschreven in dit proefschrift. Voor dit onderzoek heeft zij zes maanden in Amerika gewoond, om de eiwitexpressie van T-ALL patiënten te meten en analyseren met behulp van de reverse-phase protein microarray techniek (RPMA). Dit is uitgevoerd op het lab van Prof. Petricoin aan de George Mason University (GMU) te Manassas. Vanaf 2012 tot heden, werkt Linda als post doc bij een start-up biotechnologie bedrijf, genaamd SomantiX BV, te Amsterdam. Hier identificeert zij tumorendotheel-specifieke markers en de functionele effecten na modulatie van marker expressie, met als doel om angiogenese remmers te ontwikkelen. Zij woont momenteel in Santpoort-Zuid, is getrouwd met Mark Opdam waarmee zij een zoontje heeft (Tamo, 1 jaar).
PHD PORTFOLIO

Name PhD student: Lidwina (Linda) C. Zuurbier, MSc
Erasmus MC department: Pediatric Oncology/Hematology
Research school: Molecular Medicine
PhD period: June 2007 – June 2011
Promotor: Prof. Dr. Rob Pieters
Supervisor: Dr. Jules P.P. Meijerink
### PhD training

<table>
<thead>
<tr>
<th>General Courses</th>
<th>Year</th>
<th>ECTS</th>
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<tbody>
<tr>
<td>- Biomedical English Writing and Communication</td>
<td></td>
<td>4</td>
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<tr>
<td>- Classical methods for data-analysis (CC02)</td>
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<tr>
<th>Seminars</th>
<th>Year</th>
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<tr>
<td>- Annual Molecular Medicine Day</td>
<td>2007-2011</td>
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<tr>
<td>- Research meetings dept of pediatrics and pediatric oncology Attendance and presentations</td>
<td>2007-2011</td>
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<table>
<thead>
<tr>
<th>International and National Conferences and Presentations</th>
<th>Year</th>
<th>ECTS</th>
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<tbody>
<tr>
<td>- European Hematology Association (EHA), London, UK Poster presentation</td>
<td>2011</td>
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<tr>
<td>- Dutch Hematology Congress, Papendal, The Netherlands, Oral presentation</td>
<td>2011</td>
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<tr>
<td>- European Hematology Association (EHA), Mandelieu, France Oral presentation</td>
<td>2010</td>
<td>2</td>
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<tr>
<td>- American Association of Hematology (ASH), Orlando, USA Poster presentation</td>
<td>2010</td>
<td>1.6</td>
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<tr>
<td>- Annual Molecular Medicine Day, Rotterdam, The Netherlands Poster presentation</td>
<td>2010</td>
<td>1.6</td>
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<tr>
<td>- American Association of Hematology (ASH), New Orleans, USA Poster presentation</td>
<td>2009</td>
<td>1.6</td>
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<tr>
<td>- Annual pediatric research meeting, Rotterdam, The Netherlands Poster presentation</td>
<td>2009</td>
<td>1.6</td>
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<tr>
<td>- American Association of Hematology (ASH), San Francisco, USA Poster presentation</td>
<td>2008</td>
<td>1.6</td>
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<th>Other</th>
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<tr>
<td>- Six months lab experience at the George Mason University (GMU), Manassas, USA, Centre for applied proteomics and molecular medicine, Prof. E. Petricoin</td>
<td>2008</td>
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<tr>
<td>- European Hematology Association (EHA) Travel grant</td>
<td>2011</td>
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<tr>
<td>- American Society of Hematology (ASH) Travel grant</td>
<td>2010</td>
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<tr>
<th>Teaching</th>
<th>Year</th>
<th>ECTS</th>
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</thead>
<tbody>
<tr>
<td>- Supervision of Wasiem Sahebali, student of AVANS Hogeschool Breda. Nine months intern ship “NOTCH1 and PTEN in T-ALL” (final year Bachelor’s)</td>
<td>2009</td>
<td>10</td>
</tr>
<tr>
<td>- Supervision of Samantha Nedermeijer, 4th year medical student of ErasmusMc, 4 months investigating SHIP1 mutations in T-ALL</td>
<td></td>
<td>10</td>
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</tbody>
</table>

| Total                           |       | 48.5 |

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**Chapter 10**
DANKWOORD

Dankwoord, leden, heel erg bedankt bij het tot stand brengen van mijn proefschrift! Deze totstand komt geheel en geheel aanleiding van mijn promotie. Rood betekent aanwezig, zwart betekent niet aanwezig. De kleurniveaus zijn enkel geïnteresseerd en de map is na elkaar getekend. Bij de naam van de promotor staat ‘promotor:‘. De namen en karakteristieken van mijn promotie zijn:‘. Bedankt voor jouw begrijpendheid, inzet, kennis, diepgang, enthousiasme en humor.

* Paren: rotsen in de branding, oud collega’s en vriendinnen, lief en leed deelen we: bedankt.
* Leve afscheidsmesse, bedankt voor het eten en drinken; bedankt.
* Mark & Tano, mijn grote liefdes, ik hou ontzettend van jullie!!