The significance of *PTEN* and *AKT* aberrations in pediatric T-cell acute lymphoblastic leukemia

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The online veersion of this article has a Supplementary Appendix.

ABSTRACT

Background

PI3K/AKT pathway mutations are found in T-cell acute lymphoblastic leukemia, but their overall impact and associations with other genetic aberrations is unknown. *PTEN* mutations have been proposed as secondary mutations that follow NOTCH1-activating mutations and cause cellular resistance to γ-secretase inhibitors.

Design and Methods

The impact of *PTEN*, *PI3K* and *AKT* aberrations was studied in a genetically well-characterized pediatric T-cell leukemia patient cohort (n=146) treated on DCOG or COALL protocols.

Results

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), the opposite to that observed for 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).

Conclusions

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.

Key words: pediatric T-ALL, *PTEN*, *AKT*, NOTCH1, γ-secretase resistance, outcome.

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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.[1] 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[2-4] where cases with *TAL1* or *LMO2* aberrations share an identical expression profile and may be considered to be a single TALLMO subgroup.[4,5] These abnormalities are accompanied by other genetic aberrations, the so-called type B mutations,[6] 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 from NOTCH1, can also target various other molecules.[7] NOTCH1-activating mutations have been observed in more than 60% of T-ALL pediatric patients.[8-14] We recently observed that the incidence of NOTCH1-activating mutations is higher for *TLX3*-rearranged patients while lower for *TAL*- or *LMO*-rearranged patients.[14] NOTCH1 is a transmembrane receptor that is activated upon ligand binding,[15] and these mutations result in ligand independent activation.[13] Various studies report different prognostic consequences of NOTCH1-activating mutations.[8-12,14,16-18]

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[19] as well as by a genome-wide copy number analysis of ALL samples.[20] *PTEN* mutations were also observed in T-ALL cell lines,[21-23] and analyses of T-ALL patient samples revealed *PTEN* mutations and deletions in 5% and 15%, respectively.[19] Palomero and coworkers found an absence of *PTEN* expression in T-ALL cell lines that were resistant to γ-secretase inhibitors (GSI). Sequence analysis revealed *PTEN* mutations in 9 of 111 primary T-ALL samples, suggesting that *PTEN* mutations that follow *NOTCH1* mutations may provoke GSI resistance.[24] Various other studies showed a variable incidence of *PTEN* mutations and/or deletions in T-ALL patients (range 18-63%).[10,25,26]

PTEN acts downstream of the T-cell receptor and various other pathways. It controls the PI3K/AKT pathway by dephosphorylating PtdIns(3,4,5)P_3 (PIP_3) into PtdIns(3,4)P_2 (PIP_2). PI3-kinase (PI3K) has an opposite function and phosphorylates PIP_2 into PIP_3, which allows activation of AKT via PDK1. *PTEN*-inactivating mutations result in an overactive PI3K/AKT pathway.[21,24] Few mutations are found in *PI3K* and *AKT1* genes themselves as alternative mechanisms to activate AKT.[26] Activated AKT can act on multiple downstream targets that are involved in proliferation, cell metabolism and apoptosis.[27,28] 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.[10,25,26] Also, *PTEN*, *PI3K* or *AKT1* aberrations in relation to NOTCH1-activating mutations are unclear.[10,24] We, therefore, investigated the incidence of mono-allelic or bi-allelic *PTEN*-inactivating events and *PI3K* or *AKT1* aberrations in genetic subtypes of T-ALL, their potential downstream effects, and their relationship with clinical outcome.

Design and Methods

Patients' 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)[29,30] or ALL-9 (n=42),[31] and 74 patients enrolled on the German Co-Operative Study Group for Childhood Acute Lymphoblastic Leukemia study (COALL-97, n=74)[12] with a 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 Erasmus MC Rotterdam and the Declaration of Helsinki. Isolation of leukemia cells was as previously described;[32] all samples contained more than 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 ($sCD3^+/CD1^-$) subgroup. Patients were identified as being positive for a specific immunophenotypic marker when 25% or more of leukemic blast stained positive for it.

Statistical analysis

Statistical analysis was carried out using SPSS 15.0 software. Pearson's χ^2 or 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), event free survival (EFS) or overall survival (OS) 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. $P \leq 0.05$ (two-sided) was considered statistically significant. In a comparison of DCOG and COALL cohorts, we found no statistically significant differences between the distribution of age ($P=0.508$), male/female ratio ($P=0.51$), or distribution according to genetic subtypes ($P=0.395$) excluding potential bias in these cohorts. The 5-year EFS and OS rates for patients treated on DCOG ALL-7/8 or ALL-9 protocols were similar: DCOG ALL-7/8 (n=30) *versus* DCOG ALL-9 (n=42) was 5-year EFS=67±9% *versus* 64±7%, 5-year OS was 70±8% *versus* 67±7%, and both DCOG cohorts were regarded as a single treatment cohort. EFS and OS rates for the German COALL-97 T-ALL cohort (n=74) were: 5-year EFS=61±6%; 5-year OS=71±6%. A comparison of the impact of specified factors on EFS, OS, RFS or CIR for combined DCOG and COALL patient analyses was made in stratified analyses for the DCOG or COALL protocol.

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; *Online Supplementary 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 of 9 patients. One patient (#1959) had two mutations that occurred in *cis*. Most deletion/insertion mutations occured in exon 7, truncating PTEN in the C2-domain. Other mutations were detected in exons 5, 6 and 8. Two patients (#335 and #9963) had missense mutations, of which the R129G mutation had previously been shown to inactivate phosphatase activity.[33]

We also identified 4 patients who had intron mutations located at the 3'-end in introns 1-2, 2-3 or 4-5. However, amplification and sequencing of *PTEN* transcripts in these patients revealed no alternative *PTEN* splice isoforms.

High resolution array-CGH was performed on 113 of 146 pediatric T-ALL patients, and heterozygous *PTEN* deletions were observed in 3 (#531, #8815 and #321; *Online Supplementary Table S3*; *Online Supplementary 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 for whom there was a relatively small size of a homozygous deletion of both *PTEN* alleles (*Online Supplementary Figure S1B*). Array-CGH analysis also revealed subclonal deletions in 2 *PTEN*-mutated patients (#344 and #1959) who both carried two nonsense mutations affecting exons 5 and 7 (*Online Supplementary Table S3*; *Online Supplementary Figure S1C*). Validation by FISH demonstrated copy loss in 40% of the leukemic blasts for patient #344, for whom material was available (*Online Supplementary Figure S1D*). Taken together, 19 of the 142 pediatric T-ALL patients (13%) harbored inactivating *PTEN* aberrations, including missense and nonsense mutations, as well as deletions of the entire *PTEN* locus. Bi-allelic *PTEN* inactivation was seen in 12 of 19 patients.

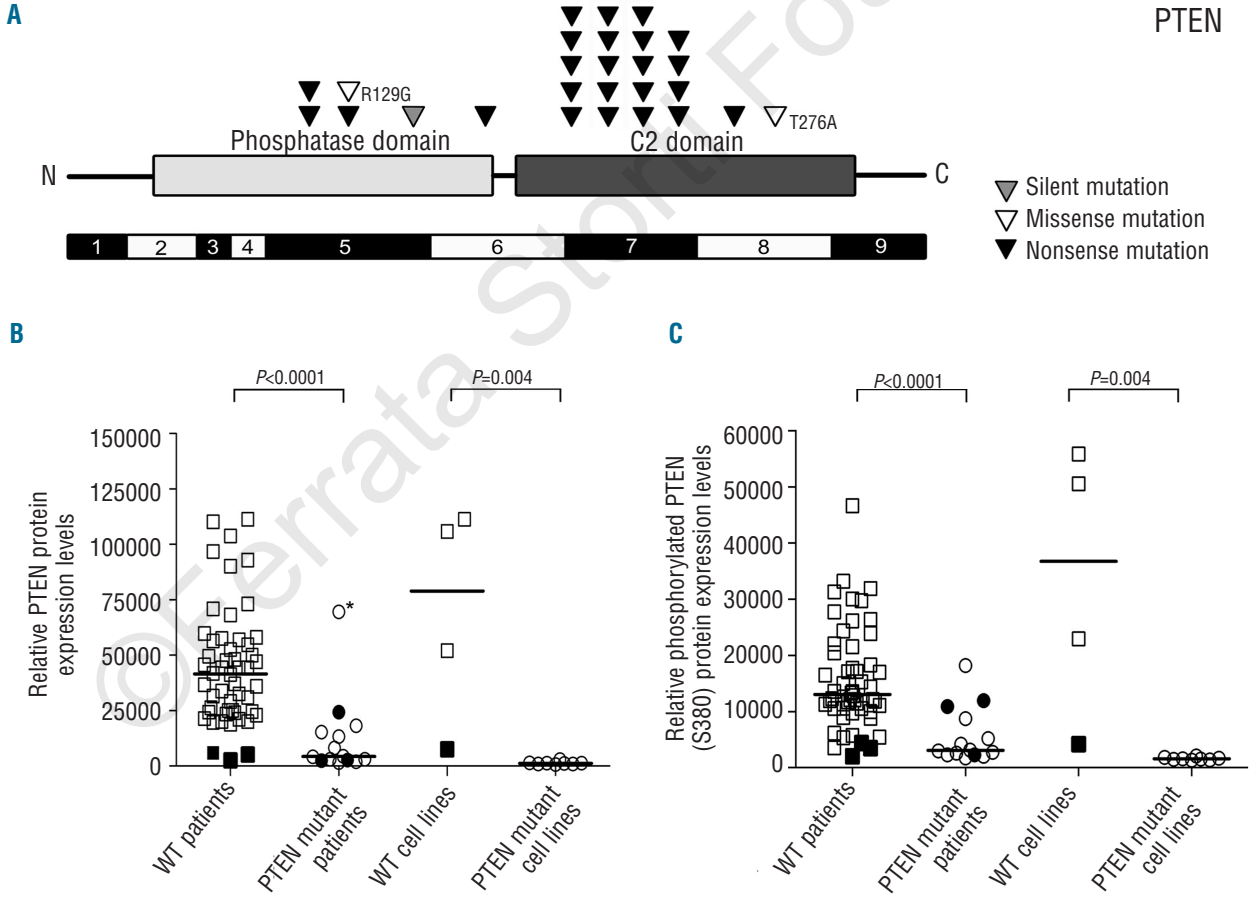

Figure 1. ***PTEN*** **aberrations result in low PTEN expression in pediatric T-ALL patients. (A) Schematic representation of identified mutations in the phosphatase- and C2-domains of the *PTEN* gene. Missense mutations are indicated by open triangles, whereas a silent mutation is presented as a filled gray triangle. Nonsense mutations due to insertions and/or deletions are indicated by a filled black triangle. (B) Total PTEN and (C) phosphorylated PTEN (S380) expression levels in wild-type and *PTEN*-mutated pediatric T-ALL patient samples and T-ALL cell lines, analyzed by reverse-phase protein microarray. Patients with a *PTEN* deletion are represented by a filled circle and patients with *PTEN* missense or nonsense mutations by an open circle. Wild-type patients that lack PTEN protein expression are indicated by a black square. *Patient sample #335; this patient bears a *PTEN* R129G missense mutation.**

PTEN protein levels in relation to the PTEN mutation status

Nonsense *PTEN* mutations result in loss of PTEN protein levels in T-ALL cell lines.[19,24] Using reverse-phase protein microarray (RPMA), total PTEN protein levels as well as phosphorylated (inactivated) PTEN protein levels (S380) were quantified. *PTEN* mutant T-ALL cell lines had significantly lower PTEN and phosphorylated PTEN levels than wild-type cell lines, and validated this technique (Figure 1B and 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 PTEN (Figure 1B; P<0.0001), as well as phosphorylated PTEN protein levels (Figure 1C; P<0.0001), were significantly lower for patients bearing inactivating *PTEN* mutations. One *PTEN*-mutated patient (#335) expressed PTEN protein (Figure 1B and C) from the mutant allele carrying the missense R129G mutation while the second allele was lost due to a frameshift insertion in exon 5. PTEN protein levels were absent or low in all other *PTEN*-mutated patients. PTEN levels were significantly lower for bi-allelic affected patients compared to mono-allelic affected patients (*Online Supplementary 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, 3 seemingly *PTEN* wild-type patient samples (#769, #8629 and #9243) and the *PTEN* wild-type cell line HPBALL lacked PTEN protein (Figure 1B and C, black squares). Array-CGH data were not available for these patients, but large *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 PTEN protein in these 3 patients and HPBALL was due to splice defects, mutations or hypermethylation of the *PTEN* promoter region. In addition, we further investigated the 7 T-ALL patients who seemed to have mono-allelic mutations or deletions (#9160, #9919, #9963, #2759, #2852, #321 and #8815). One of the 3 seemingly *PTEN* wild-type patients (#9243) and 2 of 7 patients with mono-allelic *PTEN* mutations (#2852 and #8815) demonstrated aberrant *PTEN*-splicing and lacked expression of the full-length *PTEN* isoform (Figure 2; *Online Supplementary 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 *PTEN* exon 4 to exon 6 splicing that eliminates the phosphatase domain. Miss-splicing, therefore, provides an additional mechanism to eliminate wild-type PTEN expression. So far, no explanation has been found for defective *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 *PTEN* transcript was also observed in the mono-allelic *PTEN*-deleted cell line LOUCY. 11 control T-ALL patient samples expressed the *PTEN* wild-type isoform only (Figure 2, only 2 of 11 patients are shown).

These 3 *PTEN* wild-type patients with reduced PTEN expression, as well as these 7 *PTEN* mono-allelic-mutated patients, were also investigated for *PTEN* promoter hypermethylation as a 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* (*Online Supplementary Figure S3A*), in which hypermethylation has been previously described in solid tumors and T-ALL.[34-36] However, we found no evidence for *PTEN* promoter hypermethylation (*Online Supplementary Figure S3B and C*). We also found no 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.[26] 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 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) (*Online Supplementary Table S3*). This mutation has been previously reported in a single T-ALL patient,[26] and constitutively activates AKT1.[37]

All *AKT1*-mutated patients lacked *PTEN* aberrations. Overall, *PTEN* mutations or *AKT1* mutations were identified in 25 of 142 pediatric T-ALL patients (18%), and were called the *PTEN/AKT* mutant patient group. This group also included both *PTEN* wild-type patients who lacked PTEN protein expression (#769 and #8629). Based on our findings and those reported in the literature,[19,24] more than half of T-ALL cell lines have inactivated PTEN (*Online Supplementary 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 any difference 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 (*Online Supplementary Figure S4A and B*). As AKT activation has also been described as downstream protein of the NOTCH1 pathway,[24,38] we distinguished between PTEN/AKT mutant patients, NOTCH1-activated and patients lacking PTEN/AKT or NOTCH1/FBXW7 mutations; we did not identify any significant differences in phosphorylated AKT levels or downstream AKT targets (*data 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 to be associated with younger age (Table 1; P=0.05).

Eight of 25 *PTEN/AKT* patients had *TAL1* rearrangements (Table 1; *Online Supplementary Table S5*; P=0.05), whereas only 3 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 unsupervized gene expression profiling,[5] we noticed that *PTEN/AKT* mutations were predominantly present in *TAL/LMO* cluster patients although this was not signifi-

cant, while the incidence of these mutations was significantly lower for the TLX cluster that comprises most *TLX3*- and *HOXA*-rearranged cases (*P*=0.002). No associations were observed with *PHF6* or *WT1* mutations nor with *CDKN2A/B* deletions; this is in line with previous findings as reported by Gutierrez *et al.*[26]

Initially, *PTEN* mutations were suggested to be secondary mutations following NOTCH1-activating mutations, rendering cells insensitive to γ-secretase inhibitors.[24] 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 patients.[14] Strikingly, patients carrying NOTCH1-activating mutations seemed to have a lower incidence of *PTEN/AKT* aberrations as only 10 of 90 *NOTCH1/FBXW7*-mutated patients carried *PTEN/AKT* aberrations, in contrast to 15 of 51 *NOTCH1/FBXW7* wild-type patients (Table 1; *P*=0.006). Remarkably, *PTEN/AKT*-mutated patients who had *NOTCH1/FBXW7* mutations, in particular harbored weak NOTCH1-activating mutations only (9 of 10 cases; *P*=0.011).[14,39]

So NOTCH1-activating mutations and *PTEN/AKT* mutations seem to be hits that are associated with different molecular cytogenetic T-ALL subgroups.[14] This, seems to be 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 gene[24,40] (*P*=0.01) and MUSASHI1/2 (MSI1/2; *Online Supplementary Figure S4C*; *P*=0.002) which is a repressor of the NOTCH1 negative regulator NUMB.[41] This is different in T-ALL cell lines, as 10 of 13 *PTEN/AKT*-mutated cell lines also harbor NOTCH1-activating mutations (*Online Supplementary Table S4*).

We then investigated whether *PTEN/AKT* mutations are associated with resistance to γ-secretase inhibitors as previously suggested.[24] 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 (*Online Supplementary Table S4*) were resistant to γ-secretase inhibitor treatment (*Online Supplementary Figure S5A*).[24] But four cell 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 (*Online Supplementary Figure S5B*).

Good outcome for T-ALL patients lacking PTEN/AKT *and/or* NOTCH1/FBXW7 *aberrations*

In relation to outcome, there was no difference in relapse free survival (RFS) and event free survival (EFS) rates between *PTEN/AKT* mutant patients and wild-type patients (*Online Supplementary Figures S6A and B*). In contrast to previous observations,[25,26] 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 who harbor NOTCH1-activating mutations, which were previously associated with a trend towards poor outcome,[14] 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-year CIR rate (8%) than *PTEN/AKT* and/or NOTCH1-activated patients (35%) in a stratified analysis in our cohorts (Figure 3A and *Online Supplementary S6B*; *P*=0.005). Only 2 of 36 wild-type patients relapsed *versus* 33 of 105 patients who had NOTCH1-activating and/or *PTEN/AKT* mutations (*Online Supplementary Table S6*; *P*=0.002). The 5-year 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 (*Online Supplementary Table S6*; *P*=0.03; *Online Supplementary Figure S6B*). We further investigated clinical and molecular-genetic parameters with 5-year relapse free survival (RFS) rates (*Online Supplementary Table S7*). We found improved 5-year RFS rates for male patients (*P*=0.01), but inferior RFS rates for *TLX3*-rearranged T-ALL

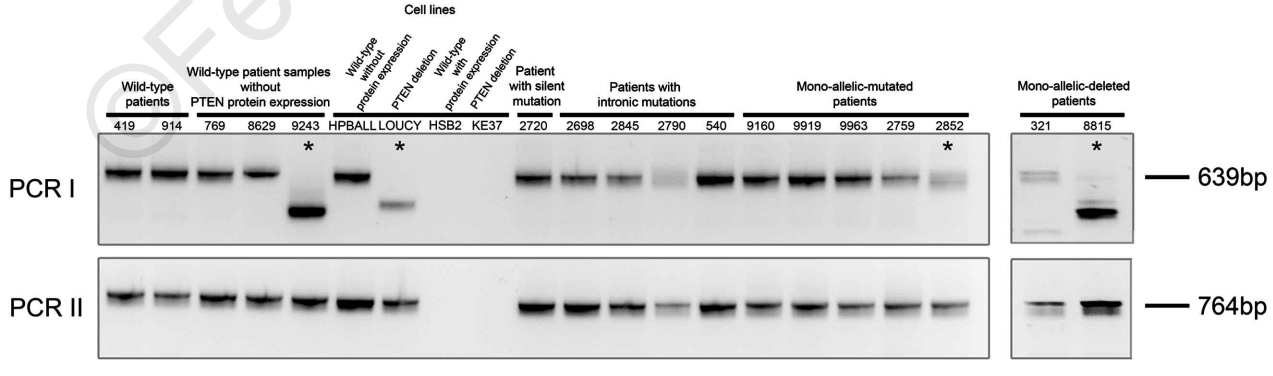

Figure 2. Defective splicing of PTEN transcripts. Analysis of alternative PTEN splicing in 2 wild-type PTEN patients and PTEN expression (#419 and #914), seven PTEN wild-type patients and cell lines without PTEN expression (#768, #8628, #9243, HPBALL, LOUCY, HSB2 and KE37), 5 patients with silent or intronic mutations (#2720, #2698, #2845, #2790 and #540) and 7 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.

(*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 (*Online Supplementary Table S8*).

Discussion

In our pediatric T-ALL patient cohort (n=146), 18% of the patients have aberrations that affect the PI3K/AKT pathway. *PTEN* aberrations were identified in approximately 16%, whereas *AKT* mutations were observed in

Table 1. Overall clinical, immunophenotypic and molecular cytogenetic characteristics of *PTEN* or *PTEN/AKT*-mutated patients *versus* wild-type patients.

	PTEN mutation /deletion					PTEN or AKT mutation/deletion + patients with a low PTEN protein expression (PTEN/AKT)				
Clinical (n=142)	**WT**		**Mut**		***P*** ▫	**WT**		**Mut**		***P*** ▫
Gender					0.60					0.97
Male	85		12			80		17		
Female	38		7			37		8		
Median age (range)	7.8 (1.1-17.8)		4.3 (2.2-15.9)			7.9 (1.1-17.8)		4.9 (2.2-15.9)		**0.05**†
Median WBC (range)	120 (2-900)		136 (5-600)			120 (2-900)		136 (5-600)		0.61†
Cytogenetics (n=142)	**WT**	**n (%)**	**Mut**	**n (%)**	***P*** ▫	**WT**	**n (%)**	**Mut**	**n (%)**	***P*** ▫
TAL1+ (n=26)	20	(77)	6	(23)	0.11	18	(69)	8	(31)	**0.05**
LMO2+ (n=14)	13	(93)	1	(7)	0.69‡	13	(93)	1	(7)	0.46‡
TLX3+ (n=28)	28	(100)	0	(0)	**0.03**‡	27	(96)	1	(4)	**0.03**‡
TLX1+ (n=7)	6	(86)	1	(14)	1‡	5	(71)	2	(29)	0.61‡
HOXA+ (n=13)	13	(100)	0	(0)	0.22‡	13	(100)	0	(0)	0.13‡
§*MEF2C*+ (n=6)	5	(83)	1	(17)	0.81	5	(83)	1	(17)	1‡
§*NKX2-1*+ (n=6)	5	(83)	1	(17)	0.81	5	(83)	1	(17)	1‡
Unknown (n=42)	33	(79)	9	(21)	0.07	31	(74)	11	(25)	0.08
Gene expression clusters (n=113)‖	**WT**	**n (%)**	**Mut**	**n (%)**	***P*** ▫	**WT**	**n (%)**	**Mut**	**n (%)**	***P*** ▫
TAL/LMO+ (n=51)	41	(80)	10	(20)	0.07	38	(75)	13	(25)	0.09
TLX+ (n=28)	28	(100)	0	(0)	**0.02**‡	28	(100)	0	(0)	**0.002**‡
Proliferative+ (n=19)	16	(84)	3	(16)	0.72‡	13	(68)	6	(32)	0.11
Immature/(ETP-ALL)+ (n=15)	13	(87)	2	(13)	1.0‡	13	(87)	2	(13)	0.73‡
***NOTCH1/FBXW7* status (n=141)**	**WT**	**n (%)**	**Mut**	**n (%)**	***P*** ▫	**WT**	**n (%)**	**Mut**	**n (%)**	***P*** ▫
Wild-type (n=51)	39	(76)	12	(24)	0.008	36	(71)	15	(29)	**0.006**
Mutant (n=90)	83	(92)	7	(8)		80	(89)	10	(11)	
Wild-type (n=51)	39	(76)	12	(24)	**0.02**‡	36	(71)	15	(29)	**0.011**‡
¶Weak activating mutation (n=62)	56	(90)	6	(10)		53	(85)	9	(15)	
#Strong activating mutation (n=28)	27	(96)	1	(4)		27	(96)	1	(4)	
***PHF6* status (n=62)**	**WT**	**n (%)**	**Mut**	**n (%)**	***P*** ▫	**WT**	**n (%)**	**Mut**	**n (%)**	***P*** ▫
Mutant	11	(100)	0	(0)	0.33‡	10	(91)	1	(9)	0.67‡
Wild-type	44	(86)	7	(14)		42	(82)	9	(18)	
***WT1* status (n=142)**	**WT**	**n (%)**	**Mut**	**n (%)**	***P*** ▫	**WT**	**n (%)**	**Mut**	**n (%)**	***P*** ▫
Mutant	16	(100)	0	(0)	0.13‡	14	(88)	2	(12)	0.74‡
Wild-type	107	(85)	19	(15)		103	(82)	23	(18)	
Del9p21 status (n=112)	**WT**	**n (%)**	**Mut**	**n (%)**	***P*** ▫	**WT**	**n (%)**	**Mut**	**n (%)**	***P*** ▫
Mutant	73	(84)	14	(16)	0.52‡	72	(83)	15	(17)	0.76‡
Wild-type	23	(92)	2	(8)		22	(88)	3	(12)	

Significant P *values are indicated in bold; all* P *values were calculated by using Pearson's* χ^2 *test, unless indicated otherwise; WT: wild-type; Mut: mutant;* P: P *value;* ▫*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 NKX2-1/NKX2-2 oncogenes that define novel genetic T-ALL subtypes;*[5] ‖ *, 113 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 unsupervized gene expression cluster analysis.*[5] *The TAL/LMO group is based on the presence of TAL1 or LMO2 rearrangements or by having a TAL/LMO expression signature;*[5] ¶*Weak NOTCH1 activating mutations are considered as mutations in the NOTCH1 heterodimerization (HD) domain or NOTCH1 PEST domain or in FBXW7;*[39] #*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 FBXW7W7.*[39]

approximately 2% of T-ALL patients. In other studies, *PTEN* mutations were identified in 5-27% of patients.[10,19,24-26] Gutierrez *et al.*[26] identified *PTEN/AKT* mutations in approximately 48% of the patients (n=44), which is considerably higher than in 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 a greater distance from these donor/acceptor sites could be 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.[42] 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,[43,44] and *PTEN* promoter hypermethylation was described for endometrial cancer, sporadic breast cancer and T-ALL,[34-36] there was no evidence of promoter mutations or promoter hypermethylation in our T-ALL patient series, so there may be additional mechanisms to inactivate PTEN in T-ALL. We cannot exclude the possibility that these mechanisms involve microRNAs, including miR-19b or miR-20a.[45] Also, a regulatory role for the *PTEN* pseudogene *PTENP1* on PTEN expression has been identified before, with *PTENP1* acting as a decoy transcript that binds miR-19b and miR-20a, resulting in elevated PTEN levels.[45] Other miRNAs have been identified that regulate PTEN expression.[8]

Mono-allelic inactivation of PTEN in cancer led to the hypothesis that PTEN is a haploinsufficient tumor suppressor gene.[46] We 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 agreement with previous findings.[24] These expression levels were still significantly higher than in 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 there is still oncogenic pressure to inactivate the second functional *PTEN* allele. This is further substantiated by subclonal *PTEN* deletions in 2 T-ALL patients who already had one dysfunctional *PTEN* allele.

Inactivation of PTEN results in ectopic activation of AKT.[19,24] However, using RPMA, we found no difference in phospho-AKT levels or phosphorylation of downstream AKT targets between *PTEN/AKT* mutant patients and patients lacking PTEN/AKT aberrations, and there were also no differences observed between *PTEN/AKT* mutant cell lines and wild-type lines. Possibly, differences in phosphorylation levels for AKT and downstream targets between *PTEN/AKT* mutant patients and wild-type patients are very subtle and difficult to identify on primary patient material by RPMA; AKT may also be regulated through other oncogenic pathways. In this respect, activation of the AKT pathway has been identified in over 75% of T-ALL cases;[47] this is well above the incidence of *PTEN/AKT* aberrations seen in this study. In T-ALL, activation of AKT has been described downstream of NOTCH1[38] and AKT may be activated upon transcriptional repression of PTEN by the NOTCH1-activated transcriptional repressor HES1.[24] So AKT activation as a consequence of *PTEN/AKT* mutations or through NOTCH1-activating mechanisms could explain the lower frequency of *PTEN/AKT* mutations in patients who have NOTCH1-activating mutations in our cohort. Furthermore, the 9 of 10 *PTEN/AKT*-mutated patients who had NOTCH1-activating mutations, only had weakly NOTCH1-activating

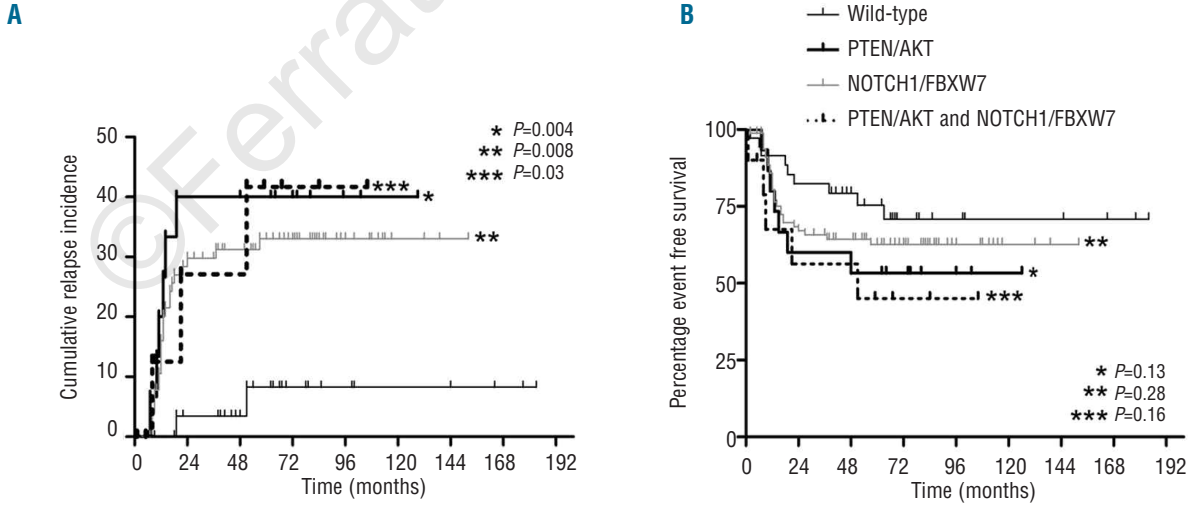

Figure 3. T-ALL patients without PTEN/AKT and/or NOTCH1-activating mutations have a good outcome. Cumulative incidence of relapse (CIR) (A) and event-free survival (EFS) (B) for DCOG and COALL pediatric T-ALL patients. Different patient groups are indicated in the legend. Log rank *P* values in a stratified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

mutations (i.e. *NOTCH1*-HD or PEST domain mutations or *FBXW7* mutations),[39] pointing to a common downstream target and, therefore, removing the need to accumulate both *PTEN/AKT* and strong NOTCH1-activating mutations. Also, in the study of Medyouf *et al.*,[48] 4 of the 6 primary T-ALL samples with PTEN-inactivating mutations were also NOTCH1-mutated and only carried weakly activating PEST domain mutations. Consequently, ICN, MYC (another NOTCH1 target gene) and the indirect NOTCH1 activator MUSASHI1/2 are expressed at lower levels in *PTEN/AKT*-mutated patients. *PTEN/AKT* mutations and NOTCH1-activating mutations may both converge on the activation of AKT. However, the associations of *PTEN/AKT* mutations with *TAL/LMO*-rearranged patients seen in this study and NOTCH1-activating mutations that are especially predominant in *TLX3*-rearranged patients[14] 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).[24] Our data show that most *PTEN*-inactivating mutations occur independently of NOTCH1-activating mutations, implying that *PTEN/AKT*-mutant leukemic cells are not sensitive towards GSIs rather than that *PTEN* aberrations would provoke γ-secretase resistance.[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 agreement with one previous study that showed that *PTEN* negative primary T-ALL cells or NOTCH1-induced T-ALL cells in mice on a *Pten* null background are as sensitive to γ-secretase inhibitors as primary T-ALL cells or NOTCH-induced tumors with unaffected PTEN loci, respectively.[48]

In the study of Gutierrez *et al.*,[26] PTEN/AKT-mutated patients did not predict for event free survival, but *PTEN* deletions seemed to be associated with early treatment failure. Jotta *et al.*[25] demonstrated poor overall survival rates for *PTEN*-mutated high-risk patients. 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 has so far not been investigated in T-ALL. Distinguishing patient 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.

In conclusion, missense or nonsense mutations or deletions affecting the *PTEN* gene occur in 13% of pediatric T-ALL patients, and may result in the activation of the AKT pathway. The AKT E17K activating mutation was observed in approximately 2% of T-ALL patients. Defective *PTEN*-splicing is an additional *PTEN*-inactivating event, but the underlying mechanism is still not fully 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 who lack *PTEN/AKT* and *NOTCH1/FBXW7* mutations demonstrated a good overall outcome.

Authorship and Disclosures

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