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

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The online version 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. T-ALL is characterized by mutually exclusive abnormalities in TAL1, LMO2, TLL3/HOX11L2, TLL1/HOX11 or HOXA10 oncogenes. Gene expression analyses supported the view that the aberrations delineate specific T-ALL subgroups where cases with TAL1 or LMO2 aberrations share an identical expression profile and may be considered to be a single TALLMO subgroup. These abnormalities are accompanied by other genetic aberrations, the 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 from 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 TLL3-rearranged patients while lower for TAL1 or LMO2-rearranged patients. NOTCH1 is a transmembrane receptor that is activated upon ligand binding, and these mutations result in ligand independent activation. Various studies report different prognostic consequences of NOTCH1-activating mutations.

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 (Tec, Atr and Tpr53) that developed T-cell lymphomas as well as by a genome-wide copy number analysis of 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 co-workers 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. Various other studies showed a variable incidence of PTEN mutations and/or deletions in T-ALL patients (range 18-65%).

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 (PIP3). PI3-kinase (PI3K) has an opposite function and phosphatase and tensin homolog (PTEN) acts downstream of this pathway by dephosphorylating PtdIns(3,4,5)P3 (PIP3) into PtdIns(3,4)P2 (PI3K/AKT pathway by mutations in pediatric T-ALL patients.

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 their potential downstream effects, and their relationship with clinical outcome.
in single exons or distributed over different exons. Resequencing 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 occurred 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.

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 (\#833) 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 (\#160, \#919, \#9963, \#2759, \#2852, \#821 and \#8315). One of the 3 seemingly PTEN wild-type patients (\#9243) and 2 of 7 patients with mono-allelic PTEN mutations (\#2852 and \#8315) 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 defective spliced to exon 4 in patient \#2852. Patient \#8315 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.\(^{25}\) To screen for such mutations, exons 12 and 13 of PIK3R1 (p65 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 155 of the 146 T-ALL patients. No mutations were identified in PIK3R1 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,\(^{23,24}\) 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 Thr380) levels, nor in the phosphorylated status of downstream AKT targets including mTOR, p70 S6 kinase, 4E-BP1, TSC2, PRA50 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/BXW7 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 unsupervised gene expression profiling,\(^7\) we noticed that PTEN/AKT mutations were predominantly present in TAL1/ML0 cluster patients although this was not signifi-
cant, while the incidence of these mutations was significantly lower for the TLX3 cluster that comprises most TLX3- and HOXA-rearranged cases (P=0.002). No associations were observed with PHE6 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=0.01).15,29 So NOTCH1-activating mutations and PTEN/AKT mutations seem to be hit 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 (IC; P=0.003), cMYC, as a prime NOTCH1-target gene,24,40 (P=0.01) and MUSASH1I2 (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 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=0.05; 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](https://example.com/figure2.png)

**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, HS82 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

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<table>
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<tr>
<th>PHF6 status (n=62)</th>
<th>WT</th>
<th>Mut</th>
<th>P</th>
<th>WT</th>
<th>Mut</th>
<th>P</th>
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<tbody>
<tr>
<td>Wild-type (n=142)</td>
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<td>0</td>
<td>0.13</td>
<td>14</td>
<td>2</td>
<td>0.74</td>
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<tr>
<td>Mutant (n=142)</td>
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<td>0</td>
<td>0.07</td>
<td>14</td>
<td>2</td>
<td>0.07</td>
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<th>Del9p21 status (n=112)</th>
<th>WT</th>
<th>Mut</th>
<th>P</th>
<th>WT</th>
<th>Mut</th>
<th>P</th>
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<tbody>
<tr>
<td>Wild-type (n=142)</td>
<td>73</td>
<td>14</td>
<td>0.52</td>
<td>72</td>
<td>15</td>
<td>0.76</td>
</tr>
<tr>
<td>Mutant (n=142)</td>
<td>73</td>
<td>14</td>
<td>0.74</td>
<td>72</td>
<td>15</td>
<td>0.76</td>
</tr>
</tbody>
</table>

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; *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; Weak NOTCH1 activating mutations are considered as mutations in the NOTCH1 heterodimerization (HD) domain or NOTCH1 FEST domain or in FBXW7; 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 FEST domain or FBXW7.
approximately 2% of T-ALL patients. In other studies, PTEN mutations were identified in 5-27% of patients. Gutierrez et al. 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. 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, 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. 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. Other microRNAs 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 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. 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. 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, and 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 and AKT may be activated upon transcriptional repression of PTEN by the NOTCH1-activated transcriptional repressor HE1. 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),\textsuperscript{36} 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 Medyousse\textit{et al.},\textsuperscript{34} 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\textsuperscript{13} 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 occurred 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.\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 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.\textsuperscript{48}

In the study of Gutierrez\textit{et al.},\textsuperscript{26} PTEN/AKT-mutated patients did not predict for event free survival, but PTEN deletions seemed to be associated with early treatment failure. Jotta\textit{et al.}\textsuperscript{35} 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.

In conclusion, missense or nonsense mutations or deletions affecting the PTEN gene occur in 18% 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.

\textbf{Authorship and Disclosures}

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

\textbf{References}


