

## ORIGINAL ARTICLE

# Clinical and molecular characterization of early T-cell precursor leukemia: a high-risk subgroup in adult T-ALL with a high frequency of FLT3 mutations

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A subgroup of pediatric acute T-lymphoblastic leukemia (T-ALL) was characterized by a gene expression profile comparable to that of early T-cell precursors (ETPs) with a highly unfavorable outcome. We have investigated clinical and molecular characteristics of the ETP-ALL subgroup in adult T-ALL. As ETP-ALL represents a subgroup of early T-ALL we particularly focused on this cohort and identified 178 adult patients enrolled in the German Acute Lymphoblastic Leukemia Multicenter studies (05/93–07/03). Of these, 32% (57/178) were classified as ETP-ALL based on their characteristic immunophenotype. The outcome of adults with ETP-ALL was poor with an overall survival of only 35% at 10 years, comparable to the inferior outcome of early T-ALL with 38%. The molecular characterization of adult ETP-ALL revealed distinct alterations with overexpression of stem cell-related genes (*BAALC*, *IGFBP7*, *MN1*, *WT1*). Interestingly, we found a low rate of *NOTCH1* mutations and no *FBXW7* mutations in adult ETP-ALL. In contrast, *FLT3* mutations, rare in the overall cohort of T-ALL, were very frequent and nearly exclusively found in ETP-ALL characterized by a specific immunophenotype. These molecular characteristics provide biologic insights and implications with respect to innovative treatment strategies (for example, tyrosine kinase inhibitors) for this high-risk subgroup of adult ETP-ALL.

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## INTRODUCTION

Early T-cell precursors (ETPs) are intrathymic c-Kit<sup>hi</sup> double-negative (DN)1 cells, which contribute to the development of T-lymphocytes.<sup>1,2</sup> These cells represent immature progenitors that have recently immigrated from the bone marrow to the thymus. In addition to their T-lymphoid potential, they carry a natural killer, dendritic, and remarkably, myeloid cell differentiation potential.<sup>3</sup>

Recently, a small subgroup of pediatric acute T-lymphoblastic leukemia (T-ALL) was described showing a gene expression profile closely linked to the expression signature of ETPs.<sup>4,5</sup> This subtype, termed ETP-ALL, is characterized by a specific immunophenotype: CD1a<sup>-</sup>, CD8<sup>-</sup>, CD5<sup>weak</sup> and expression of stem cell or myeloid markers. Moreover, ETP-ALLs are distinguished by high expression of oncogenic transcription factors, including genes involved in the pathogenesis of T-ALL like *LMO1*, *LYL1* and *ERG*. Pediatric patients with ETP-ALL showed a highly unfavorable outcome compared with typical T-ALL.<sup>4</sup> On the basis of these results, the Italian national study Associazione Italiana Ematologia Oncologia Pediatrica (AIEOP) and St-Jude Children's hospital proposed modifications in their treatment schedules for children with ETP-ALL towards a more intensive regime including allogeneic stem cell transplantation (alloSCT).<sup>4</sup> Up to date, the existence of a subtype with an ETP gene expression signature in adult patients has not yet been investigated.

Although several molecular risk factors with prognostic and potential therapeutic implications have been identified, such as mutations (*NOTCH1*) or aberrant expression (*BAALC*, *ERG*, *TLX1*,

*TLX3*),<sup>6</sup> there is still a need for more precise molecular risk stratifications to guide adapted treatment strategies. Although some study groups utilize an initial leukocyte count above 100 000/nl as an adverse prognostic factor, the German Multicenter Study Group for Adult ALL (GMALL) applies risk stratification for T-ALL based on the immunophenotype.<sup>7</sup> On the basis of the results of earlier study protocols, early (sCD3<sup>-</sup>, CD1a<sup>-</sup>) T-ALL is regarded as a high-risk subgroup and alloSCT is recommended in first complete remission (CR).<sup>7</sup> Therefore, patients with ETP-ALL, being exclusively found in the subgroup of early T-ALL, are already assigned towards alloSCT within the GMALL strategy. However, the multilineage ability of normal ETPs and the stem cell-like features of oncogenetically transformed ETP-ALL may translate in primary resistance to conventional chemotherapy. Thus, a standard protocol for lymphoblastic diseases might be insufficient and new molecular targets are warranted for specific targeted therapy in order to improve overall survival (OS) in this high-risk disease.

Although the implementation of specific targeted therapies have already been applied to high-risk B-ALL characterized by *BCR-ABL* and *CRLF2/JAK* activation, alterations of the tyrosine kinase pathway have only been identified in rare cases of T-ALL presenting with rearrangements *NUP214-ABL1* and *STRN3-JAK2*.<sup>8</sup>

In this study, we have investigated the existence of ETP-ALL among adult patients and, in particular, assessed their clinical as well as molecular characteristics. We further explored the ETP-ALL as a molecular distinct subgroup of T-ALL and identified a high

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2 frequency of *FLT3* mutations. Thus these findings may in future direct to innovative treatment strategies for this distinct T-ALL subgroup.

## PATIENTS AND METHODS

### Patients and treatment

We analyzed 178 patients classified as early T-ALL in the GMALL study trials between 1993 and 2008. The GMALL protocols include a combination of chemotherapy, radiation, and with the protocol 06/99, alloSCT was implemented for high-risk T-ALL-patients. Details of the protocols were previously described.<sup>9</sup> All patients gave written informed consent to participate in the study according to the Declaration of Helsinki.<sup>10</sup> This study was approved by the ethics board of the Johann Wolfgang Goethe-Universität Frankfurt am Main, Germany. In the GMALL study, immunophenotyping was centrally performed in the GMALL reference laboratory at the Charité University Hospital, Berlin. Immunophenotyping and subtype assignment was carried out as previously described.<sup>11-13</sup> High-risk T-ALL was defined by an immunophenotype of an early (sCD3<sup>-</sup>, CD1<sup>-</sup>) or mature (sCD3<sup>+</sup>, CD1<sup>-</sup>) T-ALL. ETP-ALL was defined as a subgroup within early T-ALL with the following immunophenotype: CD1a<sup>-</sup>, CD8<sup>-</sup>, CD5<sup>weak</sup> with expression of stem cell (CD34, HLA-DR, CD117) and/or coexpression of myeloid antigens (CD13, CD33, CD65s). Absence, positivity and weak expression of antigens were defined according to the definitions in pediatric patients.<sup>4</sup>

### Nucleic acid preparation and molecular characterization

For patients with sufficient material available, pretreatment bone marrow samples were used for DNA and total RNA extraction using TRIzol (Life Technologies, Grand Island, NY, USA) according to the manufacturer's protocol with minor modifications. Complementary DNA was synthesized using 500 ng of total RNA and avian myeloblastosis virus reverse transcriptase (RT-AMV; Roche, Mannheim, Germany) in the presence of RNase inhibitor (RNasin; Roche).

Molecular diagnostic examinations were available from in total 297 T-ALL patients (including all immunophenotypical subgroups) from the two GMALL studies 06/99 and 07/03. As far as material was available, samples were investigated by comparative real-time PCR (RT-PCR) for expression of five genes (*BAALC*, *ERG*, *IGFBP7*, *WT1* and *MN1*). mRNA expression levels for *WT1*,<sup>14</sup> *BAALC*<sup>13</sup> and *ERG*<sup>13</sup> were determined by RT-PCR as previously described. The primers for *IGFBP7* are available on request. *MN1*-primers were designed as reported.<sup>15</sup> *GUS* was used as a housekeeping gene with the exception of *ABL* in the RT-PCR for *MN1*. In 142 patient samples, the *NOTCH1* mutation status was identified by sequencing of PCR-amplified products.<sup>16,17</sup> For the *FBWX7* mutation status, exons 8 and 9 were sequenced in 121 patients samples as previously described.<sup>18</sup> *WT1* mutations analyses was performed as recently reported.<sup>14</sup> *FLT3* mutations (internal tandem duplications (ITD)/tyrosine kinase domain (TKD)) were analyzed using a commercially available *FLT3* mutation assay (*InVivoScribe* Technologies, San Diego, CA, USA) in 123 T-ALL patients. Because of lack of sufficient material, not all samples could be analyzed. No significant differences regarding immunophenotype, age or sex were found in the different subsets of patients analyzed in the different experiments.

### Statistical analyses

Differences in the clinical characteristics as well as response to induction therapy were tested by the Pearson  $\chi^2$ -test. For OS and duration of remission in the different subgroups, Kaplan-Meier curves were created and compared by the log-rank test. OS was calculated from the time-point of study entry to the time-point of death or latest follow-up (censored). Remission duration was calculated in CR patients from the time-point of first CR until relapse or last follow-up. Stem cell transplantation in first CR, death in CR, withdrawal and continuous remission were censored at the respective dates.

The statistical difference of gene expression between two independent groups was tested by the nonparametric Mann-Whitney *U*-test. Differences

in the mutation rate were analyzed by the Pearson  $\chi^2$ -test. For all tests, a *P*-value <0.05 (two-sided) was considered to indicate a significant difference. All calculations were performed using the SPSS software version 17 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism software version 5 (GraphPad Software Inc., La Jolla, CA, USA).

## RESULTS

### Patients' characteristics and clinical outcome

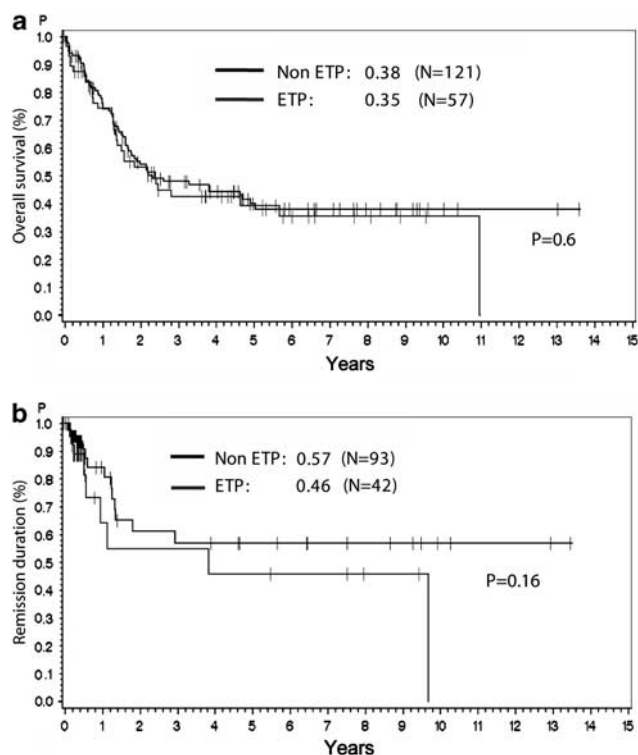
We first restricted our analysis to 178 patients diagnosed with an immunophenotype of early T-ALL who were enrolled in the GMALL trials (05/93, 06/99, 07/03) between 1993 and 2008. This cohort of early T-ALL patients represents 23% of all patients with T-ALL enrolled in the mentioned studies.<sup>7</sup> All samples were clearly positive for cytoplasmic CD3. Applying the immunophenotypic ETP-ALL profile (CD1a<sup>-</sup>, CD8<sup>-</sup>, CD5<sup>weak</sup> with expression of stem cell or myeloid markers), 57 patients (32% of early T-ALL) were classified as ETP-ALL, corresponding to 7.4% of all adult T-ALL patients. Notably, none of the early T-ALL cases fulfilled the EGIL criteria for biphenotypic leukemia.

The clinical characteristics were similar between the group of ETP-ALL and non-ETP early T-ALL regarding sex, age, white blood cell count, and central nervous system involvement (Table 1). However, a lower frequency of patients with a mediastinal mass at diagnosis was found among patients with ETP-ALL compared with those with non-ETP early T-ALL (28% vs 47%; *P* = 0.02). Patients of both groups were treated equally in the three GMALL trials and the rate of patients receiving an alloSCT in first CR did not differ between ETP-ALL and non-ETP early T-ALL (59% vs 60%, respectively). Slightly fewer patients with ETP-ALL achieved a CR after induction therapy (42 of 53 patients; 79%) compared with patients with non-ETP early T-ALL (93 of 113 patients; 82%;

**Table 1.** Characteristics of patients enrolled in the three GMALL study trials 05/93, 06/99 and 07/03 with the diagnosis of early T-ALL

	ETP-ALL (n = 57) (%)	Non-ETP early T-ALL (n = 121) (%)	P
<b>Sex</b>			
Male	47 (82.5)	84 (69.4)	0.07
Female	10 (17.5)	37 (30.6)	
<b>Age</b>			
15-35	27 (47.4)	68 (56.2)	0.27
36-65	30 (52.6)	53 (43.8)	
<b>WBC count (n = 163)</b>			
< 30/nl	32 (62.7)	71 (63.4)	0.94
> 30/nl	19 (37.3)	41 (36.6)	
<b>Mediastinal mass (n = 162)</b>			
No	37 (72.5)	59 (53.2)	0.02
Yes	14 (27.5)	52 (46.8)	
<b>CNS involvement (n = 150)</b>			
No	42 (91.3)	100 (96.2)	0.22
Yes	4 (8.7)	4 (3.8)	
<b>Response to induction</b>			
CR	42 (79.2)	93 (82.3)	0.65
PR/failure	5 (9.4)	12 (11.7)	
Death in induction	6 (11.3)	8 (7.8)	

Abbreviations: CNS, central nervous system; CR, complete remission; ETP-ALL, early T-cell precursors-acute lymphoblastic leukemia; PR, partial remission; T-ALL, acute T-lymphoblastic leukemia; WBC, white blood cell. Comparison between ETP-ALL and early T-ALL with a non-ETP immunophenotype.



**Figure 1.** Kaplan-Meier analyses for OS (a) and remission duration (b) for patients with ETP-ALL and early T-ALL with a non-ETP immunophenotype. Patients were enrolled in the GMALL study trials GMALL 05/93, GMALL 06/99 and GMALL 07/03.

n.s.; Table 1). A total of 14 patients died during the induction therapy, 6 in the group of ETP-ALL, 8 in the group of non-ETP early T-ALL. Regarding all high-risk early T-ALL patients in the three study trials, the probability of survival at 10 years was comparably poor in patients with ETP-ALL and non-ETP early T-ALL (35% vs 38%, Figure 1a). In patients with early T-ALL, more patients remained in CR in the group of non-ETP early T-ALL than in the group of ETP-ALL (57% vs 46%, n.s., Figure 1b) with a follow-up of 9 years.

#### Aberrant gene expression in ETP-ALL

To further characterize the ETP-ALL on the molecular level, we analyzed the expression of selected genes known to be involved in the pathogenesis and with prognostic implications in T-ALL in samples with sufficient material. We first compared ETP-ALL, as a new subgroup, to all remaining T-ALL subtypes including thymic, mature and early T-ALL. As previously described, high expression of *BAALC* and *ERG* predicted an unfavorable outcome in adults with T-ALL.<sup>10,13</sup> Quantitative RT-PCR assays revealed that *BAALC* was 5.34-fold highly expressed in ETP-ALL compared with all remaining T-ALL ( $P < 0.001$ , Figure 2a). *ERG* only showed a slight, however not significantly elevated expression (1.33-fold,  $P = 0.12$ , Figure 2a). *IGFBP7* is a stem cell-associated gene, which is highly associated with *BAALC* and overexpressed in early T-ALL<sup>19</sup> and among the genes highly overexpressed in pediatric ETP-ALL.<sup>4</sup> Similar to *BAALC*, it was significantly upregulated in ETP-ALL (3.53-fold,  $P = 0.003$ , Figure 2a). The gene *WT1* was of interest as it is widely overexpressed in AML<sup>20</sup> and as its overexpression is associated with a poor outcome in thymic T-ALL patients.<sup>14</sup> Interestingly, we found a significantly elevated *WT1* expression in ETP-ALL (4.33-fold,  $P = 0.04$ , Figure 2a). Because of the myeloid potential of ETPs and the frequent coexpression of myeloid surface antigens in ETP-ALL lymphoblasts, we further investigated

the expression of *MN1* as its overexpression was found to be associated with a shorter survival in AML without karyotypic abnormalities.<sup>15,21</sup> Here, we show that *MN1* was significantly overexpressed in the group of ETP-ALL compared with all remaining T-ALL patients (2.86-fold,  $P < 0.001$ ; Figure 2a).

Although these five genes were also upregulated in early T-ALL compared with mature or thymic T-ALL, *WT1*, *BAALC*, *IGFBP7* and *MN1* showed an even higher expression in the ETP-ALL group compared with the remaining group of non-ETP early T-ALL (Figure 2b).

#### Mutational analyses in ETP-ALL

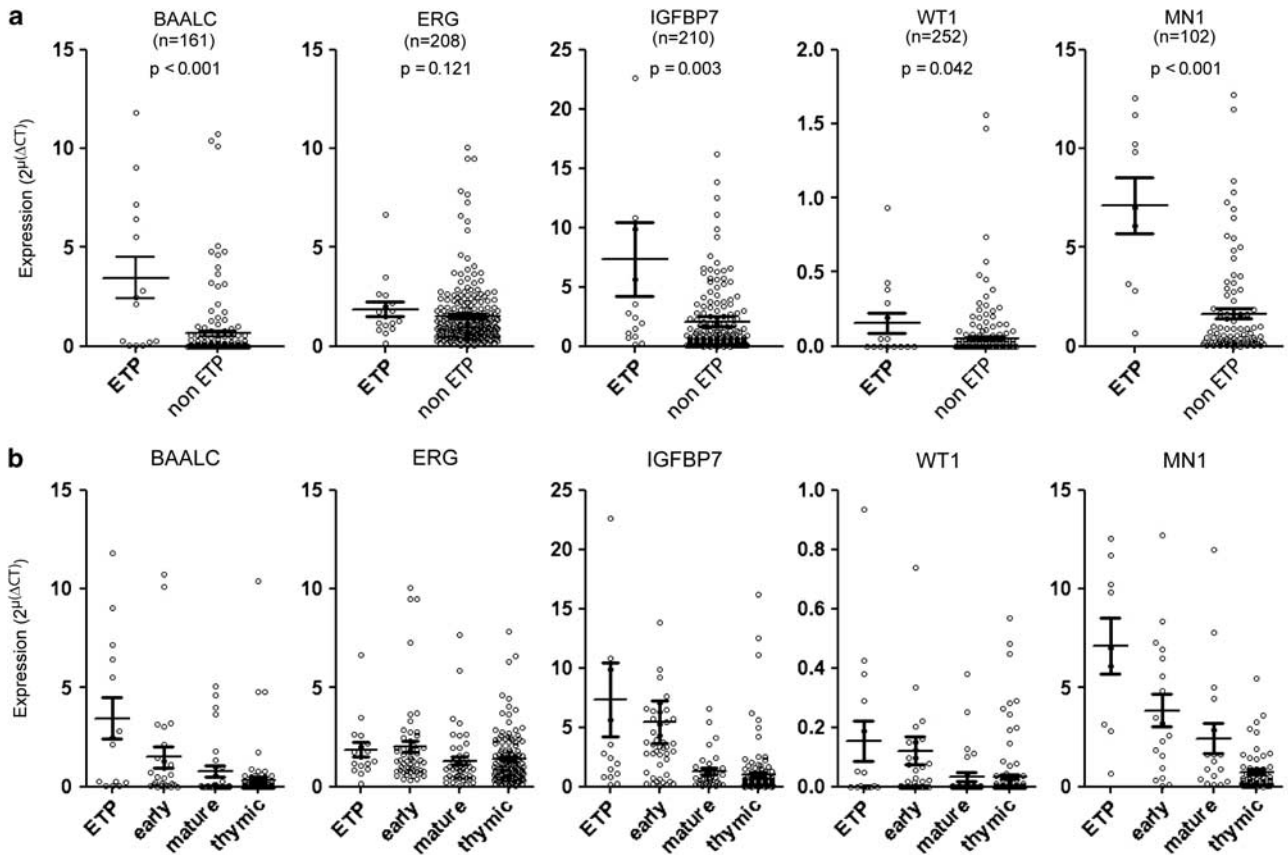
Differences in the mutation status of candidate genes between ETP-ALL and non-ETP-ALL cases were explored (Table 2). The most frequent pathogenetic mutational event in T-ALL are *NOTCH1* mutations occurring in approximately 50–70% of the cases, predominantly in thymic T-ALL<sup>22–24</sup> Although *NOTCH1* mutations have been associated with an initial good response in some studies, the prognostic impact of *NOTCH1* mutations in T-ALL remains controversial.<sup>8,25–29</sup> In 142 adult T-ALL samples analyzed, we have found a low rate of *NOTCH1* mutations in the immature subgroup of ETP-ALL ( $n = 1/14$ , 7.1%), whereas *NOTCH1* mutations were frequent (60.9%) in non-ETP T-ALL ( $n = 78/128$ ,  $P = 0.001$ , Table 2). Similar findings were observed for the tumor-suppressor gene *FBXW7* involved in *NOTCH1* signaling: no *FBXW7* mutations were found in the 14 ETP-ALL samples analyzed.

Mutations in the *WT1* gene were reported in about 8% of all T-ALL patients.<sup>14</sup> We did not observe a significant difference in the frequency of *WT1* mutations between ETP-ALL and the remaining T-ALL (Table 2).

Although *FLT3* mutations are frequent in AML (~30%) and have important prognostic and therapeutic implications, mutations for *FLT3* as ITD or in the TKD in T-ALL are very rare.<sup>30–32</sup> Interestingly, in our cohort we identified seven patients with mutations of *FLT3* that were exclusive in the cohort of early T-ALL patients and predominantly found in the subgroup of ETP-ALL patients. Five cases (4.5%) had mutations in the tyrosine kinase domain of *FLT3* (D835), four of them were in the group of ETP-ALL ( $P < 0.001$ ). Another case with an *FLT3* mutation was assigned to early T-ALL, but the immunophenotype did not fulfil the criteria of an ETP-ALL due to a weak CD8 expression. This case also showed surface expression of the myeloid antigens CD13 and the stem cell markers CD34 and CD117. ITD mutations of *FLT3* were only found in two of the 123 cases, both being in the group of ETP-ALL ( $P < 0.001$ ). In total, six of seven *FLT3*-mutations found were in the group of ETP-ALL, displaying a frequency of 37.5% within this subgroup. Interestingly, *FLT3*-mutated ETP-ALL patients showed different clinical and molecular characteristics compared with *FLT3* wild-type ETP-ALL patients. The seven patients with an *FLT3* mutation had a median age of 40 years and an initial white blood cell of 3800/ $\mu$ l. Five *FLT3*-mutated ETP-ALL patients were transplanted in first remission and the median OS was 31.3 months with six patients being still alive. Remarkably, patients with a *FLT3* mutation showed a distinct immunophenotype with positivity for CD117 (7/7 patients), CD34 (6/7 patients), CD13 (7/7 patients), and CD2 (7/7 patients). In contrast, ETP-ALL patients with a *FLT3* wild-type status had more often positivity for CD5 (7/10 patients) and CD33 (6/10 patients).

#### DISCUSSION

In T-ALL, outcome has been slightly improved in the past decades, mainly because of the implementation of alloSCT for specific subgroups.<sup>7,33</sup> However, long-term OS only reaches rates of 30–60% depending on prognostic factors and subgroups.<sup>34</sup> Further improvement remains warranted and may be achieved by enhanced risk stratification and development of novel



**Figure 2.** Expression of genes known to be correlated with outcome in T-ALL and AML. Analysis of samples with sufficient RNA was carried out by RT-PCR. For each entity the median expression is shown by a horizontal bar with the s.e. of the mean. **(a)** Comparison between ETP-ALL and the remaining non-ETP T-ALL. **(b)** Expression in the different immunophenotype-defined subgroups of T-ALL. Early T-ALL refers to non-ETP early T-ALL.

**Table 2.** Gene mutation status compared between (a) ETP-ALL and non-ETP T-ALL and (b) ETP-ALL and non-ETP early T-ALL

	ETP-ALL (%)	Non-ETP T-ALL (%)	P		ETP-ALL (%)	Non-ETP early T-ALL (%)	P
<b>(a)</b>				<b>(b)</b>			
<i>FBXW7</i> (n = 128)				<i>FBXW7</i> (n = 50)			
wt	14 (100)	98 (86)	0.13	wt	14 (100)	27 (100)	–
mut	0 (0)	16 (14)		mut	0 (0)	0 (0)	
<i>NOTCH1</i> (n = 142)				<i>NOTCH1</i> (n = 50)			
wt	13 (92.9)	50 (39.1)	< 0.001	wt	13 (92.9)	16 (47.1)	0.003
mut	1 (7.1)	78 (60.9)		mut	1 (7.1)	18 (52.9)	
<i>FLT3</i> (ITD) (n = 123)				<i>FLT3</i> (ITD) (n = 40)			
wt	14 (87.5)	107 (100)	< 0.001	wt	14 (75)	24 (100)	0.08
mut	2 (12.5)	0 (0)		mut	2 (25)	0 (0)	
<i>FLT3</i> (D835) (n = 116)				<i>FLT3</i> (D835) (n = 40)			
wt	12 (75)	99 (99)	< 0.001	wt	12 (75)	23 (95.8)	0.05
mut	4 (25)	1 (1)		mut	4 (25)	1 (4.2)	
<i>WT1</i> (n = 227)				<i>WT1</i> (n = 63)			
wt	12 (80)	196 (92.5)	0.09	wt	12 (80)	42 (87.5)	0.47
mut	3 (20)	16 (7.5)		mut	3 (20)	6 (12.5)	

Abbreviations: ETP-ALL, early T-cell precursors-acute lymphoblastic leukemia; ITD, internal tandem duplications; mut, mutant; T-ALL, acute T-lymphoblastic leukemia; wt, wild type. Non-ETP T-ALL includes all thymic, mature and early T-ALL with a non-ETP immunophenotype. Analysis was performed as far as sufficient material was available.



treatment approaches based on the implementation of targeted therapies.

We characterized a new subgroup of adult patients with T-ALL (7.4%) with an immunophenotype of pediatric ETP-ALL.<sup>4</sup> The frequency of ETP-ALL in adult T-ALL was slightly lower than first reported for pediatric ETP-ALL, but in the range of a recent report from the Children's Oncology Group (COG).<sup>35</sup> Similar to the pediatric study, there were no differences in the clinical characteristics between the groups of ETP-ALL and early T-ALL with the exception of a lower frequency of a mediastinal mass in ETP-ALL.

Although many features are in common, there are relevant differences between adult and pediatric T-ALL, especially with respect to treatment. There are no immunophenotype-defined subgroups that are currently used for risk stratification in larger pediatric T-ALL trials.<sup>36,37</sup> In contrast, early T-ALL has been recognized as an immunophenotype-defined subtype with poor prognosis in adult T-ALL<sup>33</sup>, and since 1999, the GMALL study group in accordance with other study groups<sup>38</sup> defines early T-ALL as high-risk T-ALL<sup>39,40</sup> with consecutive recommendation for alloSCT. This led to an improvement in the OS at 5 years of 40%.<sup>7</sup> Therefore, the ETP-ALL as a subgroup of early T-ALL is already regarded as a T-ALL subgroup with poor prognosis requiring intensified therapy. In agreement with pediatric ETP-ALL, adult ETP-ALL showed an unfavorable outcome in our analysis similar to early T-ALL.<sup>7</sup> Interestingly, a high overlap to groups with an unfavorable prognosis, characterized by negativity of CD1 and positivity for CD33, and the group of early T-ALL is evident.<sup>33</sup> These T-ALLs overrepresented in early T-ALL are specifically contained in the subgroup of ETP-ALL.

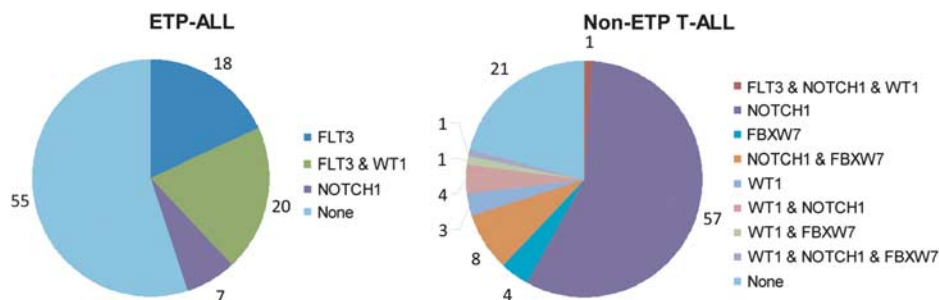
To unravel the underlying molecular alterations of ETP-ALL as a specific T-ALL subgroup, we studied the expression of candidate genes reported to be of prognostic impact. Out of these, the expression of stem cell-associated genes (*BAALC* and *IGFBP7*) and genes known to be of prognostic significance in AML (*BAALC*, *MN1*, *WT1*) are underlining the immature nature of ETP-ALL. *BAALC* is preferentially expressed in the immature T-ALL phenotypes compared with the remaining T-ALL, and its high expression has been correlated to a subgroup with poor outcome.<sup>13</sup> Moreover, T-ALL patients with high *BAALC* expression frequently showed an aberrant expression of myeloid markers. High *BAALC* expression is also linked to an immature phenotype in AML with normal cytogenetics,<sup>41</sup> to a more aggressive BCR-ABL-positive acute lymphoblastic leukemia<sup>42</sup> and is associated with an unfavorable outcome in B-precursor ALL.<sup>43</sup> This led to the hypothesis that *BAALC* is a marker for acute leukemia derived from early progenitors with multi-lineage potential. Recently, *IGFBP7* was identified as a lineage-independent *BAALC* co-expressed gene with prognostic impact and a potential link to *BAALC*.<sup>44</sup> *BAALC* and *IGFBP7* were also found as highly expressed genes within the ETP-ALL gene expression signature.<sup>4</sup> In addition, overexpression of *MN1* was recently identified to be associated with ETP-ALL, and

might also provide an indirect support for the poor prognosis of ETP-ALL.<sup>45</sup> These findings are underlined in this work as *BAALC*, *IGFBP7* and *MN1* were upregulated in ETP-ALL, probably originating from T-cell progenitors retaining myeloid differentiation potential. This multilineage potential is further strengthened by the overexpression of the molecular marker *WT1* in ETP-ALL, a gene known to be of unfavorable prognosis in AML as well as in a subgroup of T-ALL.<sup>46-50</sup> Interestingly, a recent work identified a subgroup in adult T-ALL (~10%) with myeloid characteristics using gene expression profiling with limited clinical data and no mutations or mutational events.<sup>51</sup> Up to which degree this group overlaps with ETP-ALL has to be determined in further studies.

In addition to these particular gene expression patterns, we found a clear difference in mutation events of genes known to be involved in the pathogenesis of T-ALL or AML. The high rate of *NOTCH1* mutations in T-ALL with a frequency of about 50%<sup>26,27,52,53</sup> makes the *NOTCH1* signaling pathway an interesting candidate for targeted therapies by the implementation of  $\alpha$ -secretase inhibitors.<sup>54,55</sup> Whether *NOTCH1* mutations could initiate leukemia alone<sup>56-58</sup> or are mainly secondary effects in human T-ALL<sup>59</sup> is discussed controversially.<sup>60,61</sup> Interestingly, only one *NOTCH1* mutation was found in the small group of analyzed ETP-ALL, thus demonstrating a clear pathogenetic difference to non-ETP T-ALL (61% *NOTCH1* mutations) as ETP-ALL is yet the only subgroup that lacks *NOTCH1* mutations. Additionally, in the group of ETP-ALL, mutations of the less frequent mutated tumor suppressor gene *FBXW7* were not found, again supporting a different pathogenesis for the group of ETP-ALL, likely independent from the activated *NOTCH1* pathway. Therefore, targeted therapies implementing  $\gamma$ -secretase inhibitors would presumably be less effective in ETP-ALL lacking mutational activated *NOTCH1* signaling.

Most remarkably, we found a high rate of *FLT3* mutations in ETP-ALL with a not yet reported high incidence of 37.5%, albeit in a small cohort (16 patients with ETP-ALL). Thus, with respect to the *FLT3*, *NOTCH1*/*FBXW7* and *WT1* mutation status, ETP-ALL shows a clearly different mutational profile compared with non-ETP T-ALL (Figure 3) indicating a distinct biological entity.

With respect to *FLT3* mutations, we found in contrast to AML more frequently TKD than ITD mutations. This finding is in line with data from a murine bone marrow transplantation model, where mice that received a transplant of *FLT3*-ITD-transduced bone marrow cells developed myeloproliferative diseases, whereas a transplant of *FLT3*-TKD-transduced bone marrow cells induced lymphoid disorders.<sup>62</sup> More recently, it was shown that a subset of common myeloid progenitors (*FLT3*<sup>+</sup> *CD150*<sup>-</sup>) has the potential to develop into T cells.<sup>63</sup> Although *FLT3* mutations in T-ALL are generally very rare (1-3%),<sup>30</sup> *FLT3* mutations were proposed to be frequently found in *CD117*<sup>+</sup> T-ALL patients,<sup>32,64</sup> an antigen frequently expressed on ETP-ALL lymphoblasts. Although *FLT3* mutation screening is clinically not indicated in unselected newly diagnosed T-ALL, ETP-ALL as a distinct subgroup



**Figure 3.** Distribution of mutations (*FBXW7*, *NOTCH1*, *FLT3*, *WT1*) in percent in ETP-ALL ( $n = 16$  on the left) and non-ETP T-ALL ( $n = 212$  on the right). Not all T-ALL samples tested for *WT1* were also tested for *NOTCH1* and *FLT3* mutations in the cohort of non-ETP T-ALL.

defined by immunocytology should now be prospectively tested for *FLT3* mutations. Moreover, these findings result in enlarged treatment options for this group with poor outcome<sup>65</sup> including tyrosine kinase inhibitors (TKI), which are currently investigated in trials for the treatment in AML. Importantly, similar to myeloid cells, T-ALL cell lines transfected with an *FLT3* ITD expression construct showed particular sensitivity to tyrosine kinase inhibition further justifying the use of TKI in *FLT3*-mutated ETP-ALL (data not shown). As ETP-ALL in pediatric patients is associated with high minimal residual disease levels after induction therapy,<sup>32</sup> the use of TKI may be efficient to lower the leukemic blast burden before alloSCT in minimal residual disease-positive patients. Furthermore, TKI could be implemented in the treatment of a relapse after alloSCT.

We identified the specific subgroup of ETP-ALL based on the definition of a certain immunophenotype in adult T-ALL. Overall, the outcome of ETP-ALL is comparably poor to early T-ALL. Therefore, intensified treatment protocols including alloSCT, as already implemented in the therapy of adult T-ALL, seems warranted for these high-risk patients. Moreover, as ETP-ALL has distinct molecular features with a high rate of *FLT3* mutations, these data point to new targeted therapy options including TKI for these high-risk T-ALL patients.

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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