NOTCH1 activation clinically antagonizes the unfavorable effect of PTEN inactivation in BFM-treated children with precursor T-cell acute lymphoblastic leukemia

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ABSTRACT

Despite improvements in treatment results for pediatric T-cell acute lymphoblastic leukemia, approximately 20% of patients relapse with dismal prognosis. *PTEN* inactivation and *NOTCH1* activation are known frequent leukemogenic events but their effect on outcome is still controversial. We analyzed the effect of *PTEN* inactivation and its interaction with *NOTCH1* activation on treatment response and long-term outcome in 301 ALL-BFM treated children with T-cell acute lymphoblastic leukemia. We identified *PTEN* mutations in 52 of 301 (17.3%) of patients. In univariate analyses this was significantly associated with increased resistance to induction chemotherapy and a trend towards poor long-term outcome. By contrast, patients with inactivating *PTEN* and activating *NOTCH1* mutations showed marked sensitivity to induction treatment and excellent long-term outcome, which was similar to patients with *NOTCH1* mutations only, and more favorable than in patients with PTEN mutations only. Notably, in the subgroup of patients with a prednisone- and minimal residual disease (MRD)-response based medium risk profile, *PTEN*-mutations without co-existing *NOTCH1*-mutations represented an MRD-independent highly significant high-risk biomarker. Mutations of *PTEN* highly significantly indicate a poor prognosis in T-ALL patients who have been stratified to the medium risk group of the BFM-protocol. This effect is clinically neutralized by *NOTCH1* mutations. Although these results have not yet been explained by an obvious molecular mechanism, they contribute to the development of new molecularly defined stratification algorithms. Furthermore, these data have unexpected potential implications for the development of *NOTCH1* inhibitors in the treatment of T-cell acute lymphoblastic leukemia in general, and in those with a combination of *PTEN* and *NOTCH1* mutations in particular.

Introduction

Despite recent advances in the treatment of pediatric precursor T-cell acute lymphoblastic leukemia (T-ALL),[1] this entity still remains a challenge because relapses carry a particularly poor prognosis.[2,3] Conceptually, it would, therefore, be helpful to develop a molecular risk profile, which would enable stratification of patients early after diagnosis.[4] It is known that the differentiation stage of the T-ALL clone[5-7] or the activation of defined leukemogenic pathways[8] may play a role in prognosis. Furthermore, in patients treated on the ALL-BFM 2000 protocol, we have shown that the activation of the *NOTCH1* receptor pathway signifies a favorable prognosis,[9,10] although in the context of other protocols this effect was not seen.[11,12]

In addition, inactivating mutations of the tumor suppressor phosphatase and tensin homolog (*PTEN*) are known to occur in a variety of tumors and to disturb signaling networks including the PI3K-AKT pathway. Activation of the PI3K-AKT pathway is known to play a particular role in T-ALL.[8,13] The loss of *PTEN* function thus represents a candidate mechanism to modulate the aggressiveness of T-ALL. Indeed, deletions and point mutations but also posttranslational mechanisms of *PTEN* inactivation, have previously been associated with poor treatment response in some studies with a small number of patients, although this has not been found in other studies.[13-17] The clinical effect of oncogene activation and tumor suppressor gene inactivation can depend on the treatment strategy.[11,12,18,19] Therefore, we analyzed the incidence of *PTEN* point mutations and the effect of *PTEN* inactivation on clinical outcome in what is the largest cohort so far of 301 children with T-ALL who were treated on the ALL-BFM 2000 protocol.

Studies in cell lines have revealed that *NOTCH1* can inhibit *PTEN* function via the transcriptional inhibitor *HES1*.[20,21] We have, therefore, analyzed the clinical interaction of *PTEN* inactivation and *NOTCH1* pathway activation. We show in univariate analyses that *PTEN* inactivated leukemias tend to be resistant to induction treatment and that affected patients show an unfavorable long-term outcome. When combined

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with *NOTCH1*-mutation status, *PTEN* mutated and *NOTCH1* non-mutated patients show a significantly inferior outcome than the remaining cohort. Interestingly, this unfavorable effect of *PTEN* inactivation is counterbalanced clinically by the simultaneous presence of activating *NOTCH1* mutations. These data have unexpected and potentially profound implications for the development of future treatment and stratification strategies in general, and for the use of *NOTCH1* inhibitors in particular.

Design and Methods

Patients' clinical characteristics

From August 1999 through February 2008, a total of 545 patients with T-ALL were eligible for treatment in the multicenter ALL-BFM 2000 trial; no non-Hodgkin's lymphoma patients were included. This study was approved by the institutional review board of the Hannover Medical School and other participating institutions. Informed consent was obtained in accordance with the Declaration of Helsinki. This trial enrolled pediatric patients up to 18 years of age from 70 different treatment centers in Germany, Austria and Switzerland. The subjects were selected on the basis of availability of sufficient amounts of DNA for molecular analysis. There was no significant difference in clinical parameters (age, gender, white blood cell count at diagnosis, prednisone response, MRD at Day 78) between this subgroup of patients and the entire ALL-BFM 2000 cohort.[1]

Mononuclear cells were isolated from bone marrow (BM) samples and stored in liquid nitrogen or at -80°C until DNA extraction. All BM samples contained a blast percentage of 80% or more. Immunophenotyping was carried out as previously described,[22] and the subclassification of T-ALL was performed according to the guidelines of the European Group for Immunological Characterization of Leukemias (EGIL).[23]

Early *in vivo* response to prednisone, defined as the cytoreduction to a 7-day prednisone treatment prophase and a single dose of intrathecal methotrexate on Day 1, served to assess the effect of early treatment.[24] According to prednisone response, patients were classified into good responders (PGR: <1000 blasts/microliter at Day 8) or poor responders (PPR: ≥1000 blasts/microliter at Day 8). Treatment response was further defined by determination of minimal residual disease (MRD) kinetics that were assessed at 2 different time points: at Days 33 and 78 of treatment, respectively.[25-28] Allele-specific oligonucleotide-polymerase chain reaction (PCR) protocols were used for quantitative detection of leukemic clone-specific immunoglobulin and T-cell receptor gene rearrangements on a LightCycler instrument (Roche Diagnostics, Mannheim, Germany).[29] An unfavorable MRD status (≥10^{-4}) was defined by the presence of at least one leukemic cell in 10^4 cells, whereas a favorable MRD status (< 10^{-4}) was defined as the absence of detectable leukemic cells in 10^4 cells.[9] For treatment stratification, the BFM-ALL protocol distinguishes the standard risk group (negative MRD on Days 33 and 78), the high-risk group (MRD at least one leukemic cell in 10^3 cells on Day 78) and the intermediate-risk group (all others). Complete remission (CR) was defined as less than 5% blasts in the regenerating BM, the absence of leukemic blasts in the peripheral blood and cerebrospinal fluid, and no evidence of localized disease. Relapse was defined as recurrence of lymphoblasts or localized leukemic infiltrates at any site.

Mutational analysis of diagnostic samples for PTEN and NOTCH1 mutations

PCR amplification of exon 7 of *PTEN* genes was performed with primary genomic DNA. The analysis of all other exons was performed on genomic DNA after whole genome amplification (primer sequences are shown in *Online Supplementary Table S1*). The mutations that have been identified were confirmed in primary DNA. Sequencing of *NOTCH1* has been performed as described previously.[10] PCR-amplified fragments were sequenced by GATC biotech (Konstanz, Germany) and analyzed by mutation surveyor software for identification of mutations and cross checked manually.

Statistical analysis

Event-free survival (EFS) was defined as the time from diagnosis to the date of last follow up in complete remission or first event. Events were resistance to therapy (non-response), relapse, secondary neoplasm (SN), or death from any cause. Failure to achieve remission due to early death or non-response was considered as events at time zero. Survival was defined as the time of diagnosis to death from any cause or last follow up. The Kaplan-Meier method was used to estimate survival rates, differences were compared with the two-sided log rank test. Cox's proportional hazards model was used for uni- and multivariate analyses. Cumulative incidence (CI) functions for competing events were constructed by the method of Kalbfleisch and Prentice, and were compared with the Gray's test. Results are presented as estimated probability of 5-year EFS (pEFS) and estimated cumulative incidence of relapse (pCIR) with standard error (± SE). Differences in the distribution of individual parameters among patient subsets were analyzed using Fisher's exact test for categorized variables and the Mann-Whitney-U test for continuous variables. Logistical regression was used to analyze the effect of mutations on response variables (prednisone response, MRD). All statistical analyses were conducted using the SAS program (SAS-PC, v. 9.1, SAS Institute Inc., Cary, NC, USA).

Results

The clinical and immunological characteristics of the patient cohort analyzed here is comparable to that of the entire cohort of T-ALL patients included in the BFM-ALL 2000 study (Table 1).[1] *PTEN* mutations have previously been identified in approximately 20% of children with T-ALL and reported to signify a poor treatment response in studies with small patient numbers.[13-16] In the cohort of 301 patients studied here, we found a total of 58 mutations in 52 patients (17.3%). The majority (52 of 58) of the mutations were detected in the mutational hotspot in exon 7, which is consistent with previously reported findings.[14] The other mutations were detected in exons 1, 4 and 5 (*Online Supplementary Table S2*). Four patients had 2 mutations either both in exon 7 or in exons 4 and 7 or 5 and 7, respectively. One patient had 3 mutations: 2 in exon 7 and one in exon 1. In these patients, the diagnostic strategy we used did not allow the distinction between bialleleic compound or monoallelic double mutations to be made. In a previous study, we had also found large deletion mutations in 4 of 72 patients for whom sufficient DNA for array-CGH analyses were available (including 44 from the cohort analyzed here). Three of these patients (one with a homozygous and 2 with a heterozygous deletion) showed a poor early treatment response, whereas one patient with a heterozygous deletion showed a favorable early treatment response.[8] Because the array-CGH analyses could only be performed in a subset of the entire cohort, we did not include these previously reported data in the statistical analyses of this report. However, the

observed poor treatment response in 3 of these 4 patients is consistent with the clinical effect of *PTEN* point mutations in the entire cohort (see below).

All mutations identified in the current study were small deletions or insertions which resulted either in nonsense mutations or frameshift mutations with downstream premature stop codons (48 insertions of up to 13 nucleotides, 8 deletions of up to 17 nucleotides) (*Online Supplementary Table S2*). The affected mRNAs may thus be targets of nonsense mediated decay quality control thus limiting the total expression of the encoded proteins[30,31] or may code for inactive C-terminally truncated proteins.[15] The presence of these mutations was significantly associated with the absence of activating *NOTCH1* mutations. There was no significant correlation with patient age, gender, white blood cell count at the time of diagnosis, or T-cell immunophenotype (Table 1).

We next analyzed the influence of *PTEN* mutations on early treatment response and long-term outcome. Prednisone response was available for all the 52 patients with *PTEN* mutations and for 242 of 249 patients without *PTEN* mutations. Patients with *PTEN* mutations showed a poor prednisone response significantly more frequently than those patients without *PTEN* mutations (P=0.007), with an odds ratio in the univariate analysis of 2.4 (95%CI: 1.3-4.4, P=0.006; Table 2). Furthermore, in a multivariate analysis including variables known to be associated with prednisone response (gender, age at diagnosis, presenting WBC count at diagnosis and T-cell immunophenotype), the negative effect of *PTEN* mutations retained its significant effect (odds ratio 2.6, 95%CI: 1.3-5.2, P=0.005; Table 2).

MRD data on Day 33 were available for 272 patients (47 *PTEN* mutated, 225 *PTEN* non-mutated) and in 274 patients at the end of the induction phase on Day 78 (46 *PTEN* mutated and 228 *PTEN* non-mutated). On Day 33, only 6% of the patients with *PTEN* mutations showed a favorable MRD response as compared to 29% of *PTEN* non-mutated patients (P=0.0007). On Day 78, 43% of the

Table 1. Clinical and immunological characteristics of the study cohort of 301 children with T-ALL.

Characteristic	PTEN Mutation			
	All (% of the total cohort)	Mutated (%)	Non-mutated (%)	P*
Gender				
Male	224(74)	44(20)	180(80)	P=0.08
Female	77 (26)	8(10)	69(90)	
Age at diagnosis				
< 10 years	159 (53)	21(13)	138(87)	P=0.06
≤10 years	142 (47)	31(22)	111(78)	
Presenting WBC count at diagnosis				
< than $10x10^9$/L	42 (14)	7(17)	35(83)	P=0.3
$10x10^9$/L < $50x10^9$/L	83 (28)	12(15)	70((85)	
$50x10^9$/L < $100x10^9$/L	58 (19)	7(12)	51(88)	
≥$100x10^9$/L	118 (39)	26(22)	93(78)	
T-cell immunophenotype**				
Pro	11 (4)	2(18)	9(82)	P=0.8
Pre	50 (17)	8(16)	42(84)	
Cortical	197 (65)	33(17)	164(83)	
Mature	38 (13)	9(24)	29(76)	
T not further classified	5 (2)	0 (0)	5(100)	
NOTCH1 mutations				
Mutated	150 (50)	19 (13)	131 (87)	P=0.05
Non-mutated	151 (50)	33 (22)	118 (78)	
MRD-risk classification				
High risk	53 (18)	14 (26)	41 (74)	P=0.06
Medium risk	154 (51)	28 (18)	126 (82)	
Standard risk	44 (14)	3 (6)	39 (94)	
Data not available	50 (17)	7 (14)	43 (86)	
Total-risk classification***				P=0.004
High risk	133 (44)	33 (25)	100 (75)	
Medium risk	126 (42)	17 (26)	109 (74)	
Standard risk	42 (14)	2 (5)	40 (95)	
Data not available	0	0	0	

*For PTEN mutated patients, n = 52; for PTEN non-mutated patients, n = 249. WBC: white blood cell. *P χ^2 test. **Pro (cyCD3$^+$, CD7$^+$) Pre (cyCD3$^+$, CD2$^+$ and/or CD5$^+$ and/or CD8$^+$), cortical (CD1a$^+$), mature (CD1a-, sCD3$^+$). cyCD3$^+$: cytoplasmic CD3$^+$; sCD3$^+$: surface CD3$^+$. ***The total risk classification combines prednisone response and MRD data.*

Table 2. Effect of inactivating PTEN mutations on early treatment response in ALL-BF M 2000 treated children with T-ALL.

Prednisone response (Day 8)‡	PPR(%)	PGR(%)	Data not available	P*	Univariate RR (95%CI)	P*	Multivariate RR† (95%CI)	P*
	119	175	7					
PTEN mutated	30(58)	22(42)	0	0.007	1.00§	0.006	1.00§	0.005
PTEN non-mutated	89(37)	153(63)	7		2.4(1.3-4.4)		2.6(1.3-5.2)	

MRD (Day 33)	Unfavorable, ≥10-4 (%)	Favorable, <10-4 (%)	Data not available		Univariate or (95%CI)	P*	Multivariate or (95%CI)	P*
	204	68	29					
PTEN mutated	44(94)	3(6)	5		1.00§	0.003	1.00§	0.001
PTEN non-mutated	160(71)	65(29)	24	0.0007	9.2(2.2-39.1)		11.0(2.5-48.5)	

MRD (Day 78)								
	115	159	27					
PTEN mutated	26(57)	20(43)	6		1.00§	0.02	1.00§	0.05
PTEN non-mutated	89(39)	139(61)	21	0.03	2.1(1.1-4.0)		2.0(1-4.1)	

*For PTEN mutated patients, n = 52; for PTEN non-mutated patients, n = 249. RR: relative risk; CI; confidence interval; MRD: minimal residual disease. *P χ^2 test. †Adjusted for gender, age at diagnosis ‡PPR (prednisone poor response), ≥ 1000 leukemic blasts/mL of peripheral blood, PGR (prednisone good response), <1000 leukemic blasts/mL of peripheral blood on treatment Day 8. §Reference category.*

patients with a *PTEN* mutation achieved a favorable MRD response compared to 61% in the *PTEN* non-mutated group (*P*=0.03; Table 2). Logistical regression analysis showed that patients with *PTEN* mutations carried a 9.2-fold higher of not achieving a favorable MRD level on Day 33 (95%CI: 2.2-39.1; *P*=0.003) and a 2.1-fold higher risk on Day 78 (95%CI: 1.1-4.0; *P*=0.02), respectively. In a multivariate analysis with variables known to be associated with treatment response (gender, age at diagnosis, presenting WBC count at diagnosis and T-cell immunophenotype), the negative effect of *PTEN* mutation retained its significant effect on Day 33 (odds ratio 11.0, 95%CI: 2.5-48.5; *P*=0.001) and on Day 78 (odds ratio 2.0, 95%CI: 1.0-4.1; *P*=0.05) (Table 2). These effects resulted in *PTEN* mutated patients to be stratified into the high risk group significantly more frequently. Four out of the 5 patients with 2 or 3 *PTEN* mutations showed an unfavorable early treatment response.

These differences between *PTEN* mutated and non-mutated patients in early treatment response were maintained as a trend towards an inferior pEFS of 0.72 *vs*. 0.82 (*P*=0.11, Figure 1A). There was also a slight trend towards a higher pCIR in *PTEN* mutated patients of 0.17 when compared to the pCIR of 0.11 observed in non-mutated patients (*P*=0.34, Figure 1B).

The unfavorable effect of inactivating PTEN *mutations is clinically neutralized by activating* NOTCH1 *receptor mutations*

We have previously shown that activating *NOTCH1* mutations are associated with a favorable early treatment response and long-term outcome in the same cohort of ALL-BFM 2000 treated patients who were analyzed here.[9,10] The *NOTCH1*-downstream target *HES1* is a negative transcriptional regulator of *PTEN* and thus indirectly stimulates the PI3K-AKT pathway,[13] which may ultimately lead to a clinical synergism between activating *NOTCH1* and inactivating *PTEN* mutations. We tested this hypothesis, by analyzing the clinical interaction of *PTEN* and *NOTCH1* mutations. We thus grouped the patients according to their *PTEN* and *NOTCH1* mutational status and compared their early treatment response and long-term outcome. In 19 of the 52 leukemias with *PTEN* mutations, concomitant activating *NOTCH1* mutations, either in the heterodimerization or in the PEST domains, were identified. The *PTEN* and *NOTCH1* mutation status was available in 294 patients with known prednisone response, in 272 patients with known MRD level on Day 33, and 274 patients with known MRD levels on Day 78 (Figure 2). For prednisone response and for Day 33 MRD, the unfavorable effect of *PTEN* mutations was maintained regardless of the presence of *NOTCH1* mutations. Furthermore, the presence of *PTEN* mutations neutralized the known favorable effect of *NOTCH1* mutations[9,10] because patients with both mutations show a similar poor early treatment response to those patients with a *PTEN* mutation only (Figure 2A and B). These data on early treatment response are thus consistent with a dominant clinical effect of *PTEN* inactivation over *NOTCH1* activation but do not support the notion of a clinically relevant synergism. By contrast, at the end of induction on Day 78, favorable MRD responses were observed most commonly in the *NOTCH1*-mutated groups regardless of the presence of *PTEN* mutations, whereas the group with *PTEN* mutations only was the least favorable, and the group with neither mutation was intermediate (Figure 2C). Therefore, at the end of induction the favorable effect of *NOTCH1* receptor activation was clinically dominant over the unfavorable effect of *PTEN* inactivation.

We next performed a subgroup analysis of long-term outcome of the four possible *PTEN/NOTCH1* combinations. The subgroup with *PTEN* mutations but no *NOTCH1* mutations showed a significantly lower pEFS (0.62±0.09) than the rest of the cohort (pEFS 0.83±0.02; *P*=0.005) (Figure 3A) and a strong trend for a higher pCIR (0.23 *vs.* 0.11; *P*=0.07) (Figure 3B). Subgroups with *NOTCH1* mutations, with or without *PTEN* mutations, were the most favorable (pEFS 0.87 and 0.89; pCIR 0.06 and 0.07) (Figure 4A), whereas the group with neither mutation was intermediate (pEFS 0.77, pCIR 0.16) (Figure 4B). Notably, subgroup analyses showed that the unfavorable *PTEN*-effect was restricted to the medium risk group with a good prednisone response and an intermediate MRD-response. The 14 patients with *PTEN*-mutations in

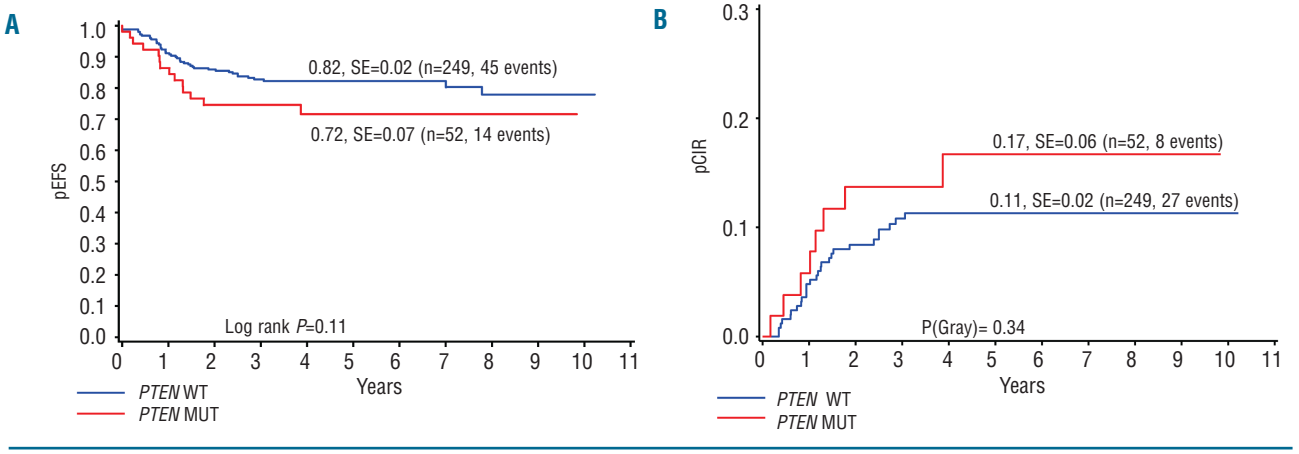

Figure 1. Univariate analysis shows a trend towards poor long-term outcome in children with T-ALL. Kaplan-Meier estimate of pEFS (A) and pCIR (B) in *PTEN* mutated and *PTEN*-non-mutated patients treated on the ALL-BFM 2000 protocol

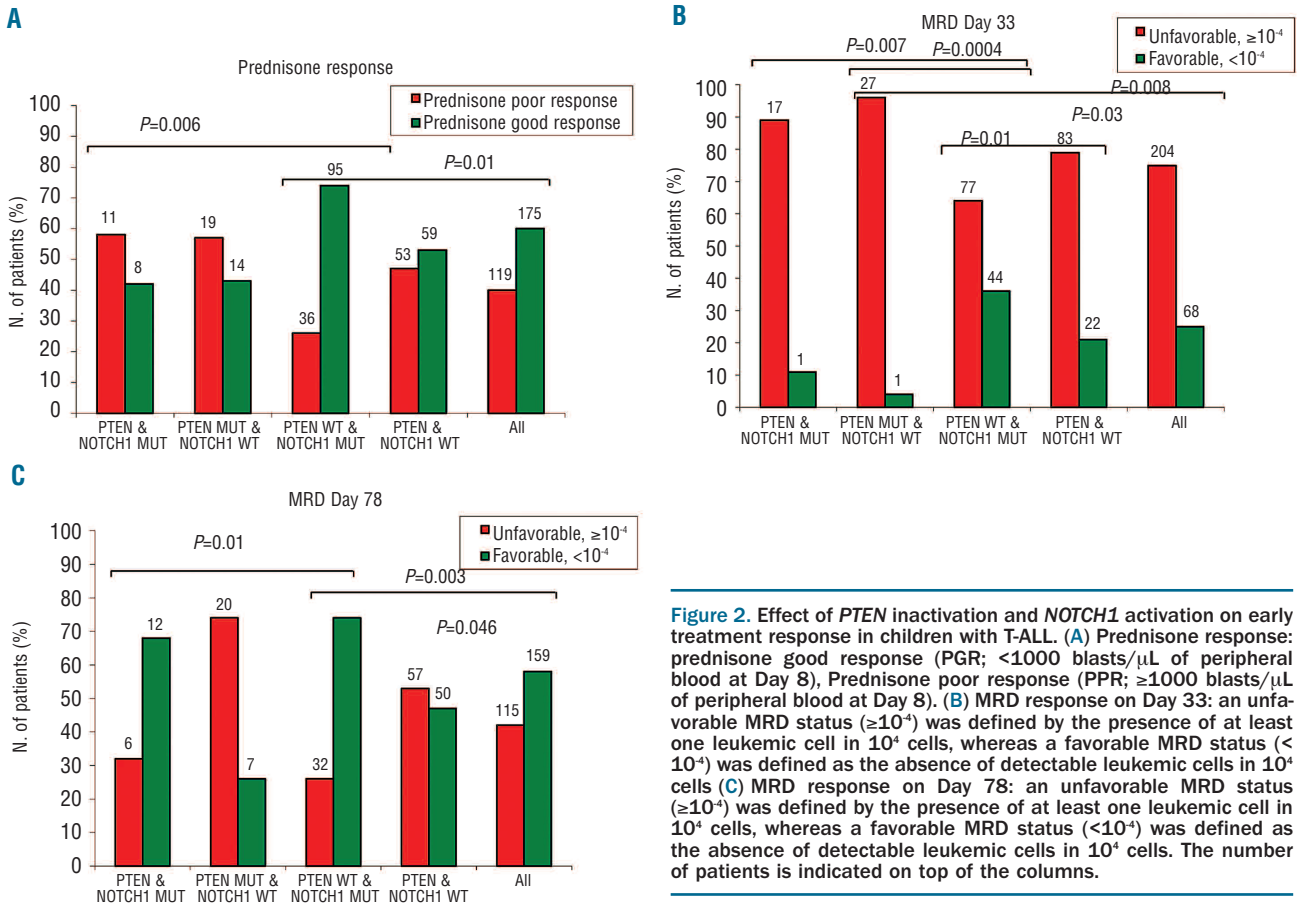

Figure 2. Effect of *PTEN* inactivation and *NOTCH1* activation on early treatment response in children with T-ALL. (A) Prednisone response: prednisone good response (PGR; <1000 blasts/μL of peripheral blood at Day 8), Prednisone poor response (PPR; ≥1000 blasts/μL of peripheral blood at Day 8). (B) MRD response on Day 33: an unfavorable MRD status (≥10^{-4}) was defined by the presence of at least one leukemic cell in 10^4 cells, whereas a favorable MRD status (<10^{-4}) was defined as the absence of detectable leukemic cells in 10^4 cells (C) MRD response on Day 78: an unfavorable MRD status (≥10^{-4}) was defined by the presence of at least one leukemic cell in 10^4 cells, whereas a favorable MRD status (<10^{-4}) was defined as the absence of detectable leukemic cells in 10^4 cells. The number of patients is indicated on top of the columns.

this group (total n=154 patients), showed a highly significantly worse outcome (pEFS 0.51) than those with a *NOTCH1* but no *PTEN*-mutation (n=85; pEFS 0.89; *P*=0.0008) or with neither a *NOTCH* nor a *PTEN* mutation (n=55; pEFS 0.88; *P*=0.0026) (Figure 4C). By contrast, there were no significant differences between the subgroups in the conventionally defined high-risk group (total n=53 patients) including 12 patients with a *PTEN* mutation only (Figure 4D). Given the small size of the subgroups, the direct comparison of the difference in outcome between the *PTEN*-only mutated patients in either the HR- or the MR-group was not statistically significant. In the standard risk group (total n=43 patients), there was only one of the 6 patients with a *PTEN*-mutation and no *NOTCH1*-mutation with a good prednisone response and negative MRD findings on Days 33 and 78 of induction.

Taken together, these results define a subgroup of approximately 10% of the large and important medium-risk group with a poor prognosis and reveal a clinical activity of *NOTCH1*, which neutralizes the negative effect of *PTEN* inactivating mutations in this subgroup of children with T-ALL treated on the ALL-BFM 2000 protocol.

Discussion

Signaling networks including the PI3K/AKT pathway control a variety of cellular functions including differentiation, cell growth, metabolism and differentiation.[32] As one of its functions, the phosphatase *PTEN* counterbalances the stimulatory effect of PI3K by dephosphorylating PIP3 and thus functions as a key negative regulator of this pathway.[33] In addition to the other pathways that are involved in leukemogenesis, dysregulation of this pathway represents one of the most common events in tumorigenesis in general,[34] and has also been identified to play an important part in T-ALL in particular.[8,35-37] In T-ALL, both large deletions and inactivating small deletions and small insertions have been identified in a subset of approximately 15%-20% of patients.[8,13-16] In addition, posttranslational modifications such as phosphorylation and oxidation have been described to also limit *PTEN* function in T-ALL.[16] The effect of *PTEN* inactivation on clinical outcome has been reported in studies with smaller numbers of patients and shown to be variable and possibly dependent on the type of mutation.[8,14,15,17] It is one of the important results of this study that in a large and clinically well-defined cohort of patients, inactivation of *PTEN* is not only associated with early treatment resistance but also with a poor long-term prognosis. This differs from previously reported results showing no prognostic effect of *PTEN* mutations.[14,17] This difference may be explained by the modulating effect of *NOTCH1* activation reported here and also by differences in treatment protocols. Differences in treatment have previously been suggested to play an important role in defining the effect of molecular risk factors in T-ALL.[10] More specifically, the differences between the outcome of patients with *PTEN*-

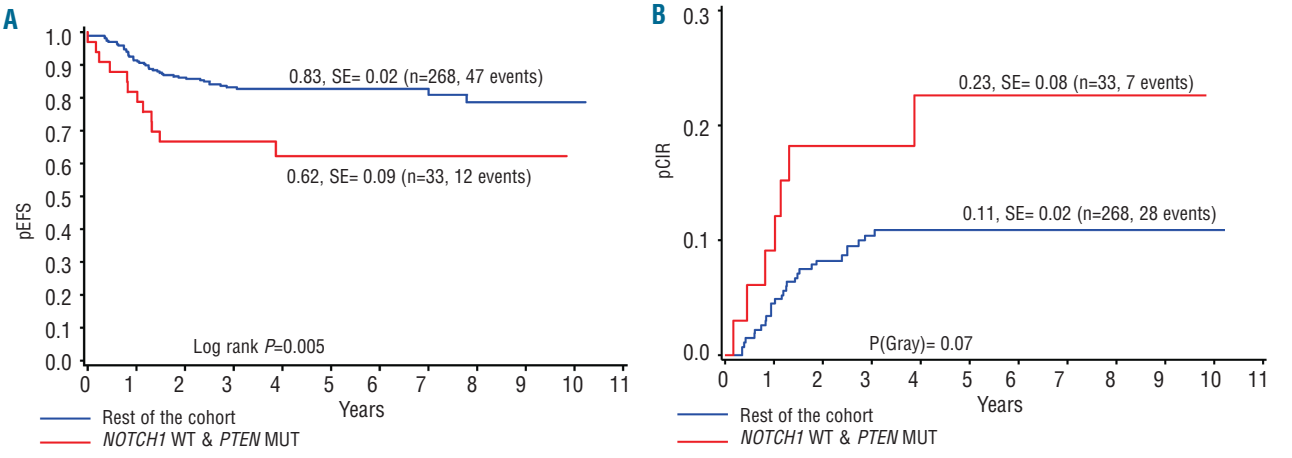

Figure 3. ***NOTCH1*** **activation neutralizes the unfavorable effect of** ***PTEN*** **inactivation on long-term outcome in children with T-ALL. Kaplan-Meier estimate of pEFS (A) and pCIR (B) in** ***PTEN*** **mutated and** ***NOTCH1*** **non-mutated patients compared to the rest of the cohort.**

mutations but no *NOTCH1* mutations, who have either been treated in the high-risk or in the medium-risk arm of the BFM protocol suggest (with the limitation of small sizes of the subgroups) that the unfavorable effect of *PTEN* inactivation may potentially be neutralized by more intensive treatment (Figure 4). The *NOTCH1* pathway is a key regulator of blood cell differentiation. While the importance of *NOTCH1* activity in T-cell development has been known for some time,[38-40] *NOTCH* has recently emerged to also function in myeloid, megakaryocyte and erythroid development.[41-43] Gain of *NOTCH1* function is a hallmark of 50%-60% of children with T-ALL,[9,44] and is thought to function as an initiating event and a progression factor in T-ALL.[45-47] Therefore, one might have predicted that activating *NOTCH1* mutations should have an unfavorable effect on treatment response and long-term outcome. Interestingly, however, the clinical association of *NOTCH1* activating mutations with treatment outcome is dependent on the protocol used. In ALL-BFM 2000 type protocols that are used in central Europe, Scandinavia and Japan, *NOTCH1* gain of function is associated with a favorable effect,[48-50] whereas in other protocols, either no effect or the expected unfavorable effect was observed.[11,12] On the level of cell biology, the *NOTCH* and the PI3K/AKT pathways are known to interact in more complex signaling networks, which is highlighted by one of the major downstream targets of *NOTCH1*, the T-cell lymphomagenesis potentiating protein, *HES1*.[51] *HES1* is known to down-regulate *PTEN* activity and thus to activate the PI3K/AKT pathway.[13] This poses the interesting question of how the two signaling pathways interact in clinical terms. The second important finding in the large cohort of patients analyzed here is that early during induction the unfavorable association of *PTEN* loss of function with response dominates over that of *NOTCH1* gain of function and that this effect is reversed later. The MRD response at the end of induction and the long-term outcome of patients with both, *NOTCH1* activating and *PTEN* inactivating mutations are indistinguishably favorable when compared to patients with *NOTCH1* mutations only. By contrast, patients with *PTEN* mutations but without *NOTCH1* mutations in the conventionally defined medium-risk group represent a particularly unfavorable subgroup. Taken together, these data demonstrate that *NOTCH1* activation and *PTEN* inactivation do not synergize on a clinical level in ALL-BFM 2000 treated patients. In contrast, when considering long-term outcome, *NOTCH1* activation is associated with a neutralizing activity of the unfavorable effect of *PTEN* inactivation. The clinical association between *NOTCH1* activation, *PTEN* inactivation and clinical outcome is surprising in the light of experimental data obtained from an analysis of cultured *NOTCH1* activated cell lines and an analysis of the cell lines in xenotransplanted mice, which indicated a molecular synergism between *NOTCH1* activation and *PTEN* inactivation.[52] The discrepancy between the clinical association and molecular findings can be explained either by an unknown molecular factor that is associated with the presence of *NOTCH1* mutations in primary patient samples but not in cell lines, or by a complex influence of the treatment given on the clinical effect of activated oncogenic networks. Further experimental studies will have to address this important enigma, for example in xenotransplanted primary T-ALL, to shed light on the mechanistically unexplained variable effects of *NOTCH1* activation in the context of different treatment protocols.[10-12]

On a more practical level, the data presented here identify *PTEN*-mutated leukemias without activating *NOTCH1* mutations as a subgroup with a particularly unfavorable prognosis with a pEFS of only 0.62. By contrast, T-ALLs with activating *NOTCH1* mutations, with or without *PTEN* mutations, show a favorable prognosis with a pEFS of 0.87 and 0.89, respectively. Interestingly, subgroup analyses showed that the effect of *PTEN* mutations in patients without *NOTCH1* mutations is restricted to patients who have been stratified to the medium-risk group (pEFS 0.51) and have not received intensified high-risk treatment. This combination of biomarkers thus defines a subgroup of patients with an unfavorable prognosis who may benefit from a new molecular stratification algorithm in future T-ALL protocols and from treatment intensification.

Interestingly, the effect of *PTEN* mutations is particular-

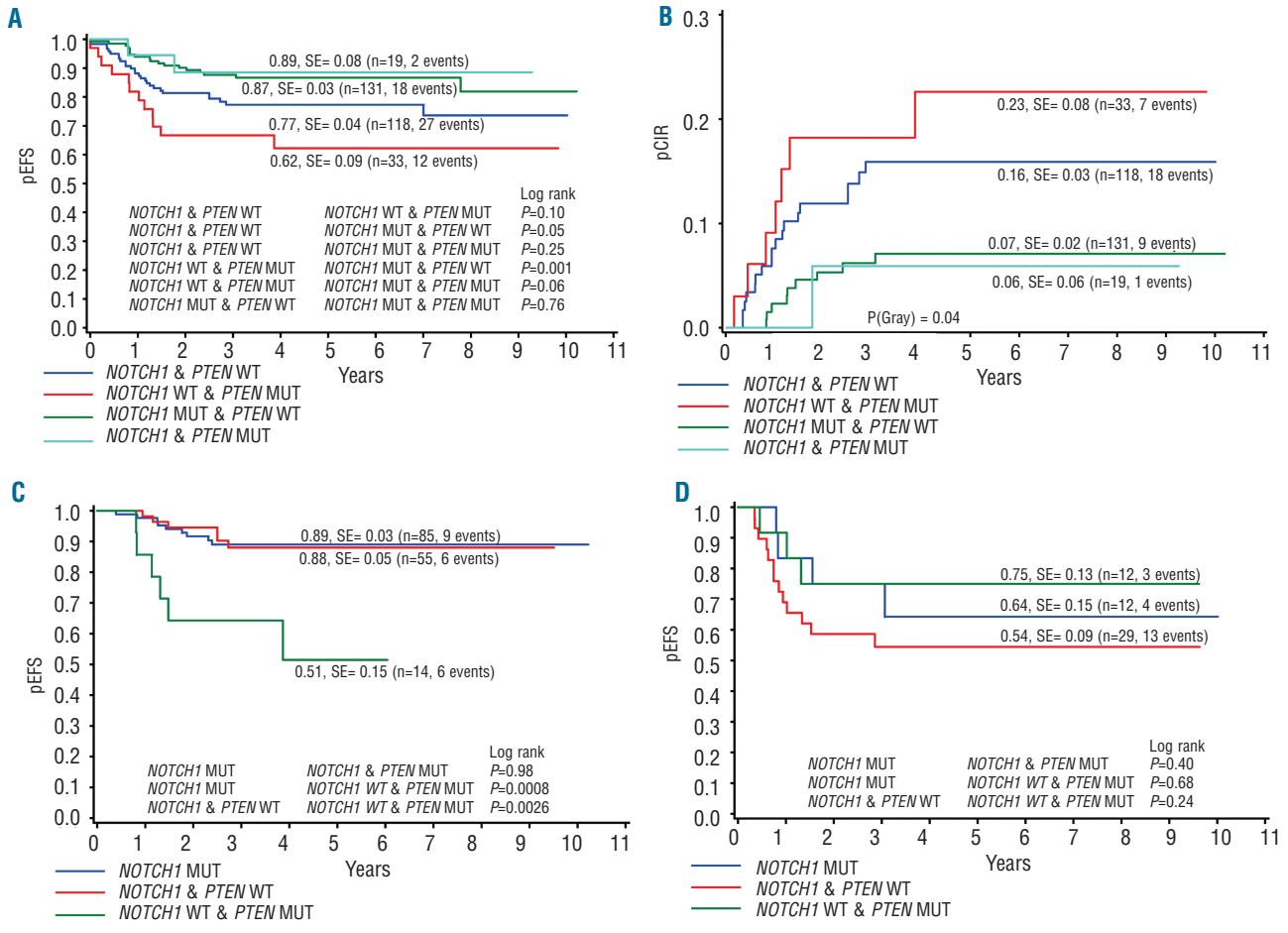

Figure 4. ***PTEN*** **only mutated patients in the BFM-2000 medium-risk group show the worst long-term outcome. (A) Kaplan-Meier estimate of pEFS in the four different combinations of** ***PTEN***, ***NOTCH1*** **genotypes (total cohort n=301). (B) pCIR in the four different combinations of** ***PTEN***, ***NOTCH1*** **genotypes (total cohort n=301) (C) Kaplan-Meier estimate of pEFS in** ***NOTCH1*** **and** ***PTEN*** **mutated,** ***NOTCH1*** **and** ***PTEN*** **non-mutated, and** ***NOTCH1*** **non-mutated and** ***PTEN*** **mutated patients stratified into the medium risk group (n=154) (D) Kaplan-Meier estimate of pEFS in** ***NOTCH1*** **and*PTEN*** **mutated,** ***NOTCH1*** **and** ***PTEN*** **non-mutated, and** ***NOTCH1*** **non-mutated and** ***PTEN*** **mutated patients stratified into the high-risk group (n=53).**

ly evident on Day 8 when prednisone response is assessed and on Day 33 MRD. By contrast, the neutralizing effect of *NOTCH1* mutations becomes apparent on Day 78 MRD and in long-term survival. These observations suggest that drugs introduced between Days 33 and 78 of the protocol (cyclophosphamide, 6-mercaptopurine, cytarabin) may be particularly important in mediating the differences of treatment response between early and later time points. These data also suggest that it may be beneficial to introduce these drugs earlier during induction.

Finally, the data presented here have unexpected and potentially profound implications for the development of *NOTCH1* inhibitors for clinical use in T-ALL. Based on the role of *NOTCH1* activity in T-ALL leukemogenesis[53,54] and the discovery of the common occurrence of activating *NOTCH1* mutations in T-ALL,[44] the therapeutic inhibition of the *NOTCH1* pathway has been a compelling perspective.[55] The data of this study suggest that some patients, and notably those with a combination of *PTEN* inactivation and *NOTCH1* activation, may not benefit from the use of *NOTCH1* inhibitors in multimodal treatment regimens, although the mechanism of the unexpected clinical interactions between *NOTCH1* activation and *PTEN* inactivation remains to be explained.

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