

ORIGINAL ARTICLE

The favorable effect of activating *NOTCH1* receptor mutations on long-term outcome in T-ALL patients treated on the ALL-BFM 2000 protocol can be separated from *FBXW7* loss of functionC Kox^{1,2,3}, M Zimmermann⁴, M Stanulla⁵, S Leible¹, M Schrappe⁵, W-D Ludwig⁶, R Koehler⁷, G Tolle¹, OR Bandapalli^{1,2}, S Breit¹, MU Muckenthaler^{1,2} and AE Kulozik^{1,2}¹Department of Pediatric Oncology, Hematology and Immunology, Angelika Lautenschläger Children's Hospital, University of Heidelberg, Heidelberg, Germany; ²Molecular Medicine Partnership Unit, Heidelberg, Germany; ³European Molecular Biology Laboratory, Heidelberg, Germany; ⁴Department of Pediatric Hematology and Oncology, MH Hannover, Hannover, Germany; ⁵Department of Pediatrics, University Medical Center Schleswig-Holstein, Kiel, Germany; ⁶Department of Hematology, Oncology and Tumorimmunology HELIOS Klinikum Berlin-Buch, Berlin, Germany and ⁷Institute of Human Genetics, University of Heidelberg, Heidelberg, Germany

Precursor T-cell acute lymphoblastic leukemia (T-ALL) remains an important challenge in pediatric oncology. Because of the particularly poor prognosis of relapses, it is vital to identify molecular risk factors allowing early and effective treatment stratification. Activating *NOTCH1* mutations signify a favorable prognosis in patients treated on ALL-BFM protocols. We have now tested if *NOTCH* pathway activation at different steps has similar clinical effects and if multiple mutations in this pathway function synergistically. Analysis of a validation set of 151 T-ALL patients and of the total cohort of 301 patients confirms the low relapse rate generally and the overall favorable effect of activating *NOTCH1* mutations. Subgroup analysis shows that the *NOTCH1* effect in ALL-BFM is restricted to patients with rapid early treatment response. Inactivation of the ubiquitin ligase *FBXW7* is associated with rapid early treatment response and synergizes with *NOTCH1* receptor activation. However, the effect of *FBXW7* inactivation is separable from *NOTCH1* activation by not synergizing with *NOTCH1* mutations in predicting favorable long-term outcome, which can probably be explained by the interaction of *FBXW7* with other clients. Finally, the comparison with other European protocols suggests that the *NOTCH* effect is treatment dependent generally and may depend on the intensity of central nervous system-directed therapy specifically.

Leukemia (2010) 24, 2005–2013; doi:10.1038/leu.2010.203;
published online 14 October 2010

Keywords: T-ALL; *NOTCH1*; *FBXW7*; ALL-BFM

Introduction

Acute lymphoblastic leukemia (ALL) represents the most common malignant disease in children. Precursor T-cell ALL (T-ALL) accounts for ~10–15% of pediatric leukemias.¹ Although the prognosis of ALL in children, in general and of T-ALL in particular, has dramatically improved in recent years, the prognosis of relapsed T-ALL remains dismally poor.² Therefore, it is important to distinguish patients with a high risk (HR) of relapse and who likely benefit from more intensive treatment from those patients with a favorable risk profile

in whom treatment intensity may potentially be reduced.¹ In the ALL-Berlin-Frankfurt-Münster (BFM) 2000 protocol, the response to early induction therapy is assessed by measuring the prednisone response after the first week of treatment.³ In patients with a poor prednisone response treatment is intensified, which has markedly improved treatment outcome in pediatric ALL. In this ALL-BFM protocol response assessment to induction treatment is refined by measuring the minimal residual disease (MRD) kinetics.^{4,5} Nevertheless, conceptually, it would be preferable to identify high-risk patients already at diagnosis, which would enable early stratification and intensification of induction therapy. At the same time, it may be possible to identify those patients, who can be cured with less intensive treatment. In T-ALL, genetic risk factors that are common enough to stratify patients have only recently been identified. Activating mutations of the transmembrane receptor *NOTCH1* occur in >50% of all childhood T-ALL patients⁶ and have previously been shown to signify a favorable prognosis in the context of the ALL-BFM protocol-based treatment⁷ and subsequently also in other similar protocols,^{8–10} although not in others.^{11–17} Under physiological conditions, *NOTCH1* is activated by ligand binding, followed by proteolytic cleavages that liberate the intracellular part of *NOTCH1* (ICN1) in the cytoplasm. ICN1 is then translocated to the nucleus and, in concert with other transcriptional activators, induces the expression of target genes that include important oncogenic pathways, such as c-MYC.¹⁸ The effect of ICN1 is limited by ubiquitination and degradation. This process is regulated by the E3 ubiquitin ligase, *FBXW7*, that binds to the C-terminal PEST domain of ICN1.^{19,20} Interestingly, inactivating mutations of *FBXW7* have recently been found to be common in T-ALL.^{21–23} These mutations are expected to increase the activity of the *NOTCH1* pathway in a similar manner as the *NOTCH1*-PEST mutations do.^{21,23}

In this study, we show in a large group of 301 children with T-ALL, who were treated on the ALL-BFM 2000 protocol that (1) the overall favorable effect of *NOTCH1* receptor activation on long-term outcome can be confirmed in a larger data set, (2) this effect is restricted to those patients with a rapid early treatment response, (3) inactivating *FBXW7* mutations occur in 14% of all T-ALL and are associated with a remarkably favorable prednisone response, but (4) that these *FBXW7* mutations do not have a significant effect on long-term outcome by themselves and (5) do not detectably synergize with *NOTCH1* receptor mutations to specify a group of patients with a particularly

Correspondence: Professor AE Kulozik, Department of Pediatric Oncology, Hematology and Immunology, Angelika Lautenschläger Children's Hospital, University of Heidelberg, Im Neuenheimer Feld 430, D-69120 Heidelberg, Germany.
E-Mail: Andreas.Kulozik@med.uni-heidelberg.de
Received 15 April 2010; revised 7 July 2010; accepted 20 July 2010; published online 14 October 2010

favorable long-term outcome. Finally, a systematic comparison of different European treatment protocols suggests that the variable effects of NOTCH mutations on prognosis likely depends on differences in treatment generally and on the type and intensity of central nervous system (CNS)-directed therapy specifically.

Materials and methods

Patients and samples

From August 1999 through February 2008, a total of 545 patients with T-ALL were eligible for treatment in the multi-center ALL-BFM 2000 trial (no non-Hodgkin's lymphoma patients 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. This study includes a subgroup of 301 patients from Germany and Switzerland. The subjects were selected on the basis of availability of sufficient amounts of DNA for molecular analysis. The group includes 150 of the 157 patients whose NOTCH1 receptor mutation status has been reported previously.⁷ In all, 7 of these 157 patients were excluded from the analysis presented in this study, because of either lack of available DNA (2), protocol violations (3) or age > 18 years (2). In all, 151 patients have not been reported previously regarding NOTCH1 status. The clinical parameters (age, gender, white blood cell count at diagnosis, prednisone response, MRD at day 78) of this subgroup of patients did not differ significantly from the entire ALL-BFM 2000 cohort.

Mononuclear cells were isolated from bone marrow samples and stored in liquid nitrogen or at -80°C until DNA extraction. All bone marrow samples contained a blast percentage of 80% or more. Immunophenotyping was carried out as previously described,²⁴ and the subclassification of T-ALL was performed according to the guidelines of the European Group for Immunological Characterization of Leukemias.²⁵ The presence of TEL/AML1, BCR/ABL and MLL/AF4 fusion transcripts was analyzed as described previously.^{26,27}

Early *in vivo* response to prednisone, defined as the cytoreduction (number of peripheral blood blasts per microliter on day 8) 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.³ According to prednisone response, patients were classified into good responders (< 1000 blasts/ μl at day 8) or poor responders (≥ 1000 blasts/ μl at day 8). Treatment response was further defined by determination of MRD kinetics that were assessed at two different time points (TPs), at days 33 (TP1) and 78 (TP2) of treatment, respectively.^{5,28-30} As previously described, allele-specific oligonucleotide-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).³¹ At both TPs, an unfavorable MRD status ($\geq 10^{-4}$) 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.⁷ Complete remission was defined as $< 5\%$ blasts in the regenerating bone marrow, 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 NOTCH1 and FBXW7

Sequencing of NOTCH1 has been performed as described previously.⁷ Sequencing of FBXW7 was carried out following PCR amplification of exons 9 and 10 that have previously been reported to be the most frequently mutated regions. Primer sequences for exon 9 are: forward 5'-ccaggccagagctatcataac-3'; reverse 5'-agacaaaacgctatggcttcc-3'; and for exon10 are: forward 5'-aaccttgactaaatctaccatggt-3'; reverse 5'-ctggatcagcaatttgacagt-3'. PCR-amplified fragments were sequenced by GATC biotech (Konstanz, Germany).

Statistical analyses

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 or death from any cause. Failure to achieve remission because of the 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. The Cox 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-years EFS (pEFS) and estimated CI of relapse (pCIR) with \pm s.e. Differences in the distribution of individual parameters among patient subsets were analyzed using the Fisher's exact test for categorized variables and the Mann-Whitney *U*-test for continuous variables. Logistic 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, Version 9.1, SAS Institute Inc, Cary, NC, USA).

Results

The analysis of an extended group of BFM patients with T-ALL confirms the favorable effect of NOTCH1 receptor mutations on early treatment response and long-term outcome

Previously, we reported the analysis of 157 T-ALL patients, who were enrolled in the ALL-BFM study and demonstrated that NOTCH1 mutations represent an independent molecular marker that specifies a large group of patients with a favorable early treatment response and excellent long-term outcome.⁷ We have now extended this analysis to study a large group of 301 patients enrolled in the ALL-BFM 2000 study. The group includes 150 of the 157 patients from our previous report and a validation set of 151 patients who have not been included in our previous analysis. As reported previously, the NOTCH1 mutational status correlates with the common cortical immunophenotype; these factors remain independent in a multivariate analysis. We identified 150 (50%) T-ALLs with heterozygous somatic NOTCH1 mutations, 94 (31.2%) in the heterodimerization domain, 29 (9.6%) in the transactivation domain-PEST domain and 27 (9%) in both. The distribution of mutations was similar in both sets (data not shown).

Prednisone response was available for 149/150 patients with NOTCH1 mutations, and for 145/151 patients without NOTCH1 mutations (Table 1). It was observed that patients with NOTCH1 mutations show a better prednisone response

Table 1 Activating NOTCH1 mutations signify favorable early treatment response in children with T-ALL

	PPR (%)	PGR (%)	Data not available	P [‡]
<i>Prednisone response (day 8)^a</i>				
NOTCH1 mutated	47 (32)	102 (68)	1	P = 0.002
NOTCH1 non-mutated	72 (50)	73 (50)	6	
	Unfavorable (%)	Favorable (%)	Data not available	P [‡]
<i>MRD (day 33)^b</i>				
NOTCH1 mutated	91 (66)	47 (34)	12	P = 0.003
NOTCH1 non-mutated	106 (82)	23 (18)	22	
<i>MRD (day 78)^b</i>				
NOTCH1 mutated	37 (26)	103 (74)	10	P < 0.0001
NOTCH1 non-mutated	76 (57)	58 (43)	17	

Abbreviations: MRD, minimal residual disease; PGR, prednisone good response; PPR, prednisone poor response; T-ALL, T-cell acute lymphoblastic leukemia.

[‡]P-value; Fisher's test.

^aPPR: ≥ 1000 leukemic blood blasts/ μ l on treatment day 8; PGR: $< 1000/\mu$ l.

^bUnfavorable MRD: $\geq 10^{-4}$; favorable MRD: $< 10^{-4}$.

than those patients without NOTCH1 mutations ($P=0.002$); Table 1).

The induction phase of the ALL-BFM 2000 protocol clears MRD more effectively in NOTCH1-mutated patients than in patients without a NOTCH1 mutation.⁷ MRD data on day 33 were available in 267 patients (138 NOTCH1 mutated, 129 NOTCH1 non-mutated) and in 274 patients on day 78 (140 NOTCH1 mutated and 134 NOTCH1 non-mutated). On both days of MRD measurements, NOTCH1-mutated patients showed a more favorable response ($P=0.003$, respectively, $P<0.0001$; Table 1).

As reported previously, long-term outcome of the NOTCH1-mutated group was significantly better (pEFS 87%) than in the non-mutated group (pEFS 74%; $P=0.005$; Figure 1a). The two very late events in the NOTCH1-mutated group are second malignancies. Furthermore, the likelihood for developing a relapse was only 7% in the mutated group but 17% in the non-mutated group ($P=0.007$; Figure 1b). A separate analysis of the second group of patients who had not been reported previously confirms the significant difference in pCIR and the trend toward better EFS⁷ (Figures 1c and d). Overall, the results obtained by the analysis of a large group of patients confirm that activating NOTCH1 mutations are strongly associated with favorable early treatment response and also with excellent long-term outcome.

Clinical data on the presence of initial CNS involvement were available in 254 patients (no information about CNS involvement for the other 47 patients). The incidence of CNS involvement was similar in NOTCH1-mutated and NOTCH1-non-mutated patients (12/127 (9%) versus 18/127 (12%)). Furthermore, the incidence of relapses with CNS involvement was low and similar in NOTCH1-mutated and -non-mutated patients both, in the entire group (pCIR $2 \pm 1\%$ versus $4 \pm 2\%$), and also in the high-risk subgroup of patients (pCIR $3 \pm 3\%$ versus $5 \pm 3\%$).

The favorable effect of NOTCH1 receptor activation is higher in the standard and intermediate risk groups

The large cohort of patients now enabled us to perform subgroup analysis and we thus assessed whether the effect of NOTCH1 mutations differs in the conventionally defined risk groups that are stratified according to *in vivo* treatment response. The standard risk group (SR) of the ALL-BFM 2000

protocol is defined by a prednisone good response and a negative MRD result on days 33 and 78, whereas HR patients either show a PPR, an MRD response of $\geq 10^{-3}$ on day 78 or both. The intermediate risk group (MR) regroups all remaining patients.^{32,33} In addition, patients who are BCR/ABL or MLL/AF4 positive are classified as HR. In our cohort of 301 T-ALL patients, 168 patients were thus stratified to the SR or MR groups, whereas 133 patients were stratified to the HR group.

When comparing patients with or without NOTCH1 mutations in the SR/MR group, the NOTCH1-mutated group had an excellent pEFS of 95%, which compared with 83% in the non-mutated group ($P=0.02$; Figure 2a). There is also a trend in the relapse rate, although the number of relapses is low. Specifically, the pCIR was only 4% (4/99) in the NOTCH1-mutated but 11% (7/69) in the NOTCH1-non-mutated group (Figure 2b; $P=0.11$). By contrast, in the HR group no difference in pEFS was observed between NOTCH1-mutated (72%) and -non-mutated patients (66%; Figure 2c), although there was a non-significant trend toward a lower rate of relapse in the NOTCH1-mutated compared with the NOTCH1-non-mutated group ($P=0.13$; Figure 2d). In a Cox regression analysis of EFS, including the variables risk group SR/MR versus HR, NOTCH1 mutation or an interaction term (NOTCH1 mutations in SR/MR versus other) and stem cell transplantation as time dependent variable, the interaction was significant (relative risk = 0.32, 95% confidence interval 0.12–0.86, $P=0.024$). This was not the case for relapse risk, but the simple effect of NOTCH1 was significant (relative risk 0.43, 95% confidence interval 0.20–0.92). Therefore, the favorable effect of NOTCH1 mutations seems to be restricted to those leukemias with a genetic profile that already specifies a good response to the ALL-BFM treatment strategy. By contrast, NOTCH1 activation does not seem to override the genetic profile that specifies the empirically defined HR group.

FBXW7 mutations predict good prednisone and early MRD response but do not signify favorable late MRD response or long-term outcome

Inactivating FBXW7 mutations have previously been reported to be common in T-ALL^{21–23} and are expected to activate the NOTCH pathway. We hypothesized that FBXW7 mutations may have a similar clinical effect as activating NOTCH1 mutations

and that these two types of mutations may synergize to sensitize leukemia cells for treatment. We have thus determined the *FBXW7* mutation status and analyzed its effect on treatment efficacy. We identified heterozygous *FBXW7* mutations in

42 (14%) of 301 T-ALL samples (Table 2). All of these mutations are located in exons 9 and 10 of the gene, which encode the C-terminal-binding site between the ligase and the substrate. These mutations are predicted to disrupt the binding between *FBXW7* and ICN1. In the N-terminal part of the protein, *FBXW7* contains a homodimerization domain that is still functional in the mutated form of the ubiquitin ligase. Therefore, the mutated monomer sequesters the product of the normal allele in a non-functional dimer and is thus thought to function in a dominant-negative manner.^{21,34}

We grouped the patients according to their *FBXW7* mutational status and compared their early treatment response (assessed by prednisone response and MRD kinetics). Most patients with *FBXW7* mutations responded well to prednisone (88%), but only 55% in the *FBXW7*-non-mutated group ($P=0.0003$; Table 2). The MRD kinetics at both TPs were available in 39/42 patients with *FBXW7* mutations. In this group, a favorable MRD response on days 33 and 78 was observed in 16/39 and in 28/39 patients. On day 33, the difference between the *FBXW7*-mutated and *FBXW7*-non-mutated groups was significant ($P=0.03$), whereas *FBXW7* mutations lost their significant predictive value on day 78, although there was still a trend ($P=0.08$). Figure 3a shows that patients with or without *FBXW7* mutations have a similar pEFS (76% compared with 81%, $P=0.48$) and a similar pCIR (10% compared with 13%, $P=0.6$, Figure 3b).

These data indicate that *FBXW7* mutations confer a high sensitivity of T-ALL blasts to glucocorticoids and possibly intrathecal methotrexate, but also that the difference in treatment response between mutated and non-mutated cells is leveled by the drugs that are used later during induction, intensification and re-induction.

NOTCH1 and FBXW7 mutations function together to result in excellent early treatment response but not to generate a more favorable long-term outcome

Because activating mutations of the *NOTCH1* receptor and inactivating mutations of the E3 ligase that targets the active ICN1 for degradation by the proteasome are expected to function in a similar manner on the NOTCH pathway, we hypothesized that these two types of mutations may synergize clinically. Furthermore, it has been reported previously that T-ALL mutations in the *NOTCH1*-PEST domain and of *FBXW7* are mutually exclusive.^{21,22} We also observed this relationship in the cohort of patients analyzed here, although there was one exception of a patient with a *NOTCH1*-PEST and a *FBXW7* mutation. In all the other 29 patients with a mutation in both genes, the *NOTCH1*-heterodimerization domain was mutated. This almost complete mutual exclusiveness of *FBXW7* and

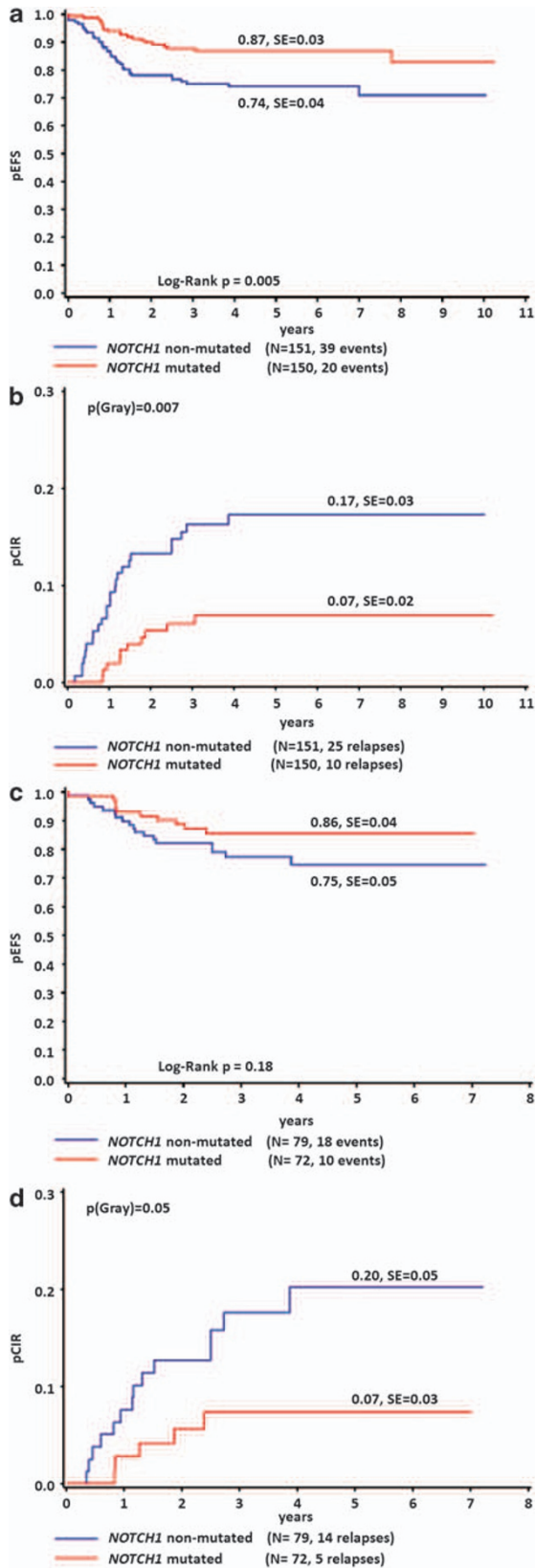
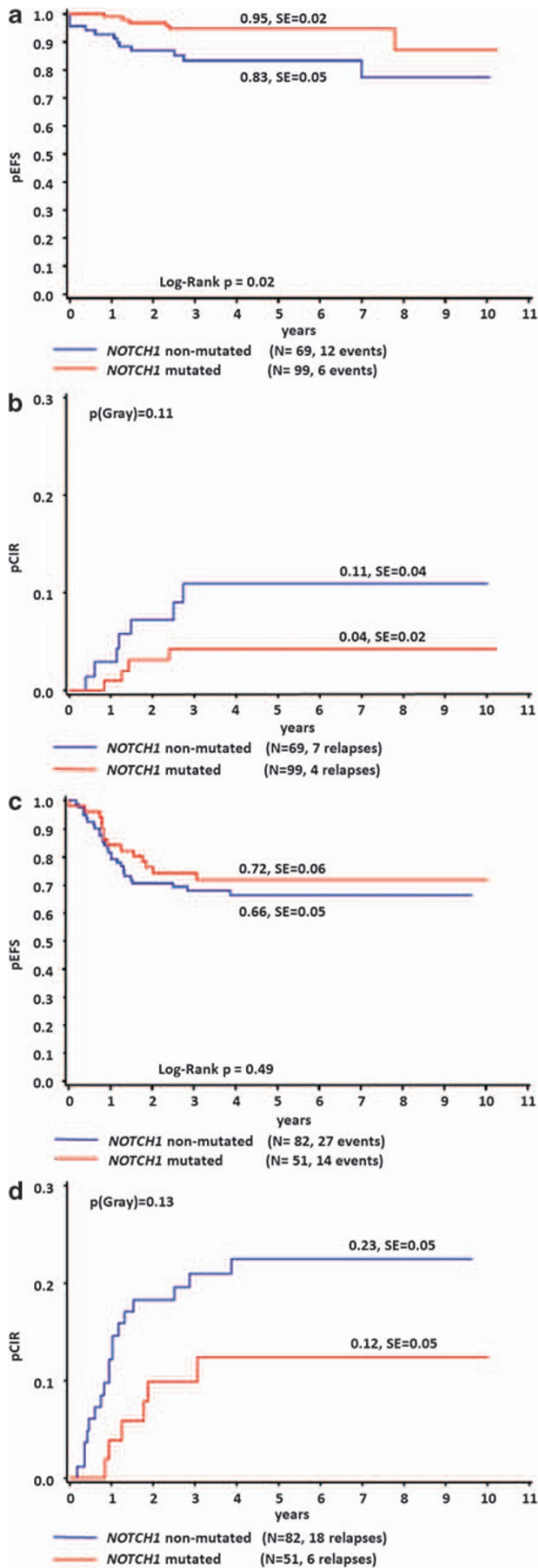


Figure 1 Activating *NOTCH1* mutations predict an excellent long-term outcome in T-ALL. (a) Kaplan-Meier estimate of the pEFS in *NOTCH1*-mutated and *NOTCH1*-non-mutated patients from the total cohort ($n=301$). (b) pCIR in *NOTCH1*-mutated and *NOTCH1*-non-mutated patients from the total cohort ($n=301$). (c) Kaplan-Meier estimate of the pEFS in *NOTCH1*-mutated and *NOTCH1*-non-mutated patients from the validation set ($n=151$). (d) pCIR in *NOTCH1*-mutated and *NOTCH1*-non-mutated patients from the validation set ($n=151$). The 18 events in the *NOTCH1*-non-mutated group were 14 relapses, 3 deaths in continuous complete remission and 1 death before complete remission. The 10 events in the *NOTCH1*-mutated group were 5 relapses, 4 deaths in continuous complete remission and 1 death before complete remission.

NOTCH1-PEST mutations suggests that no additional selection advantage is provided by a *NOTCH1*-PEST and *FBXW7* double mutation and that the biological function in T-ALL of both types of mutation is similar.



In adults with T-ALL, *FBXW7* mutations have previously been reported to add significant prognostic value to the *NOTCH1* mutational status.¹⁰ Therefore, we next assessed, if the combination of both mutations is also beneficial in the context of the pediatric ALL-BFM study. We compared the outcome of the 30 (10%) patients with mutations in both, *NOTCH1* and *FBXW7* genes, to the 120 (40%) patients with mutations in *NOTCH1* only, to the 12 (4%) patients with *FBXW7* mutations only, and to those 139 (46%) without mutation in either gene. Prednisone response data were available in 294 patients. In the group of 30 patients with the combination of *NOTCH1* and *FBXW7* mutations, only 2 (7%) showed a PPR, whereas the group of 119 patients with a *NOTCH1* mutation only or the group of 12 patients with a *FBXW7* mutation only showed a PPR in 45 (38%) and 3 (25%), respectively (Table 3). The 133 patients with neither mutation (for which prednisone response was available) showed a PPR in 69 (52%). In a logistic regression analysis both *NOTCH1* and *FBXW7* mutations had a significant influence on the prednisone good responder rate ($P=0.01$ and 0.001).

We next analyzed the MRD kinetics after 33 (available in 267 patients) and 78 days (available in 274 patients) of induction treatment. On day 33, the group with mutations in both genes showed a significantly lower MRD load (16/29; 55% favorable) when compared with the groups with either a *NOTCH1* (31/109; 28% favorable) or a *FBXW7* mutation (0/10 favorable), or with neither mutation (23/119; 19% favorable). On day 78, the group with the *NOTCH1* and *FBXW7* mutations showed a favorable MRD load in 24/30 (80%) patients, which did not differ significantly from the group with either *NOTCH1* (79/110; 72%) or *FBXW7* mutations alone (4/9; 44%). As expected, the *NOTCH1*-mutated and the double-mutated groups showed a significantly more frequent favorable MRD load than the group with neither mutation (54/125; 43%). In a logistic regression analysis *NOTCH1* but not *FBXW7* mutations had significant influence on the MRD-load at both TPs ($P=0.01$ and <0.0001 versus $P=0.09$ and 0.43).

Long-term outcome and the rate of relapse of patients with double mutations are no different to those with *NOTCH1* mutations only. Patients with *FBXW7* mutations only tend to show a less favorable outcome with 5/12 patients having had an event (one relapse, two deaths in continuous complete remission, one death before complete remission and one patient with a secondary malignancy). Unfortunately, the patient number in this group is only 12 and thus far too small for reaching meaningful conclusions in this subgroup. As expected, the advantage of the *NOTCH1*-pathway-mutated groups compared with the patients without a mutation is maintained in this subgroup analysis (Supplementary Figure 1a and 1b).

We conclude that in the context of the ALL-BFM protocol-based treatment, *NOTCH1* and *FBXW7* mutations function together to result in a favorable early treatment response. However, this additive effect is not maintained with regard to long-term outcome of children and adolescents with T-ALL.

Figure 2 The favorable effect of activating *NOTCH1* mutations on long-term outcome is restricted to the SR/MR group. (a) Kaplan-Meier estimate of the probability of pEFS in *NOTCH1*-mutated and *NOTCH1*-non-mutated SR/MR patients. (b) pCIR in *NOTCH1*-mutated and *NOTCH1*-non-mutated SR/MR patients. (c) Kaplan-Meier estimate of the pEFS in *NOTCH1*-mutated and *NOTCH1*-non-mutated HR patients. (d) pCIR in *NOTCH1*-mutated and *NOTCH1*-non-mutated HR patients.

Table 2 Inactivating FBXW7 mutations specify an excellent prednisone response, but marginal effects on MRD kinetics

	PPR (%)	PGR (%)	Data not available	P [‡]
Prednisone response (day 8)^a				
FBXW7 mutated	5 (12)	37 (88)	0	P = 0.00003
FBXW7 non-mutated	114 (45)	138 (55)	7	
	Unfavorable (%)	Favorable (%)	Data not available	P [‡]
MRD (day 33)^b				
FBXW7 mutated	23 (59)	16 (41)	3	P = 0.03
FBXW7 non-mutated	174 (76)	54 (24)	31	
MRD (day 78)^b				
FBXW7 mutated	11 (28)	28 (72)	3	P = 0.08
FBXW7 non-mutated	102 (43)	133 (57)	24	

Abbreviations: MRD, minimal residual disease; PGR, prednisone good response; PPR, prednisone poor response.

[‡]P-value; Fisher's test.

^aPPR: ≥ 1000 leukemic blood blasts/ μ l on treatment day 8; PGR: $< 1000/\mu$ l.

^bUnfavorable MRD: $\geq 10^{-4}$; favorable MRD: $< 10^{-4}$.

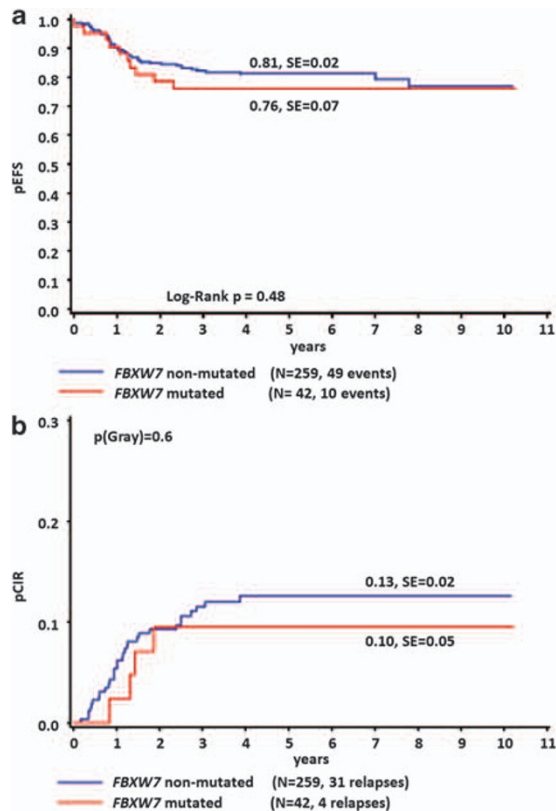


Figure 3 Inactivating FBXW7 mutations do not predict better long-term outcome in T-ALL. (a) Kaplan–Meier estimate of the pEFS in FBXW7-mutated and FBXW7-non-mutated patients. (b) pCIR in FBXW7-mutated and FBXW7-non-mutated patients.

Discussion

T-ALL in childhood and adolescence still represents one of the important challenges in pediatric oncology. Although the probability of cure has significantly improved with modern protocols and long-term disease-free survival rates of $\sim 80\%$ can now be obtained, the prognosis of relapses is still dismal. Therefore, it is critical to identify those patients at HR, who may

benefit from intensified treatment regimens. At the same time, it is important to identify those patients with a favorable risk profile, who may benefit from a reduction of treatment intensity. The measurement of the kinetics of leukemia clearance during early induction has proved to be a successful strategy to stratify patients and to improve the prognosis of HR patients in pre-B-ALL and in T-ALL.³² MRD-based stratification at later TPs has been shown to be even more effective to predict survival.³³ However, the MRD-based late identification of HR patients in the ALL-BFM 2000 study after induction treatment is a potential disadvantage of this strategy. Conceptually, it is preferable to develop molecular prognostic markers that can be studied at the time of diagnosis thus enabling a stratification of treatment from its beginning. In contrast to preB-ALL, valid and clinically useful prognostic markers in T-ALL are only beginning to emerge. The analysis of gene expression profiles in a limited number of patients suggested that the expression of homeotic genes (*HOX11*, *HOX11L2*) and transcripts that relate to the stage of maturation of the T-ALL clone (*TAL1*, *LMO1/2*, *LYL*) may be of prognostic value.³⁵ However, the value of these markers has been controversial^{12,36} and, more generally, the usefulness of mRNA-based markers is probably limited in the context of multicenter protocols, because of the influence of pre-analytical handling.³⁷

A number of mutations have previously been identified in several genes whose products are involved in oncogenic pathways, such as phosphoinositide 3-kinases–AKT and transforming growth factor- β .^{38,39} Most of these oncogenic mutations are present in only a small subset of patients, and most of them failed to stratify risk in pediatric T-ALL. In contrast, some mutations, such as deletions of the *PTEN* or the *CASP8AP2* genes, were shown to be of negative prognostic value.^{38,39} Other factors might also influence the outcome of the patients and would thus be candidates to be included as components of a multifaceted molecular risk profile of T-ALL. *NOTCH1* mutations do not make a difference for the HR patients, but other mutations might do.

Activating mutations of the *NOTCH1* receptor are common in T-ALL.⁶ However the effect of *NOTCH1* mutations on the prognosis of T-ALL has been controversial since our first report in 2006.⁷ Although the favorable effect of activating *NOTCH1* mutations on treatment outcome was confirmed by study groups with BFM-type treatment strategies,^{8,9} *NOTCH1* mutations did not predict favorable treatment response in the context of other

Table 3 Activating *NOTCH1* and inactivating *FBXW7* mutations function together to specify an excellent prednisone response and early but not late MRD

	PPR (%)	PGR (%)	Data not available	P [‡]
<i>Prednisone response (day 8)</i> ^a				
<i>NOTCH1</i> + <i>FBXW7</i> mutated	2 (7)	28 (93)	0]P<0.00001]P=0.03]P=0.13
<i>NOTCH1</i> mutated	45 (38)	74 (62)	1	
<i>FBXW7</i> mutated	3 (25)	9 (75)	0	
<i>NOTCH1</i> / <i>FBXW7</i> non-mutated	69 (52)	64 (48)	6	
	Unfavorable (%)	Favorable (%)	Data not available	P [‡]
<i>MRD (day 33)</i> ^b				
<i>NOTCH1</i> + <i>FBXW7</i> mutated	13 (45)	16 (55)	1]P=0.0003]P=0.12]P=0.21
<i>NOTCH1</i> mutated	78 (72)	31 (28)	11	
<i>FBXW7</i> mutated	10 (100)	0 (0)	2	
<i>NOTCH1</i> / <i>FBXW7</i> non-mutated	96 (81)	23 (19)	20	
<i>MRD (day 78)</i> ^b				
<i>NOTCH1</i> + <i>FBXW7</i> mutated	6 (20)	24 (80)	0]P=0.0004]P=0.00001]P=1.00
<i>NOTCH1</i> mutated	31 (28)	79 (72)	10	
<i>FBXW7</i> mutated	5 (56)	4 (44)	3	
<i>NOTCH1</i> / <i>FBXW7</i> non-mutated	71 (57)	54 (43)	14	

Abbreviations: MRD, minimal residual disease; PGR, prednisone good response; PPR, prednisone poor response.

[‡]P-value; Fisher's Test.

^aPPR: ≥ 1000 leukemic blood blasts/μl on treatment day 8; PGR: < 1000/μl.

^bUnfavorable MRD: ≥ 10⁻⁴; favorable MRD: < 10⁻⁴.

treatment protocols.^{11–17} In the light of this controversy, one of the important findings of this study is that the favorable effect of *NOTCH1* mutations is confirmed by this analysis of a large group of BFM patients. This finding suggests that the effect of *NOTCH1* mutations depends on the treatment protocol. Indeed, the long-term outcome of *NOTCH1*-non-mutated patients is similar in most modern protocols including BFM,^{16,17} whereas the generally more favorable outcome of T-ALL in BFM-type treatment strategies is caused by a particularly good outcome in the *NOTCH1*-mutated group. This may indicate that *NOTCH1*-mutated T-ALLs are particularly sensitive to the BFM strategy. More specifically, the favorable effect of *NOTCH1* mutations in BFM protocols is highly significant in the standard and intermediate risk groups, whereas *NOTCH1* mutations are neutral in the high-risk group that consists of patients with poor early treatment response. The chemotherapy in the European Organisation for Research and Treatment of Cancer (EORTC) protocols is in large parts similar to the one in BFM protocols, but EORTC does not perform cranial irradiation. Interestingly, the EORTC protocols also show a significantly better early treatment response and a trend towards better pEFS in the *NOTCH1*-mutated group with favorable early treatment response. In striking contrast to the BFM patients, EORTC HR patients with *NOTCH1* mutations have a dismal prognosis.¹⁶ Significantly, Clappier *et al.*¹⁶ report that CNS relapses are more common in the *NOTCH1*-mutated high-risk group, whereas CNS relapses are rare in BFM-treated patients in general but also in the high-risk group in particular. One could speculate that the BFM type chemotherapy alone is able to prevent CNS relapses in the *NOTCH1*-mutated good responders, but not in the poor responders. A possible mechanistic link between *NOTCH1* activation and T-ALL CNS tropism has recently been suggested by the finding that in mice the chemokine receptor CCR7 is controlled by *NOTCH1* and required for targeting leukemic cells to the CNS.⁴⁰ In this context, it is intriguing that one of the major differences between the BFM and the EORTC or the Dutch Childhood Oncology Group treatment strategies lies

in the use of cranial radiotherapy in BFM but not in EORTC or Dutch Childhood Oncology Group.^{16,17} This comparison suggests that *NOTCH1*-mutated T-ALL with poor early treatment response tend to cause CNS relapses and that this propensity may be effectively limited by cranial radiotherapy. Despite the generally debatable role of prophylactic cranial radiotherapy in childhood ALL,⁴¹ *NOTCH1*-mutated patients with T-ALL and poor early treatment response may thus constitute a subgroup of children, who may benefit from radiotherapy or other modes of intensified CNS-directed therapy.

Inactivation of *FBXW7* is expected to activate the NOTCH pathway. We thus hypothesized that T-ALL with inactivating *FBXW7* mutations would be clinically similar to those with activating *NOTCH1* mutations. Further, we hypothesized that inactivating *FBXW7* mutations and activating *NOTCH1* mutations may clinically synergize. In line with these hypotheses, this study shows that *FBXW7* mutations are associated with an excellent prednisone response and also with rapid clearance of the leukemia from the bone marrow. Furthermore, T-ALL patients with *NOTCH1*/*FBXW7* double mutations show more frequent favorable MRD at day 33 of induction than those with mutations in either *NOTCH1* or *FBXW7*. Surprisingly, however, there were no significant differences between these groups in the frequency of favorable MRD at day 78 or in the frequency of relapse and pEFS. Therefore, the effects of NOTCH pathway activation by either receptor mutations or inactivation of the E3 ubiquitin ligase are separable. Although these mutations seem to be similar and to be additive or synergistic early in treatment, *FBXW7* inactivation influences neither treatment response at the end of induction nor long-term outcome. The mechanism of the separable effect of *NOTCH1* receptor activation and *FBXW7* inactivation must remain an open question at this point. However, this difference may possibly be explained by the activation of the receptor being less pleiotropic than the inactivation of the negative regulator *FBXW7*, because this E3 ubiquitin ligase does not only interfere with the NOTCH pathway but also with other important oncogenic pathways

and proteins such as c-MYC, PSEN1, c-JUN, cyclin E1, sterol regulatory element-binding protein, mammalian target of rapamycin or Aurora-A.^{42–48}

In conclusion, in BFM protocols T-ALL patients with *FBXW7* mutations show an excellent prednisone and early MRD response. At this early time, *FBXW7* mutations function together with *NOTCH1* mutations. In contrast, this synergy is lost for long-term outcome. It will now be necessary to validate the prognostic significance of *NOTCH1* mutations in a prospective study possibly with a particular emphasis on its role to predict the necessity for prophylactic cranial radiotherapy. However, when interpreting these differences it must be borne in mind that the BFM and EORTC protocols also differ in other, potentially important details and that both studies were not designed to be directly comparable.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This study was financially supported by the Manfred Lautenschläger Foundation, the 'Tour der Hoffnung' and a grant from the German Ministry of Education and Research (BMBF, NGFN Plus). We acknowledge all participants of the ALL-BFM study. We thank Marc Remke and Sven Danckwardt for helpful discussion. We are particularly grateful to Dr Hélène Cavé and Dr Jules Meijerink for the open atmosphere in discussing unpublished data. We are grateful to the iBFM biology and diagnosis committee for fostering the comparison of the data of the BFM, DCOG/COALL and EORTC datasets before publication.

References

- Pui CH, Schrappe M, Ribeiro RC, Niemeyer CM. Childhood and adolescent lymphoid and myeloid leukemia. *Hematology Am Soc Hematol Educ Program* 2004, 118–145.
- Herold R, von Stackelberg A, Hartmann R, Eisenreich B, Henze G. Acute lymphoblastic leukemia-relapse study of the Berlin-Frankfurt-Munster Group (ALL-REZ BFM) experience: early treatment intensity makes the difference. *J Clin Oncol* 2004; **22**: 569–570.
- Riehm H, Reiter A, Schrappe M, Berthold F, Dopfer R, Gerein V et al. Corticosteroid-dependent reduction of leukocyte count in blood as a prognostic factor in acute lymphoblastic leukemia in childhood (therapy study ALL-BFM 83). *Klin Padiatr* 1987; **199**: 151–160.
- Yokota S, Hansen-Hagge TE, Ludwig WD, Reiter A, Raghavachar A, Kleihauer E et al. Use of polymerase chain reactions to monitor minimal residual disease in acute lymphoblastic leukemia patients. *Blood* 1991; **77**: 331–339.
- Flohr T, Schrauder A, Cazzaniga G, Panzer-Grumayer R, van der Velden V, Fischer S et al. Minimal residual disease-directed risk stratification using real-time quantitative PCR analysis of immunoglobulin and T-cell receptor gene rearrangements in the international multicenter trial AIEOP-BFM ALL 2000 for childhood acute lymphoblastic leukemia. *Leukemia* 2008; **22**: 771–782.
- Weng AP, Ferrando AA, Lee W, Morris IV JP, Silverman LB, Sanchez-Irizarry C et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science* 2004; **306**: 269–271.
- Breit S, Stanulla M, Flohr T, Schrappe M, Ludwig WD, Tolle G et al. Activating NOTCH1 mutations predict favorable early treatment response and long-term outcome in childhood precursor T-cell lymphoblastic leukemia. *Blood* 2006; **108**: 1151–1157.
- Park MJ, Taki T, Oda M, Watanabe T, Yumura-Yagi K, Kobayashi R et al. *FBXW7* and *NOTCH1* mutations in childhood T cell acute lymphoblastic leukaemia and T cell non-Hodgkin lymphoma. *Br J Haematol* 2009; **145**: 198–206.
- Malyukova A, Dohda T, von der Lehr N, Akhoondi S, Corcoran M, Heyman M et al. The tumor suppressor gene hCDC4 is frequently mutated in human T-cell acute lymphoblastic leukemia with functional consequences for Notch signaling. *Cancer Res* 2007; **67**: 5611–5616.
- Asnafi V, Buzyn A, Le Noir S, Baleyrier F, Simon A, Beldjord K et al. NOTCH1/*FBXW7* mutation identifies a large subgroup with favorable outcome in adult T-cell acute lymphoblastic leukemia (T-ALL): a Group for Research on Adult Acute Lymphoblastic Leukemia (GRAALL) study. *Blood* 2009; **113**: 3918–3924.
- Zhu YM, Zhao WL, Fu JF, Shi JY, Pan Q, Hu J et al. NOTCH1 mutations in T-cell acute lymphoblastic leukemia: prognostic significance and implication in multifactorial leukemogenesis. *Clin Cancer Res* 2006; **12**: 3043–3049.
- van Grotel M, Meijerink JP, van Wering ER, Langerak AW, Beverloo HB, Buijs-Gladdines JG et al. Prognostic significance of molecular-cytogenetic abnormalities in pediatric T-ALL is not explained by immunophenotypic differences. *Leukemia* 2008; **22**: 124–131.
- Larson Gedman A, Chen Q, Kugel Desmoulin S, Ge Y, LaFiura K, Haska CL et al. The impact of NOTCH1, FBW7 and PTEN mutations on prognosis and downstream signaling in pediatric T-cell acute lymphoblastic leukemia: a report from the Children's Oncology Group. *Leukemia* 2009; **23**: 1417–1425.
- Mansour MR, Sulis ML, Duke V, Foroni L, Jenkinson S, Koo K et al. Prognostic implications of NOTCH1 and *FBXW7* mutations in adults with T-cell acute lymphoblastic leukemia treated on the MRC UKALLXII/ECOG E2993 protocol. *J Clin Oncol* 2009; **27**: 4352–4356.
- Baldus CD, Thibaut J, Goekbuget N, Stroux A, Schlee C, Mossner M et al. Prognostic implications of NOTCH1 and *FBXW7* mutations in adult acute T-lymphoblastic leukemia. *Haematologica* 2009; **94**: 1383–1390.
- Clappier E, Collette S, Grardel N, Girard S, Suarez L, Brunie G et al. Prognostic significance of NOTCH1 and *FBXW7* mutations in childhood T-cell acute leukemia: results from the EORTC Children Leukemia Group. *Leukemia* [e-pub ahead of print 23 September 2010].
- Zuurbier L, Homminga I, Calvert V, Te Winkel L, Buijs-Gladdines J, Kooi C et al. Activating NOTCH1 mutations predict for initial good prednisone response but not for improved outcome in pediatric T-ALL patients treated on DCOG or COALL protocols. *Leukemia* [e-pub ahead of print 23 September 2010].
- Weng AP, Millholland JM, Yashiro-Ohtani Y, Arcangeli ML, Lau A, Wai C et al. c-Myc is an important direct target of Notch1 in T-cell acute lymphoblastic leukemia/lymphoma. *Genes Dev* 2006; **20**: 2096–2109.
- Wu G, Lyapina S, Das I, Li J, Gurney M, Pauley A et al. SEL-10 is an inhibitor of notch signaling that targets notch for ubiquitin-mediated protein degradation. *Mol Cell Biol* 2001; **21**: 7403–7415.
- Oberg C, Li J, Pauley A, Wolf E, Gurney M, Lendahl U. The Notch intracellular domain is ubiquitinated and negatively regulated by the mammalian Sel-10 homolog. *J Biol Chem* 2001; **276**: 35847–35853.
- Maser RS, Choudhury B, Campbell PJ, Feng B, Wong KK, Protopopov A et al. Chromosomally unstable mouse tumours have genomic alterations similar to diverse human cancers. *Nature* 2007; **447**: 966–971.
- O'Neil J, Grim J, Strack P, Rao S, Tibbitts D, Winter C et al. *FBW7* mutations in leukemic cells mediate NOTCH pathway activation and resistance to gamma-secretase inhibitors. *J Exp Med* 2007; **204**: 1813–1824.
- Thompson BJ, Buonamici S, Sulis ML, Palomero T, Vilimas T, Basso G et al. The SCFFBW7 ubiquitin ligase complex as a tumor suppressor in T cell leukemia. *J Exp Med* 2007; **204**: 1825–1835.
- Ludwig WD, Rieder H, Bartram CR, Heinze B, Schwartz S, Gassmann W et al. Immunophenotypic and genotypic features, clinical characteristics, and treatment outcome of adult pro-B acute lymphoblastic leukemia: results of the German multicenter trials GMALL 03/87 and 04/89. *Blood* 1998; **92**: 1898–1909.
- Bene MC, Castoldi G, Knapp W, Ludwig WD, Matutes E, Orfao A et al. Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL). *Leukemia* 1995; **9**: 1783–1786.

- 26 Schrappe M, Reiter A, Ludwig WD, Harbott J, Zimmermann M, Hiddemann W *et al.* Improved outcome in childhood acute lymphoblastic leukemia despite reduced use of anthracyclines and cranial radiotherapy: results of trial ALL-BFM 90. German-Austrian-Swiss ALL-BFM Study Group. *Blood* 2000; **95**: 3310–3322.
- 27 Viehmann S, Borkhardt A, Lampert F, Harbott J. Multiplex PCR—a rapid screening method for detection of gene rearrangements in childhood acute lymphoblastic leukemia. *Ann Hematol* 1999; **78**: 157–162.
- 28 Hansen-Hagge TE, Yokota S, Bartram CR. Detection of minimal residual disease in acute lymphoblastic leukemia by *in vitro* amplification of rearranged T-cell receptor delta chain sequences. *Blood* 1989; **74**: 1762–1767.
- 29 van Dongen JJ, Seriu T, Panzer-Grumayer ER, Biondi A, Pongers-Willemse MJ, Corral L *et al.* Prognostic value of minimal residual disease in acute lymphoblastic leukaemia in childhood. *Lancet* 1998; **352**: 1731–1738.
- 30 Willemse MJ, Seriu T, Hettlinger K, d’Aniello E, Hop WC, Panzer-Grumayer ER *et al.* Detection of minimal residual disease identifies differences in treatment response between T-ALL and precursor B-ALL. *Blood* 2002; **99**: 4386–4393.
- 31 van der Velden VH, Hochhaus A, Cazzaniga G, Szczepanski T, Gabert J, van Dongen JJ. Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: principles, approaches, and laboratory aspects. *Leukemia* 2003; **17**: 1013–1034.
- 32 Moricke A, Reiter A, Zimmermann M, Gadner H, Stanulla M, Dordelmann M *et al.* Risk-adjusted therapy of acute lymphoblastic leukemia can decrease treatment burden and improve survival: treatment results of 2169 unselected pediatric and adolescent patients enrolled in the trial ALL-BFM 95. *Blood* 2008; **111**: 4477–4489.
- 33 Conter V, Bartram CR, Valsecchi MG, Schrauder A, Panzer-Grumayer R, Moricke A *et al.* Molecular response to treatment redefines all prognostic factors in children and adolescents with B-cell precursor acute lymphoblastic leukemia: results in 3184 patients of the AIEOP-BFM ALL 2000 study. *Blood* 2010; **115**: 3206–3214.
- 34 Hao B, Oehlmann S, Sowa ME, Harper JW, Pavletich NP. Structure of a Fbw7-Skp1-cyclin E complex: multisite-phosphorylated substrate recognition by SCF ubiquitin ligases. *Mol cell* 2007; **26**: 131–143.
- 35 Ferrando AA, Neuberg DS, Staunton J, Loh ML, Huard C, Raimondi SC *et al.* Gene expression signatures define novel oncogenic pathways in T cell acute lymphoblastic leukemia. *Cancer Cell* 2002; **1**: 75–87.
- 36 Chiaretti S, Li X, Gentleman R, Vitale A, Vignetti M, Mandelli F *et al.* Gene expression profile of adult T-cell acute lymphocytic leukemia identifies distinct subsets of patients with different response to therapy and survival. *Blood* 2004; **103**: 2771–2778.
- 37 Breit S, Nees M, Schaefer U, Pfoersich M, Hagemeyer C, Muckenthaler M *et al.* Impact of pre-analytical handling on bone marrow mRNA gene expression. *Br J Haematol* 2004; **126**: 231–243.
- 38 Gutierrez A, Sanda T, Grebliunaite R, Carracedo A, Salmena L, Ahn Y *et al.* High frequency of PTEN, PI3K, and AKT abnormalities in T-cell acute lymphoblastic leukemia. *Blood* 2009; **114**: 647–650.
- 39 Remke M, Pfister S, Kox C, Toedt G, Becker N, Benner A *et al.* High-resolution genomic profiling of childhood T-ALL reveals frequent copy-number alterations affecting the TGF-beta and PI3K-AKT pathways and deletions at 6q15-16.1 as a genomic marker for unfavorable early treatment response. *Blood* 2009; **114**: 1053–1062.
- 40 Buonamici S, Trimarchi T, Ruocco MG, Reavie L, Cathelin S, Mar BG *et al.* CCR7 signalling as an essential regulator of CNS infiltration in T-cell leukaemia. *Nature* 2009; **459**: 1000–1004.
- 41 Pui CH, Campana D, Pei D, Bowman WP, Sandlund JT, Kaste SC *et al.* Treating childhood acute lymphoblastic leukemia without cranial irradiation. *N Engl J Med* 2009; **360**: 2730–2741.
- 42 Koepf DM, Schaefer LK, Ye X, Keyomarsi K, Chu C, Harper JW *et al.* Phosphorylation-dependent ubiquitination of cyclin E by the SCFFbw7 ubiquitin ligase. *Science* 2001; **294**: 173–177.
- 43 Yada M, Hatakeyama S, Kamura T, Nishiyama M, Tsunematsu R, Imaki H *et al.* Phosphorylation-dependent degradation of c-Myc is mediated by the F-box protein Fbw7. *EMBO J* 2004; **23**: 2116–2125.
- 44 Li J, Pauley AM, Myers RL, Shuang R, Brashler JR, Yan R *et al.* SEL-10 interacts with presenilin 1, facilitates its ubiquitination, and alters A-beta peptide production. *J Neurochem* 2002; **82**: 1540–1548.
- 45 Nateri AS, Riera-Sans L, Da Costa C, Behrens A. The ubiquitin ligase SCFFbw7 antagonizes apoptotic JNK signaling. *Science* 2004; **303**: 1374–1378.
- 46 Sundqvist A, Bengoechea-Alonso MT, Ye X, Lukiyanchuk V, Jin J, Harper JW *et al.* Control of lipid metabolism by phosphorylation-dependent degradation of the SREBP family of transcription factors by SCF(Fbw7). *Cell Metab* 2005; **1**: 379–391.
- 47 Mao JH, Kim IJ, Wu D, Climent J, Kang HC, DelRosario R *et al.* FBXW7 targets mTOR for degradation and cooperates with PTEN in tumor suppression. *Science* 2008; **321**: 1499–1502.
- 48 Mao JH, Perez-Losada J, Wu D, Delrosario R, Tsunematsu R, Nakayama KI *et al.* Fbxw7/Cdc4 is a p53-dependent, haplo-insufficient tumour suppressor gene. *Nature* 2004; **432**: 775–779.



This work is licensed under the Creative Commons Attribution-NonCommercial-No Derivative Works 3.0 Unported License. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-nd/3.0/>

Supplementary Information accompanies the paper on the Leukemia website (<http://www.nature.com/leu>)