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Early Relapse in ALL Is Identified by Time to Leukemia in NOD/SCID Mice and Is Characterized by a Gene Signature Involving Survival Pathways

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SUMMARY

We investigated the engraftment properties and impact on patient outcome of 50 pediatric acute lymphoblastic leukemia (ALL) samples transplanted into NOD/SCID mice. Time to leukemia (TTL) was determined for each patient sample engrafted as weeks from transplant to overt leukemia. Short TTL was strongly associated with high risk for early relapse, identifying an independent prognostic factor. This high-risk phenotype is reflected by a gene signature that upon validation in an independent patient cohort (n = 197) identified a high-risk cluster of patients with early relapse. Furthermore, the signature points to independent pathways, including mTOR, involved in cell growth and apoptosis. The pathways identified can directly be targeted, thereby offering additional treatment approaches for these high-risk patients.

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most frequent malignant disease in children and adolescents. Although intensification of multiagent chemotherapy regimens and advances in supportive care have led to improvement of remission induction and long-term survival over the last decades presently achieving cure rates of over 80%, about 20% of the patients suffer from relapse associated with an inferior prognosis (Pui and Evans, 2006; Schrappe et al., 2000a, 2000b). Particularly, in 10% of all patients, relapse occurs at early time points associated with a substantially reduced survival of 5% or less (Gaynon et al., 1998; Henze et al., 1991; Lawson et al., 2000). Stratification of patients into therapy regimens of different intensity is based on the individual risk for relapse by using prognostic factors including cytogenetics and response to therapy to define different risk groups (high-risk, HR and non-high risk, non-HR) (Pui et al., 2004a; Schrappe et al., 2000b; Silverman and Sallan, 2003). Leukemia cell clearance in response to steroid treatment is evaluated and has been used as a prognostic marker for almost 20 years, demonstrating inferior survival for patients responding poorly to prednisone (Gajjar et al., 1995; Riehm et al., 1987; Schrappe et al., 1998, 2000a, 2000b). In addition, detection of residual leukemia cells at submicroscopic levels

Significance

Treatment of pediatric ALL is increasingly successful, achieving cure rates of over 80%. Relapse is associated with poor outcome; however, the majority of relapsed patients is initially stratified into low-risk groups based on current stratification markers. This emphasizes the need for additional factors for upfront identification of high-risk patients. In this study we describe a strong association of the engraftment phenotype found in a series of transplanted primary pediatric ALL cells in a mouse in vivo model and patients with early relapse. Gene expression profiling revealed a set of genes associated with this aggressive phenotype providing a potential strategy to identify these high-risk patients. Most importantly, pathways regulating cell growth are identified, providing targets for alternative therapeutic strategies.

after remission induction therapy (minimal residual disease, MRD) qualifies for HR treatment (Chessells et al., 1995; Conter et al., 2010; Flohr et al., 2008; Steinherz et al., 1996; van Dongen et al., 1998). Early identification of patients with high risk for relapse has led to improved outcome. However, two-thirds of patients encountering relapse were initially stratified into non-HR groups (Schrappe et al., 2000a). This limitation of prognostic factors emphasizes the need to identify novel parameters that ideally also reflect the functional biology of the disease for the development of molecular-based risk stratification and identification of therapeutic targets.

Although leukemia cells cannot sufficiently be cultured in vitro, xenograft models can overcome these limitations. Transplantation of primary leukemia cells into NOD/SCID mice results in recipients exhibiting leukemia recapitulating the human disease (Baersch et al., 1997; Borgmann et al., 2000; Lock et al., 2002; Nijmeijer et al., 2001). We adopted the NOD/SCID/huALL model and observed that rapid development of leukemia in NOD/SCID mice engrafted with primary ALL cells is characteristic for patients with early relapse.

RESULTS

NOD/SCID/huALL

Leukemia cells isolated from diagnostic samples of 50 patients with pediatric de novo B cell precursor (BCP) ALL were transplanted onto NOD/SCID mice (1×10^7 cells per recipient). Recipient mice were investigated regularly for the presence of leukemia and sacrificed at manifestation of disease. Leukemia was confirmed by detection of a high percentage of human leukemia cells in the peripheral blood of the mice and infiltration of bone marrow and spleen by leukemia cells using flow cytometry. Of 50 samples, 29 led to manifestation of overt leukemia during the observation time of 20 weeks, whereas 21 transplants did not manifest disease within this time. Engraftment of leukemia was also monitored in different organ compartments at different time points. As exemplified for two samples, leukemia was consistently first detected in bone marrow and spleen followed by the occurrence of ALL cells in peripheral blood (Figure 1A).

Time to Leukemia

When we analyzed leukemia engraftment of different samples, we observed a prominent difference in time elapsing from transplantation to manifestation of leukemia. Rapid onset of leukemia-related morbidity was observed in some samples (4–6, 8, and 9 weeks after transplant) in contrast to other samples, giving rise to leukemia after a substantially longer period of time (12–26 weeks). No onset of leukemia was observed between 9 and 12 weeks after transplant, suggesting distinct engraftment phenotypes of different leukemia cell samples. The time from transplant to leukemia was defined as "Time To Leukemia" (TTL) and quantified for each leukemia sample. A threshold of 10 weeks was used to distinguish the rapid from the slow engraftment phenotype: a TTL of less than 10 weeks was designated TTL^{short}, and longer time periods were classified as TTL^{long} (Figure 1B).

Additional cutoff points representing intervals at 16 and 21 weeks (see Figure S1 available online) and leukemia characteristics (ALL-BFM high-risk stratification, TEL/AML1 fusion, prednisone-poor response (PPR), age, and white blood cells at diagnosis) were tested to divide the cohort. Age (unfavorable: 0–1 and older than 9 years; favorable: 1–9 years) and hyperleukocytosis at diagnosis (above or below 50 white blood cells [1000/μl]) were classified according to St. Jude's Total Therapy Study XIIIB protocol criteria (Pui et al., 2004b). Time to overt leukemia (in the recipient mice) and relapse-free survival (of the

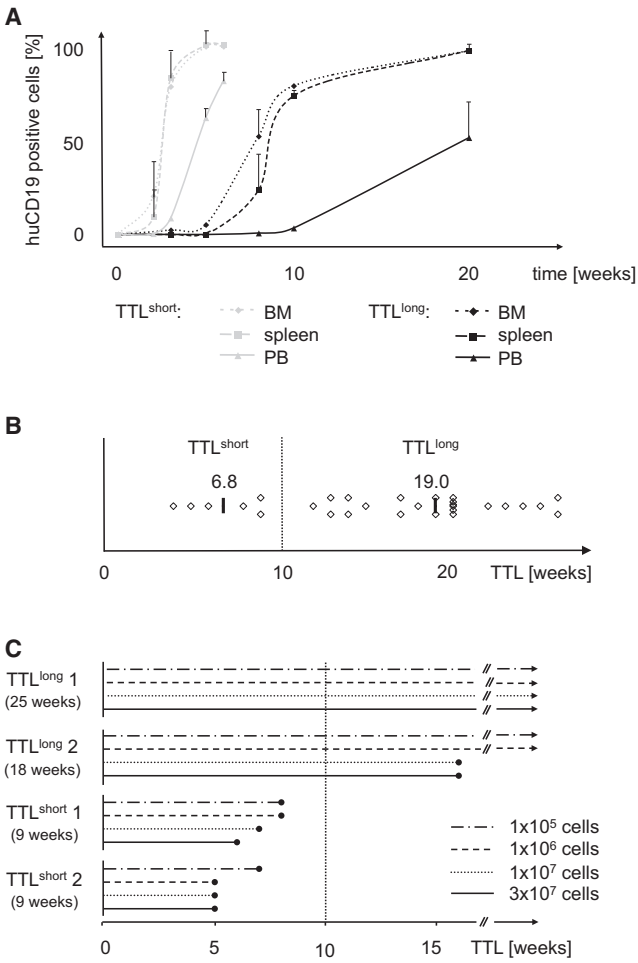

Figure 1. Distinct Engraftment Properties of NOD/SCID/huALL

(A) In vivo expansion of leukemia cells in different organ compartments of recipients over time. Two NOD/SCID/huALL samples (TTL^{short}, light gray; TTL^{long}, dark gray) were transplanted into recipient mice (n = 3 per group), and the proportions of huCD19 positive ALL cells were detected in bone marrow (rhombs, dotted lines), spleen (squares, dashed lines), or peripheral blood (triangles, solid lines) by flow cytometry. Data are given as mean of triplicates with standard deviation.

(B) TTL of engrafted transplants (n = 29). Each data point represents the mean TTL in weeks for every individual leukemia transplanted (n = 2 recipients). Some samples lead to disease within a short period of time (TTL^{short}, n = 6, mean 6.8 weeks) in contrast to samples showing leukemia manifestation after a longer time period (TTL^{long}, n = 23, mean 19.0 weeks; t test p = 2.8 e-8).

(C) NOD/SCID/huALL samples (n = 4) with either a TTL^{long} (25 or 18 weeks) or TTL^{short} (9 weeks) phenotype were retransplanted onto subsequent recipients injecting 1×10^5, 1×10^6, 1×10^7, or 3×10^7 cells per animal (n = 2 per group), and TTL was assessed for each recipient.

See also Figure S1 and Tables S1–S4.

patients) were compared between the subgroups divided according to the different cutoff points and pretreatment and clinical response features by Kaplan-Meier analysis and log rank test. Resulting p values were adjusted for multiplicity (Bonferroni's method) with the most distinct grouping according to the lowest adjusted significant p value. The 10-week cutoff point was shown to be most discriminatory with respect to engraftment and relapse-free survival (Tables S1 and S2).

Of the 50 samples transplanted, 21 samples did not expand in vivo to substantial cell numbers within the observation time. Leukemia manifestation after week 20 was not analyzed systematically in our study. However, two leukemias were followed up for a prolonged time and showed disease manifestation with a TTL of 36 and 44 weeks, suggesting that eventually all of the transplanted leukemia cell samples lead to overt leukemia if the observation time is long enough. The samples showing no overt leukemia at week 20 were included into the TTL^{long} group.

Stability, Consistency, and Independency of the TTL Phenotype

TTL was in all cases assessed after transplantation of 1×10^7 of primary patient leukemia cells. Sets of primary leukemia samples obtained at diagnosis were transplanted in parallel (1×10^7 cells), and TTL was assessed for both recipients of each leukemia sample. Similar TTL values were observed within the replicates of 21 primary leukemia samples (Table S3). Furthermore, two leukemia samples obtained at relapse were transplanted as replicates and compared to the respective matching diagnosis samples. Similar TTL values were also observed for the replicates of the relapse samples. Moreover, similar TTL was seen comparing diagnosis-relapse pairs (Table S3), indicating stability of the TTL phenotype, even at leukemia relapse.

We also analyzed the consistency of TTL in subsequent recipients. Leukemia cells of diagnostic patient samples and the consecutive xenograft passages were transplanted in parallel onto secondary and tertiary recipients using the identical dose of 1×10^7 cells for each recipient. TTL was assessed for both recipients of each passage, and similar TTL values were observed constantly within the replicates. Thus, TTL phenotypes did not change and remained TTL^{short} or TTL^{long} between diagnosis and consecutive passages in the NOD/SCID/huALL model (Table S3).

Also, later passages were investigated. Representative xenograft samples for TTL^{long} and TTL^{short}, as characterized upon initial transplantation of the diagnostic sample, were retransplanted onto primary, tertiary, or sixth recipients. The dose of 1×10^7 cells per recipient, as used throughout the study, recapitulated the TTL phenotype of the respective leukemia remaining stable also after up to five passages (Figure 1C; Table S4).

We also addressed whether the TTL phenotype might be influenced by varying numbers of cells transplanted. Higher numbers of TTL^{long} leukemia cells (3×10^7 cells per animal) did not accelerate engraftment, nor did a decrease of cell numbers (1×10^6, 1×10^5 cells per mouse) prolong TTL^{short}, demonstrating that the TTL phenotypes are largely independent from cell numbers transplanted and represent an intrinsic feature of the individual leukemia (Figure 1C; Table S4).

TTL and Patient Outcome

Six patients exhibited TTL^{short} and 44 TTL^{long}. Five of these six patients with TTL^{short} encountered medullar relapse within 24 months after diagnosis. This corresponds to the expected proportion of about 10% of patients at very high risk/with early relapse.

We analyzed relapse-free survival of the patients whose leukemia cells were used for transplant. Patients whose transplanted cells showed a TTL^{long} phenotype revealed a marked superior relapse-free survival compared to TTL^{short} patients. Most interestingly, all relapses of the patients characterized by TTL^{short} occurred early within the first 2 years after diagnosis (Figure 2A). Thus, the TTL^{short} phenotype is characteristic for patients at high risk for early relapse.

Because the majority of relapse patients originates from the low-risk groups, we analyzed whether TTL identifies patients who would encounter an early relapse despite stratification into low-risk groups. Therefore, the analysis was focused on the non-high risk group patients (n = 43: TTL^{short} n = 4, TTL^{long} n = 39). A clear inferior relapse-free survival of TTL^{short} patients was observed; all four non-HR patients characterized by TTL^{short} developed early medullar relapse within 24 months after diagnosis (Figure 2B). This demonstrates that TTL is associated with patient outcome independent of risk stratification.

Twenty-one leukemia samples did not lead to leukemia in the recipients within the observation time and were, therefore, included into the TTL^{long} group. We also analyzed whether this non-engraftment subgroup would be associated with a distinct patient outcome. Most importantly, the group of patients whose leukemia cells upon transplant did not lead to leukemia within 20 weeks of observation exhibited further increased superior relapse-free survival, even achieving 100% survival in the non-HR group (Figures 2C and 2D).

A superior relapse-free survival was also observed if only patient samples leading to overt leukemia (all risk groups, n = 29; non-high risk groups, n = 25) were analyzed, and patients whose leukemia cells did not engraft were excluded (Figures 2E and 2F). Furthermore, a significant correlation of TTL (mouse model) and remission duration (patients) was observed analyzing engrafted samples of relapsed patients (n = 9, all risk groups; n = 7, non-HR groups) (Figures 2G and 2H).

In summary the TTL phenotype is highly associated to patient survival, i.e., TTL^{short} identifies patients with early relapse, and TTL^{long} is characteristic for superior relapse-free survival of up to 100%, regardless of stratification into risk groups.

Prognostic Impact of TTL

Different prognostic factors have been implemented in different treatment protocols to stratify patients based on their individual risk. We compared the prognostic impact of TTL to established prognostic factors: poor leukemia cell clearance in response to steroid treatment (PPR); hyperleukocytosis (highly elevated white blood cell counts >50 [1000/μl] in the peripheral blood at diagnosis); and unfavorable age (age at diagnosis younger than 1 and older than 9 years) were included (Pui et al., 2004b). By multivariate Cox's regression, patients with TTL^{short} exhibited a 45-fold increased risk for relapse in contrast to no significant elevated risk for patients with PPR, high white blood cell count at diagnosis, or unfavorable age in this cohort (Table 1).

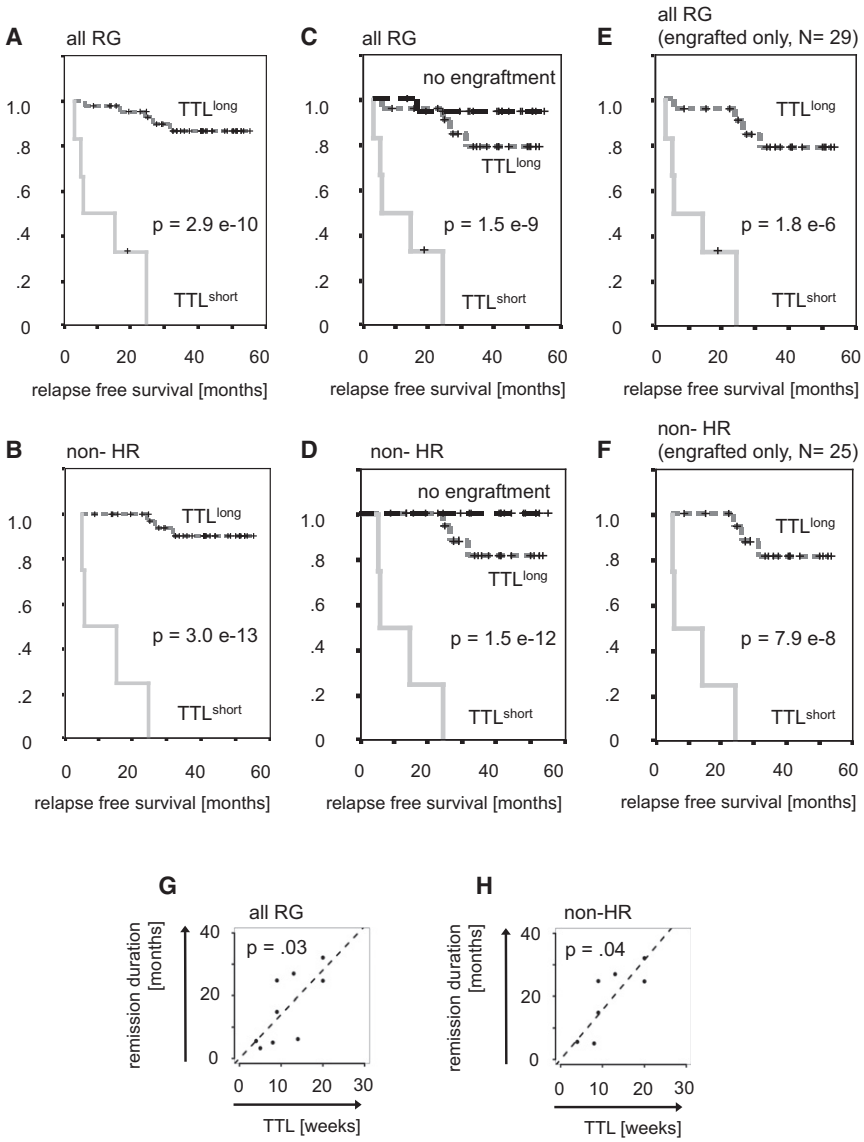

Figure 2. Superior Relapse-Free Survival of Patients with TTL^{long}

Relapse-free survival (RFS) of patients with TTL^{long} or TTL^{short} phenotypes (for A–F, P values by log rank test).

(A) All RG (n = 50: TTL^{long} n = 44; TTL^{short} n = 6; p = 2.9 e-10).

(B) Non-HR stratified patients only (n = 43: TTL^{long} n = 39, TTL^{short} n = 4; p = 3.0 e-13).

(C) RFS of patients with TTL^{long}, TTL^{short}, or with no leukemia manifestation within 20 weeks after transplant; all RG (n = 50: non-engrafted n = 21; TTL^{long} n = 23; TTL^{short} n = 6; p = 1.5 e-9).

(D) Non-HR (n = 43: non-engrafted n = 18; TTL^{long} n = 21; TTL^{short} n = 4; p = 1.5 e-12).

(E) Analysis of the group of patients whose samples engrafted, all RG (n = 29: TTL^{long}, n = 23; TTL^{short}, n = 6; p = 1.8 e-6).

(F) Non-HR (n = 25: TTL^{long}, n = 21; TTL^{short}, n = 4; p = 7.9 e-8).

(G) Spearman correlation of TTL (mouse) to time from diagnosis to relapse (remission duration) of relapsed patients; all RG (n = 9, rho = 0.731, p = 0.03).

(H) Non-HR (n = 7, rho = 0.782, p = 0.04).

See also Figure S2..

Also, in comparison to additional thresholds to determine TTL (16 and 21 weeks), TTL^{short} defined by the 10-week cutoff was the most significant prognostic factor (Table S5).

Genetic alterations are common in pediatric ALL and might be associated with favorable or unfavorable outcome and are, therefore, used for treatment stratification. The presence of BCR/ABL or MLL/AF4 fusion genes qualifies for HR stratification (Flohr et al., 2008; Schrappe et al., 2000a). In the group studied, one patient was positive for MLL/AF4 and displayed a TTL^{short} phenotype. No patients positive for BCR/ABL were included. TEL/AML1 fusion is associated with favorable outcome (Pui et al., 2004a). Most interestingly, all 19 patients positive for TEL/AML1 showed TTL^{long}. In addition, only one of the 50 patients included in this study met criteria for MRD-based high-risk stratification. However, this patient was high-risk stratified due to PPR and showed TTL^{long}. Pretreatment characteristics and response features such as age, gender, peripheral leukocyte count at diagnosis, and achievement of remission at the end of induction therapy (day 33) are not associated with TTL^{long} or TTL^{short} (Table 2).

TTL Is Confirmed in an Independent Leukemia Subset

To evaluate the significance of the TTL phenotype, an independent subset of cell bank BCP-ALL samples (four pairs matched for age, gender, and low-risk group classification either showing early relapse or not) was transplanted. Patients with early relapse always revealed a shorter TTL than patients with late or no relapse. However, transplant of one frozen cell bank sample derived from a patient with early relapse showed a "borderline" TTL of 13 weeks (Figure S2A). TTL was significantly associated with the time from diagnosis to relapse (remission duration) (Figure S2B), confirming the prognostic value of TTL.

Gene Expression Analysis of Xenograft Leukemia

To gain insight into molecular mechanisms responsible for distinct in vivo leukemia proliferation, gene expression profiles were analyzed using a human whole-genome array approach. ALL cells isolated from leukemia-bearing recipients (TTL^{long} or TTL^{short}) were investigated. Cytogenetic abnormalities leading to fusion genes and involving transcription factors are recognized by specific transcription profiles (Yeoh et al., 2002) and might overcast other differences in gene regulation. Therefore, the analysis has been focused on xenografts derived from patients without known gene fusions and also sufficient

Table 1. Prognostic Relevance of TTL

No. = 50	n	p	Risk Ratio (relapse)	CI
TTL^{short}	6	6.25 e-5	45.08	6.98–290.94
PPR	6	0.05	5.61	0.99–31.70
Unfavorable age	18	0.11	3.34	0.76–14.73
Hyperleukocytosis	16	0.51	1.68	0.36–7.86

Multivariate analysis on relapse-free survival (Cox regression, n = 50) including different risk factors: TTL^{short} phenotype; PPR; hyperleukocytosis (leukocyte count at diagnosis higher than 50 [1000/μl]); and unfavorable age (0–1 and >9 years); CI, confidence interval. See also Table S5.

follow-up time (n = 12: TTL^{short} n = 5, TTL^{long} n = 7) (Table S6). The expression profiles were analyzed employing a model-free shrinkage estimate of the variance across genes ranking the data set ("shrinkage t" statistic; Opgen-Rhein and Strimmer [2007]). Seventy-three genes (88 probe sets) were identified to be differentially regulated between TTL^{short} and TTL^{long} (FDR < 5%): 51 genes upregulated and 22 downregulated in TTL^{short} (Figure 3A and Table 3). Among the probe sets with the highest fold changes, two genes involved in regulation of mammalian target of rapamycin (mTOR) signaling were identified: *DDIT4L* and *RHEB*. *DDIT4L* codes for DNA-damage-inducible-transcript-4-like, a molecule negatively regulating mTOR. Consistent with this function (i.e., inhibiting mTOR, a central molecule regulating cell proliferation and survival), *DDIT4L* is upregulated in TTL^{long} (inhibition of mTOR) and downregulated in TTL^{short} (lack of mTOR inhibition). *RHEB*, coding for the positive mTOR regulator Ras homolog enriched in brain, was identified to be upregulated in TTL^{short} leukemia by two probe sets. In line with its mTOR-activating function, *RHEB* was found to be upregulated in TTL^{short}/early-relapse leukemia.

In addition, two genes coding for molecules involved in regulation apoptosis were identified displaying high fold changes: *PDE4A* and *DAPK1* (Table 3). The type 4 cyclic AMP phosphodiesterase PDE4A downregulates cAMP levels leading to impaired apoptosis sensitivity. In line, *PDE4A* is upregulated in TTL^{short}/early-relapse leukemia. *DAPK1* codes for the proapoptotic death-associated protein kinase and is congruent with its apoptosis-sensitizing effect, highly expressed in TTL^{long}/good prognosis leukemia.

Table 2. Patient Characteristics of De Novo BCP-ALL Samples Transplanted

		Total		TTL^{long}		TTL^{short}	
		No.	%	No.	%	No.	%
Total		50	100	44	100	6	100
Gender	Female	26	52	23	52	3	50
	Male	24	48	21	48	3	50
Age	1–9 years	32	64	29	66	3	50
	0–1 and >9 years	18	36	15	34	3	50
Immunophenotype	pro-B ALL	1	2	0	–	1	17
	c-ALL	36	72	33	75	3	50
	pre-B ALL	13	26	11	25	2	33
Fusion gene	TEL/AML1	19	38	19	44	0	–
	BCR/ABL	0	–	0	–	0	–
	MLL/AF4	1	2	0	–	1	17
	MLL/ENL	2	4	1	2	1	17
	No	26	52	22	50	4	66
	Not done	2	4	2	4	0	–
Hyperleukocytosis	<50 (1000/μl)	16	32	12	27	4	67
	>50 (1000/μl)	34	68	32	73	2	33
Prednisone response (PR)	Good	44	88	39	89	5	83
	Poor	6	12	5	11	1	17
MRD	MRD-HR	1	2	1	2	0	–
	MRD-non-HR	41	82	36	82	5	83
	No MRD available	8	16	7	16	1	17
Remission at day 33	Yes	50	100	44	100	6	100
Final risk groups	Non-HR	43	86	39	89	4	67
	HR	7	14	5	11	2	33

Differential Regulation of Identified Genes in Xenograft ALL

The differential regulation of transcripts identified in gene array analysis was analyzed by quantitative RT-PCR. ALL xenograft samples (n = 24: n = 12 used for array analysis and n = 12 without analyzed gene profile; no known gene fusions, n = 9 TTL^{short}, n = 15 TTL^{long}) were investigated. A significant differential regulation of *PDE4A*, *DDIT4L*, and *RHEB* in TTL^{short} versus TTL^{long} leukemia was confirmed (Figures 3B–3D). Additionally, also *FRAP1* coding for mTOR was analyzed and itself found to be significantly upregulated in TTL^{short} (Figure 3E). Moreover, transcript levels of the mTOR regulators *DDIT4L*, *RHEB*, and *FRAP1* itself were also highly associated with TTL (Table S7).

Application of the Xenograft Signature on an Independent Patient Cohort

To corroborate the relevance of the specific expression profile identified in xenograft leukemia samples, the TTL signature was applied onto profiles of an independent cohort of pediatric patients with BCP-ALL (n = 197, samples isolated at diagnosis; Table S8) that has also been treated according to a BFM-based protocol (AIEOP-LLA-2000). This independent set of patient profiles was analyzed with respect to the best distinction into subgroups according to the signature by a cluster number estimation procedure for k-means via repeated clusterings on resampled versions of the data including a correction for random partitions (Kraus and Kestler, 2010). Based on the 88 probe sets, two robust clusters were identified within the 197 patients (Figure S3A).

Most importantly, patients grouped into the TTL^{short} cluster displayed a significantly inferior relapse-free survival in contrast to patients clustering with the TTL^{long} profile (log rank test, p = 2.5 e-4) (Figure 3F).

A Classifier Based on the TTL Signature Identifies Patients with Early Relapse

The TTL^{short} phenotype and corresponding expression signature are strongly associated with early-relapse leukemia. For this reason we also analyzed whether, based on the TTL profile,

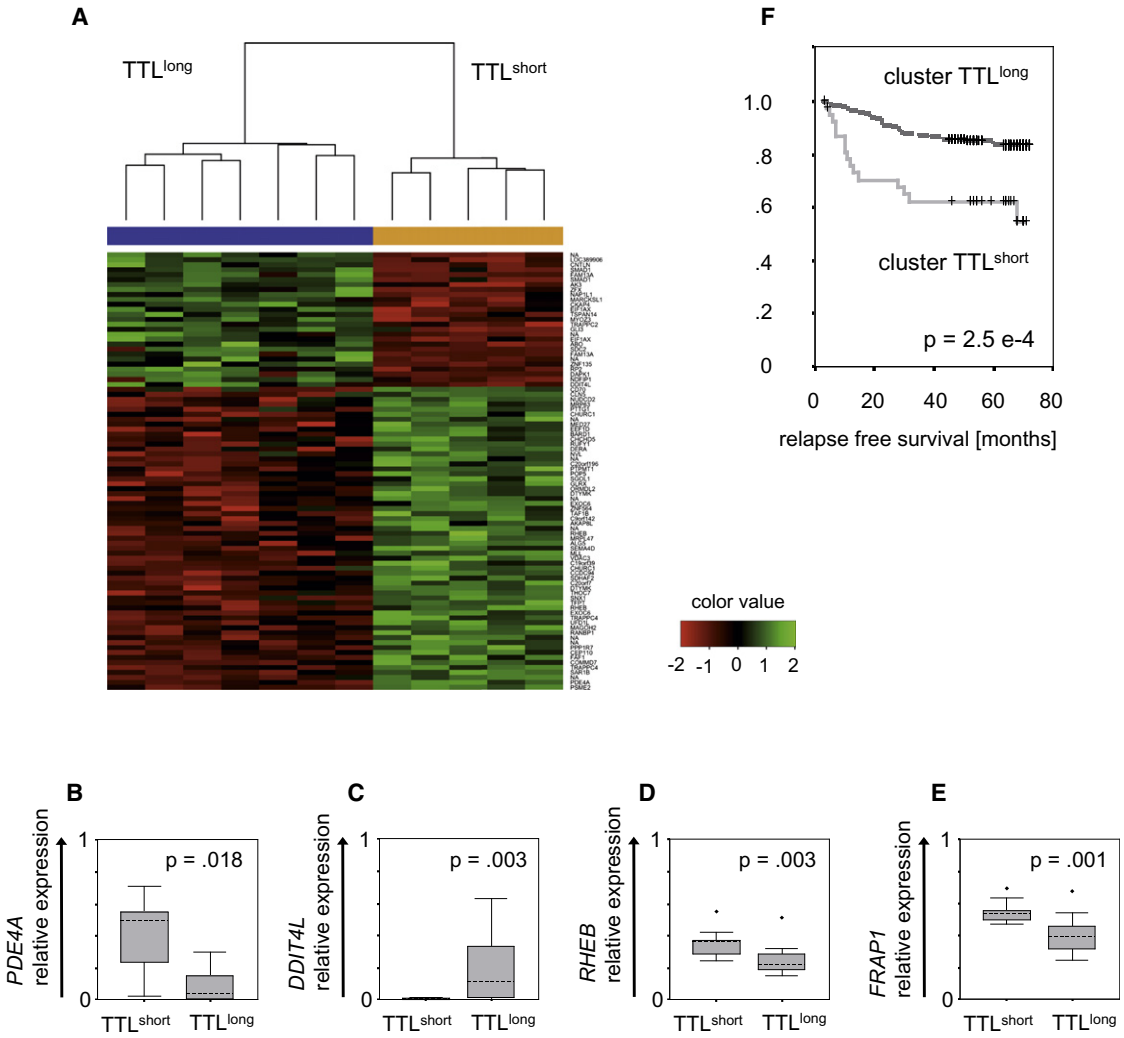

Figure 3. The TTL^{short} Signature Is Associated with Poor Outcome

(A) Signature of 88 differentially regulated probe sets (73 genes; "shrinkage t" statistic, FDR < 5%) in xenograft leukemia. Unsupervised cluster analysis of xenograft samples according to the TTL signature showing two clusters of TTL^{long} and TTL^{short} xenograft leukemia samples.

(B–E) Differential expression of individual genes in xenograft ALL samples (qRT-PCR; n = 24: TTL^{short} n = 9, TTL^{long} n = 15; Wilcoxon rank sum test); (B) *PDE4A*: p = 0.018; (C) *DDIT4L*: p = 0.003; (D) *RHEB*: p = 0.003.; and (E) *FRAP1*: p = 0.001.

(F) Significant inferior relapse-free survival of patients of an independent BCP-ALL patient cohort (n = 197) who cluster with the TTL^{short} profile. Clustering into two groups according to the TTL signature as determined by cluster number estimation analysis (see also Figure S3 and Tables S6–S8).

a classifier could be obtained to identify patients with early relapse. A classifier was generated by conjunction of threshold decisions on different genes using a set-covering machine (SCM) with data-dependent rays (Kestler et al., 2006; Marchand and Shawe-Taylor, 2002). Starting from the whole signature of 88 probes sets, the SCM reduced the number of genes employed in the decision to a set showing best early-relapse prediction. Utilizing 20 probe sets (including *RHEB* and *DAPK1*) (Figure S3B), all patients with early relapse were identified. The set was refined to further improve the robustness, yielding a subset of five probe sets (Figure S3C). Applying this rule to the 197 patients, all 26 early-relapse cases are detected (100% sensitivity), with an overall classification accuracy of 75%. Cross-validating (leave-one-out) this classifier achieved a sensitivity of 77% and an accuracy of 72%.

Cell Death Is Predominantly Induced in TTL^{short} but Not TTL^{long} Leukemia Cells

An activated (*DDIT4L*^{low}/*RHEB*^{high}/*FRAP1*^{high}) mTOR pathway was found to be characteristic for the TTL^{short} phenotype. Additionally, *PDE4A*^{high} was also associated with TTL^{short} leukemia. Therefore, inhibition of mTOR or PDE4A might successfully target TTL^{short}/early-relapse leukemia (Figures 4A and 5A). Xenograft ALL samples (TTL^{short} or TTL^{long}) isolated from recipient mice with overt leukemia were treated with either the mTOR inhibitor rapamycin or the phosphodiesterase inhibitor rolipram, and cell death was analyzed. Cell death was found to be increased upon ex vivo rapamycin treatment in most TTL^{short} (activated mTOR pathway) ALL cells, but not in TTL^{long} leukemia samples (Figure 4B). Treatment with rolipram also showed a stronger effect on the TTL^{short} (*PDE4A*^{high})

Table 3. Gene Signature Associated with the TTL Phenotype

Upregulated in TTL^{short}			Downregulated in TTL^{short}		
Probe Set	Gene Symbol	FC	Probe Set	Gene Symbol	FC
204735_at	PDE4A	3.480	227798_at	SMAD1	−5.526
226736_at	CHURC1	2.072	210993_s_at	SMAD1	−5.441
229814_at	NA	1.821	203139_at	DAPK1	−4.497
236443_at	NA	1.638	228057_at	DDIT4L	−3.809
222556_at	ALG5	1.345	217800_s_at	NDFIP1	−3.409
226643_s_at	NUDCD2	1.301	202973_x_at	FAM13A	−2.754
213409_s_at	RHEB	1.247	227376_at	GLI3	−2.427
206508_at	CD70	1.194	217047_s_at	FAM13A	−2.216
208844_at	VDAC3	1.188	206142_at	ZNF135	−1.798
244110_at	MLL	1.110	1559005_s_at	CNTLN	−1.781
223479_s_at	CHCHD5	1.070	212158_at	SDC2	−1.605
221995_s_at	MRP63	1.050	200998_s_at	CKAP4	−1.361
214395_x_at	EEF1D	1.046	213955_at	MYOZ3	−1.313
229525_at	THOC7	1.023	224151_s_at	AK3	−1.208
217426_at	NA	1.017	200644_at	MARCKSL1	−1.128
233924_s_at	EXOC6	1.009	201016_at	EIF1AX	−1.062
227364_at	NA	0.995	205191_at	RP2	−1.039
1553690_at	SGOL1	0.993	201019_s_at	EIF1AP1	−1.038
223210_at	CHURC1	0.975	201017_at	EIF1AX	−0.986
235878_at	TAF1B	0.878	219351_at	TRAPPC2	−0.938
204084_s_at	CLN5	0.854	216929_x_at	ABO	−0.804
227352_at	C19orf39	0.851	208655_at	NA	−0.786
216554_s_at	NA	0.846	214678_x_at	ZFX	−0.783
226259_at	EXOC6	0.843	221002_s_at	TSPAN14	−0.753
209276_s_at	GLRX	0.824	213864_s_at	NAP1L1	−0.681
203270_at	DTYMK	0.823	1564129_a_at	NA	−0.650
203554_x_at	PTTG1	0.821	1556102_x_at	LOC389906	−0.598
233380_s_at	RUFY1	0.821	–	–	–
235063_at	C20orf196	0.819	–	–	–
218064_s_at	AKAP8L	0.795	–	–	–
221598_s_at	MED27	0.785	–	–	–
219324_at	NOL12	0.777	–	–	–
223481_s_at	MRPL47	0.759	–	–	–
218556_at	ORMDL2	0.756	–	–	–
225901_at	PTPMT1	0.746	–	–	–
226070_at	C9orf142	0.726	–	–	–
204839_at	POP5	0.723	–	–	–
205345_at	BARD1	0.708	–	–	–
218102_at	DERA	0.702	–	–	–
224815_at	COMMD7	0.673	–	–	–
207877_s_at	NVL	0.662	–	–	–
203528_at	SEMA4D	0.659	–	–	–
205642_at	CEP110	0.653	–	–	–
1554482_a_at	SAR1B	0.652	–	–	–
217958_at	TRAPPC4	0.644	–	–	–
1553984_s_at	DTYMK	0.639	–	–	–
222894_x_at	C20orf7	0.638	–	–	–
212491_s_at	DNAJC8	0.635	–	–	–
218996_at	TFPT	0.627	–	–	–
1553957_at	ZNF564	0.627	–	–	–
223048_at	SDHAF2	0.620	–	–	–
217959_s_at	TRAPPC4	0.620	–	–	–
201214_s_at	PPP1R7	0.618	–	–	–
201452_at	RHEB	0.599	–	–	–
209103_s_at	UFD1L	0.597	–	–	–
204335_at	CCDC94	0.589	–	–	–
217692_at	MAGOH2	0.579	–	–	–
201716_at	SNX1	0.570	–	–	–
218080_x_at	FAF1	0.555	–	–	–
201762_s_at	PSME2	0.520	–	–	–
221915_s_at	RANBP1	0.519	–	–	–

Differentially regulated genes comparing TTL^{short} and TTL^{long} xenograft expression profiles (88 probe sets, 73 genes) identified by "shrinkage t" statistic, FDR < 5%. Fold change (FC) is given as logarithm (base 2), and probe sets upregulated in TTL^{short} (n = 61, 51 genes) display a positive, downregulated probe sets (n = 27, 22 genes) a negative fold change value.

samples in contrast to TTL^{long} (*PDE4A*^{low}) leukemia cells (Figure 5B).

These data on primary pediatric ALL showing an effect of both inhibitors, predominantly on TTL^{short} leukemia samples, point to a potential use as targets for directed therapy in patients at high risk for early relapse.

DISCUSSION

We identified in vivo proliferation of de novo BCP-ALL cells transplanted into NOD/SCID mice to be of prognostic and predictive value for patient outcome. While slow engraftment (TTL^{long}) is associated with favorable outcome, TTL^{short} is found in patients with poor prognosis. This biological phenotype is associated with a distinct cellular phenotype characterized by a specific gene signature identifying patients with early relapse and pointing to pathways regulating cell proliferation and apoptosis.

Consistent with previous reports, leukemia cells engrafted in our model of NOD/SCID/huALL retained the immunophenotypic and clinical characteristics of the primary leukemia (Baersch et al., 1997; Borgmann et al., 2000; Kamel-Reid et al., 1989; Lock et al., 2002) and the proportion of leukemia cells in peripheral blood correlated with infiltration in different organs (Lock et al., 2002; Nijmeijer et al., 2001). The observed engraftment phenotype remained stable not only upon transplant of identical cell numbers of the same leukemia sample but was also consistent during consecutive passages.

Intriguingly, we found a most significant correlation of in vivo leukemia growth and overall patient outcome (ALL-BFM therapy). Patients whose leukemia cells showed a short time to overt leukemia in the recipient animals (TTL^{short}) relapsed early

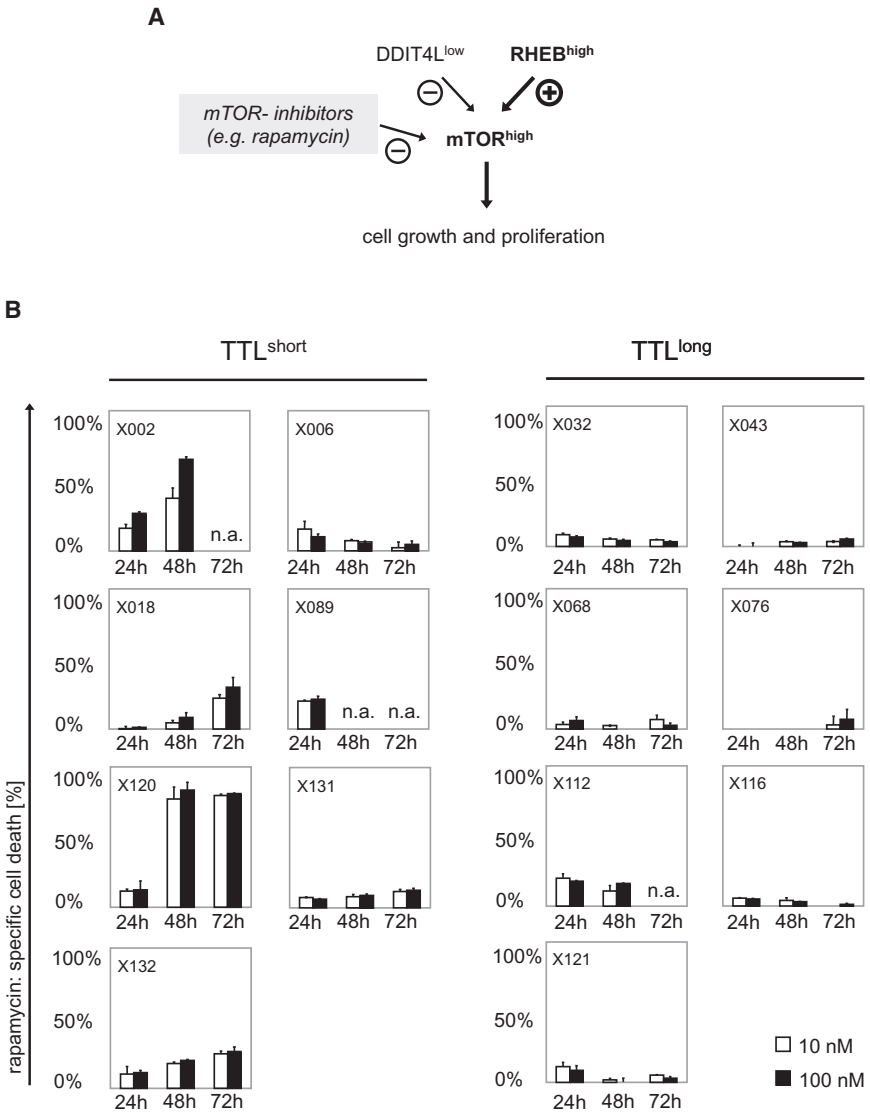

Figure 4. mTOR as Potential Therapeutic Target for TTL^{short}/Early-Relapse Leukemia

(A) TTL^{short} is characterized by an activated mTOR pathway (*DDIT4L*^{low}/*RHEB*^{high}/*FRAP1*^{high}) serving as potential target for directed therapy of TTL^{short}/early-relapse leukemia.

(B) Specific cell death of xenograft ALL upon ex vivo treatment with rapamycin (10 nM, white columns; 100 nM, black columns) for 24, 48, or 72 hr. Time points with >90% spontaneous cell death were not analyzed (n.a.). Data are given as mean of triplicates with standard deviation.

within 24 months while still on therapy, a feature repeatedly shown to be associated with poor outcome (Gaynon et al., 1998; Henze et al., 1991). Most relapses occur in non-high risk patients initially responding to treatment (Schrappe et al., 2000a), indicating that these patients are not identified by the current stratification strategies. When we focused our analysis on this low-risk group only, we also observed a clear-cut inferior survival of patients with TTL^{short} in this non-HR patient group. A putative prognostic impact of in vivo leukemia growth on patient outcome was investigated in a number of studies, so far with conflicting results. This might be explained by the small number of patients studied, the use of less permissive recipients, or because leukemia cells from patients at relapse were used (Kamel-Reid et al., 1991; Lock et al., 2002; Uckun et al., 1995, 1998).

Pretreatment characteristics such as age, white blood cell count at diagnosis, but also initial treatment response were not significantly associated with increased relapse risk. Most importantly, patients with TTL^{short} had a 45-fold increased relapse risk, demonstrating the power of TTL as an independent prognostic factor. TEL/AML1 fusion is associated with favorable outcome (Pui et al., 2004a); concordantly with this, all patients positive for TEL/AML1 (n = 19) exhibited TTL^{long} in our xenotransplant model. One patient with TTL^{long} included in our study was high-risk stratified based on MRD; all five patients with TTL^{short} with available information were not detected by MRD as high risk. Impaired reduction of leukemia cells after treatment as assessed by MRD is likely to be due to resistance. Despite relapse, patients with TTL^{short} are not characterized by poor treatment response. By transplanting different numbers of TTL^{short} and TTL^{long} leukemia cells, we found that engraftment properties are indeed intrinsic to the leukemia cell itself and are retained independently of the cell number transplanted. Also, NOD/SCID/huALL established from cryopreserved leukemia cells in the test set showed an identical correlation of TTL and time to relapse as NOD/SCID/huALL established from fresh samples in our study. These results are in line with observations showing the engraftment ability to be an inherent property also of AML cells transplanted (Monaco et al., 2004; Pearce et al., 2006).

We identified a specific TTL-gene profile with robust clustering and inferior relapse-free survival of the TTL^{short} signature group if applied onto an independent patient cohort. Signatures associated with treatment response and relapse in pediatric ALL have been previously reported (Bhojwani et al., 2006; Cario et al., 2005; Flotho et al., 2007; Holleman et al., 2004; Lugthart et al., 2005; Yeoh et al., 2002); however, based on the TTL profile, a classifier was also obtained, robustly identifying this very high-risk group of patients with early relapse.

Importantly, these findings also point to activated pathways associated with poor outcome indicating additional therapeutic targets. mTOR is a central downstream switch integrating diverse pathways involved in cell growth and survival (Brown et al., 1994). DDIT4L negatively regulates the mTOR pathway

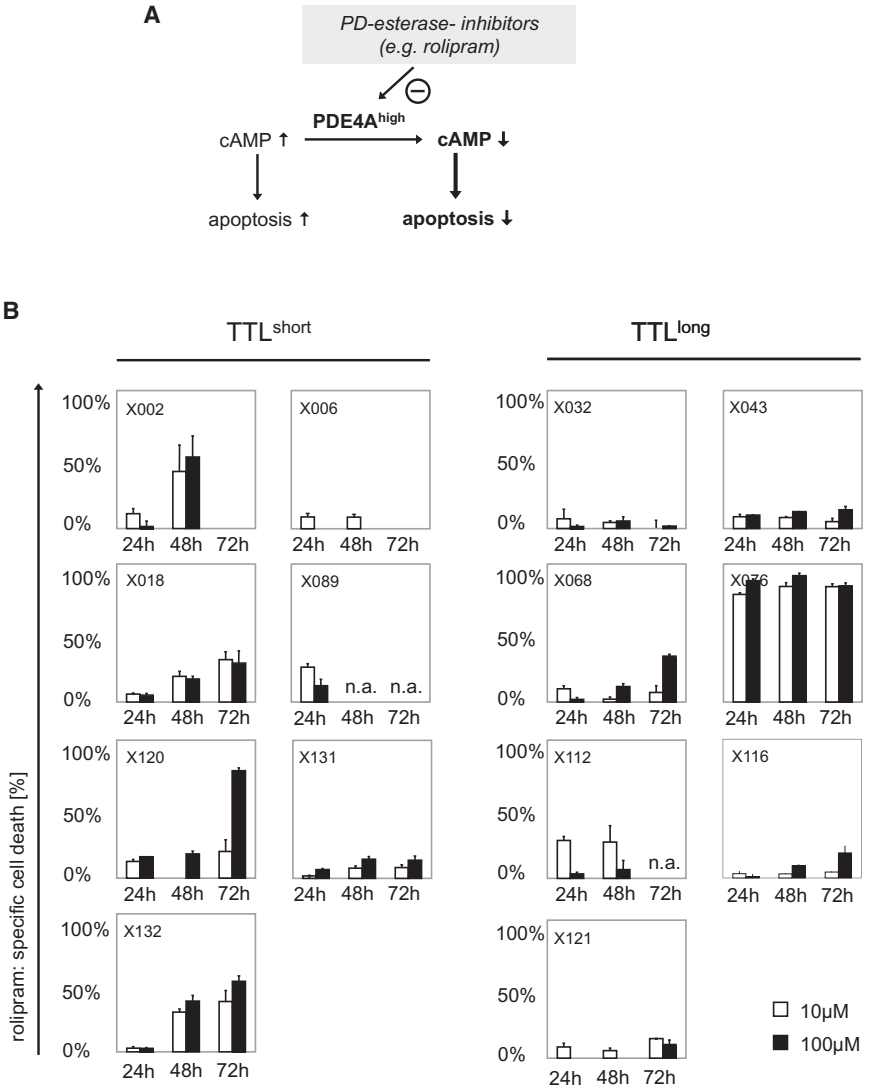

Figure 5. Inhibition of PDE4A Induces Cell Death Predominantly in TTL^{short}/Early-Relapse Leukemia

(A) Predominant effect of phosphodiesterase inhibitors on TTL^{short}/early-relapse leukemia associated with high PDE4A.

(B) Specific cell death of xenograft leukemia samples ex vivo incubated with rolipram (10 μM, white columns; 100 μM, black columns) for 24, 48, or 72 hr. Time points with >90% spontaneous cell death were not analyzed (n.a.). Data are given as mean of triplicates with standard deviation.

inhibiting cell growth and facilitating cell death (Corradetti et al., 2005) and is highly expressed in TTL^{long}, suggesting a more efficient control of mTOR in this subgroup. Vice versa, leukemias showing downregulated *DDIT4L* might lack repression of mTOR, thereby exhibiting TTL^{short} and impaired outcome.

Most interestingly, RHEB, directly binding to the mTOR complex and thereby enabling mTOR activation (Long et al., 2005), was identified in the TTL signature by two probe sets. *RHEB* is upregulated in TTL^{short}, accounting for increased activation of the mTOR pathway. Furthermore, upregulation of *FRAP1* (mTOR) itself was also strongly associated with TTL^{short}, underlining the relevance of this pathway positively regulating cell proliferation for early-relapse leukemia.

Taken together, the TTL^{short} phenotype is characterized by *DDIT4L*^{low}/*RHEB*^{high}/*FRAP1*^{high}, indicating an activated mTOR pathway in the TTL^{short}/early-relapse leukemias. These results have important therapeutic implications because the activated mTOR pathway can be directly targeted inhibiting growth of TTL^{short} leukemia. Rapamycin and related mTOR inhibitors have been shown to inhibit leukemia growth and induce apoptosis in BCP-ALL in vitro and in vivo alone or in combination with established chemotherapeutic drugs (Brown et al., 2003; Crazzolara et al., 2009; Teachey et al., 2006, 2008), and similarities of drug-associated signatures identified rapamycin to induce glucocorticoid sensitivity in ALL cells (Wei et al., 2006). In fact, TTL^{short} leukemias were more sensitive to ex vivo rapamycin treatment than TTL^{long} samples. The individual xenograft leukemias showed heterogeneous sensitivities suggesting additional mechanisms responsible for the TTL phenotype. Indeed, additional genes involved in cell proliferation and apoptosis were identified in the TTL signature.

PDE4A downregulates cAMP levels, leading to impaired apoptosis sensitivity (Jiang et al., 1996), and was, in line with this function, found to be upregulated in TTL^{short} leukemia. Inhibition of PDE4A activity using compounds like rolipram resulted in increased glucocorticoid sensitivity and apoptosis in leukemia cells (Meyers et al., 2007; Moon and Lerner, 2003; Ogawa et al., 2002). Treatment of xenograft leukemia samples with rolipram in fact induced cell death predominantly in TTL^{short} leukemia cells.

DAPK1, a tumor suppressor gene whose expression is lost by epigenetic silencing in a number of different tumors, including hematopoietic malignancies (Esteller et al., 1999; Katzenellenbogen et al., 1999; Kissil et al., 1997; Raval et al., 2007; Sanchez-Cespedes et al., 2000), codes for a serine/threonine kinase that positively regulates apoptosis and suppresses tumor progression in vivo (Cohen et al., 1999; Deiss et al., 1995; Inbal et al., 1997). Consistent with these reports, *DAPK1* was identified to be downregulated in TTL^{short}, suggesting that TTL^{short} cells evade from cell death by loss of this proapoptotic regulator. Along this line, we previously reported that deficient apoptosis signaling via the intrinsic pathway is a feature of poor prognosis in primary pediatric ALL and AML (Meyer et al., 2006, 2008). In contrast to its proapoptotic and tumor-suppressing function, upregulated *DAPK1* expression was reported in pediatric ALL

samples resistant to in vitro prednisolone treatment (Holleman et al., 2006). However, TTL is independent of glucocorticoid resistance, with five of six patients with TTL^{short} being prednisone-good responders, indicating that the engraftment phenotype of primary ALL cells reflects constitutional hallmarks of the leukemia.

Taken together, the proliferative capacity of primary ALL cells in NOD/SCID mice appears to reflect intrinsic properties of the leukemia cells and determines relapse in pediatric ALL. TTL^{short} is characteristic for patients with early relapse and associated with a specific gene profile. The biological impact of our observations in the xenotransplant model could be demonstrated in an independent group of patients and led to identification of distinct pathways involved in regulation of cell growth and apoptosis that can be targeted by well-known drugs such as rapamycin. Thus, our NOD/SCID/huALL model provides a powerful tool to identify prognostic factors in acute leukemia at a cellular and molecular level, leading to rational targets for therapeutic intervention strategies.

EXPERIMENTAL PROCEDURES

Detailed information with respect to methods and materials used is available in the Supplemental Experimental Procedures.

Patients and Leukemia Samples

Leukemia samples were obtained at diagnosis from pediatric patients with de novo BCP-ALL after informed consent was given in accordance with the institution's ethical review board (Ethikkommission Universität Ulm). Patients were enrolled into the ALL-BFM (Schrappe et al., 2000a) or the AIEOP LLA 2000 study protocol (Conter et al., 2000); both studies are performed in accordance to the Declaration of Helsinki and are registered at http://clinicaltrials.gov (ALL BFM 2000: NCT00430118; and AIEOP LLA 2000: NCT00613457).

NOD/SCID/huALL and TTL

All experimental animal studies were conducted according to the national animal welfare law (Tierschutzgesetz) and were approved by the appropriate authority (Regierungspräsidium Tübingen, Germany, experiment number 775). Patient samples were isolated from diagnostic specimens and consisted of more than 90% leukemia cells. Unconditioned NOD/LtSz-scid/scid mice were transplanted by intravenous injection of 1×10^7 cells per recipient throughout the study. As an exception, different numbers were injected if the impact of the cell dose was analyzed. TTL was determined for each patient sample transplanted as weeks from transplant to clinical leukemia manifestation. ALL cells were detected, and leukemia was confirmed in peripheral blood, bone marrow, or spleen by flow cytometry (Lock,et al., 2002). Mice without evidence for disease at week 20 after transplant were killed, and absent leukemia infiltration of spleen or bone marrow confirmed no leukemia.

Gene Expression and Statistical Analysis

Gene expression analysis was carried out on xenograft leukemia samples with sufficient follow-up time and negative for known cytogenetic abnormalities (n = 12: TTL^{short} n = 5 and TTL^{long} n = 7) isolated from recipients with overt leukemia. Expression profiles of the validation cohort (n = 197) were obtained from diagnostic specimens from patients treated according to the AIEOP LLA 2000 protocol. Expression was analyzed using the Human Genome U133 Plus 2.0 Array platform. Gene expression microarray files (Affymetrix .CEL files) were generated using the GCOS 1.4 or 1.2 software (Affymetrix). Arrays have been normalized using robust multiple-array average (RMA) (Gautier et al., 2004). Expression data were analyzed using Bioconductor package for R (v2.11.1). Differentially expressed genes have been calculated by the shrinkage T-statistic (Opgen-Rhein and Strimmer, 2007). Multiple comparison results were controlled by maintaining a FDR < 0.05 (Strimmer, 2008). The signature of 88 differentially expressed probe sets was applied to profiles of 197 independent patients with BCP-ALL. A robust clustering method based on the K-means algorithm has been applied to estimate the correct number of clusters in this data set (Kraus and Kestler, 2010). Kaplan-Meier analysis has been performed of relapse-free survival in the two clusters obtained from the cluster analysis. Classification into TTL^{short} and TTL^{long} classes was computed with a SCM algorithm (Kestler et al., 2006; Marchand and Shawe-Taylor, 2002), and a leave-one-out method has been used to validate the classifier.

All other statistical analyses were carried out using SPSS 11.0 software (SPSS, Munich, Germany) or "R" (v. 2.11.1) (R-Development-Core-Team, 2010); p values of <0.05 were considered significant in all tests carried out in this study.

Ex Vivo Treatment of Xenograft Leukemia

Leukemia cells were isolated from recipient animals as described and incubated with either rapamycin (10 and 100 nM) or rolipram (10 and 100 μM) diluted in dimethyl sulphoxide (DMSO) or DMSO alone, and cell viability was assessed by flow cytometry (forward/side scatter criteria).

ACCESSION NUMBERS

Data have been deposited in NCBI's Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo) and are accessible through GEO Series accession number GSE13576.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and eight tables and can be found with this article online at doi:10.1016/j.ccr.2010.11.014.

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