Clinical Cancer Research

Epigenetic Silencing Affects L-Asparaginase Sensitivity and Predicts Outcome in T-ALL

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Abstract

Purpose: Biological explanation for discrepancies in patient-related response to chemotherapy depending on the underlying oncogenic events is a promising research area. TLX1- or TLX3-deregulated T-cell acute lymphoblastic leukemias (T-ALL; TLX1/3$^+$) share an immature cortical phenotype and similar transcriptional signatures. However, their prognostic impacts differ, and inconsistent clinical outcome has been reported for TLX3. We therefore hypothesized that the overlapping transcriptional profiles of TLX1$^+$ and TLX3$^+$ T-ALLs would allow identification of candidate genes, which might determine their distinct clinical outcomes.

Experimental Design: We compared TLX1$^+$ and TLX3$^+$ adult T-ALL outcome in the successive French national LALA-94 and GRAALL-2003/2005 multicentric trials and analyzed transcriptomic data to identify differentially expressed genes. Epigenetic regulation of asparagine synthetase (*ASNS*) and *in vitro* L-asparaginase sensitivity were evaluated for T-ALL cell lines and primary samples.

Results: We show that TLX1$^+$ patients expressed low levels of *ASNS* when compared with TLX3$^+$ and TLX-negative patients, due to epigenetic silencing of *ASNS* by both DNA methylation and a decrease of active histone marks. Promoter methylation of the *ASNS* gene correlated with L-asparaginase sensitivity in both T-ALL cell lines and patient-derived xenografts. Finally, *ASNS* promoter methylation was an independent prognostic factor for both event-free survival [HR, 0.42; 95% confidence interval (CI), 0.24–0.71; $P = 0.001$] and overall survival (HR, 0.40; 95% CI, 0.23–0.70; $P = 0.02$) in 160 GRAALL-2003/2005 T-ALL patients and also in an independent series of 47 LL03-treated T lymphoblastic lymphomas ($P = 0.012$).

Conclusions: We conclude that *ASNS* methylation status at diagnosis may allow individual adaptation of L-asparaginase dose.

Introduction

T-cell acute lymphoblastic leukemias (T-ALL) are aggressive and highly heterogeneous malignancies that predominate in the 10- to 39-year age group, where they account for 20% of ALL. Genome-wide expression (1–3) assays led to the identification of several oncogenic T-ALL subgroups, namely the immature/Early Thymic Precursor (ETP; LyL1, MEF2C), late cortical (TAL1), and early cortical (TLX1/3 and NKX2.1) clusters (Supplementary Fig. S1). T-cell leukemia homeobox genes *TLX1* and *TLX3* belong to the NKL subclass of homeodomain (HD) proteins and contain a highly conserved HD known to be involved in DNA–protein and protein–protein interactions. Both of these genes are frequently deregulated in T-ALL (TLX1/3$^+$ T-ALL), predominantly by involvement in chromosomal translocations, which lead to constitutive expression.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Despite recent advances, "personalized" medicine does not currently benefit most patients individually, and classical chemotherapies still remain the cornerstone of first-line treatment. We report, using a leukemic "proof-of-concept" model, the implication of epigenetic modulation in individual patient-related drug response. Methylation status may therefore allow individual adaptation of chemotherapy dose.

During embryonic development, HOX proteins, in general, and TLX, in particular, repress transcriptional events. Physiologic expression of TLX1 and TLX3 is restricted to embryonic development (4, 5), and no specific function of these genes has been reported in T-cell lymphopoiesis. We recently demonstrated that TLX1/3 proteins repress TCRα rearrangements (6), leading to the early cortical maturation arrest observed in TLX1/3$^+$ T-ALLs. Despite the highly related oncogenic functions and structural features of these proteins, TLX1/3$^+$ T-ALLs are paradoxically heterogeneous in their clinical outcome. Although several adult and pediatric clinical trials have shown that T-ALLs overexpressing TLX1 display a favorable prognosis (7–13), data regarding the prognostic impact of TLX3 are more conflicting (1, 14, 15). The molecular explanation of this heterogeneity is unknown. We therefore hypothesized that the differences in transcriptional profiles of TLX1$^+$ and TLX3$^+$ T-ALLs would allow identification of candidate genes which might determine their distinct clinical outcomes. We identified asparagine synthetase gene (*ASNS*) repression by hypermethylation in TLX1$^+$ T-ALLs and demonstrated that *ASNS* methylation, but not expression, could represent a prognostic biomarker in T-ALLs treated within the GRAALL (Group for Research in Adult Acute Lymphoblastic Leukemia) trials.

Materials and Methods

Patients and treatments

Adult patients (15–60 years old), included in three successive French ALL cooperative group trials (LALA-94, GRAALL-2003, and GRAALL-2005) with T-ALL, defined according to the 2008 WHO classification, were analyzed. The LALA-94 protocol was a multicenter phase III trial, which enrolled 239 T-ALL from June 1994 to January 2002 (13). Of 97 patients with material available for oncogenic analysis, 25 demonstrated TLX1/3 overexpression. Ninety-seven LALA-94 patients were included in this study purely on the basis of material availability and were representative of the entire group (Supplementary Table S1; Supplementary Figs. S2 and S3). The GRAALL-2003 protocol was a multicenter phase II trial, which enrolled 76 adults with T-ALL between November 2003 and November 2005 of whom 50 had sufficient diagnostic tumor material available (16). The multicenter randomized GRAALL-2005 phase III trial was very similar to the GRAALL-2003 trial, with the addition of a randomized evaluation of an intensified sequence of hyperfractionated cyclophosphamide during induction and late intensification (17). Between May 2006 and May 2010, 337 adults with T-ALL were randomized in the GRAALL-2005, of which 185 had available diagnostic material. TLX1/3 overexpression was found in 77 of the 235 GRAALL T-ALL samples (Supplementary Fig. S2). All samples contained >80% blasts. Phenotypic and oncogenetic characteristics were as described (7, 9). Trial registration: GRAALL-2003 and GRAALL-2005 trials were registered at http://www.clinicaltrials.gov as #NCT00222027 and #NCT00327678, respectively. The LALA94, GRAALL-2003, and GRAALL-2005 protocols are briefly described in Supplementary Table S2. Informed consent was obtained from all patients at enrollment. All trials were conducted in accordance with the Declaration of Helsinki and approved by local and multicenter research ethical committees.

TLX1 inactivation

MISSION TRC shRNA Target Set vectors for TLX1 (TRCN0000014995) were purchased from Sigma Chemicals. Knockdown of the *TLX1* endogenous RNA transcripts was performed by transduction of the ALL-SIL cell line as described (6).

Genetic inactivation of ASNS

Two different SiRNA, SiRNA-1 and SiRNA-2, and a scrambled universal negative control RNA duplex (SR300319, Origene) were used to perform transient genetic knockdown of *ASNS*. SiRNA sequences are specified in Supplementary Table S3.

Microarray gene expression profiling analyses

The Affymetrix U133A microarray analyses (3) were performed on the quantile-normalized data using dChip1.3 (http://www.dchip.org) software. Locus Link symbols and ID (https://www.ncbi.nlm.nih.gov/gene) were used when the information was available to label genes corresponding to the probe sets. The Affymetrix U133 plus 2.0 microarrays analyses (2, 6) were performed with R version 3.1.1 using the "Affy" package from Bioconductor 3.1. The probe intensities were $\log_2$ transformed and normalized using RMA methodology. Identification of differentially expressed genes was performed by Significance Analysis of Microarrays, using 1000 permutations and an FDR threshold of 5%. The Custom-designed Agilent microarray covering all protein coding genes (33,128 mRNA probes, Human Sureprint G3 8 × 60k Agilent microarrays) analyses (18) were performed with R v3.1.1. (R Foundation for Statistical Computing). Expression data were normalized using the VSN-package (Bioconductor release 3.1) in R. Differential expression analysis was performed in R using Limma package. The genes were said to be differentially expressed if there was more than one Log_2 fold change in expression between the two conditions with a P value less than or equal to 0.05.

ChIP-seq and RNA-seq

Data of H3K4me3 chromatin immunoprecipitation sequencing (ChIP-seq) and RNA-seq in ALL-SIL and CCRF-CEM cell lines and H3K4me3 and H3K36me3 ChIP-seq in 1 TLX1$^+$ and 3 TLX$^-$ patients were generated and processed within the framework of the Blueprint consortium and visualized using the Integrated Genome Browser (http://bioviz.org/igb/). ChIP antibodies used wereH3K4me3: catalog number C15410003-50 (diagenode) and H3K36me3: catalog number C15410192 (diagenode).

Western blot

Immunoblotting was performed with primary antibodies to ASNS (ref. 19, kindly provided by D. Bouscary, Cochin Hospital, Paris) and Actin (Abcam) followed by bovine anti–mouse-IgG-HRP (Santa Cruz Biotechnology) secondary antibodies.

Quantification of ASNS by qRT-PCR

qRT-PCR was performed using the Master SYBR-Green reagent kit. Primers sequences used were: ASNS-Forward: 5′-GAAATGAGAATTCCAAAGAATGG-3′ and ASNS-Reverse: 5′-CTTTTGGTCGCCAGAGAATCT-3′. The change-in-threshold (-ΔΔCt) method was used to quantify transcript levels following PCR amplification. *ASNS* levels were calculated relative to the reference genes *ABL1* or *GAPDH*.

DNA methylation study

Global DNA methylation was assessed by Methylated DNA Immunoprecipitation (MeDIP) assay in a series of 24 T-ALLs, including 6 TLX1-positive cases and 3 human *thymii*. Briefly, methylated DNA was immunoprecipitated as described previously (20) using 2 μg of sonicated genomic DNA. MeDIP samples were directly subjected to labeling and hybridization on a custom human promoter array (Agilent; ref. 6), following the manufacturer's instructions. Finally, median-normalized $\log_2$ enrichment ratios (MeDIP/Input) were calculated for each probe using CoCAS software (21) and visualized using IGB tool (http://bioviz.org/igb). Direct *ASNS* methylation levels were analyzed by Methylation Sensitive-Multiple Ligation-dependent Probe Assay (MS-MLPA) with custom probes (Supplementary Table S4) and SALSA MLPA P200 Reference-1 probemix and EK1 reagent kits from MRC-Holland, according to manufacturer's recommendations. Data were analyzed with the Coffalyser software (MRC-Holland).

Patients analyzed for global DNA methylation have clinicobiological characteristics similar to patients not included in this analysis (Supplementary Table S5).

Statistical analysis

Comparison of continuous and categorical variables between subgroups was performed by Mann–Whitney test and Fisher exact test, respectively. Censored data [i.e., overall survival (OS), event-free survival (EFS), and disease-free survival (DFS)] were analyzed using Cox models. OS and EFS were calculated from the date of prephase initiation. The Kaplan–Meier method was used to estimate the probability of survival. Events accounting for EFS were induction failure, first hematologic relapse, and death from any cause in first complete remission (CR). DFS was calculated from the date of CR achievement. Events accounting for DFS were first hematologic relapse and death from any cause in first CR. In some analyses, outcome variables were censored at allogeneic hematopoietic stem cell transplant (HSCT) time. Univariate and multivariate analyses assessing the impact of categorical and continuous variables were performed with a Cox model. Proportional-hazards assumption was checked before conducting multivariate analyses. Interaction between trial (LALA-94 vs. GRAALL-2003/2005) effect and TLX ($TLX1^+$ vs. $TLX3^+$) status was tested by introducing an interaction term in the multivariable model comprising three covariates: Trial, TLX1/3 status, and the interaction term (Trial x TLX1/3). Statistical analyses were performed with STATA software (STATA 12.0 Corporation). All tests were two-sided with a significance level of 0.05.

Results

$TLX3^+$ adult T-ALL outcome is improved by an intensive, pediatric-inspired, chemotherapy regimen

In order to compare the therapeutic response of $TLX1^+$ and $TLX3^+$ adult T-ALLs, we first analyzed their outcome within the successive French LALA-94 and GRAALL-2003/2005 multicenter trials. The main difference in chemotherapy was a global increase in dose intensity in the GRAALL trials with, notably, addition of an intensive asparaginase sequence during induction therapy (6,000 U/m^2/day; see Supplementary Table S2 for details) which was repeated during delayed intensification, 12 weeks after CR. TLX overexpression was demonstrated in 25 of 97 (26%) and 77 of 235 (33%) LALA94 and GRAALL03/05 T-ALLs, respectively, including 65 (64%) $TLX1^+$ and 37 (36%) $TLX3^+$ cases (Supplementary Fig. S2).

As previously reported, $TLX3^+$ patients were slightly younger [28 years (15–55) vs. 35 years (17–57), $P = 0.03$] and had higher initial white blood cell (WBC) counts [59 G/L, range (2–308) vs. 23 G/L, range (4–294), $P = 0.03$] and less frequent cortical immunophenotypes compared with $TLX1^+$ T-ALL cases. *NOTCH1*, *FBXW7*, *RAS*, *PTEN*, and *NUP214-ABL* alterations were found at similar incidences in both groups (Supplementary Table S6).

CR was achieved in 65 of 65 $TLX1^+$ patients (100%) and 34 of 37 $TLX3^+$ patients (92%, $P = 0.05$). For $TLX3^+$ patients, the CR rate was 80% (8/10) in the LALA-94 trial versus 96% (26/27) in the GRAALL trials ($P = 0.17$). Because eligibility for HSCT differed among trials, survival analyses were censored at the time of HSCT. As shown in Fig. 1, 3-year EFS was 0% versus 66% [95% confidence interval (CI), 39–84] for $TLX3^+$ patients treated on the LALA-94 and GRAALL trials, respectively ($P = 0.001$). In $TLX1^+$ patients, although a trend, this difference was not significant (3-year EFS: 46%, 95% CI, 19–70 vs. 65%, 95% CI, 46–79 in LALA-94 vs. GRAALL trials, $P = 0.16$). Similarly, 3-year OS was 11% (95% CI, 2–72) versus 82% (95% CI, 66–100) for $TLX3^+$ patients treated on the LALA-94 and GRAALL trials, respectively ($P < 0.001$). This difference was not statistically significant in $TLX1^+$ patients, with a 3-year OS of 60% (95% CI, 39–95) versus 80% (95% CI, 68–95) for LALA-94 and GRAALL patients, respectively ($P = 0.15$). Moreover, there was a significant interaction, in a multivariable model between the trial and the TLX1 and TLX3 groups for EFS ($P = 0.05$) and a trend for OS ($P = 0.07$), suggesting that $TLX3^+$, but not $TLX1^+$, patients preferentially benefited from the more intensified GRAALL protocols. We therefore hypothesized that the overlapping transcriptional profiles of $TLX1^+$ and $TLX3^+$ T-ALLs would allow identification of candidate genes which might determine their distinct clinical outcomes.

Low asparagine synthetase transcript expression in $TLX1^+$ T-ALL

In an attempt to identify the molecular mechanism underlying the disparity in response to treatment between $TLX1^+$ and $TLX3^+$ T-ALLs, differentially expressed genes in three data sets were compared. We first took advantage of public transcriptomic data of T-ALL patients (3) to establish a list of differentially expressed transcripts in $TLX1^+$ ($n = 11$) as compared with $TLX3^+$ ($n = 18$) patients. This list (Supplementary Table S7) contains 259 unique genes ($P < 0.05$, fold change = 2). Two other transcriptomic data sets were obtained by RNAi inactivation of TLX1 (18) and TLX3 (6) in the TLX overexpressing ALL-SIL (TLX1) and DND41 (TLX3) cell

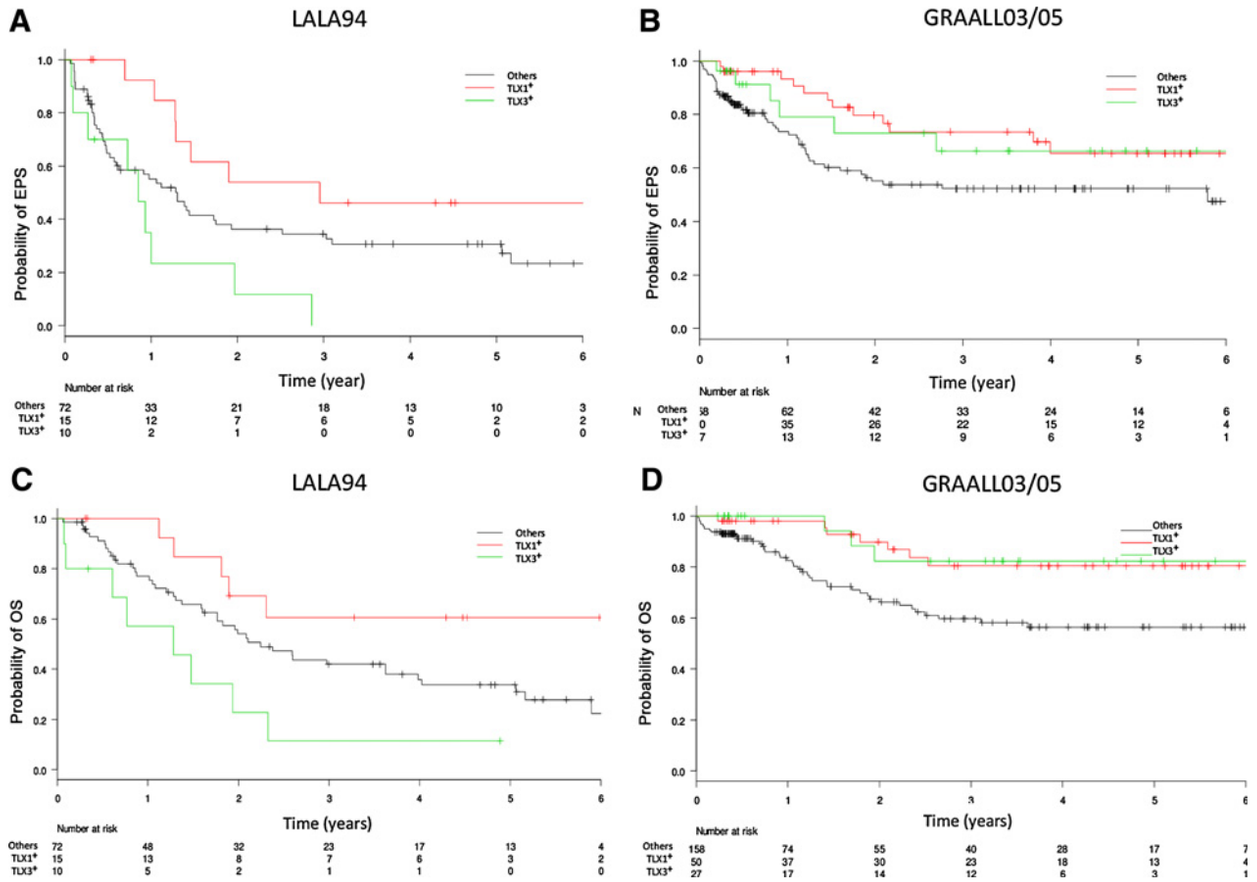

Figure 1.

Impact of *TLX1* and *TLX3* deregulation and treatment era on adult T-ALL patient outcome. Kaplan–Meier graphs for (**A–B**) EFS and (**C–D**) OS censored at HSCT in LALA-94 and GRAALL-2003/2005, respectively, are shown according to oncogenetic status (TLX1$^+$ vs. TLX3$^+$ vs. others). A significant impact of treatment is shown within the TLX3$^+$ subgroup.

lines. This identified 929 and 1,259 (Supplementary Table S7) differentially expressed genes ($P < 0.05$, fold change = 2) in ALL-SIL and DND41, respectively, after TLX inactivation. Comparison of these three lists of differentially expressed genes identified only 4 overlapping genes (Fig. 2A). Among those, the *ASNS* gene was the only one which was less expressed in TLX1$^+$ compared with TLX3$^+$ patients. *ASNS* was, moreover, upregulated after TLX1 siRNA inactivation, as confirmed in an independent ALL-SIL cell line (Fig. 3E and F). Conversely, *ASNS* was downregulated after TLX3 shRNA inactivation. Of note, *ASNS* was the third most differentially expressed gene between TLX1 and TLX3 T-ALL ($P = 0.0025$) and demonstrated significantly lower expression in TLX1$^+$ compared with TLX3$^+$ and TLX-negative patients in independent published gene expression profiling and RNA-seq analyses on purified blast cells (refs. 2, 22; Fig. 2B and C). These observations were confirmed, despite a larger overlap, using 86 unpurified samples from GRAALL-treated T-ALL including 19 TLX1$^+$ and 12 TLX3$^+$ cases (Fig. 2D).

Promoter hypermethylation leads to asparagine synthetase silencing in TLX1$^+$ T-ALLs

To explore the mechanism underlying *ASNS* low-level transcription in TLX1$^+$ T-ALL, we performed global methylation analyses by MeDIP-array in 24 primary T-ALLs, including 6 TLX1$^+$ cases. *ASNS* promoter methylation was significantly higher in TLX1$^+$ cases compared with other T-ALL cases (Fig. 3A and B). To validate this observation, 153 adult T-ALLs were analyzed by an in-house custom MS-MLPA assay which clearly demonstrated significantly higher *ASNS* promoter methylation in TLX1$^+$ cases ($n = 33$) as compared with TLX1$^-$ ($n = 120$), including 25 TAL1$^+$ and, importantly, 22 TLX3$^+$ cases (Fig. 3C and D). Interestingly, we observed a decrease of *ASNS* promoter methylation in the ALL-SIL cell line transduced with shRNA-TLX1 (fold change = −0.14 relative to shRNA mock; Fig. 3E and F), suggesting a role of TLX1 in the methylation status of *ASNS* promoter methylation. An increase of *ASNS* expression and concomitant decrease of *ASNS* promoter methylation were also observed following a 5-day exposure of the ALL-SIL cell line to the 5-azacytidine hypomethylating agent, supporting the relationship between *ASNS* expression and promoter methylation (Fig. 3F). To provide additional support for the epigenetic silencing of ASNS in TLX1$^+$ blasts, we analyzed active histone marks in several T-ALL cell lines and primary blasts. The TLX$^-$ CCRF-CEM cell line, which displays high *ASNS* expression and low-level *ASNS* promoter methylation (Fig. 4A and B), demonstrated significant enrichment of H3K4-me3-active marks within the *ASNS* promoter region, as compared

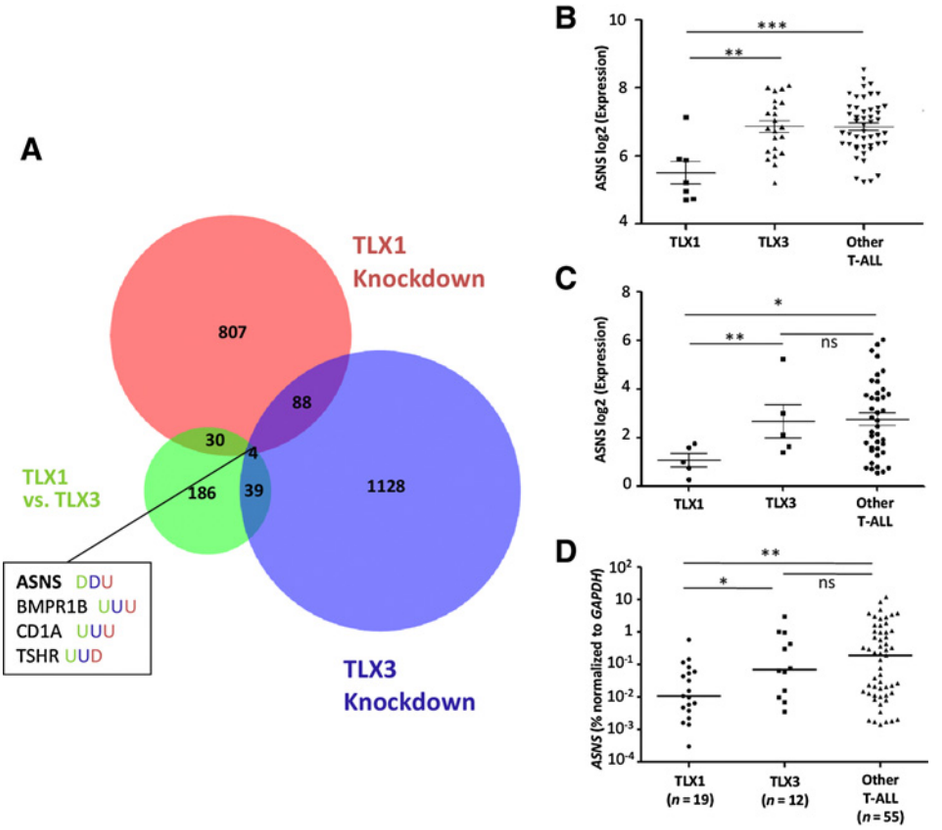

Figure 2.
The *ASNS* gene is expressed at lower levels in TLX1$^+$ T-ALL. **A,** Venn diagram comparing three transcriptomic signatures: (i) genes differentially expressed between *TLX1*$^+$ and *TLX3*$^+$ T-ALL (ref. 3; green circle), (ii) genes differentially expressed after *TLX1* siRNA inactivation in the *TLX1* overexpressing ALL-SIL cell line (ref. 18; red circle), and (iii) genes differentially expressed after T*LX3* shRNA inactivation in the *TLX3* overexpressing DND41 cell line (ref. 6; blue circle). Four genes overlap, including the *ASNS* gene, which is the only one to be expressed at lower levels in TLX1$^+$ patients (**D**), downregulated after TLX3 inactivation (D) and upregulated after TLX1 inactivation (U). **B,** Public gene expression profiling (2) and (**C**) RNA-seq data (22) showing significantly reduced *ASNS* expression in TLX1$^+$ patients compared with TLX3$^+$ and TLX$^-$ patients. **D,** *ASNS* expression normalized to *GAPDH* by RTQ-PCR in an adult T-ALL series included in the GRAALL 2003/20005 trials, including 26 TLX1$^+$, 10 TLX3$^+$, and 55 TLX$^-$ cases. TLX1$^+$ patients expressed significantly less *ASNS* than other patients.

with the TLX1$^+$ ALL-SIL cell line (Supplementary Fig. S4A). ChIP-seq analysis performed on primary T-ALL cells (one TLX1$^+$ and three TLX$^-$) also showed a decrease of H3K4me3 and H3K36-me3-active marks within the promoter and the gene body of *ASNS*, respectively, in the TLX1$^+$ case as compared with the 3 TLX$^-$ cases (Supplementary Fig. S4B).

In an attempt to identify the mechanism of *ASNS* repression by TLX1, we analyzed TLX1 ChiP-seq data in the ALL-SIL cell line (18), which failed to demonstrate direct TLX1 binding on *ASNS* (data not shown). Altogether, these results suggest epigenetic silencing of *ASNS* in TLX1$^+$ cases by both DNA methylation and a decrease of active histones marks.

ASNS hypermethylation leads to L-asparaginase sensitivity

We then hypothesized that the favorable outcome of TLX1$^+$ T-ALL could be associated with the low ASNS protein expression via *ASNS* promoter methylation. In order to test L-asparaginase *in vitro* sensitivity according to *ASNS* status, the TLX1$^+$ ALL-SIL (high-level *ASNS* promoter methylation) and the CCRF-CEM (lowest-level *ASNS* methylation level) T-ALL cell lines were exposed to increasing doses of native *Escherichia coli* L-asparaginase (Kidrolase, EusaPharma; 0–25 UI/mL) for 48 hours, followed by a cell viability assay. As expected, ALL-SIL was more sensitive to L-asparaginase as compared with CCRF-CEM (Fig. 4A–D). Then two different *ASNS* siRNA, SiRNA-1 and SiRNA-2, were used to determine the relationship between ASNS protein expression levels and L-asparaginase sensitivity in the CCRF-CEM cell line. SiRNA-1 was more efficient compared with SiRNA-2, yielding a decrease of *ASNS* expression of 63% and 37%, respectively (Fig. 4E and F). Decreased *ASNS* expression led to a proportional increase in sensitivity to L-asparaginase (Fig. 4G), with LC_{50} values at 72 hours of 0.48, 0.23, and 0.017 UI/mL for CCRF-CEM siRNA-mock, CCRF-CEM siRNA-2, and CCRF-CEM siRNA-1, respectively compared with less than 0.001 UI/mL for ALL-SIL, suggesting, as expected, an inverse correlation between *ASNS* expression and L-asparaginase sensitivity. As L-asparaginase efficacy correlates with exogenous asparagine depletion, this assumes that the leukemic cells are incapable of synthesizing asparagine. We then reasoned that if the T-ALL cells are perfectly capable of synthesizing asparagine (i.e., by ASNS protein expression due to hypomethylated *ASNS* promoter), they should be essentially insensitive to asparaginase therapy. TAL1$^+$ cases harbor the lowest methylation levels (Fig. 3D). This suggests that the TAL1$^+$ subgroup of T-ALL should be L-asparaginase insensitive or require the highest level of L-asparaginase, potentially not reached in the adult GRAALL protocol. In line with this, as shown in Supplementary Fig. S5, TAL1$^+$ GRAALL-treated cases benefitted less from GRAALL intensification than the TLX3$^+$ cases. Moreover, we evaluated *in vitro* L-asparaginase sensitivity of 8 T-ALL cells from patient-derived primary T-ALL xenografts (PDX) in NSG mice. Importantly and as expected, PDXs with hypomethylated *ASNS* promoters (UPNT-374 methylation ratio 0.08, M106 methylation ratio 0.33, and UPNT-420 methylation ratio 0.25) and high *TAL1* expression (Supplementary Fig. S6) were strongly less sensitive to L-asparaginase than those with hypermethylated *ASNS* promoters (UPNT-670 methylation ratio 0.86, UPNT-565 methylation ratio 1, M152 methylation ratio 1.04, and M149 methylation ratio 1.04; Fig. 4H).

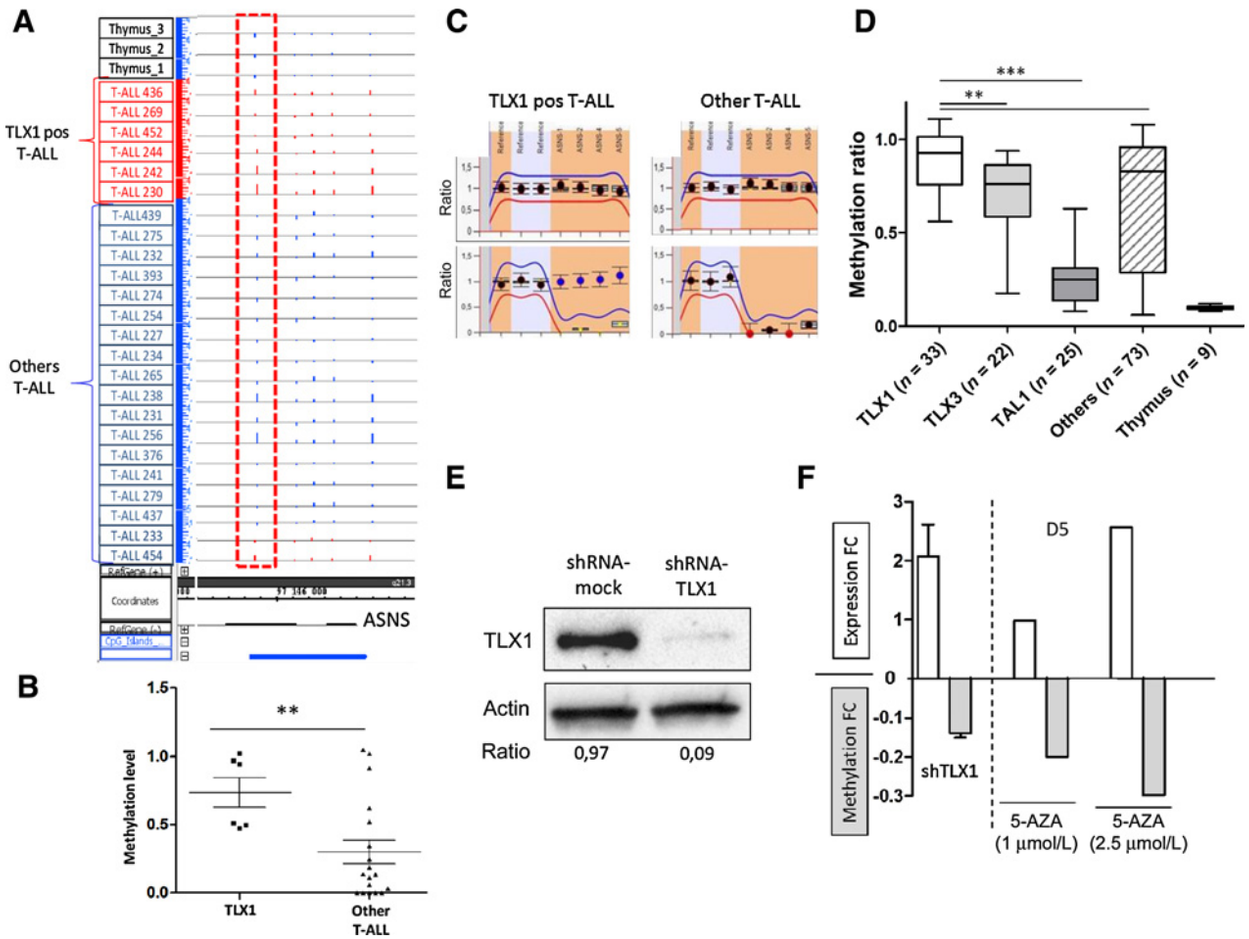

Figure 3.

Hypermethylation of the *ASNS* promoter in TLX1$^+$ cases. **A,** MeDIP-array data focused on *ASNS* promoter in 3 *thymii*, 6 TLX1$^+$ cases (in red), and 18 TLX1$^-$ cases (in blue), including two TLX3$^+$ cases (T-ALL #233 and T-ALL #454). **B,** Diagram of MeDIP-array data focused on CpG in the dashed box. **C,** Examples of MS-MLPA profiles: left, a TLX1$^+$ case; right, a TLX$^-$ case. Signals for three reference probes and four probes located on the *ASNS* promoter (ASNS-1, ASNS-2, ASNS-4, and ASNS-6) are shown. Top part, profiles of control MLPA analysis; bottom part, profiles with HhaI restriction endonuclease showing *ASNS* promoter methylation ratios. **D,** *ASNS* promoter methylation by MS-MLPA in GRAALL patients according to oncogenotype (TLX1$^+$, TLX3$^+$, TAL1$^+$, and others). **E,** Western blots for expression of TLX1 and the actin control (bottom). **F,** Fold change (FC) of *ASNS* expression and promoter methylation in the ALL-SIL cell line transduced with shRNA-TLX1 relative to shRNA-mock and in the ALL-SIL cell line exposed to 5-azacytidine during 5 days (1 µmol/L and 2.5 µmol/L) relative to cells treated with vehicle.

These data strongly support an inverse correlation between *ASNS* promoter methylation (leading to decrease ASNS expression) and *in vitro* sensitivity to L-asparaginase.

ASNS methylation, but not expression, is an outcome predictor in adult T-ALL

ASNS expression and promoter methylation by MS-MLPA were then evaluated for 86 and 160 T-ALL patients, respectively, from the GRAALL03/05 trials. No significant correlation between *ASNS* expression and promoter methylation was observed (Supplementary Fig. S7A), probably due to high-level *ASNS* expression by both normal peripheral blood lymphocytes and bone marrow normal residual cells (Supplementary Fig. S7B). We used two strategies to purify blast cells, (1) blast- and nonblast cells sorting and (ii) PDX, for *ASNS* quantification. As for T-ALL cell lines (Supplementary Fig. S7E), we then observed a significant correlation confirming this hypothesis (Supplementary Fig. S7C and S7D). *ASNS* expression levels had no prognostic impact on EFS or OS (Fig. 5A and B). In striking contrast, *ASNS* promoter methylation levels strongly correlated with outcome. When considering the methylation ratio as a continuous variable, a higher ratio was significantly associated with better EFS (HR, 0.33; 95% CI, 0.17–0.64; $P = 0.001$) and OS (HR, 0.25; 95% CI, 0.12–0.50; $P < 0.001$). Patients in the lower methylation tertile T1 had a significantly lower EFS (3-year EFS: 36%, 95% CI, 23–49) and OS (3-year OS: 43%, 95% CI, 30–56) than patients in the T2 and T3 tertiles, who displayed comparable outcome (3-year EFS: 67%, 95% CI, 57–75, $P < 0.001$; 3-year OS: 75%, 95% CI, 66–83, $P < 0.001$; Fig. 5C and D). Similar results were observed after censoring outcome at the time of allogenic stem cell transplantation (data not shown). Of note, the T1 tertile subgroup was significantly associated with a low rate of NOTCH1 pathway mutated status and unfavorable classifier

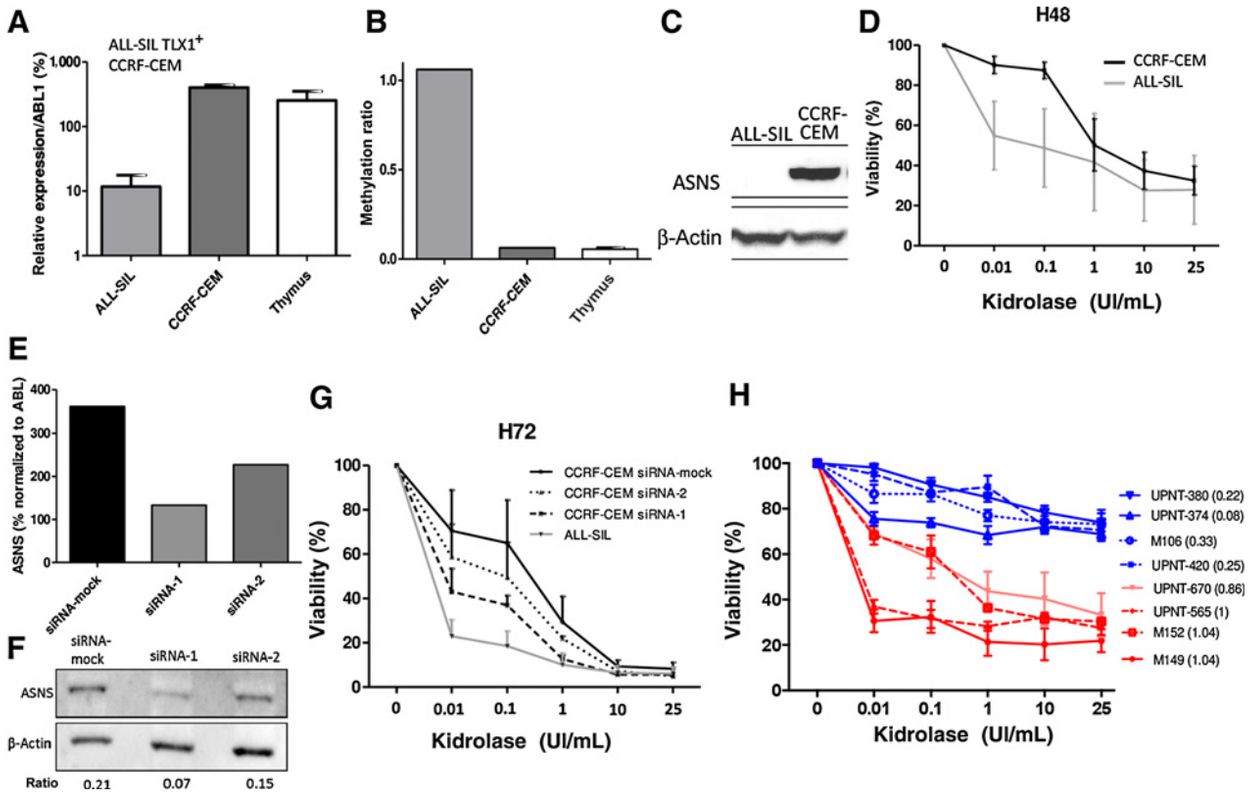

Figure 4.

L-Asparaginase sensitivity according to *ASNS* expression and methylation ratios. **A,** *ASNS* expression normalized to *ABL1* evaluated by RTQ-PCR in the ALL-SIL and CCRF-CEM cell lines and in normal thymus. **B,** *ASNS* promoter methylation ratio by MS-MLPA in ALL-SIL and CCRF-CEM cell lines and in normal thymus. **C,** Western blot for ASNS or actin expression in ALL-SIL and CCRF-CEM cell lines. **D,** Sensitivity experiments of ALL-SIL and CCRF-CEM T-ALL cell lines upon L-asparaginase (Kidrolase) exposure. Results are expressed as the relative number of surviving cells, as compared with an internal control (NaCl). **E,** Decrease of *ASNS* expression after *ASNS* siRNA inactivation in the CCRF-CEM cell line. Two independent siRNA were used, siRNA-1 and siRNA-2, with a decrease of *ASNS* expression of 63% and 37%, respectively, compared with SiRNA control (SiRNA-mock). **F,** Western blot for ASNS or actin expression in CCRF-CEM cell line after *ASNS* siRNA inactivation; ASNS/actin protein ratios are indicated below the blot. **G,** Sensitivity experiments of ALL-SIL cell line and CCRF-CEM cell line after ASNS siRNA inactivation upon L-asparaginase (Kidrolase) exposure. **H,** *In vitro* sensitivity experiments of primary T-ALL xenografted in NSG mice upon L-asparaginase (Kidrolase) treatment. Eight primary T-ALLs were exposed during 72 hours, and apoptosis was analyzed by FACS after Annexin V/propidium iodide staining. *ASNS* promoter methylation was analyzed by MS-MLPA, and ratios are noted in brackets. T-ALL with higher methylation ratios (in red) appears more sensitive to L-asparaginase than T-ALL with lower methylation ratios (in blue).

(Supplementary Table S8). It was also less likely to achieve CR, but did not demonstrate other early resistance to treatment as assessed by prednisone response, bone marrow blast clearance, or minimal residual disease (MRD) after induction (Supplementary Table S8). After adjustment for age, WBC, central nervous system (CNS) involvement, early therapeutic responses, and the NOTCH1/FBXW7/RAS/PTEN molecular classifier, *ASNS* promoter methylation status was confirmed to be an independent prognostic indicator for both EFS (HR, 0.42; 95% CI, 0.24–0.71; $P = 0.001$) and OS (HR, 0.40; 95% CI, 0.23–0.70; $P = 0.02$; Supplementary Table S9). We also demonstrated a significant prognostic impact of *ASNS* promoter methylation in an independent series of 47 adult T lymphoblastic lymphomas (T-LBL) included in the French LL03 protocol (23) which used similar L-asparaginase schedules to the GRAALL trial (Fig. 5E). Overall, these data suggest that the methylation status of the *ASNS* promoter could represent a biomarker of L-asparaginase response and outcome of adult T-ALL/T-LBL.

Discussion

Despite recent insights into the molecular and cellular mechanisms responsible for T-ALL onset and progression, survival rates remain around 50% in adults, justifying the search for novel therapeutic options or more adapted/personalized regimens. Extensive clinical data support the benefit of more intensive L-asparaginase treatment in pediatric ALL (24–28), and the GRAALL has reported a significant improvement in the outcome of adults with BCR-ABL–negative ALL using a pediatric-inspired intensified treatment protocol (16). The LALA-94 and GRAALL trials mostly differed in the use of L-asparaginase, with no L-asparaginase in LALA-94 induction versus 48,000 UI/m^2 cumulative dose in GRAALL induction, and 16 times more L-asparaginase in the whole GRAALL protocol (144,000 UI/m^2) than in the LALA-94 (9,000 UI/m^2; refs. 13, 16).

The *ASNS* gene, which encodes for asparagine synthetase, is an aminotransferase enzyme that catalyzes the biosynthesis of the amino acid asparagine (Asn) from aspartic acid (Asp). Most

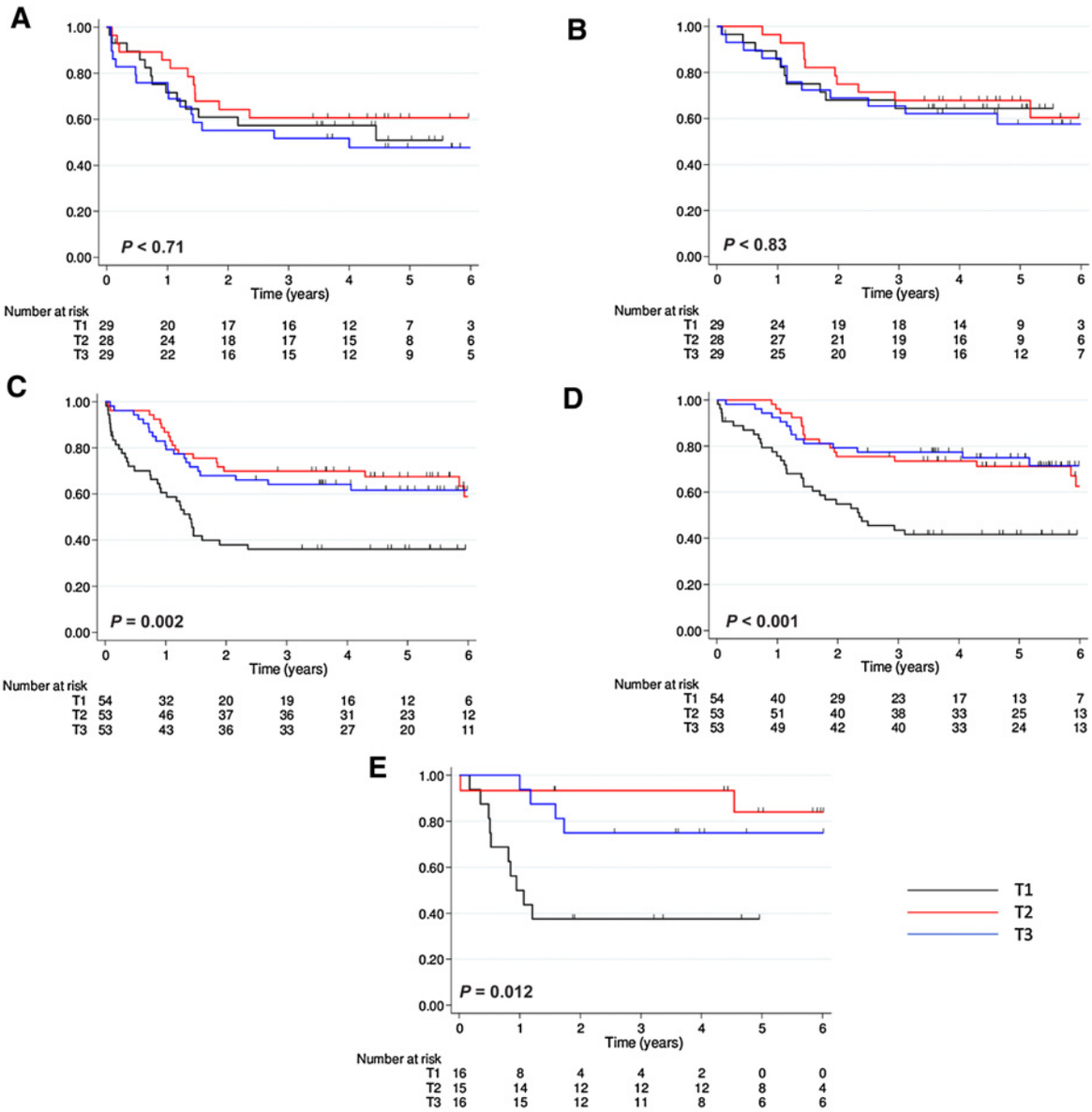

Figure 5.

Outcome of patients according to *ASNS* expression and methylation ratio. **A** and **B,** Kaplan–Meier graphs according to *ASNS* expression tertiles for EFS and OS, respectively, for patients included in GRAALL03-05 trial. **C** and **D,** Kaplan–Meier graphs according to *ASNS* methylation tertiles for EFS and OS, respectively, for patients included in GRAALL03-05 trial. **E,** EFS according to methylation tertiles for patients included in the LL03 lymphoblastic lymphoma trial.

tissues contain sufficient ASNS activity to maintain satisfactory asparagine levels and upregulate the enzymatic activity in response to asparagine depletion (29, 30). Primary ALL cells and many ALL cell lines exhibit a particularly low level of *ASNS* expression (31), and as such, are usually sensitive to asparagine depletion. This relatively low *ASNS* expression in lymphoblasts is the basis for the use of L-asparaginase, leading to the depletion of plasma asparagine, during chemotherapy in ALL and is now an essential component of most pediatric therapeutic protocols.

Asparaginase has been recently added to adult ALL trials as a pediatric-inspired approach. Despite the benefits of this strategy, these dose-intense regimens are far more toxic in adults, and cumulative incidence of nonrelapse mortality is as high as 40% in patients over 55 years (Huguet and colleagues, ASH 2016). L-asparaginase is responsible for hepatic toxicity, thrombosis, hypoalbuminemia, and denutrition. There is a consequent need for tailored L-asparaginase use, based on leukemia biology and patient-related conditions.

It is noteworthy that L-asparaginase is the drug with the most heterogeneous usage within different ALL cooperative groups. We hypothesized that prognosis could be related to differences in L-asparaginase dosage in therapeutic protocols and to differential sensitivity between $TLX1^+$ and $TLX3^+$ patients based on their ASNS activity. Although it is unknown if the total cumulative

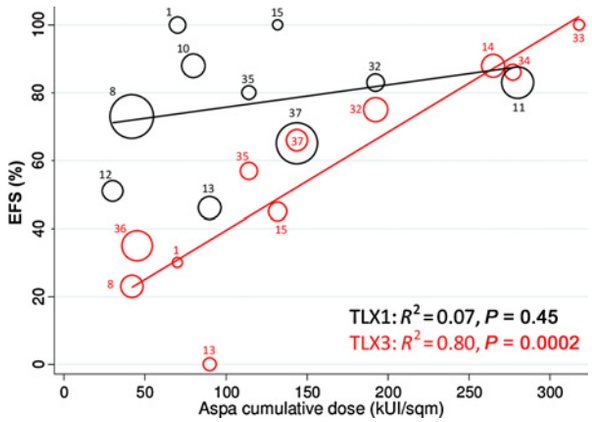

Figure 6.

Reported 2- to 5-year EFS in published series of adult and pediatric TLX1$^+$/3$^+$ patients according to the cumulative amount of asparaginase. Mean asparaginase dose was calculated in case of paper reporting about a mix of patients included in various trials. Each circle represents a published series. Circle sizes are proportional to the number of TLX$^+$ subjects reported. The numbers next to the circles correspond to the references reporting the respective trials.

amount of L-asparaginase is more relevant than the overall time spent with effective depletion, these data are difficult to assess and are not commonly reported. We therefore analyzed published outcomes of TLX patients as a function of their cumulative L-asparaginase treatment. After excluding duplicates and articles that did not specify the chemotherapy regimen used, we found 14 publications (1, 8, 10–15, 32–37) that report on the survival of TLX3 and TLX1 patients. TLX1 overexpression is constantly associated with favorable EFS and OS in all reported trials (Fig. 6) and seems to be independent of L-asparaginase cumulative dose. Conversely, the outcome of TLX3$^+$ patients is highly heterogeneous between different trials, but demonstrates an approximately "linear" correlation between L-asparaginase cumulative dose and outcome, suggesting that this could account for the variable prognostic impact of TLX3$^+$ (and potentially other TLX1-negative) T-ALLs. Of course, it is likely that the protocols included in this analysis might have many other differences, and further investigations are required to address a definitive conclusion.

The mechanisms of L-asparaginase resistance remain unclear, and data from studies on the importance of ASNS are conflicting. In leukemic cell lines (38, 39), correlations between *ASNS* transcriptional expression and L-asparaginase sensitivity seem reliable. It has been shown that drug-selected L-asparaginase–resistant cell lines exhibit a high expression of *ASNS* (40) and that overexpression of exogenous ASNS protein could result in an L-asparaginase–resistant phenotype in the absence of drug selection (40). Concerning primary ALL, data remain unclear, and the role of ASNS expression with regard to L-asparaginase resistance may vary among genetic subtypes and, as suggested by others, may have little or no relevance to drug sensitivity *in vivo* (38, 41–45). Moreover, most studies correlated *ASNS* expression and L-asparaginase sensitivity at a transcriptional level, but the relationship between ASNS mRNA and intracellular protein concentrations has not been established. It has been reported (46) that the correlation between ASNS protein levels and IC_{50} was much better than that between the IC_{50} and *ASNS* mRNA content, suggesting that transcriptional expression might not be the best marker of ASNS activity. In addition, we found that normal blood and bone marrow cells express high level of *ASNS*. As such, mRNA content could be accurately correlated to ASNS activity in ALL cell lines but not in primary ALLs, because of potential contamination with residual normal blood or bone marrow cells. The fact that *ASNS* methylation is not dependent on exponential PCR amplification, unlike transcript quantification, probably contributes to its greater predictive value and a more reliable and accurate way to assess ASNS activity. Advantage in methylation could also be explained by the lower disruption of input of contaminating cells at the genome level (two low methylation copies per contaminating normal cell) than at the mRNA level (multiple mRNA copies per contaminating normal cell).

No study has specifically focused on the role of ASNS in T-ALL; the majority of studies were conducted in B-cell precursor (BCP)-ALL (38, 42–45, 47). One study did, however, report higher *ASNS* expression in T-ALL as compared with BCP-ALL (48). Therefore, it would be interesting to study whether *ASNS* methylation could also be a biomarker of L-asparaginase response in BCP-ALL. Amino acid metabolism is essential for cancer cell proliferation in general and not only for ALL. L-asparaginase is also used in chemotherapy protocols to treat NK/T-cell lymphomas and other aggressive malignancies such as soft-tissue sarcomas or gliomas. *In vivo*, genetic silencing of ASNS in mouse sarcoma cells combined with depletion of plasma asparagine by L-asparaginase inhibited tumor growth. It could therefore be advantageous to combine asparagine depletion and ASNS inhibition for tumors which exhibit high ASNS activity. Despite recent advances in "personalized" medicine, targeted therapies do not currently benefit most ALL patients, and classical chemotherapies still remain the cornerstone of first-line treatment. Although L-asparaginase management optimization mainly relies on monitoring of immune inactivation of the drug and adaptation of effective asparagine depletion, we report for the first time on a leukemic cell biological explanation for discrepancies in patient-related outcomes to this major ALL drug. If these observations are confirmed in larger scale and independent T-ALL trials, *ASNS* methylation status at diagnosis may allow individual adaptation of L-asparaginase total dose, potentially with a reduced incidence of side effects.

Disclosure of Potential Conflicts of Interest

F. Huguet reports receiving speakers' bureau honoraria from Amgen, Bristol-Myers Squibb, Incyte, Jazz Pharma, Novartis, and Pfizer, and is a consultant/advisory board member for Novartis. N. Ifrah is a consultant/advisory board member for Pfizer, Bristol-Myers Squibb, and Novartis. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Touzart, E. Lengliné, M. Belhocine, S. Spicuglia, C. Graux, N. Ifrah, E. Macintyre, N. Boissel, V. Asnafi
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Other (provided important materials): F. Pflumio

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