**Bim Polymorphisms: Influence on Function and Response to Treatment in Children with Acute Lymphoblastic Leukemia**

Vincent Gagné, Julie Rousseau, Malgorzata Labuda, Bahram Shariﬁ-Askari, Ivan Brukner, Caroline Lavergnière, Francesco Ceppi, Stephen E. Sallan, Lewis B. Silverman, Donna Neuberg, Jeffery L. Kutok, Daniel Sinnett, and Maja Krajinovic

**Abstract**

**Purpose:** Corticosteroids induce apoptosis in the malignant lymphoid cells and are critical component of combination therapy for acute lymphoblastic leukemia (ALL). Several genome-wide microarray studies showed major implication of proapoptotic Bim in mediating corticosteroid-related resistance in leukemia cells.

**Experimental Design:** We investigated Bim gene polymorphisms and their association with childhood ALL outcome, and the mechanism underlying the observed finding.

**Results:** Lower overall survival (OS) was associated with Bim C29201T located in Bcl-2 homology 3 (BH3) domain (P = 0.01). An association remained signiﬁcant in multivariate model (P = 0.007), was more apparent in high-risk patients (P = 0.004) and patients treated with dexamethasone (P = 0.009), and was subsequently conﬁrmed in the replication patient cohort (P = 0.03). RNA analysis revealed that C29201T affects generation of γ isoforms (γ1) that lack proapoptotic BH3 domain. The phenotypic effect was minor suggesting the influence of additional factors that may act in conjunction with Bim genotype. Combined analysis with Mcl gene polymorphism (G-486T) revealed profound reduction in OS in individuals with both risk genotypes (P < 0.0005 in discovery and P = 0.002 in replication cohort) and particularly in high-risk patients (P ≤ 0.008).

**Conclusions:** Increased expression of prosurvival Mcl1 and presence of Bim isoforms lacking proapoptotic function might explain marked reduction of OS in a disease and dose-dependent manner in ALL patients carrying Bim- and Mcl1-risk genotypes.

**Introduction**

Corticosteroids induce apoptosis and cell-cycle arrest in the majority of malignant lymphoid cells (1). Consequently, they are critical component of combination chemotherapy regimens used in the treatment of lymphoid malignancies, including childhood acute lymphoblastic leukemia (ALL; refs. 2, 3). Despite their clinical importance, the mechanism underlying molecular basis of corticosteroid-induced apoptosis and corticosteroid-related resistance are not fully understood. Corticosteroids mediate their effect via glucocorticoid receptor (4, 5); the resulting complex recruits either coactivator or corepressor proteins thereby inducing or repressing the expression of a large number of target genes (4, 6–8). A subpopulation of childhood ALL cases fail to respond to corticosteroid treatment and one of the underlying mechanisms is a change in glucocorticoid receptor expression (5, 9, 10). Other mechanisms are also involved in the resistance to cell death induced by these drugs. A number of studies including genome-wide expression profiling have attempted to identify critical glucocorticoid-regulated genes, which may undergo altered expression before the onset of apoptosis (11–14). The proapoptotic protein that was upregulated by glucocorticoids in several models of corticosteroid-induced apoptosis is Bim, the Bcl-2 homology 3 (BH3)-only molecule (11, 15, 16). Bim is expressed in hematopoietic, epithelial, neuronal, and germ cells and was found frequently upregulated in childhood leukemia samples following corticosteroid exposure (11, 13). It is a member of Bcl-2 family that induces the mitochondrial apoptosis pathway by either opposing the prosurvival proteins of this family or by binding to the proapoptotic Bcl-2 members directly activating their proapoptotic functions (17, 18). Change in Bim expression seems to influence sensitivity to corticosteroid in ALL and, as shown in primary ALL...
Translational Relevance

Acute lymphoblastic leukemia (ALL) is the most frequent malignancy of childhood. The treatment of pediatric ALL has greatly improved in the past 4 decades due to the introduction of effective combination risk-adapted therapies. However, therapy resistance in a significant number of children still is a major obstacle to successful treatment. Intensive treatment has also significant short-term side effects and long-term consequences. Identification of genetic component underlying this variability would allow traditional treatment to be complemented by genotype-based drug dose adjustment. Genome-wide experiments pointed to Bim gene as a major proapoptotic gene mediating corticosteroid-related effects and resistance in ALL. Here we analyzed genetic variations in the gene Bim and try to explain an observed association through functional study and gene–gene interaction with other components of apoptotic machinery. The study provides a new insight into the Bim pharmacogenetics and its role with the respect to ALL.

Patients and Methods

Study population and endpoints in the analysis

The study population consisted of 348 Caucasian children (97.5% of patients are of French-Canadian origin from the similar geographical region) diagnosed with ALL (15, 19–21). Bim is encoded by gene BCL2L11 and 3 major mRNA isoforms and Bim protein products (BimEL, BimS and BimL) exist (15). Moreover, it is extensively regulated at transcriptional and posttranscriptional level. The changes in expression may also be affected by genetic polymorphisms.

Here we report the analysis of Bim polymorphisms and their association with ALL disease outcomes in 2 patient populations. Further functional analysis based on mRNA and cellular viability assays was also conducted to give an insight into the underlying mechanism of significant association.

Table 1. Characteristic of ALL patients in the test (QcALL) and validation (DFCI) cohorts

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<th>DFCI</th>
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</tr>
<tr>
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</tr>
<tr>
<td>Male</td>
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</tr>
<tr>
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<tr>
<td>&lt;10</td>
<td>276 (79.3)</td>
<td>228 (81.7)</td>
</tr>
<tr>
<td>≥10</td>
<td>72 (20.7)</td>
<td>51 (18.3)</td>
</tr>
<tr>
<td>WBC, ×10^9/L</td>
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</tr>
<tr>
<td>&lt;50</td>
<td>286 (82.2)</td>
<td>226 (81.0)</td>
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<tr>
<td>&gt;50</td>
<td>62 (17.8)</td>
<td>53 (19.0)</td>
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<tr>
<td>Cell type</td>
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<tr>
<td>B</td>
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<td>256 (91.8)</td>
</tr>
<tr>
<td>T</td>
<td>29 (8.3)</td>
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<td>Risk groups</td>
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<td>257 (92.1)</td>
</tr>
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<td>Total</td>
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<td>279 (100)</td>
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due to the population stratification, similar to discovery cohort, only Caucasians (self-reported, $n = 279$; DFCI 95-01, $n = 95$; and 2000-01, $n = 184$) were included in the analysis.

The characteristics of patients for both test and validations set are provided in Table 1.

**Genotyping**

Thirty-two polymorphisms located in regulatory and coding gene regions were selected from National Center for Biotechnology Information (NCBI) single nucleotide polymorphism (SNP) databases (http://www.ncbi.nlm.nih.gov/SNP). Selected polymorphisms were analyzed in 60 healthy unrelated adults (of the same ethnic background as patients) to estimate allele frequency, linkage disequilibrium, and haplotype phase (Fig. 1). Eight tagSNPs (sufficient to define common haplotypes) with minor allele frequency (MAF) $\geq 5\%$ were retained for analysis in patients. Primers and probes used for amplification and genotyping of these polymorphisms are shown in Supplementary Table S1. dbSNP numbers along with SNP positions for the polymorphisms genotyped only in controls are given in Supplementary Table S2. The subset of samples was genotyped in duplicate to ensure genotype reproducibility. Genotyping was conducted by allele-specific oligonucleotide hybridization as previously described (26).

**Statistics**

The estimates of linkage disequilibrium and haplotype phase in control individuals were obtained by PHASE software, version 2.0 (27). The tagSNPs were selected based on linkage disequilibrium information ($r^2$; Fig. 1) using Haploview software. Association of genotypes with ALL outcome was assessed by EFS and OS. Survival differences, estimated by Kaplan–Meier analysis for patients with different genotypes, were assessed using a log-rank test. Times to event or a death were measured as the time between diagnosis and the event of interest. For censored cases, it represented the time from diagnosis to date last known alive without an event; for longer follow-up durations, all times were truncated at 5 years posttreatment. For the polymorphism associated with a disease outcome, the analyses were performed on the subset of patients genotyped.
also conducted following stratification by risk group and corticosteroid type. The HR (with a 95% confidence interval, CI) for genetic variants was estimated by Cox regression analysis. Cox regression was also used to estimate multivariable HR in the presence of common prognostic factors [age, sex, presenting white blood cell (WBC) count, immunophenotype] and treatment protocol (listed in Table 1), as described previously (25).

mRNA analysis

Total RNA from prednisolone (75 μmol/L prednisolone) and dexamethasone treated (2.8 × 10⁻⁴ μmol/L) lymphoblastic cell lines (LCL) of HapMap subjects of European origin (CEU) extracted with Qiagen kit was reverse transcribed using 2 μmol/L oligo dT and random primers (mixed at 1:2 molar ratio) and M-MLV enzyme (Invitrogen) according to the protocol provided by the enzyme supplier. Quantitative PCR was carried out using the Syber Green detection system (Applied Biosystems). The expression was measured by relative quantification normalized to B2-microglobulin (primers: TACTCTCTTTCTGGCCTG and GGATGGATGAAACCCAGACA) and the calculation was conducted using the comparative cycle threshold (Cₜ) method (28). Pair of primers TGCGGAGAAGGTTAGACCAAT and GCCATAAAAATCTTAAGCCAGT targeting alternatively spliced exon 3 was used to amplify non-BH3-containing γ isoforms (refs. 29, 30; Fig. 1). For remaining Bim isoforms, primers GAGATATGGATCGCCCAAGA and CAATGCATTCTCCACACCAG were used to amplify last 2 exons common of all isoforms except γ. When appropriate, relative mRNA levels were log transformed and the difference in relation to genotypes was analyzed by t-test or ANOVA, or expressed as quartile distribution and difference according to genotype were assessed by χ² test.

Semiquantitative analysis of 3 major RNA isoform (BimS, BimL and BimEL; Fig. 2) was carried out by using primers described in (31), whereas γ1 and γ2 assessment was carried out by same PCR primers described earlier for total γ estimation. PCR condition included 0.4 to 1 μmol/L for each primer, 0.2 mmol/L of each dNTP, 2.5 mmol/L MgCl₂, and 50 ng of cDNA, and amplification at 95/3 minutes, 30 to 40 cycles of 94/30 seconds, 55 to 58/30 seconds, 72/30

![Figure 2. Schematic representation of Bim gene. The Bim isoforms and its NCBI reference sequence number including major and minor isoforms with and without BH3 domain are given below genomic structure. Exonic and coding sequence is represented by open and gray boxes, respectively. SNPs are represented by gray dots. The black and gray arrows indicate the transcription and translation start site, respectively. The figure is not to scale. Modified from refs. 29, 31, 33, and 54.](image-url)
seconds to 1 minute. Amplified PCR products were analyzed by QIAxcel system (QIAGEN, http://www.qiagen.com); QIAxcel DNA High Resolution Kit (1,200; fragment size range of 15 bp–3 kb) was chosen to recognize size differences of amplicons; electropherogram and gel images were generated for each lane; Biocalculator software was used for data export and analysis. Relative contribution of each isoform (expressed as ratio of normalized areas under the curve) was analyzed as quartile distribution in relation to genotype using $\chi^2$ test.

The sequence of the 2 $\gamma$ isoforms ($\gamma_1$ and $\gamma_2$) were confirmed by Sanger sequencing. Genotyping of LCLs was conducted as described earlier.

Cellular proliferation assay

LCLs were cultured in RPMI medium supplemented with 15% FBS and antibiotics (100 IU/mL penicillin; 100 $\mu$g/mL streptomycin). The experiments were carried out to determine the concentrations lethal to 50% of the cells (IC$_{50}$) during 48 hours incubations time by curve fitting the percentage of cell death growth versus controls. Around $5 \times 10^4$ cells/well were seeded and submitted to 7 different concentrations of prednisolone ranging from $2.8 \times 10^{-3}$ to 750 $\mu$mol/L and 8 different concentrations of dexamethasone ranging from $2.8 \times 10^{-3}$ to $560 \mu$mol/L and tested in duplicate. Cell Proliferation Reagent WST-1 (Roche; 10 mL/well) was added to the cells and the optical density was measured at 450 nm with a Spectra MAX 180 (Molecular Devices). After correction for the culture media by using a background control in duplicate, the relative cell survival was calculated as (OD drug-exposed well)/(mean OD control wells) $\times 100%$. Log-transformed values or quartile distribution was compared in relation to genotypes.

Results

Thirty-two polymorphisms located in the region of potential functional importance (regulatory and coding regions) were genotyped in 60 controls of Caucasian origin to estimate allelic frequency (Fig. 1A). Polymorphisms with MAF $\geq 5%$ were analyzed for pairwise linkage disequilibrium (Fig. 1B); 8 tagSNPs were identified (sufficient to define 6 common haplotypes, arbitrarily named t1 to t6; Fig. 1C) and retained for the analysis in patients.

The analysis in the discovery cohort revealed no association with EFS (Table 2). In contrast, the analysis showed significant association between OS and synonymous

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$^aP$ value estimated by log-rank test using additive (A), dominant (D), and recessive (R) model; nc, not calculated due to low genotype count.
Polymorphisms of Bim Gene and ALL Outcome

**Figure 3. Relationship between Bim C29201T and OS in ALL patients.** A, OS according to C29201T genotypes in discovery group (QcALL). OS curves are given for CC, CT, and TT genotype. The number of all patients in each curve (with the number of patients that died in brackets) is indicated next to the curve. OS difference between genotype groups is estimated by log-rank test and P value is given on the plot. Genotype-associated risk (univariable HR with 95% confidence interval (CI, in brackets) is indicated below plot). B, OS curves according to Bim C29201T genotypes in high-risk patients and patients treated with dexamethasone. OS for QcALL patients who received dexamethasone (left plot), or who were assigned to high risk (right plot), with (black line), and without (gray line) TT29201 genotype. C, OS curves according to genotypes of Bim C29201T polymorphism in replication cohort. Left plot is OS for all patients of replication cohort (DFCI). Curves are represented for CC, CT, and TT genotypes. Right plot is OS for patients with and without TT genotype who received dexamethasone.

C29201T replacement (C29201T Ile65Ile) located in BH3 domain of exon 8 (P = 0.01; HR = 1.9; 95% CI, 1.1–3.1; Fig. 3A and Table 2) with lowest OS seen in patients with C29201T genotype. The association remained significant (HR = 2.1; 95% CI, 1.2–3.7; P = 0.007) with inclusion of typical prognostic factor in Cox regression model (see section Patients and Methods). Given that patients were administered either prednisone or dexamethasone during the intensification and continuation treatment phases and that doses differed between standard- and high-risk patients, C29201T was subsequently analyzed following stratification by corticosteroid type and risk groups. Lower OS for TT29201 individuals was noted in patients who received dexamethasone (P = 0.009; HR = 6.3; 95% CI, 1.3–31.4; Fig. 3B) as well in patients who were assigned to high-risk group irrespective of type of corticosteroid received (P = 0.004; HR = 3.6; 95% CI, 1.3–10.4; Fig. 3B). The T29201 allele defines haplotype ‘1’, and the same result as for the T allele, was obtained when this haplotype was analyzed for an association with OS (not shown). The analysis for C29201T variation was subsequently conducted in the replication cohort (DFCI group of patients). The significant association between genotype groups and OS was seen (P = 0.03; HR = 1.9; 95% CI, 1.0–3.4; Fig. 3C), and remained significant in multivariable analysis (HR = 2.4; 95% CI, 1.2–4.7; P = 0.01). Reduction in OS was observed for both CI and TT individuals; the TT29201 individuals had nevertheless the lowest EFS among patients who received dexamethasone (P = 0.04; Fig. 3C).

The C29201T polymorphism seems to affect exonic splicing element in BH3 domain, as based on F-SNP prediction tool (32), introducing additional binding site to serine- and arginine-rich (SR) protein SRp55. We therefore conducted quantitative and semiquantitative mRNA analysis in lymphoblastoid cell lines (LCL) following addition of prednisone or dexamethasone to investigate whether it may affect the total Bim mRNA levels (except γ1, levels of major BimS, BimL and BimEL isoforms, or levels of γ isoforms (total and γ1/γ2 ratio), which lack BH3 domain and correlate with reduced apoptotic function (29, 33). Bim gene organization with respective mRNA isoforms is schematically presented in Fig. 2. The influence of genotypes on sensitivity to corticosteroid was tested by in vitro cellular viability assay following addition of either prednisone or dexamethasone to LCLs. There was no relation between C29201T genotypes and mRNA levels when either total or major individual isoforms were quantified. In contrast, nonsignificant increase in γ isoform formation was noted in LCLs carrying...
T29201 allele after addition of dexamethasone, which seems to be due to an increase in γ1-γ2 ratio (Fig. 4, P = 0.03). There was no significant change in cellular viability in relation to C29201T genotype.

Given that the functional effect of a C29201T substitution was relatively minor, we further explored whether the effect seen in clinical setting may arise through the interaction with other genes of apoptotic pathway. Bim/Mcl1 complex plays a role of a regulatory “node,” critical for controlling cell apoptosis in response to multiple signals (34). We recently reported that 3 Mcl1 promoter SNPs in perfect linkage disequilibrium were associated with higher promoter activity and lower OS in the QCALL cohort (35). The combined effect between Bim C29201T and Mcl1 G-486T variations (one of SNPs in linkage disequilibrium) showed that the effect on ALL outcome was potentiated and present only in individuals carrying both risk genotypes. Marked reduction in OS was seen in discovery (P < 0.0005; HR = 9.6; 95% CI, 3.7–25.2; Fig. 5A) and replication cohort (P = 0.002; HR = 4.2; 95% CI, 1.8–10.0; Fig. 5A) in individuals with combined genotypes. The association was limited to high-risk patients (P < 0.0005 and P = 0.008, respectively; Fig. 5B).

**Discussion**

We showed that C29201T substitution located within BH3 domain of Bim gene was associated with inferior ALL outcome independently from typical prognostic factors, as showed by reduced overall survival in 2 patient cohorts. Bim plays a critical role in development and homeostasis of the lymphoid system (36). It is a critical regulator of apoptosis in normal and malignant cells, a tumor suppressor in B-cell malignancies (37), and confers resistance of normal and malignant lymphocytes to corticosteroids (38). Several lines of evidence suggest that corticosteroid resistance in ALL was associated with attenuated induction of Bim. Resistance to corticosteroid was associated with a failure to induce the Bim when xenograft ALL cells were exposed to dexamethasone (19, 20). Reduction of Bim mRNA and protein levels by RNA interference in corticosteroid-sensitive ALL cells reduced the activation of caspase-3 and increased cellular viability following corticosteroid exposure (15). Significantly, lower Bim expression correlated with poor early prednisone response and event free survival in pediatric ALL patients (39). Bim expression and function is regulated by a variety of mechanisms from transcriptional and posttranscriptional regulation to posttranslational modification and epigenetic silencing (40–42). The Bim gene sequence alterations may as well affect mRNA splicing, the rate of transcription, and protein level, affecting the cellular fate and response to corticosteroid treatment. Interestingly, C29201T is predicted (32) to introduce splicing enhancer element for SRp55 protein (also known as SR splicing factor SRSF6). SRs have an important part in splice-site selection through association with splicing enhancers and silencers (43, 44). It has been shown that change in Bim splicing is mediated by SRp55 in melanoma cells (45). Moreover, SRSF1 overexpression promoted alternative splicing of Bim to produce γ isoforms (29). In our study, the T29201 allele was not associated with the change of overall Bim expression or preferential transcription of any of major mRNA isoforms. However, change in γ1-γ2 ratio was noted in individuals with T29201 allele after addition of dexamethasone to LCLs. γ isoforms include an alternatively spliced exon 3 instead of BH3 containing one, thus lacking the BH3 domain and the apoptotic function (29). Change in Bim alternative splicing in favor of γ isoforms generation was consistent with the delay in apoptosis during mammery epithelial cell transformation (29). Interestingly, expression of Bim γ1, but not γ2, promoted a decrease in apoptosis.

The functional changes in studied LCLs were relatively minor suggesting that either subtle changes may have important phenotypic consequences or Bim may cooperate with other factors leading to phenotypic changes observed in clinical setting. Our recent study (35), which analyzed functional SNPs in promoter region of 11 intrinsic apoptosis genes with ALL susceptibility and ALL outcome, showed an association between Mcl1 promoter polymorphisms only and reduced OS in high-risk ALL patients (35). Here we assessed combined Mcl1/Bim effect, which showed marked reduction in OS, only for individuals with both at risk genotypes. The preferential association between Bim and Mcl1 in regulating cell death signals was reported in several studies. Mutation analysis revealed that the extent to which Mcl1 mutants were able to exert their antiapoptotic effects in hemopoietic cells correlated with their ability to associate with Bim (46). Bim phosphorylation was associated with reduced Bim/Mcl1 binding in chronic lymphocytic leukemia (CLL; ref. 34). Low Mcl1 expression and high Bim/Mcl1 ratio correlated with a favorable response to novel BH3 mimetic navitoclax in CLL patients (47). Disruption of Bim/Mcl1 complex is a mechanism used by granzyme B and TRAIL-induced caspase to induce a mitochondrial apoptotic cascade (48, 49). The Bim/Mcl1 association with ALL...
outcome seems to be limited to high-risk patients. Risk-dependent change in Bim expression was also reported by others. Significantly lower Bim expression was detected in high-risk childhood ALL patients who exhibited slow responses to induction regimen compared with patients who responded rapidly (50). Similar response was noted in our cohort. Four patients of our studied cohort carrying both risk genotypes (5 patients died from progressive disease among 7 with such combined genotypes; see Fig. 5) experienced induction failure. Higher expression of Mcl1 along with the presence of Bim isoforms lacking BH3 domain in patients with combined genotype may possibly explain association with poor outcome, particularly in high-risk patients with less favorable features of presenting disease who require higher corticosteroid doses. However, the regulation of the affinity of Bim for each prosurvival protein is complex and interaction between them in addition to expression level may play a key role in neoplastic cell survival (51).

Finally, several Bim polymorphisms were previously shown to play a role in susceptibility to non-Hodgkin lymphoma (30). Interestingly one of these variants (intronic rs686952) was in perfect linkage disequilibrium with C29201T variation as based on our analysis in HapMap individuals. Similarly, the deletion polymorphism resulting in BIM γ formation, that had a profound effect on the sensitivity to tyrosine kinase inhibitors in chronic myeloid leukemia, was recently reported (33). This polymorphism is limited to East-Asian population and could not be assessed in this study, but suggests importance of Bim gene variations and alternative Bim γ isoforms in mediating resistance to variety of drugs.

In conclusion, we reported the association between C29201T polymorphism in BH3 domain of Bim gene with reduced OS in 2 childhood ALL cohorts, possibly arising through the generation of Bim isoforms lacking BH3 domain. The effect seems to be dependent on interaction with the risk genotype in Mcl1 gene. It is potentiated in high-risk patients and thus likely influenced by more progressive disease requiring higher corticosteroid doses. The strength of the study comes from the fact that Bim induction has been shown to shape corticosteroid-related resistance in ALL. It identifies for the first time the polymorphism in the Bim gene (supported with functional assessment and analysis of 2 independent cohorts of reasonable sample size) and importantly, points to the role of an interaction between apoptosis gene variations in modulating therapeutic responses in ALL. There are several limitations to the study, including small number of patients with the combined gene effects, limited mechanistic insight into the Bim-related resistance in ALL and limited number of factors investigated. The other mechanism may affect the Bim function, and many other genetic (52, 53) and nongenetic factors contribute to complex pattern of responsiveness to corticosteroid. In the light of this complexity, this finding should be regarded as only one of the components of multifactorial corticosteroid resistance in pediatric ALL, which adds to our understanding how genetic variations modulate therapeutic responses in ALL.
Disclosure of Potential Conflicts of Interest

J.L. Kutok is employed (other than primary affiliation; e.g., consulting) as a Senior Director, Biology and Translational Research in Infinity Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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References


Correction: *Bim* Polymorphisms: Influence on Function and Response to Treatment in Children with Acute Lymphoblastic Leukemia

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Reference


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Bim Polymorphisms: Influence on Function and Response to Treatment in Children with Acute Lymphoblastic Leukemia

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