Expression of SMARCB1 modulates steroid sensitivity in human lymphoblastoid cells: identification of a promoter SNP that alters PARP1 binding and SMARCB1 expression

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Although cure rate of childhood acute lymphoblastic leukemia (ALL) has surpassed 80%, drug resistance remains a major cause of treatment failure. We previously identified a panel of 33 genes differentially expressed in prednisolone sensitive versus resistant ALL cells from newly diagnosed children. Here we used bioinformatics to identify resistance genes most likely to contain single nucleotide polymorphisms (SNPs) in their promoter region. The highest priority gene was SMARCB1, a core member of the SWI/SNF complex which promotes glucocorticoid effects through nucleosome remodeling. We identified several SNPs in the SMARCB1 promoter in lymphoblastoid cells from 90 individuals in the Centre d’Etude du Polymorphisme Humain (CEPH) panel. Among these SNPs, the $228G>T$ SNP (allele frequency 9.4%) was the only one that significantly increased reporter activity in human ALL cell lines. Furthermore, we identified nuclear protein poly (ADP-ribose) polymerase family, member 1 (PARP1) as a nuclear protein binding to the SMARCB1 promoter and showed that the $228$ SNP significantly altered PARP1 binding affinity. The $228G>T$ SNP altered SMARCB1 mRNA and protein levels and a positive association was found between the SMARCB1 mRNA level and both the $228$ genotype and prednisolone sensitivity in CEPH cell lines. Finally, knockdown experiments performed in human ALL cell lines confirmed that lower SMARCB1 expression increased prednisolone resistance. In summary, we provide functional evidence that SMARCB1 is involved in prednisolone resistance and identified a promoter SNP that alters the level of SMARCB1 mRNA and protein expression and the binding of PARP1 to the SMARCB1 promoter.

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common childhood cancer, representing nearly one-third of all pediatric malignancies. Over the past four decades, the cure rate for childhood ALL has dramatically improved due to better diagnosis and treatment, and the long-term disease-free survival now exceeds 80% (1). However, despite this progress, cancer remains the leading cause of death by disease in US children and drug resistance remains a major obstacle to curing the remaining patients.

Synthetic glucocorticoids (GCs), such as dexamethasone and prednisolone, are pivotal in the treatment of childhood ALL as well as many inflammatory or autoimmune diseases. These agents effectively induce G1 cell cycle arrest and apoptosis in lymphoid cells (2,3). GC-induced apoptosis is mediated through interaction of GCs with the glucocorticoid receptor (GR). GR is a member of the nuclear receptor
superfamily and is a modular protein with three functionally active domains (i.e. an activation, a DNA binding and a steroid binding domain) (4). In the absence of ligand, the GR is held in an inactive cytosolic complex consisting of two molecules of heat shock protein 90 and several immunophilins such as FKBP51. Upon ligand binding, the GC–GR complex translocates to the nucleus where it regulates the expression of a plethora of genes via both DNA binding-dependent and DNA binding-independent mechanisms (4).

Despite numerous investigations, the molecular basis of GC resistance remains poorly understood in general (5) and in particularly in ALL (6). Mutations that alter GC response (7) have been identified in all functional domains of the GR protein. Other studies have shown decreased expression of the GR or dysfunction of the GR-associated proteins such as heat shock protein or immunophilins as a potential mechanism of GC resistance. Finally, defective signaling of GC target genes has been reported to lead to GC resistance (6,8,9).

Recently, using a genome-wide approach to assess gene expression in ALL cells, we identified a panel of 33 genes differentially expressed in prednisolone sensitive versus resistant primary leukemia cells from newly diagnosed children with ALL (10). This revealed that SMARCB1, a core member of the SWI/SNF chromatin remodeling complex (11–13), was expressed at a significantly higher level in primary ALL cells that exhibit the greatest sensitivity to prednisolone.

In the current study, we aimed to provide functional evidence that SMARCB1 is involved in prednisolone resistance and to determine whether single nucleotide polymorphisms (SNPs) in the SMARCB1 promoter region affect transcription and thus prednisolone sensitivity. We utilized human CEPH (Centre d’Etude du Polymorphisme Humain) cell lines to identify candidate regulatory SNPs (rSNPs) in the SMARCB1 promoter and to assess their relation to SMARCB1 expression as well as prednisolone sensitivity. CEPH cell lines are EBV-transformed lymphoblastoid cell lines derived from healthy individuals of Western and Northern European ancestry, which have proven to be a useful tool for identifying polymorphisms related to drug sensitivity, including the influence of genetics on sensitivity to anticancer agents (14–16). The current studies showed that differences in expression of SMARCB1 significantly influence prednisolone cytotoxicity and identified a promoter SNP that is significantly related to SMARCB1 mRNA and protein expression. We also identified PARP1 as the primary nuclear protein binding to the SMARCB1 promoter and showed that the promoter polymorphism significantly alters PARP1 binding.

RESULTS
Identification of SNPs in the SMARCB1 promoter

Using a bioinformatics approach, SMARCB1 was identified as the highest priority candidate gene discriminating prednisolone resistance, which was likely to have promoter SNPs influencing expression. We found 20 SNPs within 5 kb upstream of the transcription start site (TSS) that affected nine putative transcription factor (TF) binding sites within ~1 kb of the TSS. We sequenced lymphoblastoid cells from 90 CEPH individuals and found seven polymorphic sites, of which five were

<table>
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<tr>
<th>Allele</th>
<th>SNP</th>
<th>MAF (%)</th>
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</thead>
<tbody>
<tr>
<td>−228</td>
<td>G/T</td>
<td>9.4</td>
</tr>
<tr>
<td>−117</td>
<td>C/T</td>
<td>3.9</td>
</tr>
<tr>
<td>117</td>
<td>C/T</td>
<td>11.1</td>
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<tr>
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<td>G/T</td>
<td>9.4</td>
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<tr>
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<td>352</td>
<td>G/T</td>
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</tr>
</tbody>
</table>

MAF, minor allele frequency.

Figure 1. Reporter activity of the SMARCB1 promoter containing various polymorphisms. Three SMARCB1 promoter constructs were designed and inserted upstream of the luciferase gene, specific for either the −117 T, −228 T or the wild-type (WT) allele. The above results were derived from three different human leukemia cell lines (MOLT-4, 697 and Reh). The promoterless pGL4 was used as the baseline control (‘empty’). Values represent the mean of two independent experiments performed in triplicate. Relative luciferase activity and standard errors are shown for each construct. Values that differ significantly from those of the wild-type construct are indicated as ‘*’ (P < 0.01, t-test).

Increased SMARCB1 promoter activity in cells with the variant −228T allele

To determine the functional effects of the two SNPs (−117 C > T, −228 G > T) on transcriptional regulation, we used the luciferase reporter gene to measure promoter activity. Three human leukemia cell lines (MOLT-4, 697 and Reh) were transiently transfected with the promoter constructs or the empty vector. As depicted in Figure 1, luciferase activity was significantly greater for the −228 T variant promoter construct compared with the wild-type construct in all three cell lines [3-fold higher in MOLT-4 and Reh cells, 2-fold higher in 697 cells (P = 0.0003, 0.001, 0.0004; Students t-test)]. In contrast, the −117 T showed no significant effect on
transcriptional activity compared with the wild-type promoter construct (Fig. 1).

The −228 G > T SNP alters nuclear protein binding to the SMARCB1 promoter

Nuclear protein extracts from MOLT-4 cells were subjected to binding to oligonucleotides representing the promoter region of either the −228 G allele (wild-type) or the −228 T allele (variant) by electrophoretic mobility shift assays (EMSAs). Specificity of the protein–oligonucleotide interaction was shown for both T and G alleles by reduced intensity of the shifted complex upon addition of increasing amounts of the respective unlabeled oligonucleotide probe. However, at lower concentrations of the competitor probe, the G allele oligonucleotide probe did not compete as efficiently as the T allele oligonucleotide probe, suggesting that the −228 G > T SNP increases nuclear protein-binding affinity (Fig. 2A, C).

Western blot analysis was used to assess the quality of nuclear and cytoplasmic protein extract with antibodies against heme-oxygenase 2 (34 kDa), a cytoplasmic marker, and against cyclin E2 (45 kDa), a nuclear marker. Good quality was assured by the presence of cyclin E2 only in the nuclear protein extract, without contamination of cytoplasmic protein (Fig. 2B).

Isolation and identification of DNA-binding protein

To identify the DNA-binding protein(s), double-stranded 28mer oligonucleotides flanking the −228 G and T variants were coupled to magnetic bead particles and used to enrich protein(s) from MOLT-4 nuclear extract. The purified protein(s) were subjected to SDS–PAGE and a unique band of ~120 kDa was visualized for both the −228 G and the T alleles (Fig. 3A). The band was then excised and subjected to protein identification by assisted laser desorption/ionization (MALDI)-time-of-flight (TOF)/TOF mass spectrometry. Figure 3B shows the mass spectrum of tryptic peptides, with PARP1 identified by a total sequence coverage of 38% (Fig. 3C) and MASCOT total protein score of 276 (score of 77 is significant at 95% confidence level). The precursor ion mass accuracy was 45 p.p.m. RMS error. Four MS/MS spectra were assigned to the protein (Table 2).

Increased SMARCB1 mRNA and protein expression in CEPH individuals with the −228 G>T SNP

To assess the effects of the −228 SNP on expression, we determined SMARCB1 genotypes and protein levels in lymphoblastoid CEPH cells and used the publicly available gene expression data from the Affymetrix Focus array (17). The −228 T/T genotype was associated with increased SMARCB1 expression when compared with wild-type genotype (−228 G/G) at both the mRNA level (Fig. 4, N = 56, \( P = 0.046 \) Wilcoxon rank-sum test and \( P = 0.166 \) Kruskal–Wallis test) and the protein level (Fig. 4B, \( N = 63, P = 0.012 \) Kruskal–Wallis test). In contrast, no association was found for the −117 T/T genotype with mRNA expression (\( P = 0.24 \) Wilcoxon rank-sum test and \( P = 0.48 \) Kruskal–Wallis test) or SMARCB1 protein levels (\( P = 0.68 \) Wilcoxon rank-sum test and \( P = 0.68 \) Kruskal–Wallis test).
Moreover, the $-228$ SNP was not in LD with any other promoter SNPs identified by sequencing (Fig. 5, Table 1), indicating that the $-228$ SNP has an independent effect on SMARCB1 expression. The $-352$ G>T SNP was not associated with the expression of SMARCB1 mRNA or protein ($P = 0.82$ and $P = 0.59$, respectively).

Association of SMARCB1 and PARP1 expression with prednisolone sensitivity

SMARCB1 expression was compared in CEPH cells that were either sensitive, intermediate or resistant to prednisolone, with asparaginase used as a negative control. This revealed that SMARCB1 expression was significantly inversely related to prednisolone IC$_{50}$ ($\rho = 0.27$, $P = 0.047$ Spearman’s rank test), but was not correlated to asparaginase IC$_{50}$, as depicted in Figure 6A, where SMARCB1 expression was inversely related to prednisolone sensitivity ($P = 0.013$ Kruskal–Wallis) but not to asparaginase sensitivity ($P = 0.48$ Kruskal–Wallis).

Because we identified PARP1 as a putative transactivator of SMARCB1 regulation, we assessed the SMARCB1/PARP1 mRNA ratio for its association with prednisolone sensitivity. This revealed that the SMARCB1/PARP1 ratio was significantly inversely related to prednisolone sensitivity (Fig. 6B, $P = 0.030$), whereas the ratio was not inversely related to asparaginase sensitivity (Fig. 6B, $P = 0.05$, Wilcoxon rank-sum test).

**Table 2.** MS/MS spectra and MASCOT result

<table>
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<tr>
<th>Start–end</th>
<th>Observed</th>
<th>Mr(expected)</th>
<th>Mr(calculated)</th>
<th>Delta</th>
<th>Miss</th>
<th>Sequence</th>
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<tr>
<td>270–282</td>
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<td>1398.77</td>
<td>1398.71</td>
<td>0.06</td>
<td>0</td>
<td>QQVPGESAILDR</td>
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<tr>
<td>453–467</td>
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<td>1623.86</td>
<td>1623.80</td>
<td>0.06</td>
<td>0</td>
<td>VVSEDFLQDVASTK</td>
</tr>
<tr>
<td>487–496</td>
<td>1065.62</td>
<td>1065.58</td>
<td>1065.58</td>
<td>0.03</td>
<td>0</td>
<td>AEPVEVVAPR</td>
</tr>
<tr>
<td>866–878</td>
<td>1377.80</td>
<td>1376.79</td>
<td>1376.74</td>
<td>0.05</td>
<td>0</td>
<td>TTNFAGILSQGLR</td>
</tr>
</tbody>
</table>

Total ion score 125; total protein score 276; confidence interval 100%.
SMARCB1 was identified as the candidate gene most likely to contain functionally important rSNPs. SMARCB1 is a core member of the SWI/SNF chromatin-remodeling complex (12,13), which we previously found to be expressed at significantly higher levels in steroid-sensitive ALL cells (10). In prior studies, the SWI/SNF complex was shown to increase glucocorticoid effects in yeast and human cancer cell lines (22). Additionally, it was shown that GR mediated the stimulation of SWI/SNF nucleosome disruption, suggesting that the GR can increase accessibility of nucleosomal DNA to TFs. Furthermore, nucleosome disruption mediated by the SWI/SNF complex is required for initiation of GC target gene activation (23). Finally, SMARCB1 is required for full chromatin remodeling activity of the SWI/SNF complex (22–27). In summary, there is evidence that SMARCB1 is important to execute prednisolone effects, via the nucleosome-disrupting activity of the SWI/SNF complex.

Although there have been no functional genetic polymorphisms identified in the core members of this complex, we identified a functional rSNP in the SMARCB1 regulatory region (−228 G > T) that was associated with higher SMARCB1 expression at both mRNA and protein levels. Consistent with this finding were luciferase experiments performed in T- and B-lineage ALL cell lines that further support a functional role of this rSNP in leukemia cells. The SMARCB1 regulatory region was previously identified as spanning nucleotide −192 to −436 relative to the first ATG (28) and only the −228 SNP is located in this region. Linkage analysis showed that the −228 SNP is not in LD with other polymorphisms in the promoter region, indicating an independent function of the −228 SNP. Furthermore, gel-shift experiments (EMSA), performed with nuclear protein extracts from ALL cell lines, showed increased binding affinity of the −228 SNP to the nuclear protein PARP1 in human ALL cells.

PARP1 is a highly conserved protein that contains three main domains [i.e. an N-terminal DNA-binding domain, a central automodification domain and a C-terminal catalytic adenine nucleotide (NAD) + -binding domain] (29). PARP1 was originally identified as a DNA damage sensor involved in DNA repair and apoptosis, binding to both single and double-stranded DNA breaks (30). However, there is growing evidence that its biological role is more complex and includes various important functions such as regulation of gene transcription (31). In this study, we identified PARP1 as a transactivator of the SMARCB1 promoter that binds to a specific cis-element encompassing the −228 SNP (32–35). Moreover, PARP1 mRNA expression has been associated with prednisolone sensitivity in primary ALL cells (36) and depletion of PARP1 has been shown to decrease drug-induced apoptosis (37–40). Potential mechanisms include modification of proteins involved in apoptosis such as p53 (41), depletion of cellular NAD+ and adenosine triphosphate (ATP) pools (42), facilitation of oligonucleosomal DNA fragmentation (43) and up-regulation of P-glycoprotein in PARP1-depleted cells (44). The current study reveals a new mechanism by which PARP1 can modulate sensitivity of human cancer cells to chemotherapy, via modulation of SMARCB1 expression. It should be noted that the −228 SNP was not associated with prednisolone sensitivity at a statistically significant level, which may be due to limited

Knockdown of SMARCB1 induces GC-resistance

To assess the influence of SMARCB1 on prednisolone sensitivity, we analyzed cell viability by MTT drug-sensitivity assay in human Jurkat and NALM-6 ALL cell lines that were transduced with an shRNA against SMARCB1 as described in Materials and Methods. Knockdown efficiency of SMARCB1 protein was verified by western blot analysis (Fig. 7A). SMARCB1 knockdown increased prednisolone resistance 4.5-fold in Jurkat cells and 7.9-fold in NALM-6 cells relative to the control shRNA (IC50 = 312 versus 69 nM and 1.3 × 10−2 versus 1.65 × 10−3 in knock-down versus non-target control; (Fig. 7B).

DISCUSSION

On the basis of genes we previously identified as differentially expressed in prednisolone sensitive versus resistant primary ALL cells (10), our goal was to determine whether genetic rSNP in the promoters of these genes influence interindividual differences in their expression (18–20). To date, most pharmacogenomic studies have focused on coding SNPs, with relatively few attempting to identify and evaluate rSNPs (21).

On the basis of bioinformatic analyses of reported SNPs in the 5’ region of genes associated with prednisolone resistance,
statistical power or because the genotype–expression–phenotype relationship is affected by additional factors.

SMARCB1 expression was significantly lower in steroid-resistant CEPH cells, consistent with our previous findings in primary ALL cells (10). This was functionally confirmed by stable expression of shRNA against SMARCB1 which lowered SMARCB1 expression and increased steroid resistance in human ALL cells. Furthermore, as we identified PARP1 to be a transactivator of SMARCB1 expression, we found a significant association between the ratio of SMARCB1/PARP1 and prednisolone sensitivity in CEPH cells. In conclusion, we provide functional evidence that SMARCB1 is involved in prednisolone resistance and identified an rSNP in the promoter region of SMARCB1 that increases its expression through modulation of PARP1 binding affinity and alters prednisolone sensitivity in CEPH cells.

In conclusion, we provide functional evidence that SMARCB1 is involved in prednisolone resistance and identified an rSNP in the promoter region of SMARCB1 that increases its expression through modulation of PARP1 binding affinity and alters prednisolone sensitivity. These findings provide mechanistic insights into interindividual differences in SMARCB1 expression and establish a definitive effect of SMARCB1 on steroid sensitivity in human leukemia cells.

MATERIALS AND METHODS

Bioinformatics

We identified the promoter region of genes that we had previously shown to be differentially expressed in prednisolone-resistant and sensitive ALL (10). In our initial scan, the promoter region was defined as 5000 bases immediately upstream of each gene’s TSS or of the start of translation (if TSS unknown) and 50 bases of the 5′-UTR based on the human genome assembly (version hg17) by the University of California at Santa Cruz (UCSC). The program TransFac was used to scan these promoter regions (version 9.4) and combined with positions of polymorphisms annotated in the dbSNP database (build 125). To further prioritize our experiments, we rank-ordered candidate genes within this group according to four successive criteria: (1) largest number of SNPs in putative TF-binding sites, (2) highest number of TF sites affected, (3) SNP in a strong (S) versus weak (W) affinity region of the TF binding sequence (i.e. heavier weighted base position in the matrix) and (4) SNPs closest to the TSS. The observed distribution of these scores for the prednisolone resistance candidate genes is shown in Table 3. Of note, SMARCB1 emerged as the highest priority gene for further investigation.

Human cell lines

CEPH cell lines were obtained from the Coriell cell repositories (http://ccr.coriell.org) or the University of Chicago via the Pharmacogenetics Research Network. For genotyping, we included the CEPH trios involving 90 individuals with Northern and Western European ancestry. MOLT-4, Reh, 697, NALM-6 and Jurkat (MOLT-4, Jurkat and NALM-6 American Type Culture Collection, Rockville, MD, USA; Reh and 697 German Collection of Microorganism and Cell Cultures, Braunschweig, Germany) were maintained in RPMI 1640 medium (BioWhittaker, Walkersville, MD, USA), supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) and 1% L-glutamine, 200 mM (BioWhittaker).

SMARCB1 promoter sequencing

We used GenBank Accession no. AP000349 to design primers (28). Forward primer: 5′-CAC ATC CGC TGT TGT CAT TTT GCC C-3′ and reverse primer: 5′-GAT CAT GTA GAA CTC GCC GTC GTC C-3′ were used to amplify a 721 bp fragment of the SMARCB1 promoter region. Briefly, 10 ng genomic DNA was mixed with 50 μl reaction volume containing 15 pmol of each primer, 0.2 mM of each dNTPs, 1 × PCR reaction buffer with DMSO, 1.5 mM MgCl₂ and 1 μl of GC-RICH PCR system enzyme mix (Roche, Indianapolis, IN, USA). PCR amplification was done by heating to 95°C for 3 min, five cycles with annealing temperature 62°C, 5 cycles with annealing temperature 59°C, 25 cycles
with annealing temperature 57°C, followed by a final extension step at 72°C for 7 min. PCR fragments were purified before sequencing by Shrimp Alkaline Phosphatases and Exo-nuclease I (USB Corporation, Cleveland, OH, USA). After direct sequencing of PCR-amplified DNA using ABI Big Dye terminator reagents 3.0 on an ABI PRISM 3700 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), we used the Polyphred program to detect SNPs at: 2485, 2427, 2352, 2319, 2228 and 2117 (location relative to start codon).

Western blot analysis

Nuclear and cytoplasmic protein extract derived from the MOLT-4 ALL cells were obtained using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL, USA). Proteins (25 μg) were separated on 4–12% SDS-PAGE (NuPAGE 4–12% Bis-Tris gel, Invitrogen) and electrotransferred onto PVDF membranes (Invitrogen). Filters were incubated for 2 h in Tris-buffered saline containing 1% Tween-20 (TBST) and 5% non-fat dry milk, then incubated with primary antibody in milk/TBST overnight at 4°C on a shaker. Filters were washed twice for 15 min in TBST before incubation with the secondary antibody conjugated with horseradish peroxidase in milk/TBST. After three final washes, filters were exposed after the enhanced chemiluminescence western blotting detection reagent (ECL plus, Amersham Biosciences, Piscataway, NJ, USA) to an autoradiographic film (Hyperfilm ECL, Amersham). Quantification of SMARCB1 protein was assessed by western blotting, using GAPDH for within-gel normalization and a standard (NALM-6) for between-gel normalization.

HO-2 antibody was purchased from Sigma (St Louis, MO, USA), cyclin E2 and SMARCB1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

In vitro sensitivity (MTT) assay

We determined in vitro drug sensitivity in 88 individuals of the CEPH population for prednisolone and asparaginase as

Figure 6. SMARCB1 mRNA expression and sensitivity to prednisolone in CEPH cell lines. CEPH cell lines were classified into three groups: sensitive, intermediate and resistant cells to prednisolone or asparaginase. (A) SMARCB1 mRNA expression (available in 54 CEPH cell lines) was plotted against three sensitivity categories for each drug (S, I, R). (B) SMARCB1/PARP1 mRNA expression ratio in 54 CEPH cell lines was plotted against three sensitivity categories for each drug. Significance was evaluated using the Kruskal–Wallis rank-sum test. The box represents the 25–75% quartiles, the line in the box represents the median and whiskers represent the range. S, sensitive (lowest quartile for IC₅₀); I, intermediate (middle two IC₅₀ quartiles); R, resistant (highest IC₅₀ quartile).
well as shRNA containing cell lines for prednisolone using a modification of the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazoliumbromide) assay described previously (10). Briefly, cells in mid-log phase were plated on day 0, exposed to six serial dilutions of prednisolone (20.72–1678.33 μM) and of asparaginase (0.003–100 IU/ml) on day 1 and analyzed after 48 h incubation on day 3. For each cell line, at least duplicate LC50 values (drug concentration lethal for 50% of the cultured cells) were estimated. The median LC50 value was used for assessing cell sensitivity.

**Table 3.** Promoter polymorphisms rank-order of prednisolone resistance genes

<table>
<thead>
<tr>
<th>Rank</th>
<th>Prednisolone-resistance gene</th>
<th>SNPs</th>
<th>TF sites</th>
<th>Strong or weak</th>
<th>Location from TSS</th>
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<tbody>
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<td>1</td>
<td>SMARCB1</td>
<td>7</td>
<td>9</td>
<td>S</td>
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<tr>
<td>2</td>
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See text for details.

**Figure 7.** shRNA-mediated SMARCB1 depletion induced prednisolone resistance. (A) Western blot analysis evaluating knockdown efficiency of NALM-6 and JURKAT that were stably transduced with control shRNA or SMARCB1 shRNA (shSMARCB1_4 and shSMARCB1_5). (B) Viability of stably transduced NALM-6 and JURKAT after 3 days of prednisolone treatment, as determined by in vitro sensitivity (MTT) assay. Each point represents the results of two parallel experiments (mean ± S.E.).

**Reporter constructs and site-directed mutagenesis**

Human genomic DNA samples were amplified to generate a 1522 bp fragment (−1510 to +12) of SMARCB1 using the forward primer 5′-TGTGTCTTCTGAATAGCATAGTTG-3′, the reverse primer 5′-GCCGCAATGATGATGATGATGATG-3′ and the GC-RICH PCR System kit (Roche), according to manufacturer’s instructions. PCR products were purified with the GeneClean III kit (Q-Biogene, Carlsbad, CA, USA). A two-step cloning strategy was applied to prevent unspecific mutations in the luciferase reporter vectors when performing site-directed mutagenesis. First, the purified PCR product was cloned into the multiple cloning region of pCR2.1 TOPO-TA vector (Invitrogen, Carlsbad, CA, USA) which was then used as template for site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to manufacturer’s instructions. Primers used for site-directed mutagenesis are shown in Table 4. This procedure introduced the specific mutations at
Table 4. Primers used for site-directed-mutagenesis of the SMARCB1 promoter

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<td>31</td>
<td>−228 G &gt; T</td>
</tr>
<tr>
<td>−228 F</td>
<td>GCCCTTTTGGTACGGCGGGCGGTCA</td>
<td>31</td>
<td>−228 G &gt; T</td>
</tr>
<tr>
<td>−228 R</td>
<td>TGACCGCGCCGCGCCGTCGAAACAAAAGGC</td>
<td>31</td>
<td>−228 G &gt; T</td>
</tr>
</tbody>
</table>

position −117 C > T and −228 G > T. Wild-type and mutated fragments were subcloned into the XhoI/HindIII sites of the promoterless eukaryotic expression vector pGL4.11 (Promega, Madison, WI, USA) and verified by DNA sequencing.

Luciferase reporter assay

For the reporter assay, human MOLT-4, 697 and Reh ALL cells (1 × 10^6 cells) were electroporated with 10 μg (MOLT-4 and Reh cell lines) or 5 μg (697 cell line) of the different reporter plasmid constructs described above using the Amaxa electroporation system (Amaxa Biosystem, Winooski, VT, USA) and used to normalize the results, and pGL4 wild-type luciferase activity was used as a reference (100%). Data shown are representative of two independent experiments performed in triplicates. Differences in activities among the various promoter constructs were evaluated using t-test.

Electromobility shift assays

Oligonucleotides used for this assay were the −228 T allele: forward 5’-CTTTTGTGGTCGCGCCGCGGTCT-3’ and reverse 5’-GAGCGCCGCGCCGCACTCGCTCAAACAAAAAG-3’ and the −228 G allele: forward 5’-CTTTTGTGGTCGCGCCGCGGTCT-3’ and reverse 5’-GAGCGCCGCGCGCCGCGTTCA-3’. Double-stranded oligonucleotides of the G and T alleles were generated by heating the complementary oligonucleotides in a buffer solution (50 mM Tris–HCl pH 8, 20 mM MgCl₂) at 100°C for 10 min and then slowly cooling to room temperature to allow perfect annealing. Double-stranded oligonucleotides were end-labeled by using [γ-32P]dATP (Perkin Elmer, Wellesley, MA, USA) and T4 polynucleotide kinase (Promega). Un-incorporated label was removed with MicroSpin G-25 Columns (Amersham Biosciences). Specific activity was determined by liquid scintillation counter (Beckman, Fullerton, CA, USA).

To determine the effect of the SNP on protein binding, we used a competition method. A constant amount of labeled wild-type oligonucleotide probe and nuclear protein extract was mixed with increasing amounts of unlabeled mutant oligonucleotide probe. The same was done using a constant concentration of mutant labeled oligonucleotide probe and nuclear protein extract with increasing amounts of the unlabeled wild-type oligonucleotide probe. EMSAs were performed with 5 μg of MOLT-4 total nuclear extract. Proteins were incubated with 1X binding buffer (0.2 μg of poly(dl–dC) in 10 mM Tris–HCl pH 8, 40 mM KCl, 0.05% NP-40, 0.8% glycerol, 10 mM DTT and 50 μM ZnCl₂), 3 × 10⁵ c.p.m. of 32P-labeled double-stranded oligonucleotide probe with or without unlabeled competitor probe, either one allele or the other allele, in varying concentrations (50×, 100×, 200×, 300×, 400×). Reaction mixtures were incubated at room temperature for 20 min and then subjected to gel electrophoresis (4% polyacrylamide gel in 0.5X TBE running buffer at 100 V for 2 h). The gels were then exposed overnight to a phosphoimager screen, scanned by the Storm 860 Phospholmager (Molecular Dynamics, Sunnyvale, CA, USA) and then analyzed by the ImageQuant software. In every case, at least two independent experiments were performed.

DNA affinity purification of SMARCB1-binding proteins

The proteins binding to the SMARCB1 DNA element were purified using streptavidin-coated Dynabeads M-280 (Invitrogen). Briefly, double-stranded oligonucleotides for the −228 G and T alleles (see EMSA Assay) were 5’ end labeled with biotin and coupled to streptavidin magnetic beads. Fifty micrograms of MOLT-4 total nuclear extract was applied to the DNA–magnetic beads complex and the binding reaction was performed for 30 min at room temperature in protein-binding buffer (10 mM Tris–HCl pH 8, 40 mM KCl, 0.05% NP-40, 0.8% glycerol, 10 mM DTT and 50 μM ZnCl₂). Non-specific DNA binding was inhibited by the addition of poly(dI–dC). Subsequently, the bound proteins were separated from the supernatant by use of a magnetic separator followed by three washes with protein binding buffer. Next, the DNA-binding protein mixture was eluted from the magnetic beads by use of a high ionic strength buffer (10 mM Tris–HCl pH 8, 0.05% NP-40, 0.8% glycerol, 10 mM DTT, 50 μM ZnCl₂ and 2M KCl). The purified proteins were run on 4–20% Novex SDS–PAGE gel (Invitrogen) and the gel was stained with SYPRO Ruby staining reagent (Invitrogen).

Protein identification by MALDI-TOF/TOF

The protein was reduced and alkylated with iodoacetamide, and a tryptic digest was prepared. Mass spectrometric analysis was performed using a Model 4700 Proteomics Analyzer (Applied Biosystems). This instrument employs MALDI, in conjunction with tandem TOF mass analyzers. The digest was introduced into the instrument in a crystalline matrix of α-cyano-4-hydroxycinnamic acid also containing 2 mM ammonium citrate to suppress ionization of matrix clusters. Searches of the NCBI nr database (rev 021206) were performed with Applied Biosystem’s GPS explorer software,
which uses the MASCOT search engine. Protein assignments were made on the basis of both MS and MS/MS spectra.

RNA interference

SMARCB1 knockdown was achieved by RNA interference using a lentiviral vector-based shRNA approach from the MISSION™ TRC-Hs 1.0 library (Sigma). Lentiviral particles corresponding to the MISSION shRNA SHRS-NM_003073 target set were used as well as the MISSION Non-Target shRNA control. Specificity and efficiency of the shRNA SMARCBI procedure were controlled by western blotting, after transduction and puromycin selection in Nalm-6 and Jurkat cells.

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Conflict of Interest statement. The authors and/or their immediate family have nothing to disclose in terms of the source of funding and any affiliations with or involvement in any companies, trade associations, unions, litigants or other groups with a direct financial interest in the subject matter or materials discussed in the manuscript.

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