

## Original Article

# A facile, branched DNA assay to quantitatively measure glucocorticoid receptor auto-regulation in T-cell acute lymphoblastic leukemia

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## Abstract

Glucocorticoid (GC) steroid hormones are used to treat acute lymphoblastic leukemia (ALL) because of their pro-apoptotic effects in hematopoietic cells. However, not all leukemia cells are sensitive to GC, and no assay to stratify patients is available. In the GC-sensitive T-cell ALL cell line CEM-C7, auto-up-regulation of RNA transcripts for the glucocorticoid receptor (GR) correlates with increased apoptotic response. This study aimed to determine if a facile assay of GR transcript levels might be promising for stratifying ALL patients into hormone-sensitive and hormone-resistant populations. The GR transcript profiles of various lymphoid cell lines and 4 bone marrow samples from patients with T-cell ALL were analyzed using both an optimized branched DNA (bDNA) assay and a real-time quantitative reverse transcription-polymerase chain reaction assay. There were significant correlations between both assay platforms when measuring total GR (exon 5/6) transcripts in various cell lines and patient samples, but not for a probe set that detects a specific, low abundance GR transcript (exon 1A3). Our results suggest that the bDNA platform is reproducible and precise when measuring total GR transcripts and, with further development, may ultimately offer a simple clinical assay to aid in the prediction of GC-sensitivity in ALL patients.

**Key words** Glucocorticoid receptor, T-cell acute lymphoblastic leukemia, apoptosis, branched DNA assay

Glucocorticoids (GCs) are used in multi-drug treatment protocols for acute lymphoblastic leukemia (ALL) because they can induce cell cycle arrest and apoptosis in malignant cells without causing myelosuppression, thereby reducing tumor load<sup>[1-3]</sup>. However, not all patients benefit from the tumor-reducing effects of GCs because some lymphoblasts are resistant to their apoptosis-inducing actions<sup>[4-6]</sup>. To date, a predictive test to stratify ALL patients as GC responders and GC non-responders remains elusive, although a patient's

initial response to GC monotherapy is a strong indicator of long-term prognosis<sup>[1,4,6,7]</sup>. Regardless of the response to GCs, patients who are administered high-dose GC treatment may suffer a wide range of adverse effects such as hypertension, osteoporosis, osteonecrosis, growth retardation, diabetes mellitus, immunosuppression, and psychosocial morbidities<sup>[8-10]</sup>. Our goal was to investigate if a branched DNA/RNA hybridization technique can be developed into a simple clinical assay for predicting GC sensitivity in newly diagnosed and relapsed ALL patients, such that the use and dosage of GCs could be tailored specifically to the patient and minimize undesirable adverse effects.

GCs exert their function on cells by diffusing through the plasma membrane and binding to the cytoplasmic glucocorticoid receptor (GR). The ligand-bound GR then translocates to the nucleus where it can function as a transcriptional activator or repressor<sup>[11-16]</sup>. Most notably, activated GR binds to glucocorticoid response units within its own promoters and auto-up-regulates or

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doi: 10.5732/cjc.012.10044

auto-down-regulates transcription of its own gene<sup>[17-19]</sup>. GC-induced GR up-regulation is correlated with increased apoptosis, or sensitivity, in the CEM-C7 T-cell ALL cell line, whereas GC-mediated GR down-regulation is related with decreased apoptosis, or resistance, in the IM-9 B-lymphoblastoid cell line or mature lymphocytes<sup>[20-23]</sup>. Furthermore, differential regulation of at least two major splice variants of the GR transcript (1A3 and 1C) is lineage-specific<sup>[24]</sup>. Specifically, in CEM-C7 cells, all GR transcripts are up-regulated in response to treatment with the GC hormone analog dexamethasone (Dex), with the exon 1A3 transcript being the most robustly up-regulated (~8- to 9-fold)<sup>[20]</sup>. A Dex-sensitive pre-B-cell ALL cell line, 697, exhibits an steroid-mediated up-regulation in exon 1C- containing transcripts, whereas exon 1A3 transcripts are down-regulated<sup>[24]</sup>. These differential regulation patterns are potentially suitable markers of GC sensitivity.

This study compared two assays, the branched DNA (bDNA) assay and the quantitative reverse transcription-polymerase chain reaction (QRT-PCR), for their ability to accurately measure GR transcript concentrations in 4 ALL model cell lines (CEM-C7, CEM-C1, 697, and IM-9) and 4 fresh T-cell ALL patient samples. With each assay we used probe sets that were specific for either exon 1A3 transcripts or exon 5/6 transcripts (total GR). The results suggest that the bDNA assay has the potential, with additional development, to assist in decisions regarding steroid-therapy in ALL patients.

## Materials and Methods

### Cell culture and treatment

The human CEM-C7 and CEM-C1 cell lines (gifts from Dr. E. Brad Thompson, University of Texas Medical Branch, Galveston, TX, USA), human 697 cell line (a gift from Dr. Noreen M. Robertson, Drexel University School of Medicine, Philadelphia, PA, USA), and IM-9 cell line (ATCC, Manassas, VA, USA) were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 2 mmol/L L-glutamine (Invitrogen, Carlsbad, CA). Cells were cultured in 5% CO<sub>2</sub> air at 37°C and treated with either Dex at a final concentration of 1 mmol/L or with vehicle alone, ethanol (EtOH), at a concentration of 1 μL/mL. No ethanol-related effects were observed at the concentration used.

### Patient samples

All patient bone marrow samples were obtained at initial diagnosis by the Pediatric Hematology/Oncology Department at Children's Hospital of New Orleans with IRB approval. Patient immunophenotypes were

determined by the Children's Hospital Immunology Lab. Lymphoblasts were isolated via centrifugation over Ficoll-Paque™ (GE Healthcare, Piscataway, NJ) according to manufacturer's instructions. Isolated lymphoblasts were cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS and 2 mmol/L L-glutamine. Patient samples were treated with the same dosages of Dex or EtOH and incubated at the same temperature and atmospheric conditions as the cell lines.

### Quantitative reverse transcription-polymerase chain reaction

Total RNA was prepared using Trizol® Reagent (Invitrogen, Carlsbad, CA). The primers and Taqman® probes used for each GR transcript (exon 1A3 and exon 5/6) were described previously<sup>[20]</sup>. The Taqman® One-Step RT-PCR reagents (Applied Biosystems, Foster City, CA) or iScript™ One-Step RT-PCR Kit for Probes (Bio-Rad, Hercules, CA) were used for QRT-PCR of transcripts. For measuring 18S rRNA, a eukaryotic 18S rRNA endogenous control (Applied Biosystems) was used. The QRT-PCR was performed on an ABI Prism 7700 Sequence Detection System as described previously<sup>[20]</sup> or a Bio-Rad CFX96 Real-Time PCR Detection System following the manufacturer's protocol. Each transcript measurement was normalized to the 18S rRNA concentration of its respective sample. In a study designed to define the threshold level of GR needed to trigger apoptosis in cell lines, these same patient samples were analyzed an additional time using QRT-PCR, and this study yielded similar results<sup>[25]</sup>.

### Quantigene assay

Quantigene® reagents and custom exon-specific bDNA probe sets were obtained from Panomics (Fremont, CA). The probe set specific for exon 1A3 transcripts binds to locations between base pair 350 and 950 of the NM\_001018077 cDNA sequence. A probe set that binds to locations between base pair 2440 and 2920 of NM\_001018077 captures all GR transcripts containing exons 5 and 6 and serves as a measure of total GR transcript concentration. A β-actin specific probe set (Panomics, cat. No. SA-10008) was used as a normalization control, in addition to cell counts performed prior to lysis using trypan blue exclusion. Normalization to β-actin was used because the expression levels are lower than that of 18S, and therefore, the cellular lysates required less dilution to ensure that the level of luminescence was within the detection range of the luminometer. Optimization with the 18S bDNA probe set was not successful. Normalization of QRT-PCR data with 18S and β-actin showed no difference in the calculated fold-change of GR expression after hormone

treatment (data not shown). The manufacturer's instructions were followed for preparing cell lysates and for all Quantigene® assay procedures. Luminescence was detected using an Ascent Luminoskan luminometer (Thermo Electron, Franklin, MA). Single measurements of each well were recorded using an integration time of 200 ms. Two different versions of the Quantigene® bDNA assay (v1.0 and v2.0) were used. The major improvements for version 2.0 were the addition of another bDNA molecule, the pre-amplifier, which allows for greater signal amplification, and also the use of a more stable luminescent substrate. Results obtained with the two versions were comparable. All data presented after Figure 1 were obtained using version 2.0.

### Cell viability assays

Cell viability was measured via either trypan blue exclusion or flow cytometry (FACSCalibur, BD Biosciences, Franklin Lakes, NJ) using the Vybrant® Apoptosis Assay Kit #4 (Invitrogen). For the trypan blue exclusion assay, cells were suspended in a 0.4% trypan blue dye solution that cannot pass through the cell membrane if the cell is viable. Viable cells, appearing bright-white against a dark-blue background, were counted via hemacytometry. When measuring cell viability with flow cytometry and the Vybrant® Apoptosis Assay kit, cells were treated with propidium iodide and YO-PRO-1 dyes that enter through or bind to the compromised membranes of cells undergoing necrosis and apoptosis, respectively, and label them with red or green fluorescent signals. The leukemic cell survival (LCS) was calculated using the following equation for both methods:  $LCS = (\% \text{ viable cells}_{\text{Dex-treated}} / \% \text{ viable cells}_{\text{EtOH-treated}}) \times 100\%$ .

### Statistical analysis

The correlation between the bDNA assay and the QRT-PCR assay for measuring GR transcript expression was measured by simple linear regression modeling. The correlation coefficient ( $R$ ) was calculated from the coefficient of determination ( $R^2$ ) obtained with the equation of the best-fit line drawn after plotting each platform against the other. Statistical significance was determined by  $P$  values less than 0.05 ( $P = 1 - R$ ). Linear regression modeling was completed using the Microsoft Excel spreadsheet and graphing software. This analysis was completed for both cell lines and patient sample data. For the other experiments, a paired  $t$  test was used, and a  $P$  value of 0.05 or less was considered significant.

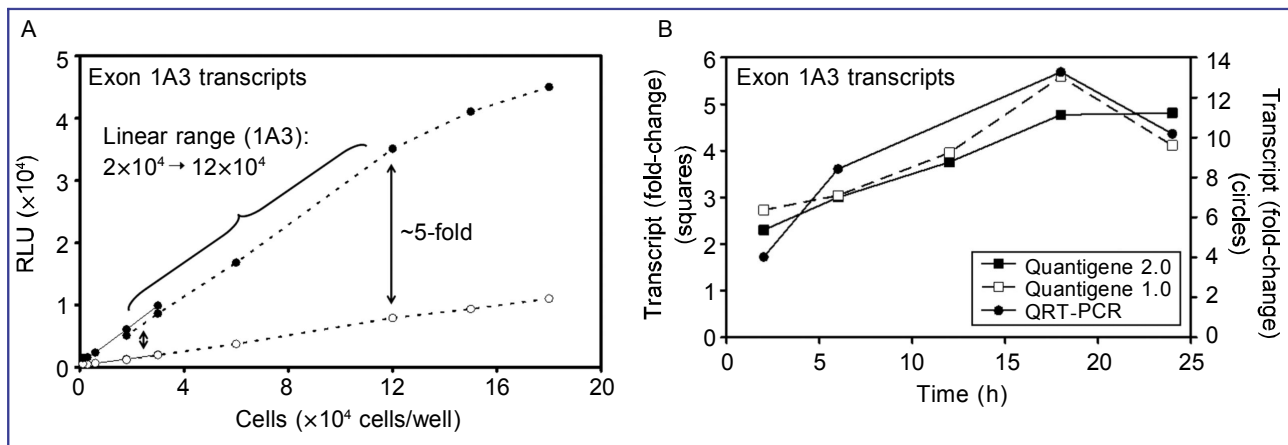
## Results

### The bDNA assay is sensitive and has a good linear range

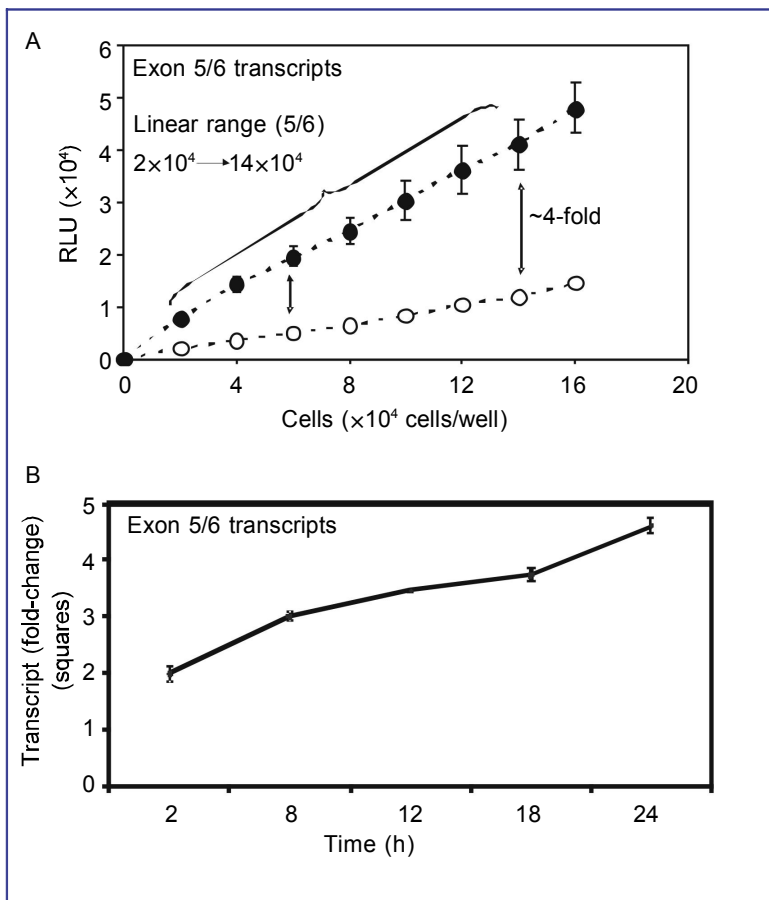
The bDNA assay is a DNA/RNA hybridization assay designed to capture RNA transcripts with specific probe sets ([http://www.panomics.com/index.php?id=QG2\\_2\\_Large](http://www.panomics.com/index.php?id=QG2_2_Large)). The use of various bDNA molecules to build the scaffold results in a large amplification of the signal from a single mRNA molecule, thus greatly augmenting the sensitivity of the assay. The final bDNA probe is conjugated to alkaline phosphatase, which produces a luminescent signal proportional to the number of RNA transcripts of interest captured on the plate when the APS-5 substrate is added. This assay presents a simple enzyme-linked immunosorbent assay (ELISA)-like workflow and requires instruments that are commonly found in a clinical laboratory. For these reasons, the bDNA platform presents a potentially facile approach to predicting GC sensitivity in ALL patients by analyzing the relative amounts of GR transcript present in lymphoblasts. Therefore, our goal was to test the assay's ability to measure GR auto-regulation in crude cellular extracts after an overnight, *ex vivo* Dex challenge test.

Preliminary optimization studies were performed for the bDNA assay. We focused initially on exon 1A3 transcripts for two reasons. First, this transcript was up-regulated in a T-ALL cell model system used in our laboratory, CEM-C7 cells. Second, previous studies using QRT-PCR showed that there is a large signal/noise ratio for exon 1A3 transcripts in CEM-C7 cells, with an 8- to 10-fold up-regulation seen upon treatment of these cells with the glucocorticoid analog Dex<sup>[20]</sup>. This robust up-regulation made the 1A3 transcript a good candidate for a clinical assay. When using the probe set specific for exon 1A3 transcripts, the linear range of the assay was from a lysate concentration of 20 000 to 120 000 cells (Figure 1A). In this range, there was an approximately 5-fold up-regulation of exon 1A3 transcripts in Dex-treated CEM-C7 cells when compared to EtOH-treated CEM-C7 cells. Dex treatment time-course experiments were performed using both QRT-PCR and the bDNA assay (Figure 1B) to optimize the time used to assay up-regulation of exon 1A3 transcripts. These preliminary data suggest that 18 h of hormone treatment in CEM-C7 cells is optimal for this transcript and both the 1.0 and 2.0 versions of the assay gave similar results (Figure 1B).

The exon 5/6 (total GR) probe set was also validated using the Quantigene 2.0 assay. The assay



**Figure 1. Optimization the Quantigene® assay for exon 1A3 transcripts using CEM-C7 cells.** A, the bDNA assay has a large linear range with respect to lysate cellular concentration when measuring exon 1A3 transcripts. Cellular lysate prepared from 1 200 to 180 000 cells was incubated in the wells of the plate as described in Materials and Methods section and Results section. The bDNA assay was then carried out according to the manufacturer’s directions. The number of cells used to prepare the lysate is plotted against the luminescence (RLU). *Open symbols*, EtOH-treated cells; *closed symbols*, Dex-treated cells; *Solid and dashed lines* signify two separate Quantigene plates and experiments that were used to cover the full range of cell concentrations tested. B, the magnitude of 1A3 transcript up-regulation by Dex was measured by Quantigene® 1.0 and 2.0 bDNA assays and QRT-PCR at 2-, 6-, 12-, 18-, and 24-h time points. Between 50 000 and 100 000 cells were used in the Quantigene assays. The data were normalized to  $\beta$ -actin (bDNA) or 18S rRNA (QRT-PCR) expression in each respective sample and are presented as the fold-change relative to the respective GR (1A3) transcript level measured in the EtOH vehicle-treated control sample.

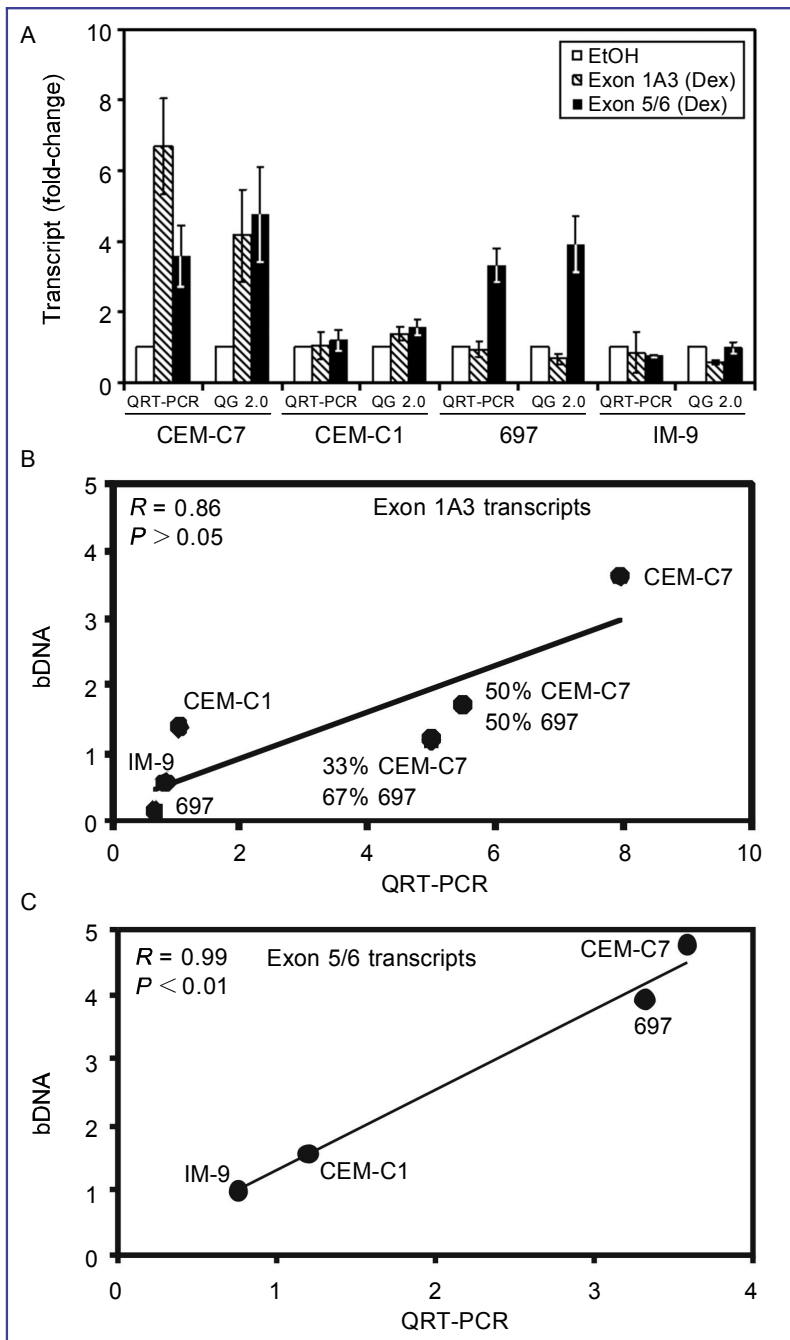


**Figure 2. Optimization the Quantigene® assay for exon 5/6 (total GR) transcripts using CEM-C7 cells.** Optimization was carried out as described in Figure 1. A, the bDNA assay has a large linear range for exon 5/6 transcripts extending over at least an order of magnitude. The number of cells used to prepare the lysate is plotted against RLU. *Open symbols*, EtOH-treated cells; *closed symbols*, Dex-treated cells. B, the magnitude of exon 5/6 transcript up-regulation by Dex was measured by the Quantigene® 2.0 bDNA assays at 2-, 6-, 12-, 18-, and 24-h time points. The data were normalized to  $\beta$ -actin expression in each respective sample and are presented as the fold-change relative to the respective total GR (exon 5/6) transcript level measured in the EtOH vehicle-treated control sample. Three separate experiments were performed. The data are presented as the average of the three experiments  $\pm$  the standard error of the mean (SEM).

was linear using extracts that contained between 20 000 and 160 000 cells per well (Figure 2A), and a linear increase in exon 5/6 transcripts was obtained for the first 24 h of steroid treatment (Figure 2B). Because the assay was linear for 18 h for both exon 1A3 and 5/6 transcripts, and because an overnight Dex challenge assay would be clinically convenient, we used 18 h of Dex treatment in subsequent experiments. A total of 60 000 cells per well, which is clearly in the linear portion of the assay curve for both exon 1A3 and exon 5/6 transcripts, was used in all subsequent bDNA assays.

**The bDNA and QRT-PCR assays correlate very well for the total GR (exon 5/6) probe set in four different cell lines**

GC-induced regulation of exon 1A3 and exon 5/6 transcripts was measured with the bDNA and QRT-PCR assays in four cell lines, two of which (CEM-C7 and 697) are sensitive to steroid-mediated apoptosis and two (CEM-C1 and IM-9) are resistant. Each assay platform measured a similar relative fold-increase or fold-decrease for each cell line (Figure 3A). The



**Figure 3. Measurements of GR transcript regulation in cell lines via the bDNA assay and QRT-PCR assays are correlated.** A, cell samples were prepared from CEM-C7, CEM-C1, 697, and IM-9 cells treated for 18 h with 1 mol/L Dex or EtOH vehicle alone. CEM-7 is a glucocorticoid-sensitive T-cell ALL cell line and the CEM-C1 cell line was derived from CEM-C7 cells and they are resistant to steroid-mediated apoptosis. 697 is a pre-B-ALL cell line that is steroid-sensitive, whereas the IM-9 lymphoblastoid cell line is resistant to hormone-mediated apoptosis. Cells were lysed for the Quantigene assay using the manufacturer’s lysis buffer and total cellular RNA samples were isolated from another aliquot of the culture for QRT-PCR using the Trizol® reagent. The average and SEM are shown for three separate experiments. The data are presented as the fold-change in GR transcript concentration as compared to the EtOH-treated control. For each cell line, there was no significant difference (via paired sample *t*-test) between QRT-PCR and QG 2.0 for the exon 5/6 measurements. The correlation between QRT-PCR and QG 2.0 when measuring the GC-mediated regulation of exon 1A3 transcripts (B) and exon 5/6 transcripts (C) was tested by calculating the correlation coefficient (*R*). Because the level of exon 1A3 transcripts was so much greater in CEM-C7 cells than in other cells, we mixed the higher expressing CEM-C7 cell extract with the lower expressing 697 cell extract to obtain points in the middle of the curve (B). The ratios of CEM-C7 extract to 697 extract was 1:1 (50% CEM-C7/50% 697) and 1:2 (33% CEM-C7/67% 697). NS denotes that the difference is not significant (*P* > 0.05).



coefficient of correlation was reasonably good when comparing the bDNA assay and the QRT-PCR assay for exon 1A3 transcripts (Figure 3B), but it did not reach statistical significance. In contrast, a very good coefficient of correlation that was significant was obtained for exon 5/6 transcripts (Figure 3C). The superiority of the exon 5/6 transcript probe in the Quantigene assay was borne out in later experiments (see below).

### The bDNA assay exhibits interplate variation in the overall amplitude of luminescence

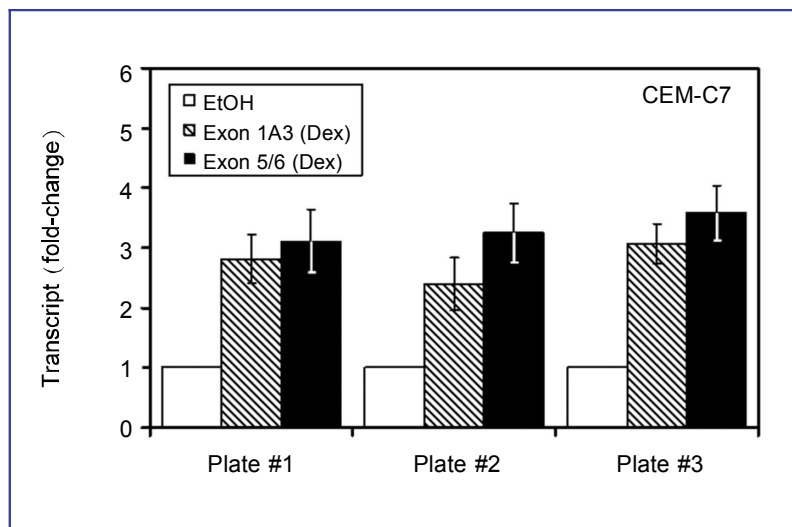
While investigating the reproducibility of the bDNA assay, we found that there could be an approximate 1.5- to 2-fold difference in the overall amplitude of luminescence between replicate plates (i.e., plates containing the same cellular lysates and assayed under the same conditions). The difference was found to be a general and global increase in the luminescence signal (for unknown reasons), because the relative difference between EtOH- and Dex-treated samples remained the same across the different plates used (Figure 4). This finding has clinical implications, because in a patient care setting, numerous plates will be used on different days and it will be necessary for all data obtained from different assays to be comparable, as clinical decisions will be made from them. To solve this issue, inclusion of a known standard will be necessary for each assay such that the interplate signal differences can be factored out. A GR transcript standard would meet this purpose nicely because it would not only serve as a signal normalizer but also allow for the determination of absolute GR transcript levels in experimental samples.

### The bDNA and QRT-PCR assays correlate well for the total GR (exon 5/6) probe set in fresh patient samples

We obtained four T-cell ALL patient bone marrow samples at the time of diagnosis. These T-cell ALL patient samples were different with respect to immunophenotype, age of diagnosis, and white blood cell count at presentation—all of which are strong prognostic indicators of childhood ALL<sup>[26,27]</sup> (Table 1). Mononuclear cells were isolated from these samples, and they were treated with either 1  $\mu$ mol/L Dex or 1  $\mu$ L/mL EtOH for 18 h. Following the 18 h treatment, the samples were processed for QRT-PCR (cell lysis and RNA extraction) and for the bDNA assay (cell lysis only). As was seen with the cell lines (Figure 3), there was a significant correlation between the bDNA assay and the QRT-PCR assay only when measuring the GC-mediated regulation of exon 5/6-containing GR transcripts, and not with the exon 1A3 assays (Figure 5). Many more patient samples must be tested to make firm conclusions about the correlation between the bDNA assay and the QRT-PCR assay for measuring GC-mediated GR transcript regulation.

### GR regulation in four T-cell ALL patient samples requires further study due to small sample size

Every patient sample tested was sensitive to GC-mediated apoptosis, as cell viability in all cases was 35% or less after Dex treatment (Table 1). The role for hormone-mediated up-regulation of GR in steroid-mediated apoptosis remains complex. It may be important to evaluate the actual transcript level rather



**Figure 4. Interplate signal differences do not affect fold-change calculation.** Exon 1A3 and exon 5/6 transcripts were measured in CEM-C7 cells treated with either EtOH or 1 mol/L Dex for 18 h. The same sample lysates were used for analysis on three different bDNA plates, #1, #2, and #3. Error bars represent the SEM of three separate biological replicates contained in each plate. There was no significant difference (via a paired sample *t* test) between any plate for measuring fold-changes in the Dex-induced levels of either exon 1A3 or exon 5/6 transcripts.

**Table 1. Clinical data for patients with acute lymphoblastic leukemia (ALL)**

Patient code	WBC at diagnosis ( $\times 10^9$ cells/ $\mu$ L)	ALL immunophenotype	Age at diagnosis (years)	% Blasts	LCS
TALL1	207.40 (4.0–11.9)	CD2 <sup>+</sup> , CD3 <sup>-</sup> , CD4 <sup>+</sup> , CD5 <sup>+</sup> , CD7 <sup>+</sup> , CD8 <sup>+</sup>	6	91	22
TALL2	32.33 (3.7–11.7)	CD2 <sup>-</sup> , CD3 <sup>-</sup> , CD4 <sup>-</sup> , CD5 <sup>+</sup> , CD7 <sup>+</sup> , CD8 <sup>-</sup> , CD13 <sup>+</sup>	18	90	35
TALL3	323.35 (3.7–10.3)	CD2 <sup>+</sup> , CD3 <sup>+</sup> , CD4 <sup>-</sup> , CD5 <sup>+</sup> , CD7 <sup>+</sup> , CD8 <sup>-</sup>	10	98	10
TALL4	40.47 (4.5–11.9)	CD2 <sup>+</sup> , CD3 <sup>-</sup> , CD4 <sup>+</sup> , CD5 <sup>+</sup> , CD7 <sup>+</sup> , CD8 <sup>+</sup>	4	91	18

Age-matched normal white blood cell count (WBC) ranges are listed in parentheses. LCS = (% viable cells<sub>Dex-treated</sub> / % viable cells<sub>EtOH-treated</sub>)  $\times$  100%. All samples were treated for 72 h except for TALL1, which was treated for 48 h. For comparative purposes, the LCS for the CEM-C7 cell line (100% blasts) after 72 h of treatment was (16  $\pm$  3)% is a myeloid marker. All patients are males.

than the fold-increase of Dex-treated cells versus EtOH controls to help resolve this matter. Although we found a significant correlation between the two assay platforms when measuring exon 5/6 transcripts (total GR) in 4 patient samples (Figure 5), we observed notable differences for 2 samples. When the bDNA assay was used, an apparent steroid-mediated up-regulation was observed in all cases (Figure 6A). Conversely, if the QRT-PCR assay was used, an apparent GC-induced GR up-regulation occurred in patient samples TALL2 and TALL3, whereas in other patient samples (TALL1 and TALL4) there was either a slight GC-induced GR up-regulation or a lack of up-regulation (Figure 6B) although these samples were still sensitive to GC-induced apoptosis (Table 1). Irrespective of the assay used, however, TALL1 and TALL4 had higher basal (EtOH control) GR transcript levels than did TALL2 and TALL3. These data emphasize that, because of limited patient sample material, this analysis is preliminary. Many more patient samples must be analyzed to achieve conclusive results regarding steroid regulation of GR transcript levels.

### GR transcript up-regulation is important for steroid-mediated apoptosis in CEM-C7 cells

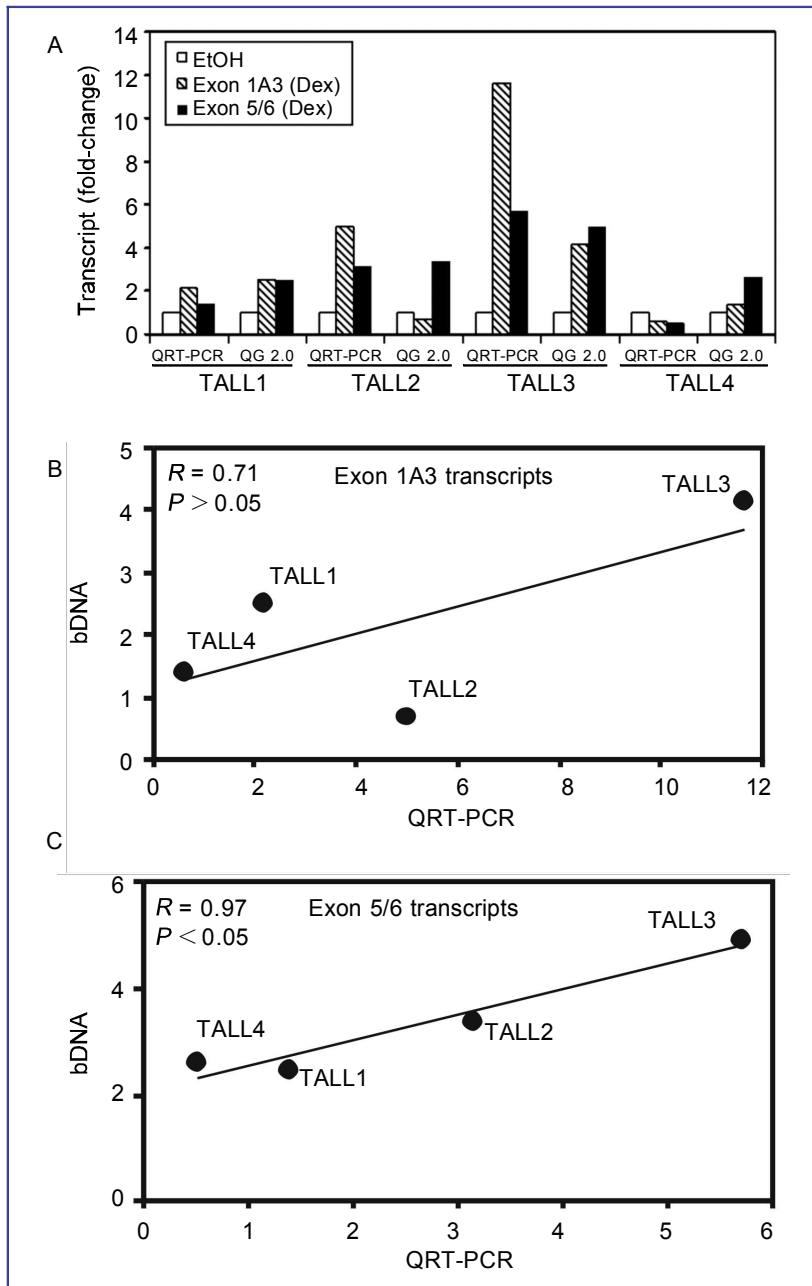
To clarify if there is a correlation between the up-regulation of GR transcripts and apoptosis after Dex treatment in CEM-C7 cells, the cells were treated with 2-fold serial dilutions of Dex starting from  $1 \times 10^{-9}$  mol/L for 18 h. RNA was isolated and QRT-PCR was performed to measure exon 1A3 and exon 5/6 transcripts in these cells. Also, cell viability was measured using the Vybrant<sup>®</sup> Apoptosis Assay Kit #4 after treating the cells with Dex for 72 h. With increasing concentrations of Dex, we observed an increase in the auto-up-

regulation of both exon 1A3 and exon 5/6 transcripts (Figure 7). Also, we observed a gradual decrease in cell viability in these cells with increasing concentrations of Dex (Figure 7), suggesting the importance of the auto-up-regulation of these transcripts in promoting apoptosis in CEM-C7 cells.

## Discussion

The goal of this study was to evaluate the ability of a DNA/RNA hybridization assay to detect and quantitatively measure the up-regulation of GR transcripts in ALL model cell lines and fresh patient samples. The large dynamic range and reproducibility of the assay suggested by preliminary optimization studies would make it quite suitable for a clinical assay, as patient samples received in a clinical setting may vary substantially in quality and blast concentration. The difficulty in getting a good correlation between the QRT-PCR and bDNA assays for exon 1A3 transcripts might be owing to their low abundance, which requires a large amplification for their detection. Alternatively, these transcripts could be more susceptible to RNase degradation in sample preparation for the bDNA assay versus QRT-PCR. While the more robust up-regulation of exon 1A3 transcripts compared to exon 5/6 transcripts made the former a more attractive candidate for the bDNA assay, at present, exon 5/6 transcripts give the most reliable results.

The preliminary patient sample data suggest that the bDNA assay could be applied to fresh patient samples when using the total GR (exon 5/6) probe set, although more samples are required to draw firm conclusions regarding the potential use of this assay for predicting if a patient is steroid-sensitive or resistant. Additional

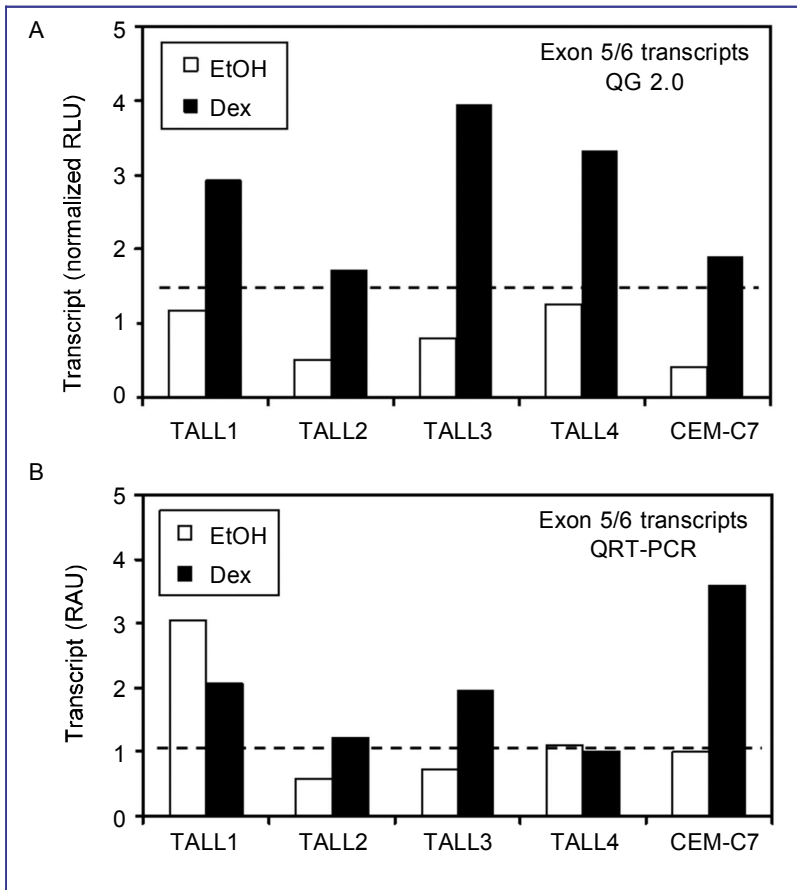


**Figure 5.** In patient samples, the bDNA and QRT-PCR assays are correlated only when measuring exon 5/6 transcripts. A, cell samples were prepared from TALL1, TALL2, TALL3, and TALL4 cells treated with 1  $\mu\text{mol/L}$  Dex or EtOH vehicle alone for 18 h. The cells were lysed for the Quantigene assay using the manufacturer's lysis buffer and total cellular RNA was isolated from another aliquot of the culture for QRT-PCR using the Trizol<sup>®</sup> reagent. Each measurement was completed only once because of the low yield of material obtained from fresh patient samples. The data are presented as the fold-change in GR transcript concentration as compared to the EtOH-treated control. The correlation between QRT-PCR and QG 2.0 when measuring the GC-mediated regulation of exon 1A3 transcripts (B) and exon 5/6 transcripts (C) was tested by calculating the correlation coefficient ( $R$ ).

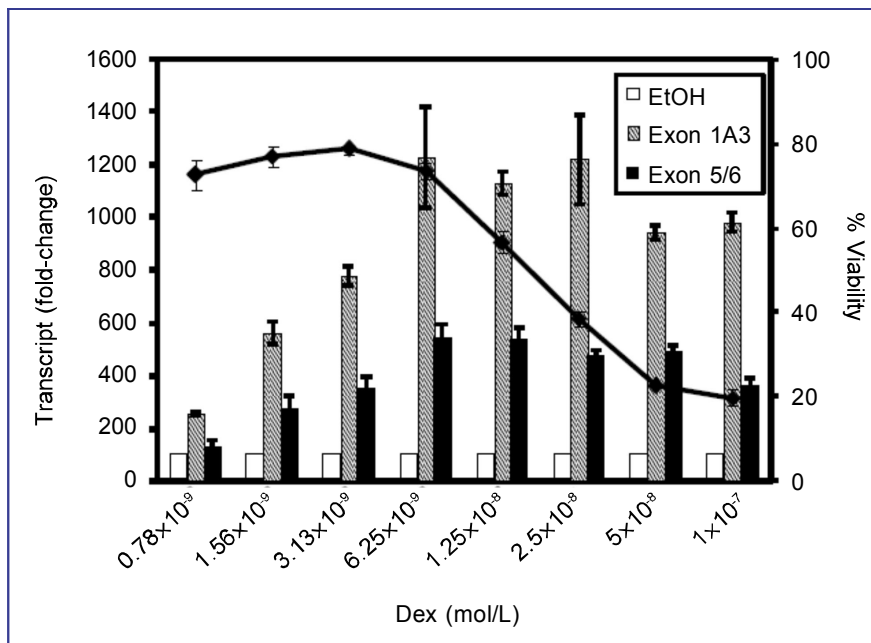
patient samples would also help determine if it is possible to accurately measure exon 1A3 transcripts with the bDNA assay. It is obvious that the differing results obtained by the two assays shown in Figure 6 cannot both be correct, as is especially evident in TALL1 and TALL4. If further studies were to confirm the validity of the QRT-PCR assay, these preliminary data could suggest that because the TALL1 and TALL4 patient samples have a relatively higher basal level of GR as compared to the others, additional up-regulation might not be required for triggering GC-induced apoptosis. Interestingly, the immunophenotype and age were similar for patients TALL1 and TALL4, and they exhibited similar

GR characteristics (higher basal level, lower or no up-regulation as measured by QRT-PCR). Our preliminary patient sample data suggest that a certain GR threshold must be reached for GC-induced apoptosis to be triggered, and this is in concert with other published studies using ALL cell lines<sup>[25,28,29]</sup>, and which is demonstrated by the data for the CEM-C7 cell line shown in Figure 7. This threshold level may exist basally or may require steroid-mediated up-regulation to be reached. It is necessary to measure GC-induced GR regulation and apoptotic responses in a greater number of fresh T-cell ALL patient samples to gather a clearer picture of these responses in leukemia patients and the





**Figure 6. Data comparison of transcript levels in various tissue samples.** The basal and Dex-induced GR transcript levels in four T-cell ALL patient samples and CEM-C7 cells were measured with the bDNA assay (A) or QRT-PCR (B) using the Quantigene probe set or Taqman probe and primers specific for GR transcripts containing exons 5 and 6. CEM-C7 basal and induced levels are the actual observed levels divided by 2, as CEM-C7 cells are known to have only one functional copy of GR<sup>[33-35]</sup>. The dashed lines indicate a potential GR threshold level that might be required for GC-induced apoptosis. The threshold level might be reached at the basal GR transcript level or could require hormonal up-regulation to be reached.



**Figure 7. Correlation between GR transcript up-regulation and apoptosis after Dex treatment in CEM-C7 cells.** CEM-C7 cells were treated with 2-fold serial dilutions of Dex starting from  $1 \times 10^{-9}$  mol/L or EtOH vehicle alone for 18 h. RNA was isolated and QRT-PCR was performed to measure exon 1A3 and exon 5/6 transcript levels. Also, cell viability was measured via flow cytometry using the Vybrant® Apoptosis Assay Kit #4 after treating the cells for 72 h with 2-fold serial dilutions of Dex starting from  $1 \times 10^{-9}$  mol/L or EtOH vehicle alone. Error bars represent the SEM of three separate experiments.

performance and reliability of the two assay platforms relative to each other. To date, we have been limited by the relative rarity of T-cell ALL and a scarcity of samples.

The contribution of GR levels and its auto-regulation to the sensitivity of ALL to hormone-induced apoptosis shows controversy. Voluminous literature shows a direct

correlation of the transcriptional response of target genes and biological responses with the GR level in many different cell types. Regarding GR levels and steroid sensitivity in ALL, one study has stated that the “absolute expression level of GR ... is linked to GC resistance in childhood ALL”<sup>[30]</sup>. However, results obtained in a subsequent study suggest that neither the baseline level of five different GR transcripts nor the up-regulation of GR transcripts by steroid is related to resistance<sup>[31]</sup>. This apparent quandary may be due to the precise manner in which the experiments were performed. In nearly all cases, percentages were used for the analyses. This included the percentage that total GR transcripts comprise each of the five transcripts and the percentage of up-regulation of GR transcripts in resistant and sensitive ALL patient samples. One possibility is that even though the percent up-regulation was substantial in resistant ALL samples, the initial basal expression levels may have been low, such that a necessary threshold level of GR was not achieved after steroid-mediated up-regulation. Conversely, a weak up-regulation of GR expression may be all that is needed in a sensitive ALL patient if the basal level of GR protein present is just below the threshold level needed for triggering apoptosis. If the QRT-PCR results that we obtained for patients TALL1 and TALL4 are valid, this would add some support to this concept. In any event, an assay that measures GR transcript regulation and absolute levels (rather than fold- or percent-increase) following an *ex vivo* Dex challenge may assist clinicians in predicting an ALL patient’s GC sensitivity status prior to treatment initiation.

There are limitations to this approach and study. As is evident from the immunophenotype, age of onset, and clinical presentations (e.g., white blood cell count), T-cell ALL is a somewhat heterogeneous disease. Thus, trying to develop an assay that is applicable for all patients to determine a clinical treatment design is challenging. Sample quality is critical, as this can affect the reliability of the results. Another important parameter is the source of the sample (bone marrow, peripheral circulation, plasmapheresis). The percentage of blast/leukemic cells in the sample must be high, as a dilution effect would occur if a significant amount of normal leukocytes were present in the sample. The four patient samples used in this study contained greater than 90% lymphoblasts. There are two notable major limitations in the current study. As mentioned previously, the first is the small number of available patient samples. T-cell ALL is a relatively rare disease, so obtaining more

samples to increase the statistical power of the analysis is a challenge. Performing a similar study with pre-B-ALL and multiple myeloma would be valuable, as these cancers are more common (which would increase sample size), and these diseases are likewise treated successfully with corticosteroids. The second major limitation is the use of the GR mRNA as the sole target gene for the assay. The use of a panel of glucocorticoid-responsive genes in the bDNA assay would provide a more robust and reliable profile of the hormone responsiveness of leukemia and raise confidence in making clinical decisions. With further developments in the areas, the use of the bDNA technique as a clinical assay could be feasible.

The bDNA assay described here offers a platform that could be easily used in a hospital laboratory because of its simple ELISA-like workflow, requirement of only common laboratory equipment (e.g., luminometer), and its ability to accurately measure transcript concentrations from crude cellular lysates. This pilot study shows that the bDNA probe set for GR transcripts containing exons 5 and 6 correlates well with the QRT-PCR measurement of the same transcripts in cell lines and in fresh patient samples. Because of the heterogeneity that is seen in T-cell ALL bone marrow samples, it is unlikely that the assay of GR transcripts alone via any assay will be sufficient to stratify, with confidence, patients into those that are responders and those that are steroid-resistant. Rather, we believe that a “molecular signature,” which involves measuring a subset (the exact number to be determined) of GC-responsive genes (including GR itself) via the bDNA assay or QRT-PCR, might provide the necessary confidence on which to base medical treatment decisions (inclusion, and at which dosage level<sup>[32]</sup>, of steroid in the treatment protocol).

## Acknowledgments

This study was supported in part by a grant from NCI (No. CA116042) to W.V. Vedeckis. We are grateful to Drs. Renee V. Gardner, Cori A. Morrison, Faisal S. Razzaqi, and Quan Zhao for their assistance in obtaining the patient samples used in this study. We also thank Dr. Beatriz Finkel-Jimenez for her assistance with flow cytometric analysis.

Received: 2012-02-15; revised: 2012-05-16;  
accepted: 2012-05-24.

## References

[1] Langebrake C, Reinhardt D, Ritter J. Minimising the long-term adverse effects of childhood leukaemia therapy. *Drug Saf*,

2002;25:1057–1077.

[2] Pearson OH, Eliel LP, Rawson, RW, et al. Adrenocorticotropic

- hormone- and cortisone-induced regression of lymphoid tumors in man: a preliminary report. *Cancer*, 1949,2:943–945.
- [3] Ploner C, Schmidt S, Presul E, et al. Glucocorticoid-induced apoptosis and glucocorticoid resistance in acute lymphoblastic leukemia. *J Steroid Biochem Mol Biol*, 2005,93:153–160.
- [4] Kaspers GJ, Pieters R, Van Zantwijk CH, et al. Prednisolone resistance in childhood acute lymphoblastic leukemia: *in vitro-vivo* correlations and cross-resistance to other drugs. *Blood*, 1998,92:259–266.
- [5] Hulleman E, Kazemier KM, Holleman A, et al. Inhibition of glycolysis modulates prednisolone resistance in acute lymphoblastic leukemia cells. *Blood*, 2009,113:2014–2021.
- [6] Den Boer ML, Harms DO, Pieters R, et al. Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. *J Clin Oncol*, 2003,21:3262–3268.
- [7] Dordelmann M, Reiter A, Borkhardt A, et al. Prednisone response is the strongest predictor of treatment outcome in infant acute lymphoblastic leukemia. *Blood*, 1999,94:1209–1217.
- [8] Hochhauser CJ, Lewis M, Kamen BA, et al. Steroid-induced alterations of mood and behavior in children during treatment for acute lymphoblastic leukemia. *Support Care Cancer*, 2005,13:967–974.
- [9] Schimmer BP, Parker KL. Adrenocorticotrophic hormone; adrenocortical steroids and their synthetic analogs; inhibitors of the synthesis and actions of adrenocortical hormones. Brunton LL, Lazo JS, Parker KL, eds. *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 11th ed. New York: McGraw-Hill, 2006:1587–1612.
- [10] de Vries MA, van Litsenburg RR, Huisman J, et al. Effect of dexamethasone on quality of life in children with acute lymphoblastic leukaemia: a prospective observational study. *Health Qual Life Outcomes*, 2008,6:103.
- [11] Webster JC, Cidlowski JA. Mechanisms of glucocorticoid-receptor-mediated repression of gene expression. *Trends Endocrinol Metab*, 1999,10:396–402.
- [12] McKay LI, Cidlowski JA. CBP (CREB binding protein) integrates NF-kappaB (nuclear factor-kappaB) and glucocorticoid receptor physical interactions and antagonism. *Mol Endocrinol*, 2000,14:1222–1234.
- [13] Schule R, Rangarajan P, Klierer S, et al. Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor. *Cell*, 1990,62:1217–1226.
- [14] Jonat C, Rahmsdorf HJ, Park KK, et al. Antitumor promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. *Cell*, 1990,62:1189–1204.
- [15] Mullick J, Anandatheerthavarada HK, Amuthan G, et al. Physical interaction and functional synergy between glucocorticoid receptor and Ets2 proteins for transcription activation of the rat cytochrome P-450c27 promoter. *J Biol Chem*, 2001,276:18007–18017.
- [16] Park SK, Beaven MA. Mechanism of upregulation of the inhibitory regulator, src-like adaptor protein (SLAP), by glucocorticoids in mast cells. *Mol Immunol*, 2009,46:492–497.
- [17] Geng CD, Vedeckis WV. Steroid-responsive sequences in the human glucocorticoid receptor gene 1A promoter. *Mol Endocrinol*, 2004,18:912–924.
- [18] Geng CD, Vedeckis WV. c-Myb and members of the c-Ets family of transcription factors act as molecular switches to mediate opposite steroid regulation of the human glucocorticoid receptor 1A promoter. *J Biol Chem*, 2005,280:43264–43271.
- [19] Geng CD, Schwartz JR, Vedeckis WV. A conserved molecular mechanism is responsible for the auto-up-regulation of glucocorticoid receptor gene promoters. *Mol Endocrinol*, 2008,22:2624–2642.
- [20] Pedersen KB, Vedeckis WV. Quantification and glucocorticoid regulation of glucocorticoid receptor transcripts in two human leukemic cell lines. *Biochemistry*, 2003,42:10978–10990.
- [21] Breslin MB, Geng CD, Vedeckis WV. Multiple promoters exist in the human GR gene, one of which is activated by glucocorticoids. *Mol Endocrinol*, 2001,15:1381–1395.
- [22] Presul E, Schmidt S, Kofler R, et al. Identification, tissue expression, and glucocorticoid responsiveness of alternative first exons of the human glucocorticoid receptor. *J Mol Endocrinol*, 2007,38:79–90.
- [23] Eisen LP, Elsasser MS, Harmon JM. Positive regulation of the glucocorticoid receptor in human T-cells sensitive to the cytolytic effects of glucocorticoids. *J Biol Chem*, 1988,263:12044–12048.
- [24] Geng CD, Vedeckis WV. A new lineage specific autoupregulation mechanism for human glucocorticoid receptor gene expression in 697 pre-B-acute lymphoblastic leukemia cells. *Mol Endocrinol*, 2011,25:44–57.
- [25] Schwartz JR, Sarvaia PJ, Vedeckis WV. Glucocorticoid receptor knock down reveals a similar apoptotic threshold but differing gene regulation patterns in T-cell and pre-B-cell acute lymphoblastic leukemia. *Mol Cell Endocrinol*, 2010,320:76–86.
- [26] Pui CH, Robison LL, Look AT. Acute lymphoblastic leukaemia. *Lancet*, 2008,371:1030–1043.
- [27] Carroll WL, Bhojwani D, Min DJ, et al. Pediatric acute lymphoblastic leukemia. *Hematology Am Soc Hematol Educ Program*, 2003,2003:102–131.
- [28] Ramdas J, Liu W, Harmon JM. Glucocorticoid-induced cell death requires autoinduction of glucocorticoid receptor expression in human leukemic T cells. *Cancer Res*, 1999,59:1378–1385.
- [29] Gruber G, Carlet M, Turtcher E, et al. Levels of glucocorticoid receptor and its ligand determine sensitivity and kinetics of glucocorticoid-induced leukemia apoptosis. *Leukemia*, 2009,23:820–823.
- [30] Tissing WJ, Lauten M, Meijerink JP, et al. Expression of the glucocorticoid receptor and its isoforms in relation to glucocorticoid resistance in childhood acute lymphocytic leukemia. *Haematologica*, 2005,90:1279–1281.
- [31] Tissing WJ, Meijerink JP, Brinkhof B, et al. Glucocorticoid-induced glucocorticoid-receptor expression and promoter usage is not linked to glucocorticoid resistance in childhood ALL. *Blood*, 2006,108:1045–1049.
- [32] Schwartz CL, Thompson EB, Gelber RD, et al. Improved response with higher corticosteroid dose in children with acute lymphoblastic leukemia. *J Clin Oncol*, 2001,19:1040–1046.
- [33] Hillmann AG, Ramdas J, Multanen K, et al. Glucocorticoid receptor gene mutations in leukemic cells acquired *in vitro* and *in vivo*. *Cancer Res*, 2000,60:2056–2062.
- [34] Powers JH, Hillmann AG, Tang DC, et al. Cloning and expression of mutant glucocorticoid receptors from glucocorticoid-sensitive and -resistant human leukemic cells. *Cancer Res*, 1993,53:4059–4065.
- [35] Ashraf J, Thompson EB. Identification of the activation-labile gene: a single point mutation in the human glucocorticoid receptor presents as two distinct receptor phenotypes. *Mol Endocrinol*, 1993,7:631–642.