Reciprocal Regulation of HSD11B1 and HSD11B2 Predicts Glucocorticoid Sensitivity in Childhood ALL

Title: Reciprocal regulation of *HSD11B1* and *HSD11B2* predicts glucocorticoid sensitivity in childhood ALL

Shuji Sai MD, PhD[1,2,3], Cristina Esteves PhD[1,4], Val Kelly[1], Kimiyoshi Sakaguchi MD, PhD[2], Rachel McAndrew[5], Sandra Chudleigh[6], Alison Spence[6], Brenda Gibson MD, PhD[6], Angela Thomas MD, PhD[5], Karen E Chapman PhD[1]

[1]University/BHF Centre for Cardiovascular Science, The Queen's Medical Research Institute, The University of Edinburgh, UK
[2]Department of Pediatrics, Hamamatsu University School of Medicine, Japan
[3]Department of Pediatrics, Teine-Keijinkai Hospital, Sapporo, JAPAN
[4]Division of Developmental Biology, The Roslin Institute, The University of Edinburgh, UK
[5]Department of Paediatric Haematology, Royal Hospital for Sick Children, Edinburgh, UK
[6]Department of Paediatric Haematology, Royal Hospital for Sick Children, Yorkhill, Glasgow, UK

Corresponding author:

Shuji Sai MD PhD, Department of Pediatrics, Teine-Keijinkai Hospital
1-12 Maeda, Teine-ku, Sapporo 006-8555, Japan
Email: shuji-sai@keijinkai.or.jp
Tel: +81 11 681 8111, Fax: +81 11 685 2196

Short title:
11β-HSD predicts glucocorticoid sensitivity in childhood ALL

Abbreviations:
ALL - Acute lymphoblastic leukemia
GR - Glucocorticoid receptor
11β-HSD - 11beta-hydroxysteroid dehydrogenase

Funding Source:
This work was supported by the Leukaemia and Cancer Children's Fund of the Edinburgh and Lothians Health Foundation. Shuji Sai was funded by the Uehara Memorial Foundation.

Financial Disclosure:
The authors have indicated they have no financial relationships relevant to this article to disclose.

Conflict-of-interest disclosure:
The authors have no potential conflicts of interest to disclose.

Key words
Glucocorticoid resistance; Leukemia; 11β-HSD

Abstract

There are few biomarkers to predict efficacy of glucocorticoid treatment in childhood acute lymphoblastic leukemia (ALL) at diagnosis. Here, we demonstrate reciprocal regulation of 11β-HSD, may predict the apoptotic response of ALL to glucocorticoid treatment. Our data may be useful to refine glucocorticoid treatment, to retain benefit whilst minimizing side-effects.

The synthetic glucocorticoids, dexamethasone and/or prednis(ol)one, form crucial first-line treatment for childhood acute lymphoblastic leukemia (ALL). The response to initial glucocorticoid treatment is a strong prognostic indicator of treatment outcome.[1] ALL cells show differing degrees of glucocorticoid sensitivity/resistance at diagnosis and glucocorticoid resistance remains a therapeutic problem.[2] Identification of biomarkers of glucocorticoid sensitivity/resistance would be useful in order to stratify therapy. This would facilitate targeting of high dose glucocorticoid therapy to those children with resistant disease, most likely to benefit. Low glucocorticoid receptor (GR) levels may be a common though hard to detect mechanism of glucocorticoid resistance.[3] Downstream markers of glucocorticoid action may be more sensitive indicators of glucocorticoid responsiveness. The glucocorticoid metabolizing enzymes, 11β-hydroxysteroid dehydrogenase types 1 and 2 (11β-HSD1 and 11β-HSD2, respectively) are regulated by glucocorticoids in a number of cell types, including ALL cells,[4,5,6] and may therefore act as biomarkers of glucocorticoid responsiveness. 11β-HSD1 predominantly regenerates active glucocorticoids (cortisol, corticosterone) from inert 11-keto forms (cortisone, 11-dehydrocorticosterone), whereas 11β-HSD2 catalyses the reverse reaction.[4] In a pilot study, we demonstrated induction of *HSD11B1* (encoding 11β-HSD1) by dexamethasone in glucocorticoid-sensitive childhood ALL, but repression in glucocorticoid-resistant samples.[7] There are few data on dexamethasone regulation of

HSD11B2 (encoding 11β-HSD2) in childhood ALL, but one report suggests down-regulation in glucocorticoid-sensitive ALL.[8] Moreover, expression of 11β-HSD2 contributes to prednisolone resistance in lymphoblastic leukemia cells.[9,10] Recently, we have shown reciprocal regulation of 11β-HSD1/2 may predict steroid sensitivity in childhood nephrotic syndrome.[11] Therefore we hypothesized that expression of both 11β-HSD1 and -2 is glucocorticoid-regulated and associated with glucocorticoid sensitivity in childhood ALL.

Methods

Patients ALL patients were recruited from the Royal Hospital for Sick Children (Edinburgh) and the Royal Hospital for Sick Children (Glasgow), UK, between November 2011-November 2015. The study was approved by the National Cancer Research Network Coordinating Centre (MREC, 10/S0709/35). Additional patients were recruited from Hamamatsu University School of Medicine, Japan between November 2011-December 2015 (project approval number, 16-38). Study protocols were approved by the ethics committees of the respective hospitals. Peripheral blood and/or bone marrow samples were obtained at diagnosis of ALL with informed consent. Diagnosis of ALL was made by pediatric hematologists based on clinical and laboratory findings. Leukemic cells were isolated by density gradient centrifugation (Ficoll-Paque PLUS; GE Healthcare Life Sciences, Little Chalfont, Bucks, UK) and duplicate or triplicate samples cultured for 24h in the presence or absence of dexamethasone (10^{-6}M). Minimal residual disease (MRD) at day 29 of remission induction was measured for UK samples by flow cytometry, as part of the UKALL2011 trial.

Cell culture CCRF-CEM (JCRB9023) and MOLT4F (JCRB0021) T-lymphoblastic leukemia cells were obtained from the Health Science Research Resources Bank (Osaka, Japan). 1.0×10^6 cells/well were seeded in triplicate in 12-well plates in RPMI-1640 supplemented with 10% fetal calf serum, penicillin (100U/mL) and streptomycin (100μg/mL) (ThermoFisher Scientific, Paisley, UK), at 37◦C, 5%CO_2. Cells were cultured with

dexamethasone (10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}M), vincristine (10^{-6}M) or methotrexate (10^{-6}M) (all Sigma-Aldrich, Gillingham, Dorset, UK). Vincristine and Methotrexate treatments were 24h.

RNA extraction and real-time qPCR RNA was extracted using Trizol (ThermoFisher Scientific). RNA (1µg) was reverse transcribed using SuperScript III (ThermoFisher Scientific) and quantified by qPCR (in triplicate) using a LightCycler (Roche, Burgess Hill, Sussex, UK) as previously described.[7,9] Mastermix and primer-probe sets for *HSD11B1*, *HSD11B2*, *NR3C1* and *18S* RNA (internal control) were from ThermoFisher Scientific.

Cell viability assay For cell lines, flow cytometry was used to measure apoptosis following annexin-V and propidium iodide staining (Annexin-V Apoptosis Detection Kit FITC, ThermoFisher Scientific). Fluorescence was measured by FACScalibur using Cellquest (Becton Dickinson Ltd, Oxford, UK) with 10,000 cells/sample acquired; data were analyzed using FlowJo software (Treestar, Ashland, Oregon, USA). For patient samples, 1×10^5 cells/well were seeded in 96-well plates and cell viability measured by MTT assay (Sigma-Aldrich, Gillingham, Dorset, UK) following addition of prednisolone or dexamethasone (10-fold dilutions: 10^{-9}M-10^{-4}M) for 96h. Samples were classified as glucocorticoid-sensitive or resistant, as previously described.[7,12]

Statistics Data from cells and patient samples were analyzed using ANOVA and Paired t-test (2-tailed) with significance set at $P<0.05$. Values are mean±SEM. Patient characteristics and gene profiles were compared using Mann-Whitney U test for age and the chi-squared test for gender, immuno-phenotype, MRD and gene expression (JMP software).

Results

We first measured levels of *HSD11B1* and *HSD11B2* mRNA (encoding 11β-HSD1 and 11β-HSD2, respectively) in glucocorticoid-sensitive CCRF-CEM lymphoblastic leukemia cells.[9] As expected, cellular apoptosis was increased following 24h incubation with dexamethasone, in a dose-dependent manner (Figure 1a). *HSD11B1* mRNA levels were increased and

HSD11B2 mRNA levels decreased by dexamethasone, in a dose dependent manner (Figure 1b) that paralleled cellular apoptosis. Consistent with previous reports,[13] levels of *NR3C1* mRNA encoding GR were increased by dexamethasone (Figure 1b). In contrast to *NR3C1* mRNA, which was already increased 2h after dexamethasone addition (Figure 1d), the increase in cellular apoptosis was apparent only 24h after addition of dexamethasone (Figure 1c). Similarly, *HSD11B1* mRNA levels only increased 24h following addition of dexamethasone, suggesting this is a late glucocorticoid response. *HSD11B2* mRNA levels were significantly reduced 24h after dexamethasone addition (Figure 1d). These data suggest that induction of *HSD11B1* and repression of *HSD11B2* are associated with cellular apoptosis. Next, we investigated the regulation of *HSD11B1* and *HSD11B2* in glucocorticoid resistant MOLT4F cells in which high expression of 11β-HSD2 contributes to prednisolone resistance.[9] Dexamethasone had no significant effect upon apoptosis in MOLT4F cells (Figure 1e). In contrast to glucocorticoid sensitive CCRF-CEM cells, neither *HSD11B1* nor *HSD11B2* mRNA levels were affected by dexamethasone in MOLT4F cells (Figure 1f), possibly secondary to the very low levels of *NR3C1* expression in these cells.[9] Interestingly, the anti-leukemic drugs vincristine and methotrexate, both of which induced apoptosis in MOLT4F cells (Figure 1e), increased *HSD11B1* and decreased *HSD11B2* mRNA levels without affecting *NR3C1* mRNA levels (Figure 1f). Thus, as in CCRF-CEM cells, induction of *HSD11B1* and repression of *HSD11B2* is associated with induction of apoptosis. These data suggest that *HSD11B1* and *HSD11B2* may be sensitive biomarkers to predict the apoptotic response of ALL cells to chemotherapy more generally, although this requires testing with vincristine and methotrexate in patient samples. Whether and how manipulation of these genes affect cell apoptosis remains to be determined.

Findings in cell lines do not necessarily translate to patient samples. To test whether *HSD11B1* and *HSD11B2* are differentially regulated in glucocorticoid sensitive and resistant

childhood ALL, we measured *HSD11B1* and *HSD11B2* mRNA levels in cells from patients, obtained at diagnosis, prior to initiation of treatment. Where sufficient cells were available, we assessed glucocorticoid sensitivity/resistance *in vitro* by methyl-thiazol-tetrazolium (MTT) assay with patients classified as GC-sensitive or resistant as previously described.[7,12] In a representative glucocorticoid-sensitive patient sample where both prednisolone and dexamethasone were effective inducers of cell death, dexamethasone treatment up-regulated *HSD11B1* and down-regulated *HSD11B2* mRNA levels (Figure 1g). In contrast, dexamethasone down-regulated *HSD11B1* and up-regulated *HSD11B2* mRNA levels in a representative glucocorticoid-resistant ALL patient sample (Figure 1h). *NR3C1* mRNA levels were unchanged following dexamethasone in both GC-sensitive and resistant ALL patient samples (Figure 1g, 1h). Across all GC-sensitive patients, irrespective of B-ALL or T-ALL subtype, *HSD11B1* was up-regulated in the majority (Table 1), whereas *HSD11B2* was down-regulated (Table 1). In contrast, in the majority of GC-resistant patients, *HSD11B1* was down-regulated and *HSD11B2* was up-regulated (Table 1). A chi-square test showed these differences between GC-sensitive and GC-resistant samples are significant.

Discussion

These data suggest that increased *HSD11B1* and/or decreased *HSD11B2* expression are associated with glucocorticoid-induced apoptosis whereas the opposite pattern, of decreased *HSD11B1* and/or increased *HSD11B2* expression is associated with resistance to glucocorticoid-induced apoptosis at diagnosis. The difference in *NR3C1* regulation between GC-resistant and GC-sensitive ALL was not significant (Table 1), consistent with previous reports that GR up-regulation is not linked to GC-resistance in childhood ALL.[3] Minimal residual disease (MRD), the most useful predictor to stratify patients,[14] tended to be associated with GC-sensitivity/resistance, but did not achieve significance (Table 1). Whether the reciprocal regulation of *HSD11B1* and *HSD11B2* is a cause or a result of GC

sensitivity/resistance remains unknown. Dexamethasone, a poor substrate for 11β-HSD, was used here, suggesting the association reflects a cell state, rather than being causative. A previous report has suggested that 11β-HSD1 and 2 influence cell proliferation with 11β-HSD1 reducing but 11β-HSD2 increasing cellular proliferation.[15] Expression of 11β-HSD1 and 11β-HSD2 is normally reciprocally regulated.[4] Many cells/tissues switch from 11β-HSD2 to 11β-HSD1 expression as they differentiate and mature. It is possible that the expression of 11β-HSD1 and 2 reflects the developmental state of ALL cells and produces opposing patterns of cell proliferation/apoptosis against GC treatment. Our findings are preliminary, qualitative rather than quantitative, and require confirmation in a larger number of patients. Nevertheless, 11β-HSD1/2 patterns may be useful biomarkers to predict which patients will respond well to glucocorticoids. This might enable glucocorticoid treatment to be tailored more to the individual child to maximize treatment benefit and minimize side-effects.

Acknowledgements: We thank Agnes Coutinho for methodological advice, Junko Oikawa for assistance with statistical analysis and Michaela Rodger for assistance with ethical approval. They have no financial relationships relevant to this article to disclose. We thank the Leukaemia and Cancer Children's Fund of the Edinburgh and Lothians Health Foundation for financial support. SS is grateful for support from the Uehara Memorial Foundation. SS, CL, VK, KS, SC and KC performed the research. SS, BG, AT and KC designed the research study. SS, RM, AS and KC analysed the data. SS and KC wrote the paper.

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Figure legend

Figure 1. ***Reciprocal regulation of HSD11B1 and HSD11B2 is associated with apoptosis in lymphoblastic leukemia cells*****.**

Glucocorticoid-sensitive CCRF-CEM cells were treated with dexamethasone (DEX, black bars) ranging from 0-10^{-6}M. (a) Apoptosis was measured by flow cytometry following staining with Annexin-V and propidium iodide. (b) Quantitative (q)PCR measurements of *HSD11B1* (left), *HSD11B2* (centre) and *NR3C1* (right) mRNA. In a time course of the effects of 10^{-6}M dexamethasone upon CCRF-CEM cells, cells were collected between 0-24h for

measurement of (c) apoptosis and (d) qPCR measurement of *HSD11B1*, *HSD11B2* and *NR3C1* mRNA. (e, f) 10^{-6}M Dexamethasone (24h) had no effect on *HSD11B1* and *2* mRNA expression, but 10^{-6}M vincristine (VCR) and 10^{-6}M methotrexate (MTX) 24h treatment increased *HSD11B1* and decreased *HSD11B2* mRNA levels in accordance with cell apoptosis in GC-resistant MOLT4F cells. Data are mean±SEM of at least three independent mRNA samples. RNA concentrations are expressed as fold-induction relative to vehicle treated cells (CON, white bars). (g, h) Left panels: qPCR measurements of *HSD11B1*, *HSD11B2* and *NR3C1* mRNA in representative patient samples. Data are the mean±SEM of duplicate (g) or triplicate (h) mRNA samples. Right panels: Viability of ALL cells following prednisolone (PRED) and dexamethasone (DEX) treatment in MTT assay. Significant effect of treatment, *p<0.05, **p<0.01.

Table legend

Table 1. *Summary of ALL patient characteristic*s

Patient characteristics were compared between groups using Mann-Whitney U test for age and the chi-squared test for gender, immuno-phenotype, gene expression and MRD. *HSD11B1*, *HSD11B2* and *NR3C1* had missing values due to insufficient sample. MRD data were only available for UK samples. Up-regulation was defined as more than 1.00-fold difference between vehicle and dex samples. Down-regulation was defined as less than 1.00-fold difference between vehicle and dex samples.

Table

Table 1. Summary of ALL patient characteristics

n =37		GC-sensitive (*n = 22*)	GC-resistant (*n= 15*)	*P* (sensitive vs resistant)
Age (years), median (range)		5.91 (1-14)	6.0 (2-12)	0.9378
Gender	Boys	55% (12/22)	53% (8/15)	0.9421
	Girls	45% (10/22)	47% (7/15)	
Immuno-phenotype	B	73% (16/22)	80% (12/15)	0.6127
	T	27% (6/22)	20% (3/15)	
HSD11B1	up-regulated	**65% (13/20)**	23% (3/13)	**<0.0185**
	down-regulated	35% (7/20)	**77% (10/13)**	
HSD11B2	up-regulated	24% (4/17)	**70% (7/10)**	**<0.0176**
	down-regulated	**76% (13/17)**	30% (3/10)	
NR3C1	up-regulated	53% (8/15)	73% (8/11)	0.3153
	down-regulated	47% (7/15)	27% (3/11)	
MRD	Low risk	67% (8/12)	29% (2/7)	0.1087
	High risk	33% (4/12)	71% (5/7)	

Figure

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