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Identification of prognostic protein biomarkers in childhood acute lymphoblastic leukemia (ALL)

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ABSTRACT

Early response to 7 days of prednisolone (PRED) treatment is one of the important prognostic factors in predicting eventual outcome in childhood acute lymphoblastic leukemia (ALL). Using proteomic tools and clinically important leukemia cell lines (REH, 697, Sup-B15, RS4;11), we have identified potential prognostic protein biomarkers as well as discovered promising regulators of PRED-induced apoptosis. After treatment with PRED, the four cell lines can be separated into resistant (REH) and sensitive (697, Sup-B15, RS4;11). Two dimensional gel electrophoresis (2-DE) and MALDI-TOF/TOF MS identified 77 and 17 significantly differentially expressed protein spots ($p<0.05$) in PRED-sensitive and PRED-resistant cell lines respectively. Several of these were validated by Western blot including proliferating cell nuclear antigen (PCNA), cofilin1, voltage-dependent anion-channel protein1 (VDAC1) and proteasome activator subunit 2 (PA28β). PCNA is a promising protein because of its important roles both in cell cycle regulation and survival control. We subsequently validated PCNA in 43 paired bone marrow samples from children with newly diagnosed ALL (Day 0) and 7 days after PRED treatment (Day 8). ROC curve analysis confirmed that PCNA was highly predictive of PRED response in patients (AUC=0.81, $p=0.007$) and most interestingly, independent of the molecular subtype, providing a promising universal prognostic marker.

Abbreviations: 2-DE, two-dimensional gel electrophoresis; ALL, acute lymphoblastic leukemia; BFM, Berlin-Frankfurt-Munster; EFS, event-free survival; GC, glucocorticoid; GR, glucocorticoid receptor; PA28β, proteasome activator subunit 2; PCNA, proliferating cell nuclear antigen; PGR, prednisolone good response; PI, Propidium iodide; PRED, prednisolone; PPR, poor prednisolone response; VDAC1, voltage-dependent anion-channel protein 1.

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1. Introduction

Childhood acute lymphoblastic leukemia (ALL) is the most common form of childhood cancer throughout the world; it alone accounts for more than 30% of childhood cancers diagnosed every year. Currently, using risk stratified, multi-agent chemotherapy, more than 80% of children with ALL in developed countries are cured [1].

Many of the ALL treatment protocols including ours Malaysia-Singapore (Ma-Spore) and the widely adopted German Berlin-Frankfurt-Munster (BFM) clinical trial utilize Day 8 prednisolone (PRED) response for risk stratification [2–4]. This comprises of 7 days of PRED and one intrathecal dose of methotrexate. Patients with PRED poor response (PPR) with Day 8 blast count more than 1000/μl, have poor 38–50% event-free survival (EFS) compared to patients with PRED good response (PGR) who achieve 80% EFS. Similarly, in our Ma-Spore ALL 2003 study, PPR patients (n=50) have 70.6% EFS compared to PGR patients (n=422) who have 83.7% EFS (p=0.001). Poor PRED responders now receive intensified therapy in order to improve their treatment outcome. Using *in vitro* 4-day drug cytotoxicity assay, cellular resistance to PRED is also associated with unfavorable treatment outcome in pediatric ALL [5]. The reason behind why the D8 *in vivo* and D4 *in vitro* PRED responses are prognostic of eventual outcome is a mystery.

Little is known about the mechanisms of PRED resistance in leukemic blasts. It may be due to the misregulation of glucocorticoid receptors (GR) [6] or defects in the glucocorticoid response genes and their cross talks. Some studies showed that multidrug-resistant gene (MDR1) and genes involved in cell cycle, drug metabolism, DNA repair, and intrinsic apoptosis pathway are involved in glucocorticoid resistance in ALL [7–9].

In this study, we aimed to identify prognostic biomarkers and potential protein regulators that underlie the prognostic significance of Day 8 PRED response by comparing the changes in the proteome of four clinically important cells lines (pre- and post-PRED treatment) representing the 4 most common subtypes of ALL: REH [ETV6-RUNX1], 697 [TCF3-PBX1], Sup-B15 [BCR-ABL1] and RS4;11[MLL-AF4]. We then validated our findings in 43 paired bone marrow samples of ALL patients at diagnosis (Day 0) and after 7 days of PRED treatment (Day 8). We surmised that the prognostic biomarkers identified will provide insights in developing novel drug target to reverse glucocorticoid resistance or used as prognostic markers to risk-stratify patients in the future.

2. Materials and methods

2.1. Cell lines

Four clinically important cells lines REH, 697, Sup-B15 and RS4;11 were used in this study. The 697 cell line was purchased from German Collection of Microorganisms and Cell Cultures (DSMZ). The other three cell lines were purchased from the American Type Culture Collection (ATCC). They were cultured and maintained according to the manufacturer's protocol.

2.2. Patients samples

We randomly selected paired bone marrow samples at diagnosis (Day 0) and after 7 days of PRED (Day 8) from 43 children with newly diagnosed B-lineage ALL (35 PGR and 8 PPR) enrolled in the Ma-Spore ALL 2003 clinical trial. Written consent was obtained and the study was approved by the Domain-specific Review Board under the National Healthcare Group (DSRB-B/04/275). Briefly, mononuclear cells were separated and harvested from bone marrow aspirates using Ficoll-Paque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) density gradient centrifugation. Thereafter, one ml of TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) was added to 10 million cells. Proteins were extracted according to manufacturer's protocol and kept in −80 °C. We compared protein fold-change in paired bone marrow samples at Day 0 (diagnosis) and Day 8. PGR patients had Day 8 peripheral blood blast count less than 1000/μl, while PPR patients had blast count more than 1000/μl. Clinical characteristics of these 43 patients are summarized in Table 1.

2.3. Cell viability assay

Modified *in vitro* MTS assay was used to determine the sensitivity of leukemia cell lines to PRED [5,10]. Cells from the REH, 697, Sup-B15 and RS4;11 cell lines were plated into 96-well plate at a concentration of 5×10^5 cells/ml. The cells were treated 1.0 or 10.0 μg/ml of PRED for 24 h or 48 h with or without Ru486 cotreatment. For cell lines REH, Sup-B15 and RS4;11, 1.0 μM Ru486 was added. 5.0 μM of Ru486 was used in cell line 697. The cell viability was evaluated by CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). Each cell line was run in duplicates and

Table 1 – Distribution of paired childhood ALL patients, 35 paired PGR patients and 8 paired PPR patients were involved in this study.

	PGR n=35	PPR n=8
Gender		
Male	18	3
Female	17	5
Age at diagnosis		
<1 year	1	2
1–9 years	30	6
≥10 years	4	0
Chromosome translocation		
Non-detected	23	7
ETV6-RUNX1	7	0
BCR-ABL1	2	0
MLL-AF4	0	0
TCF3-PBX1	2	1
MLL-ENL	1	0
Blast count at day 8		
0	12	0
0–1000	23	0
≥1000	0	8
Status		
Complete remission	33	5
Relapsed	1	0
Induction failure	1	2
Died	0	1