



# Label-free quantitative proteomics reveals differentially expressed proteins in high risk childhood acute lymphoblastic leukemia

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## ABSTRACT

Acute lymphoblastic leukemia (ALL) is the most common pediatric cancer. B-ALL is the most common type in pediatric ALL. Risk stratification is critical for setting on chemo-therapeutic regimen that has great impact on the survival rate. But the risk allocation schemas at present were not satisfied. We performed a proteomic study expecting to figure out the critical altered proteins which can indicate the risk rank. We depicted 86 differently expressed proteins in the high-risk childhood B-ALL, and 35 proteins were predicted to have directive interactions. We validated five identified proteins by immunoblot using specimens same as proteomics, and others different from that. We found the differently expressed proteins participated in pre-mRNA splicing, DNA damage response, and stress response which indicated different events happened in the high risk B-ALL. Our result provided new information for children B-ALL. It might aid more accurate risk stratification and might also be valuable to find new therapeutic targets.

**Biological significance:** To our knowledge, this is the first proteomic analysis comparing the differentially expressed proteins between high risk and low risk of childhood B-ALL by a label-free quantitative proteomics. We found in high risk B-ALL, the aberrant events happened in pre-mRNA splicing, DNA damage response, and stress response. This study reveals new insights in the high risk B-ALL, and might also be valuable to identify the high-risk more accurately, as well as to find new therapeutic targets.

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## 1. Introduction

Acute lymphoblastic leukemia (ALL) is the most common pediatric cancer [1,2]. 80–90% of pediatric ALL is B-lineage ALL [3,4]. Benefited from the advances in chemotherapy, the survival rates for childhood ALL have improved during the last decades [3]. In the developed country, the overall cure rates of newly diagnosed childhood ALL are about 85% [5]. But little progress has been made in the treatment of relapsed ALL. Now, clinical features, cytogenetic changes, and the response to treatment are used to classify the risk rank into low risk (LR), medium risk (MR) and high risk (HR). High risk is associated with poor prognosis. The therapeutic regimens are adjusted according to the risk stratification. This has improved the outcome to HR patients by the intensified treatments, and avoiding unnecessary dose for L/MR patients. However

relapses still occur in patients initially classified as low risk, which indicated the risk allocation schemas was imperfect. It prompted the search for new factors to refine the risk stratification.

Many efforts have been done to identify the risk factors. In children ALL, many genetic alterations have been found to improve the diagnosis and refine the risk stratification [6]. By high throughput genomic technologies, the biological features of relapsed leukemia were discussed [7]. And there were also increasing studies focusing on the epigenetic alteration in ALL. For example, Roman-Gomez J's study suggested the hyper-methylation of CDH1, p73, p16, p15, p57, PTEN was an independent factor in predicting disease-free survival and overall survival [8]. While genetic alterations were paid so intensive attention, Timsah et al. reported the unbalance protein expression played critical role in cancer [9]. As the ultimate executioners, we believed the roles protein played were worth to be discussed. But in childhood ALL, there are seldom reports about the protein alteration focusing in the risk criteria. We then performed a proteomic study in order to figure out the critical altered proteins in high-risk ALL patients. We expected some of them could indicate the different risk rank, and the proteomic profile might give some hint to the treatment.

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## 2. Methods

### 2.1. Patients and specimens selection

For proteomic analysis, twelve newly diagnosed B-lineage ALL patients were classified into low/medium risk group (L/MR) and high risk group (HR) by 6 patients each, and six patients with non-malignant hematological disorders were selected as non-ALL group (Table 1). While additional bone marrow specimens from 42 patients were used for further verification, including 24 cases of L/MR group and 18 cases in HR group (Table 2). The diagnosis of ALL was based on French-American-British (FAB) Cooperative Group criteria and immunophenotype scheme [10]. The clinical and biological features of all patients were provided in Table 1 and Table 2. The patients enrolled in HR group had at least one of the risk factors as following: at newly diagnosis, white blood cells (WBC) count  $\geq 100 \times 10^9/L$ ; age  $< 1$ -year old; presence of blasts in the central nervous system (CNS disease); failed in prednisone induction; MRD  $> 0.01\%$  at end of 5-weeks of remission induction. The patients relapsed were also included in HR group. The study was approved by the local ethical committee. Bone marrows were obtained for each patient after gave written informed consent.

### 2.2. Sample preparation for a label-free experiment

Bone marrows were suspended on ice in 200  $\mu L$  lysis buffer (4% SDS, 100 mM DTT, 150 mM Tris-HCl pH 8.0), then agitated using a homogenizer (Fastprep-24®, MP Biomedical) and boiled for 5 min. The samples underwent further sonication and boiling for another 5 min. The supernatant were collected by centrifugation at  $14,000 \times g$  for 15 min, then the protein was quantified with a BCA Protein Assay Kit (Bio-Rad, USA).

### 2.3. Protein digestion

Digestion of protein (250  $\mu g$  for each sample) was performed according to the FASP procedure described by Wisniewski [11]. Briefly, the detergent, DTT and other low-molecular-weight components were removed using 200  $\mu L$  UA buffer (8 M Urea, 150 mM Tris-HCl pH 8.0) by repeated ultrafiltration (Microcon units, 30 kD, Millipore, USA) facilitated by centrifugation. Then 100  $\mu L$  0.05 M iodoacetamide in UA buffer was added to block reduced cysteine residues and the samples were incubated for 20 min in darkness. The filter was washed with 100  $\mu L$  UA

buffer three times and then 100  $\mu L$  25 mM  $NH_4HCO_3$  twice. Finally, the protein suspension was digested with 3  $\mu g$  trypsin (Promega, USA) in 40  $\mu L$  25 mM  $NH_4HCO_3$  overnight at 37 °C, and the resulting peptides were collected as a filtrate. The peptide content was estimated by UV light spectral density at 280 nm using an extinctions coefficient of 1.1 of 0.1% (g/l) solution that was calculated on the basis of the frequency of tryptophan and tyrosine in vertebrate proteins.

### 2.4. Liquid chromatography (LC) - electrospray ionization (ESI) tandem MS (MS/MS) analysis by Q Exactive

The peptide of each sample was desalted on C18 Cartridges (Empore™ SPE Cartridges C18 (standard density), bed I.D. 7 mm, volume 3 mL, (Sigma)), then concentrated by vacuum centrifugation and reconstituted in 40  $\mu L$  of 0.1% (v/v) trifluoroacetic acid. MS experiments were performed on a Q Exactive mass spectrometer that was coupled to Easy nLC (Proxeon Biosystems, now Thermo Fisher Scientific). 5  $\mu g$  peptide was loaded onto a the C18-reversed phase column (Thermo Scientific Easy Column, 10 cm long, 75  $\mu m$  inner diameter, 3  $\mu m$  resin) in buffer A (2% acetonitrile and 0.1% Formic acid) and separated with a linear gradient of buffer B (80% acetonitrile and 0.1% Formic acid) at a flow rate of 250 nL/min controlled by IntelliFlow technology over 120 min. MS data was acquired using a data-dependent top10 method dynamically choosing the most abundant precursor ions from the survey scan (300–1800  $m/z$ ) for HCD fragmentation. Determination of the target value is based on predictive Automatic Gain Control (pAGC). Dynamic exclusion duration was 25 s. Survey scans were acquired at a resolution of 70,000 at  $m/z$  200 and resolution for HCD spectra was set to 17,500 at  $m/z$  200. Normalized collision energy was 30 eV and the underfill ratio, which specifies the minimum percentage of the target value likely to be reached at maximum fill time, was defined as 0.1%. The instrument was run with peptide recognition mode enabled. MS experiments were performed triplicate for each sample.

### 2.5. Sequence database searching and data analysis

The MS data were analyzed using MaxQuant software version 1.3.0.5. MS data were searched against the UniProtKB human database (2,585,998,134,919 total entries, downloaded 23/12/13). An initial search was set at a precursor mass window of 6 ppm. The search followed an enzymatic cleavage rule of Trypsin/P and allowed maximal two

**Table 1**  
Patients Information for LC-MS/MS analysis.

No.	Gender	Diagnosis	Age at diagnosis	WBC ( $10^9/L$ )	Pred <sup>a</sup> response	TEL-AML1	BCR-ABL	E2A-PBX1	MLL-AF4	MRD <sup>b</sup> (%)	Clinical outcome	Risk rank	Survival time <sup>c</sup>
L1	M	ALL-commonB	4 years	9.5	Good	—	—	—	—	0	CR	LR	2.4 years
L2	F	ALL-commonB	11 years	7.2	Good	—	—	—	—	0	CR	LR	2.4 years
L3	M	ALL-commonB	3 years	5.3	Good	+	—	—	—	0	CR	LR	2.2 years
L4	F	ALL-commonB	2 years	7.6	Good	—	—	+	—	0	CR	LR	2.2 years
L5	F	ALL-commonB	3 years	25.2	Good	+	—	—	—	0	CR	LR	2.1 years
L6	M	ALL-commonB	5 years	6.9	Good	—	—	+	—	0	CR	LR	2.1 years
H1	F	ALL-commonB	0.5 year	100	Good	—	—	—	—	0	CR	HR	2.4 years
H2	M	ALL-commonB	10 years	147	Good	—	—	—	—	3.07	CR	HR	2.8 years
H3	F	ALL-commonB	5 years	144	2.48	—	—	+	—	0	CR	HR	2.4 years
H4	M	ALL-commonB	3 years	15.3	Good	+	—	—	—	0.07	PR	HR	2.2 years
H5 <sup>d</sup>	F	ALL-commonB	2.9 years	153.7	/	/	/	/	/	/	/	HR	/
H6	M	ALL-commonB	9 years	5.4	Good	—	—	—	—	—	CR	HR	2.1 years
C1	F	ITP <sup>d</sup>	5 years										
C2	M	ITP	1.8 year										
C3	M	Anemia	1.5 year										
C4	M	Anemia	0.5 year										
C5	F	ITP	12 years										
C6	M	ITP	2.6 years										

ITP: idiopathic thrombocytopenic purpura; CR: complete remission; PR: partial remission; NR: no remission.

<sup>a</sup> Number in table mean the blast cells in peripheral blood ( $10^9/L$ ). Blast cells  $\geq 1 \times 10^9/L$  in peripheral blood after prednisone induction for 7 days is refer to a high risk factor.

<sup>b</sup> MRD (minimal residual disease) at end of 5-weeks remission induction, MRD  $\geq 1\%$  is considered to be a high risk factor.

<sup>c</sup> Survival time refers to the time from diagnosis to the data published.

<sup>d</sup> Patient H5 refused any treatment, who was classified as HR for the count of WBC at the diagnosis.

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