



**UNIVERSITI PUTRA MALAYSIA**

***IDENTIFICATION OF DIFFERENTIALLY EXPRESSED PROTEINS AS  
BIOMARKERS OF ACUTE MYELOID LEUKAEMIA***

**FATEMEH BARANTALAB**

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UNIVERSITI PUTRA MALAYSIA  
BERILMU BERBAKTI

**IDENTIFICATION OF DIFFERENTIALLY EXPRESSED PROTEINS AS  
BIOMARKERS OF ACUTE MYELOID LEUKAEMIA**

**By**

**FATEMEH BARANTALAB**

**Thesis Submitted to the School of the Graduate Studies, Universiti Putra Malaysia,  
in Fulfillment of the Requirements for the Degree of  
Doctor of Philosophy**

**January 2014**

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## **ABSTRACT**

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

### **IDENTIFICATION OF DIFFERENTIALLY EXPRESSED PROTEINS AS BIOMARKERS OF ACUTE MYELOID LEUKAEMIA**

By

**FATEMEH BERANTALAB**

**January 2014**

**Chair: Maha Abdullah, Ph.D.**

**Faculty: Medicine and Health Sciences**

Acute myeloid leukaemia (AML) is a hematopoietic malignancy characterized by aberrant proliferation of myeloid progenitor cells, coupled by a partial block in cellular differentiation. Sixty-five to 75% of younger AML patients will achieve complete remission (CR) after induction therapy, while the rate of CR is lower in elderly patients (40-50%). Most patients who achieve CR will relapse within three years and those who do not respond to induction therapy display resistance to chemotherapy. Therefore, resistance to chemotherapy is a major problem in treatment of patients with AML. Current prognostic markers include age, total white blood counts and certain chromosomal translocations. However in half of the patients, these markers are not adequate.

Unlike genomics, screening for potential prognostic markers using proteomics is less frequently conducted due to its more laborious and time-consuming nature. This includes for acute myeloid leukaemias. The aims of this thesis were to establish a two-dimensional gel electrophoresis (2-DE) method for protein extracts from peripheral blood mononuclear cells (PBMC) and plasma samples from acute myeloid leukaemia patients. This proteomics approach will be exploited to identify potential biomarkers that may be associated with resistance to chemotherapy at initial diagnosis before treatment.

Ten samples were chosen for 2-DE analysis of PBMC (7 Resistant and 3 Responsive) and plasma (6 Resistant and 4 Responsive). LC-MS/MS and MALDI-TOF/TOF were used for identification of selected PBMC and plasma proteins, respectively. Real time RT-PCR was applied to confirm proteomics results of differentially expressed proteins in PBMC. For validation of 2-DE results of plasma firstly, a monoclonal antibody was produced against selected proteins from 2-DE analysis. Western blot was used to screen hybridoma and to validate proteomic results of plasma.

HnRNP H1, ACADS and Putative Uncharacterised Protein in Bacteria were identified as

differentially expressed proteins in PBMC. ACADS and Putative Protein were up-regulated in the resistant group while and HnRNP H1 showed higher expression in the responsive group. By performing blast search of matched peptide of Putative Protein to NCBI database against homo sapiens, DNA-PK was found as a hit. This protein along with two additional HnRNPs-related proteins (HnRNP K, HnRNP A1) was used for further analysis by real time RT-PCR.

Quantitative RT-PCR with 16 AML samples (8 resistant and 8 responsive) confirmed the 2-D analysis on PBMC (using HnRNPH1, ACADS and DNA-PK and two additional HnRNPs related genes, HnRNP K and A1) at the gene expression level. HnRNP K was found to be a highly expressed in responsive group. No difference was observed in mRNA expression level of HnRNP A1 between resistant and responsive groups. ACADS and DNA-PK represented significantly higher expressions in resistant group ( $p < 0.05$ ).

In 2-DE analysis of plasma, eight proteins were differentially expressed significantly between plasma of resistant and responsive patients. Selected spots were divided into three groups on the basis of their position on 2D gel, Molecular Weight (HMW, MW=70kDa) spots were obviously detectable in three samples of resistant group but it was not seen in responsive and normal samples, while identified protein APO E (MW=36 kDa) and low molecular weight spots No. 177 and 173 (MW < 20kDa) were over-expressed in the responsive group ( $p < 0.03$ ) using Mann Whitney U test.

Protein spots HMW and No. 177 and 173 were excised from gel and used as antigen for antibody production. By western blot screening of hybridoma, clone 3-16 was selected for screening on AML samples. Signals of HMW spots were obtained by 2-DE western blotting of this hybridoma using anti-mouse IgG as a secondary antibody. The results of 2-DE western blot on 16 AML samples confirmed the results of 2-DE analysis of plasma. No antibody was found for spots No. 177 and 173.

In conclusion, gel-based proteomic approach is a good technique for selection of differentially expressed proteins and identification of potential biomarkers in AML patients with differential response to chemotherapy. HnRNP K but not HnRNP A1 may be useful to identify AML patients responsive to chemotherapy. ACADS and DNA-PK may have potential for identification of resistant patients. Furthermore, the monoclonal antibody generated in this study may be useful in differentiating resistant patient. In general, the proteins identified in this study and the generated antibody may have potential to predict response to induction chemotherapy in AML patients at diagnosis.

## **ABSTRARK**

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

### **Pengenalan dan Penyataan Perbezaan Nyata Penanda Protin Biomarkers untuk Leukaemia Mieloid Akut.**

Oleh

**FATEMEH BARANTALAB**

**January 2014**

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**Fakulti: Perubatan dan Sains Kesihatan**

Leukemia Mieloid Akut (AML) adalah malignan hematopoietik yang dicirikan oleh percambahan sel-sel leluhur mieloid yang tidak normal, ditambah pula oleh blok separa dalam pembezaan sel. 65 kepada 75% daripada pesakit AML yang lebih muda akan mencapai penyembuhan sempurna (CR) selepas terapi induksi, manakala kadar CR lebih rendah pada pesakit yang lebih berusia (40-50%). Kebanyakan pesakit yang mencapai CR akan berulang dalam tempoh tiga tahun dan pesakit yang tidak bertindak balas terhadap terapi induksi menyaksikan penentangan terhadap kemoterapi. Oleh itu, rintangan kepada kemoterapi adalah masalah besar di dalam rawatan pesakit dengan AML. Ramalan penanda semasa termasuk umur, jumlah kiraan darah putih dan perubahan kromosom tertentu. Walau bagaimanapun pada sesetengah pesakit, penanda ini adalah tidak mencukupi.

Tidak seperti genomik, pemeriksaan untuk potensi penanda ramalan menggunakan proteomik adalah kurang kerap dijalankan kerana ianya agak rumit dan memakan masa. Ini termasuklah untuk AML. Matlamat tesis ini adalah untuk mewujudkan satu gel elektroforesis dua dimensi (2-DE) dengan kaedah untuk mengekstrak protein daripada periferal sel-sel mononuklear darah (PBMC) dan sampel plasma daripada pesakit AML. Pendekatan proteomik akan diguna pakai untuk mengenal pasti penanda bio yang berpotensi supaya boleh dikaitkan dengan rintangan terhadap kemoterapi pada diagnosis awal sebelum rawatan.

10 sampel telah dipilih untuk analisis 2-DE PBMC (7 Rintangan dan 3 Responsif) dan plasma (6 Rintangan dan 4 Responsif). LC-MS/MS dan MALDI-TOF/TOF telah digunakan untuk mengenal pasti PBMC dan plasma protin dipilih, masing-masing. Masa sebenar RT-PCR telah digunakan untuk mengesahkan proteomik keputusan protein terzhahir dalam PBMC. Untuk pengesahan 2-DE keputusan plasma pertama, antibodi monoklonal dihasilkan terhadap protein dipilih daripada analisis 2-DE. Hapuskanlah Barat telah digunakan untuk menyaring hybridoma dan untuk mengesahkan keputusan

proteomik plasma.

HnRNP H1, ACADS dan Protin yang diandaikan tidak mempunyai identiti dikenal pasti sebagai protin terzhahir dalam PBMC. ACADS dan Protin Andaian diselaras dalam kumpulan rintangan sementara HnRNP H1 menunjukkan penyataan yang lebih tinggi dalam kumpulan yang responsif. Dengan melakukan carian meluas terhadap padanan peptida dengan Protin Andaian yang kemudiannya dijadikan pangkalan data NCBI terhadap manusia, DNA-PK didapati sebagai menonjol. Protin ini bersama-sama dengan dua HnRNPs tambahan yang berkaitan dengan protin (HnRNP K, HnRNP A1) telah digunakan untuk analisis lanjut oleh masa sebenar RT-PCR.

Kuantitatif RT-PCR dengan 16 sampel AML (8 rintangan dan 8 responsif) mengesahkan analisis 2-D pada PBMC (menggunkan HnRNPH1, ACADS dan DNA-PK dan dua HnRNPs tambahan yang berkaitan dengan gen, HnRNP K dan A1) pada peringkat penyataan gen. HnRNP K telah didapati sangat dinyatakan dalam kumpulan responsif. Tiada perbezaan yang diperhatikan dalam tahap mRNA ungkapan daripada HnRNP A1 antara kumpulan rintangan dan responsif. ACADS dan DNA-PK diwakili dengan ungkapan lebih tinggi dalam kumpulan rintangan ( $p < 0.05$ ).

Dalam analisis 2-DE plasma, lapan protein terzhahir yang ketara antara plasma pesakit dengan rintangan dan responsif. Kawasan-kawasan yang dipilih dibahagikan kepada tiga kumpulan berdasarkan kedudukan pada gel 2D. Berat Molekul Tinggi (HMW, MW = 70kDa) dapat jelas dikesan dalam tiga sampel kumpulan rintangan tetapi ia tidak dilihat dalam sampel responsif dan normal. Spot No.178 yang telah dikenal pasti sebagai protin APO E (MW = 36 kDa) dan berat molekul rendah pada titik No 177 dan 173 (MW <20kDa) lebih dinyatakan dalam kumpulan yang responsif ( $p < 0.03$ ) dengan menggunakan ujian Mann Whitney U.

Titik Protin HMW dan No 177 dan 173 telah dikeluarkan daripada gel dan digunakan sebagai antigen untuk antibodi monoklonal. Dengan pemeriksaan Western Blot hybridoma, klon 3-16 telah dipilih untuk pemeriksaan ke atas sampel AML. Isyarat tempat HMW diperolehi dengan 2-DE Western Blot hybridoma ini menggunakan anti-tetikus IgG sebagai antibodi pertengahan. Keputusan 2-DE Western Blot pada 16 sampel AML mengesahkan keputusan analisis 2-DE plasma. Tiada antibodi ditemui untuk tempat No 177 dan 173.

Kesimpulannya, pendekatan proteomik berasaskan gel adalah teknik yang baik untuk pemilihan protin terzhahir dan mengenal pasti penanda bio yang berpotensi dalam pesakit AML dengan respon perbezaan untuk kemoterapi. HnRNP H1 and HnRNP K tetapi bukan HnRNP A1 mungkin berguna untuk mengenal pasti pesakit AML responsif kepada kemoterapi. ACADS dan DNA-PK mungkin mempunyai potensi untuk mengenal pasti pesakit rintangan. Tambahan pula, antibodi monoklonal yang dihasilkan dalam kajian ini mungkin berguna dalam membezakan pesakit rintangan. Secara umum, protin yang dikenal pasti dalam kajian ini dan antibodi yang dihasilkan mungkin mempunyai potensi untuk meramalkan tindak balas kepada induksi kemoterapi pada pesakit AML di diagnose.

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I certify that a Thesis Examination Committee has met on 16 January 2014 to conduct the final examination of Fatemeh Baran Talab on her thesis entitled "Identification of Differentially Expressed Proteins as Biomarkers of Acute Myeloid Leukemia" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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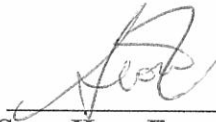
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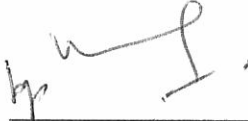
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## LIST OF ABBREVIATIONS

PBS	phosphate buffered saline
PBMC	peripheral blood mononuclear cell
BSA	bovine serum albumin
EDTA	ethylenediaminetetraacetic acid
DMEM	Dulbecco's modified Eagle's Medium
FBS	fetal bovine serum
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobuline M
MW	Molecular weight
KCL	Potassium chloride
PVDF	Polyvinylidene fluoride
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
PEG	Polyethylene glycol
TBS	Tris Buffered Saline
TBS/T	Tris Buffered Saline/Tween
T <sub>m</sub>	melting Temperature
PCR	polymerase chain reaction
CBB	Coomassie Brilliant Blue
DMSO	Dimethyl sulfoxide
2-DE	2-dimensional electrophoresis
IPG	Immobilized pH gradient
CHAPS	3-3[(3-cholamidopropyl dimethylammonio]-1propanesulfonic acid
DTT	Dithiothreitol
IEF	Isoelectric focusing
IAA	Idoacetamide
MALDI	Matrix assisted Laser Desorption Ionization
MS	mass spectrometry
m/z	mass to charge ratio
pI	Isoelectric point
TFA	Trifluoroacetic acid
ACN	Acetonitrile
MS/MS	Tandem mass spectrometry
V <sub>hour</sub>	volt/hour
Mins	minutes
ELISA	Enzyme-linked Immunosorbent Assay
PBS/T	Phosphate Buffered Saline/Tween
kDa	atomic mass unit

## CHAPTER 1

### INTRODUCTION

Acute myeloid leukaemia (AML) is a hematopoietic malignancy characterized by aberrant proliferation of myeloid progenitor cells, coupled with a partial block in cellular differentiation (Robak & Wierzbowska, 2009). Subsequently the immature leukemic blasts are accumulated in the bone marrow and eventually in the peripheral blood.

The incidence rate has been reported as 1.8/100,000 in people <65 years and 17/100,000 in people >65 years in the United States (Deschler & Lübbert, 2006).

Cytogenetic abnormalities and age are the two most important prognostic factors in AML patients. Age  $\geq 60$  years has been identified as an adverse prognostic factor in AML, and there are very few long-term survivors in this age group (Roboz, 2011). The use of chromosome abnormalities or translocations is limited as only they are detected in only 50% of AML patients.

Intensive chemotherapy is a standard treatment in AML which is divided into phases, induction, consolidation and maintenance chemotherapy. Complete remission (CR) is the aim of induction chemotherapy which is defined by presence of 5% blasts in the bone marrow.

A majority of younger AML patients (65-75%) initially respond to induction chemotherapy and achieve complete remission, while the rate of CR is lower in elderly patients (40-50%). Most patients who achieve CR will relapse within three years and those who do not respond to induction therapy display resistance to chemotherapy. Hence, resistance to chemotherapy is considered as a major problem in the treatment of these patients. Resistance to chemotherapy can be seen either at the treatment initiation when no clinical response happens, or later at the cancer recurrence, in spite of initial successful response. For current therapeutic strategies, there is a lack of biomarkers for predicting prognosis or the therapeutic response before treatment (Czibere *et al.*, 2006). In recent years, a considerable study has been directed towards the identification of biomarkers for AML treatment.

During the last decade, DNA microarray has been utilized to identify differentially expressed genes in human haematological malignancies (Lockhart & Winzeler, 2000). Gene expression profile has also been identified for all AML subtypes (Luczak *et al.*, 2012). Although microarray analysis and transcriptional profiles have a good potential to provide information on cancer classification, response to treatment, and prognosis, however, RNA expression levels often do not correlate with abundance of protein expression (Kornblau *et al.*, 2009). Moreover, microarray technologies also are not able to provide information on the abundance or the posttranslational modification such as phosphorylation, glycosylation, cleavage and redox regulation of proteins. These concerns have highlighted the significance of identifying protein profile (proteome) directly, in addition to the transcriptome.

Therefore, it is of great interest to identify biomarkers for the initial diagnosis,

chemotherapy response, detection of relapse and monitoring for minimal residual disease of AML at the level of the proteins rather than at the gene level.

Proteomics analysis is one of the most interesting approaches that facilitate the analysis of very complex protein mixtures in cells, tissue and body fluid.

**Objectives of study:**

- To establish and optimize a two-dimensional gel electrophoresis (2-DE) method for protein extracts from peripheral blood mononuclear cells (PBMC) and plasma of AML patients referred to Hospital Ampang.
- To exploit this proteomics approach to identify potential biomarkers that may be associated with resistance to chemotherapy at initial diagnosis before treatment.
- To select and identify differentially expressed protein biomarkers in plasma and PBMC from resistant and responsive AML patients.
- To produce monoclonal antibodies against differentially expressed proteins in plasma for validation of plasma proteomic results.
- To confirm PBMC proteomic results using real time RT-PCR.

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