UNIVERSITI PUTRA MALAYSIA

IDENTIFICATION OF DIFFERENTIALLY EXPRESSED PROTEINS AS BIOMARKERS OF ACUTE MYELOID LEUKAEMIA

FATEMEH BARANTALAB

FPSK(p) 2014 3
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
COPYRIGHT

All material contained within the thesis, including without limitation text, logos, icons, photographs and all other artwork, is copyright material of Universiti Putra Malaysia unless otherwise stated. Use may be made of any material contained within the thesis for non-commercial purposes from the copyright holder. Commercial use of material may only be made with the express, prior, written permission of Universiti Putra Malaysia.

Copyright © Universiti Putra Malaysia
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 upregulated 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 overexpressed 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

Pengerusi: Maha Abdullah, Ph.D.
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 diagnosa 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 terzahir 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 dikenali pasti sebagai protein terzahir 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 protein (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 (menggunakan 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 terzahir 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 dikenali pasti antara protein 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 antitetikus 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 protein terzahir 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.
ACKNOWLEDGEMENTS

First of all, all praise is dedicated to the Almighty, the Most Merciful and Most Benevolent, for bestowing me this opportunity, guidance, wisdom, and patience to pursue my doctoral journey successfully. The sincere appreciation and gratitude is presented to the following individuals whose advice, support, assistance, and encouragement were invaluable towards the success in my PhD program.

I would like to give my appreciation to Associate Prof. Dr. Maha Binti Abdullah, the Chairman of the Supervisory Committee, for her magnificent professional expertise, patience, advice, motivation, and time to make the completion of my PhD thesis a success. Her professional commitment and critical views were of great importance and taught me a world of lessons regarding research and the academic arena.

My special thanks to Associate Prof. Dr. Chong Pei Pei, a member of my Doctoral Committee, for her help to organize the use of laboratory facilities easier to me. Her cooperation is highly respected.

My special appreciation and regards goes to Prof. Dr. Seow Heng Fong, also as a member of my Doctoral Committee, for her cooperation and intellectual motivation.

Many thanks to Dr. Zainina Binti Seman, as another member of my Doctoral Committee, for her cooperation.

Special thanks covey to my lab mates and peers for their help and assistance.

I would also like to thank, the Head Department of Haematology Department of Hospital Ampang, Dato’ Dr. Chang Kian Meng and also Dr. Ong Tee Chuan for their support in this project.

Last but not the least, my special appreciation goes to my husband and my son, Younes, for their cooperation, sacrifices, supports, and patience throughout my busy schedule during my study, doing my laboratory experiments, and writing my dissertation. All in all paved the path and allowed me to complete my PhD dissertation satisfactorily. May Allah grant all His numerous blessings.
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.

Members of the Thesis Examination Committee were as follows:

**Elizabeth George, PhD**
Professor
Faculty of Medicine and Health Science
Universiti Putra Malaysia
(Chairman)

**Rozita binti Rosli, PhD**
Professor
Institute of Bioscience
Universiti Putra Malaysia
(Internal Examiner)

**Raha binti Hj Abdul Rahim, PhD**
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

**Jill Slansky, PhD**
Associate Professor
University of Colorado
United States
(External Examiner)

\[signature\]

**NORITAH OMAR, PhD**
Associate Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 21 April 2014
This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of type of degree. The members of the Supervisory Committee were as follows:

**Maha Bt Abdullah, PhD**  
Associate Professor  
Faculty of Medicine and Health Sciences  
Universiti Putra Malaysia  
(Chairman)

**Seow Heng Fong, PhD**  
Professor  
Faculty of Medicine and Health Sciences  
Universiti Putra Malaysia  
(Member)

**Chong Pei Pei, PhD**  
Associate Professor  
Faculty of Medicine and Health Sciences  
Universiti Putra Malaysia  
(Member)

**Zainina Binti Seman, PhD**  
Faculty of Medicine and Health Sciences  
Universiti Putra Malaysia  
(Member)

---

**BUJANG BIN KIM HUAT, PhD**  
Professor and Dean  
School of Graduate Studies  
Universiti Putra Malaysia  
Date:
DECLARATION

Declaration by graduate student

I hereby confirm that:

- this thesis is my original work;
- quotations, illustrations and citations have been duly referenced;
- this thesis has not been submitted previously or concurrently for any other degree at any other institutions;
- intellectual property from the thesis and copyright of thesis are fully-owned by Universiti Putra Malaysia, as according to the Universiti Putra Malaysia (Research) Rules 2012;
- written permission must be obtained from supervisor and the office of Deputy Vice-Chancellor (Research and Innovation) before thesis is published (in the form of written, printed or in electronic form) including books, journals, modules, proceedings, popular writings, seminar papers, manuscripts, posters, reports, lecture notes, learning modules or any other materials as stated in the Universiti Putra Malaysia (Research) Rules 2012;
- there is no plagiarism or data falsification/fabrication in the thesis, and scholarly integrity is upheld as according to the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) and the Universiti Putra Malaysia (Research) Rules 2012. The thesis has undergone plagiarism detection software.

Signature: ___________________     Date:  16 January 2014

Name and Matric No: Fatemeh Barantalab, GS20810
Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

Signature: Maha Bt Abdullah, PhD
Associate Professor
(Chairman)

Signature: Seow Heng Fong, PhD
Professor
(Member)

Signature: Chong Pei Pei, PhD
Associate Professor
(Member)

Signature: Zainina Binti Seman, M.D.
(Member)
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>ABSTRACT</th>
<th>iii</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACTR</td>
<td>v</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>vii</td>
</tr>
<tr>
<td>APPROVAL</td>
<td>viii</td>
</tr>
<tr>
<td>DECLARATION</td>
<td>x</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xvi</td>
</tr>
</tbody>
</table>
| LIST OF APPENDICES                                                      | xvi
| LIST OF ABBREVIATIONS                                                   | xix |

## CHAPTER

1. **INTRODUCTION**

2. **LITERATURE REVIEW**

   2.1 Acute myeloid leukaemia
   2.2 Incidence
   2.3 Aetiology
   2.4 Diagnosis and classification
   2.5 Prognosis factors
   2.6 Treatment
     - 2.6.1 Induction therapy
     - 2.6.2 Post-remission therapy
     - 2.6.3 Stem cell transplantation
     - 2.6.4 Treatment in Elderly patients
   2.7 Cytostatic Drugs
     - 2.7.1 Topoisomerase poisons
     - 2.7.2 Nucleoside analogues
   2.8 Mechanism of Drug resistance
     - 2.8.1 Drug transport
     - 2.8.2 Drug metabolism
     - 2.8.3 Drug target
     - 2.8.4 DNA damage and apoptosis
   2.9 Proteomics based on 2-D gel electrophoresis
     - 2.9.1 Two-Dimensional Gel Electrophoresis and Mass Spectrometry
     - 2.9.2 Matrix-Assisted Laser Desorption/ ionisation (MALDI)
     - 2.9.3 Electrospray ionisation (ESI)
     - 2.9.4 Mass Spectrometry Instrumentation and Protein Identification
   2.10 Proteomics in prognosis of AML
   2.11 Proteomic assessment of response to chemotherapy in AML
   2.12 Identification of biomarkers related to chemotherapy response in other cancers by proteomics
2.13 Antibody production
  2.13.1 Antibody structure and function
  2.13.2 Polyclonal Antibodies production
  2.13.3 Monoclonal Antibodies production

3 MATERIALS AND METHODS
  3.1 Study Design
  3.2 Patient samples collection
  3.3 Isolation PBMC cells and plasma from peripheral blood
  3.4 Optimization of two dimensional gel electrophoresis of PBMC proteins
    3.4.1 Optimization of sample preparation for 2-DE of PBMC proteins
    3.4.2 First dimension separation of PBMC: Isoelectric focusing (IEF)
    3.4.3 Second dimension separation of PBMC: SDS-PAGE
    3.4.4 Spot visualization
  3.5 Two dimensional gel electrophoresis of clinical and normal PBMC proteins
    3.5.1 Spot visualization of clinical and normal PBMC proteins using silver staining
  3.6 Two dimensional electrophoresis of plasma
    3.6.1 Optimization of plasma sample preparation for 2-DGE analysis
    3.6.2 First dimension separation (IEF) of clinical and normal plasma
    3.6.3 Second dimension separation of plasma: SDS-PAGE
    3.6.4 Spot visualization of plasma proteins
  3.7 Scanning and image analysis
  3.8 Identification of proteins by mass spectrophometry
  3.9 Production of monoclonal antibody against proteins in gel
    3.9.1 Preparation of antigen in Freund’s adjuvant
    3.9.2 Immunization protocol
    3.9.3 Preparation of feeder cells
    3.9.4 Fusion of hybridoma
    3.9.5 Screening and selection of hybridoma through western blot
    3.9.6 Cloning of hybridoma by limiting dilution
    3.9.7 Determination of hybridoma isotypes and subclasses
  3.10 Gene expression- Real time RT- PCR
    3.10.1 RNA extraction
    3.10.2 DNase treatment
    3.10.3 Reverse transcription
    3.10.4 Real-time PCR
    3.10.5 Analysis of data
  3.11 Statistical analysis
4 RESULTS
4.1 2-DGE analysis results of PBMC
    4.1.1 Clinical data and diagnosis
    4.1.2 Optimization of sample preparation for 2-DGE of PBMC
    4.1.3 2-DE gel Analysis of PBMC and Blast cells
    4.1.4 Identification of PBMC protein spots
4.2 Plasma 2-DE analysis
    4.2.1 Clinical data of Patients used for 2-DE plasma
    4.2.2 Optimization of 2-DE of plasma
    4.2.3 2-DE Analysis of clinical and normal plasma
    4.2.4 Identification of plasma protein spot
4.3 Antibody production
    4.3.1 Antigen preparation and immunization
    4.3.2 Primary and secondary screening of hybridoma using Western blot
    4.3.3 Limiting dilution of hybridoma clone 1/22
    4.3.4 Determination of hybridoma isotypes/classes and subclasses
    4.3.5 2-D immunoblot screening of antibody clone 3-16 on patients plasma using Anti-mouse IgG (H+L)
    4.3.6 2-D immunoblot screening of antibody clone 3-16 on patients plasma using Anti-mouse IgM (µ chain)
4.4 Gene expression Real time RT-PCR
    4.4.1 Standard Curve
    4.4.2 Quantification of Target Genes

5 DISCUSSION
5.1 Methodological recommendations for PBMC and plasma 2-D Gel Electrophoresis
5.2 PBMC 2-DE analysis on AML samples
5.3 Plasma 2-DE analysis on AML samples
5.4 Antibody production

6 CONCLUSION

FUTURE RECOMMENDATIONS
REFERENCES
APPENDICES
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>FAB classification of acute myeloid leukaemia</td>
<td>5</td>
</tr>
<tr>
<td>2.2</td>
<td>The 2008 WHO classification Of AML</td>
<td>8</td>
</tr>
<tr>
<td>3.1</td>
<td>Primers of gene and thermal cycle specification in real-time PCR</td>
<td>44</td>
</tr>
<tr>
<td>4.1</td>
<td>Clinical details of AML sample used in this study</td>
<td>46</td>
</tr>
<tr>
<td>4.2</td>
<td>Characteristics of differentially expressed proteins in resistant and responsive group of AML identified by LC/MSMS</td>
<td>53</td>
</tr>
<tr>
<td>4.3</td>
<td>Clinical details of AML sample were used for 2-DE study of plasma</td>
<td>54</td>
</tr>
<tr>
<td>4.4</td>
<td>Characteristics of protein identified from plasma 2DE analysis</td>
<td>65</td>
</tr>
<tr>
<td>4.5</td>
<td>Clinical details of AML plasma samples used for 2-D western blot probed with clone 3-16 hybridoma and anti-mouse IgG (H+L) as secondary antibody</td>
<td>75</td>
</tr>
<tr>
<td>4.6</td>
<td>Clinical details of AML sample used for 2-D western blot screening of plasma using Anti-mouse IgM</td>
<td>79</td>
</tr>
<tr>
<td>4.7</td>
<td>Clinical details of AML samples used for real time RT-PCR</td>
<td>81</td>
</tr>
<tr>
<td>4.8</td>
<td>Primers and their sequences</td>
<td>82</td>
</tr>
<tr>
<td>4.9</td>
<td>Correlation coefficient, $R^2$ values and equation for standard curve of genes used in real-time PCR method</td>
<td>84</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Classic scheme of hematopoietic hierarchy</td>
<td>3</td>
</tr>
<tr>
<td>2.2</td>
<td>Diagram of AML diagnosis strategy based on both the FAB and WHO classifications</td>
<td>6</td>
</tr>
<tr>
<td>2.3</td>
<td>Sites of action of topoisomerase poisons which inhibit DNA uncoiling and the nucleoside analogs block the formation and use of functional nucleic acids.</td>
<td>12</td>
</tr>
<tr>
<td>2.4</td>
<td>Schematic representation of different mechanisms of cytostatic drug resistance</td>
<td>14</td>
</tr>
<tr>
<td>2.5</td>
<td>Schematic diagram of 2-D Electrophoresis and mass spectrometry</td>
<td>18</td>
</tr>
<tr>
<td>2.6</td>
<td>Schematic diagram of Matrix-Assisted Laser Desorption/ionisation (MALDI) principle.</td>
<td>19</td>
</tr>
<tr>
<td>2.7</td>
<td>Schematic representation of an antibody structure</td>
<td>25</td>
</tr>
<tr>
<td>2.8</td>
<td>Representation of five classes of immunoglobulins</td>
<td>26</td>
</tr>
<tr>
<td>4.1</td>
<td>Coomassie Brilliant Blue (CBB) stained 2-DGE gels of normal PBMC</td>
<td>47</td>
</tr>
<tr>
<td>4.2</td>
<td>Comparison of 2D gel images derived from healthy PBMC with two types of IGP strips 7cm, pH 3-10 (Linear and non linear)</td>
<td>48</td>
</tr>
<tr>
<td>4.3</td>
<td>A representative 2-DE protein profile derived from (A) healthy control, (B) resistant and (C) responsive samples</td>
<td>50</td>
</tr>
<tr>
<td>4.4</td>
<td>The close-up 2-DE gel images comparing differently expressed spots</td>
<td>51</td>
</tr>
<tr>
<td>4.5</td>
<td>Comparison of proteins differentially expressed in resistant and responsive groups before (5 samples resistant and 5 samples responsive) and after transferring the two samples No. 35 and 45 from responsive group to resistant group (7 samples resistant and 3 samples responsive).</td>
<td>52</td>
</tr>
<tr>
<td>4.6</td>
<td>Comparison of optimization experiments on 2-DE of plasma</td>
<td>55</td>
</tr>
<tr>
<td>4.7</td>
<td>Comparison of 2-DE gels of plasma proteins sample undepleted (A) and immunodepleted (B) for albumin and IgG</td>
<td>57</td>
</tr>
<tr>
<td>4.8</td>
<td>Comparison of 2-D gel images of depleted healthy plasma using two types of IGP strips with same length and pH (7cm, pH3-10) but different linearity (linear and nonlinear).</td>
<td>58</td>
</tr>
<tr>
<td>4.9</td>
<td>Representative 2-DE gel images of plasma derived from (A) Resistant, (B) Responsive and (C) Healthy samples</td>
<td>62</td>
</tr>
<tr>
<td>4.10</td>
<td>The close-up 2-D gel images of differentially expressed proteins (HMW, spots No 173, 177 and 178) in plasma of resistant, responsive and healthy samples.</td>
<td>63</td>
</tr>
<tr>
<td>4.11</td>
<td>Comparison of % spot volume average of differentially expressed proteins spots (No 173, 177 and 178) in plasma of resistant (6 samples) and responsive groups (4 samples)</td>
<td>64</td>
</tr>
<tr>
<td>4.12</td>
<td>Flow chart of monoclonal antibody production</td>
<td>66</td>
</tr>
</tbody>
</table>
4.13 Representative 2-DE gel images of plasma sample derived from patients. 67
4.14 Western blot images of plasma samples from AML sample, comparing two secondary antibodies to use it for hybridoma screening. 68
4.15 Representative western blot images of primary and secondary screening with supernatant from hybridoma 69
4.16 Image of antibody producing hybridoma cells under Inverted Phase Contrast microscope 40X, 4 days after first limiting dilution. 70
4.17 Representative western blot images of selected positive clones from first, second and third limiting dilution of initial clone No. 1/22 71
4.18 Results of first and second ELISA 72
4.19 Representative 1D western blot image of antibody No. 3-16 to check background 73
4.20 Representative 2-D western blot and stained-post blot images of depleted plasma sample No. 52 74
4.21 The images of 2-D western blot comparing expression of HMW spots in resistant (8) and responsive group (8) of AML patients using anti-mouse IgG(H+L) as a secondary antibody 76
4.22 Representative western blot images to check the background of Anti-mouse IgM. 78
4.23 The representative 2-D western blot images comparing the pattern of spots in resistant (8) and responsive (5) group using anti-mouse IgM as a secondary antibody. The arrows showing the big spot which was expressed in 75% of resistant and 60% of responsive group. Negative of sample 43 also is included in image for comparing. 80
4.25 Representative melting curves for B2M and DNA-PK 84
4.26 mRNA expression of three identified genes (HnRNP H1, DNA-PK, ACADS) from 2DE analysis of PBMC and another two HnRNP- related proteins (HnRNP A1, HnRNP K) in resistant (N=8) vs. responsive (N=8) samples using real-time PCR. 85
# LIST OF APPENDICES

<table>
<thead>
<tr>
<th>Appendix</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Solutions and reagents</td>
<td>116</td>
</tr>
<tr>
<td>B</td>
<td>2-D gel images of PBMC</td>
<td>119</td>
</tr>
<tr>
<td>C</td>
<td>Mascot search results of PBMC spots</td>
<td>124</td>
</tr>
<tr>
<td>D</td>
<td>2-D gel image of plasma</td>
<td>130</td>
</tr>
<tr>
<td>E</td>
<td>Mascot search results of plasma spot</td>
<td>136</td>
</tr>
<tr>
<td>F</td>
<td>Images of 2-D western blot and stained-post blot which immunoblotted with Anti-mouse IgG (H+L) as a secondary antibody</td>
<td>138</td>
</tr>
<tr>
<td>G</td>
<td>Images of 2-D western blot and stained-post blot which immunoblotted with Anti-mouse IgM as a secondary antibody</td>
<td>142</td>
</tr>
<tr>
<td>H</td>
<td>Standard curve, real-time PCR amplification plot and melting curve of genes</td>
<td>145</td>
</tr>
</tbody>
</table>
# LIST OF ABBREVIATIONS

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


effects: insight into the function of eukaryotic initiation factor 5A. *Oncogene*, 22(31), 4819-4830.


human colon cancer cells with induced 5-fluorouracil resistance. *Cancer Research, 65*(8), 3162-3170.


Wolfe, L., Jethva, R., Oglesbee, D., & Vockley, J. *Short-Chain Acyl-CoA Dehydrogenase Deficiency*.


