UNIVERSITI PUTRA MALAYSIA

HAEMATOLOGY, GENETICS AND MOLECULAR EPIDEMIOLOGY OF DELETIONAL ALPHA THALASSAEMIA IN MALAYSIA

ALIZA BINTI MOHD YACOB

FPSK(m) 2012 17
HAEMATOLOGY, GENETICS AND MOLECULAR EPIDEMIOLOGY OF DELETIONAL ALPHA THALASSAEMIA IN MALAYSIA

By

ALIZA BINTI MOHD YACOB

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science
Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of
the requirement for the degree of Master of Science

HAEMATOLOGY, GENETICS AND MOLECULAR EPIDEMIOLOGY OF
DELETIONAL ALPHA THALASSAEMIA IN MALAYSIA

By

ALIZA BINTI MOHD YACOB

October 2011

Chairman: Professor Elizabeth George, M.D
Faculty: Medicine and Health Sciences

Alpha thalassaemia is the most common autosomal recessive single gene disorder in
Southeast Asia, encountered in increasing numbers all over the world and heterogenous.
Deletions of all four alpha thalassaemia genes (---/-) result in fetuses mostly die before
or shortly after birth known as Hb Barts hydrops foetalis syndrome. It also caused
serious maternal complications in pregnancies in which without medical care half were
estimated to die. Deletions of 3 alpha thalassaemia genes (---/-α) is known as HbH
disease with moderate anaemia, usually was similarly seen in the Malays and Chinese.
In contrast, antenatal diagnosis for alpha thalassaemia reported Hb Barts hydrops
foetalis (---/-) mostly in the Chinese. Thalassaemia studies among blood donors of
91.3% Malays found out that 30% were anaemic with all donors had a negative H-
inclusion test, which is usually positive with double deletions (---/αα). Malaysia is a
multiethnic country with mix marriages and 4.5% of Chinese-Malaysian is carriers of α0
thalassaemia (−/−). Therefore the purpose of this study was to determine the current deletional alpha thalassaemia status and deletional alpha thalassaemia burden in our population. Changes in deletional alpha thalassaemia gene frequency may change the deletional alpha thalassaemia burden. In this study haematology, genetics and molecular epidemiology of deletional alpha thalassaemia were looked at and carrier detection of common deletions was carried out. This will identify phenotypic characteristics, type of deletions and genotypes present, determine prevalence, estimate disease burden of deletional alpha thalassaemia and determine foetal genotype from pregnancies at risk of deletional Hb Barts hydrops foetalis in the country.

Standard haematology protocol, DNA studies and statistical analysis of qualitative and quantitative were applied. A cross sectional study was carried out on 405 samples. These were 238 EDTA blood from blood donors from Universiti Putra Malaysia, 15 DNA of 5 Hb Barts hydrops foetalis (−/−−) and 10 alpha thalassaemia spouses (−/−αα) from Universiti Malaya Medical Centre, 72 EDTA blood from HbH disease (−−/−α) families from Institute for Medical Research and 80 DNA from blood donors from National Blood Bank. Blood count/blood film, HPLC, haemoglobin electrophoresis and multiplex PCR for detecting the 5 most common gene deletions were carried out. These are the common −SEA in Southeast Asia, the ethnic origins −THAI and −FIL, the world common single deletions −α3.7 and −α4.2. Multiplex PCR using validated primers was developed using samples from families of HbH disease, detected the −SEA, −THAI, −FIL and −α3.7 giving 5 genotypes. This was compared with conventional method using samples from National Blood Bank and gave 100% accuracy, sensitivity and specificity
for --SEA, --FIL, --THAI and -α^{3.7} were also detected. In blood donors from Universiti Putra Malaysia, MCV < 80 fL was identified in 16.5% (with exclusion of Indians), deletional α–thalassaemia prevalence was 7.4% (3.5% double and 3.9% single deletions), equally distributed among Malays and Chinese. The most common double deletion detected was --SEA and -α^{3.7} for single followed by -α^{4.2}. The prevalence was 5.3% in the Malays with the most common was -α^{3.7} (4.3%), 8.5% in the Chinese with 5.4% --SEA indicating an increase from 4.5% α^0 thalassaemia (--/--) and 0.4% in others. All the --SEA were with MCV≤68 fL and MCH <22 pg. The projected number of pregnancies each year at risk of deletional Hb Barts hydrops foetalis syndrome and HbH disease are 30 and 120 in Malays, 250 and 150 in Chinese, 640 of both and on average 600 and 669 respectively. A typical α–thalassaemia phenotype was observed in HbH disease, and carriers of α^0 and α^+ thalassaemia. Real-time quantitative PCR (TaqMan®) with validated primers was optimized for --SEA. Fetal genotype values of α^{SEA-BRN} were 0.6 to 1.6 for (--SEA/--SEA), 0.2 to 0.5 for (--SEA/αα) and 0 for (αα/αα) giving distinctly different values. The methods used were simple, reliable and useful in monitoring allele status. Identified carriers can be counseled and DNA from pregnancies at risk for (--/--) can be analysed. These will help in increasing awareness among carriers, bringing down deletional α–thalassaemia births and eventually lower the disease burden in Malaysia.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai mematuhi keperluan untuk ijazah Master Sains

HEMATOLOGI, GENETIK DAN EPIDEMOLOGI MOLEKUL ALPHA THALASEMIA DELESI DI MALAYSIA

Oleh

ALIZA BINTI MOHD YACOB

Oktober 2011

Pengerusi: Profesor Elizabeth George, M.D
Fakulti: Perubatan dan Sains Kesihatan

Alfa talasemia adalah kecacatan gen tunggal autosomal resesif paling lazim berlaku di Asia Tenggara, bilangannya semakin meningkat di seluruh dunia dan heterogenus. Delesi kesemua empat gen alfa talasemia (\(\alpha^{+}\)/\(\alpha^{-}\)) mengakibatkan fetus kebanyakkannya meninggal dunia sebelum atau sejurus selepas dilahirkan dan dikenali sebagai sindrom Hb Barts hydrops foetalis. Ia juga menyebabkan komplikasi maternal yang serius pada kandungan di mana tanpa penjagaan perubatan separuh dijangka meninggal dunia. Delesi 3 gen alfa talasemia (\(\alpha^{+}/\alpha^{-}\)) dikenali sebagai penyakit HbH dengan anemia sederhana, selalunya dilihat samarata pada kedua-dua orang Melayu dan Cina. Walaubagaimanapun, diagnosis antenatal untuk penyakit alfa talasaemia melapurkan sindrom Hb Barts hydrops foetalis (\(\alpha^{+}/\alpha^{-}\)) kebanyakkannya pada orang Cina. Kajian talasemia dikalangan penderma darah yang 91.3% Melayu mendapati 30% adalah anemik dan kesemua penderma adalah negatif ujian H-inklusi, yang selalunya positif

v
dengan 2 delesi (\(-/\alpha\alpha\)). Malaysia adalah negara berbilang kaum dengan perkahwinan campur dan 4.5% daripada orang Cina-Malaysia adalah pembawa \(\alpha^0\) talasemia (\(--/\)). Oleh sebab itu tujuan kajian ini adalah untuk menentukan status terkini penyakit alfa talasemia delesi dan beban penyakit alfa talasemia delesi dalam populasi kita. Perubahan pada frekuensi gen penyakit alfa talasemia delesi boleh mengubah beban penyakit alfa talasemia delesi. Dalam kajian ini hematologi, genetik dan epidemiologi molekul penyakit alfa talasemia delesi dilihat dan pengesan pembawa delesi lazim dijalankan. Ini akan mengenalpasti sifat fenotip, jenis delesi dan genotip yang hadir, menentukan prevalen, menganggar beban penyakit alfa talasemia delesi dan menentukan genotip fetus pada kandungan berisiko sindrom Hb Barts hydrops fetalis delesi di negara ini.

Protokol hematologi piawai, kajian DNA dan analisis statistik kualitatif dan kuantitatif diaplikasikan. Kajian rentas dijalankan ke atas 405 sampel. Ini adalah 238 darah EDTA penderma darah dari Universiti Putra Malaysia, 15 DNA daripada 5 sindrom Hb Barts hydrops foetalis (\(--/--\)) dan 10 pasangan alpha talasemia (\(-/\alpha\alpha\)) dari Pusat Perubatan Universiti Malaya, 72 darah EDTA ahli keluarga penyakit HbH (\(--/\alpha\)) dari Institut Penyelidikan Perubatan dan 80 DNA penderma darah dari Tabung Darah Negara. Kiraan darah/filem darah, HPLC, elektroforesis hemoglobin dan multiplex PCR untuk mengesan 5 delesi gen lazim dijalankan. In adalah delesi lazim --SEA di Asia Tenggara, mengikut asal etnik--THAI, --FIL, tunggal lazim di dunia -\(\alpha^{3.7}\) dan -\(\alpha^{4.2}\). PCR Multiplex menggunakan primer yang disahkan dibangunkan menguna sampel ahli keluarga pesakit
penyakit HbH, mengesan --SEA, --THAI, --FIL dan -α3.7 dan memberi 5 genotip. Ini dibandingkan dengan kaedah konvensional menggunakan sampel dari Tabung Darah Negara dan memberi 100% betul, sensitif dan spesifik untuk --SEA, --FIL, --THAI dan -α3.7 juga dikesan. Pada penderma darah Universiti Putra Malaysia, MCV<80fL dikenalpasti pada 16.5% (eksklusif orang India), prevalen α–talasemia delesi ialah 7.4% (3.5% --SEA dan 3.9% delesi tunggal) dan ditaburkan samarata diantara orang Malau dan Cina. --SEA adalah delesi kembar dan -α3.7 adalah delesi tunggal paling lazim dikesan diikuti -α4.2. Prevalen ialah 5.3% pada orang Melayu dengan -α3.7 (4.3%) paling lazim, 8.5% pada orang Cina dengan 5.4% --SEA iaitu menunjukkan peningkatan daripada 4.5% α0 talasemia (--/--) dan 0.4% pada bangsa lain. Semua --SEA mempunyai MCV≤68 fL dan MCH <22 pg. Unjuran kandungan setiap tahun berisiko sindrom Hb Barts hydrops foetalis delesi dan penyakit Hb H delesi ialah 30 dan 120 pada orang Melayu, 250 dan 150 pada orang Cina, 640 pada keduanya dan secara purata 600 dan 669. Fenotip tipikal α–talasemia diperhatikan dari penyakit HbH, dan pembawa α0 dan α+ talasaemia. Kuantitatif PCR Real-time (TaqMan) menggunakan primer yang disahkan dioptima untuk (--SEA). Nilai genotip fetus αSEA-BRN ialah 0.6 ke 1.6 untuk (--SEA/--SEA), 0.2 ke 0.5 untuk (--SEA/αα) dan 0 untuk (αα/αα) memberi perbezaan nilai yang jelas. Kaedah-kaedah ujian yang digunakan adalah ringkas, boleh diharap dan berguna untuk monitor status alel. Pembawa yang dikenalpasti dapat diberi kaunseling dan DNA dari kandungan berisiko (--/-- alpha talasemia boleh dianalisis. Ini membantu meningkatkan kesedaran dikalangan pembawa, menurunkan kelahiran alfa talasaemia delesi dan seterusnya mengurangkan beban penyakit ini di Malaysia.
ACKNOWLEDGEMENTS

In the name of Allah Most Gracious, Most Merciful.

My sincere thanks to Professor Dr. Elizabeth George for her guidance, Professor Dr. Abdul Manaf Ali for his technical advice and support and Dr. Zubaidah Zakaria for her professional support.

My gratitude to Dr. Nor Asiah Muhamad from IMR for her advice on medical biostatistical and epidemiology, Prof. Dr. Mary Anne Tan from UMMC for providing samples, Professor Dr. Abdul Rahman Omar and Dr. Daud for their technical support, Dr. Teo Guan Young for his statistical guidance and SPSS application, Dr. Goh Yong Meng for his statistical lectures and Ms. Normi Mustapha for her guidance on the application of Open Epi.

My special thanks to my senior colleagues Dr. Puteri J Noor, Dr. Chin Yuet Meng, Ms. Ten Seow Keoh and Dr. Rahimah Ahmad, staff of Haematology Unit IMR Mrs. Nor Rizan Kamaluddin and others, staff of Faculty of Medicine and Health Sciences UPM Mr. Quek, Mrs. Amrina, Mrs. Safarina and Mr. Roslaini, and staff/members of Molecular and Cell Biology Laboratory UPM Nancy, Safdi, Lina, Mus, Nora, Fiza, Faizah, Zuhaida, Ainul, Rohaya, Dr. Asmah, Mashitoh and others for their continuous support.

Finally, I would like to extend my appreciation to my husband, mother, father, children and siblings for their patience and sacrifices.

May all your kindness be rewarded with more kindness, Amin.
I certify that a Thesis Examination Committee has met on 18 October 2011 to conduct the final examination of Aliza binti Mohd Yacob on her thesis entitled “Haematology, Genetics and Molecular Epidemiology of Deletional Alpha Thalassaemia in Malaysia” in accordance with the Universities and University College 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 Master of Science.

Members of the Thesis Examination Committee were as follows:

**Fauziah binti Othman, PhD**  
Professor  
Faculty of Medicine and Health Sciences  
Universiti Putra Malaysia  
(Chairman)

**Patimah binti Ismail, PhD**  
Professor  
Faculty of Medicine and Health Sciences  
Universiti Putra Malaysia  
(Internal Examiner)

**Rosline Hassan, M.D**  
Assoc. Professor  
Faculty of Medicine  
UniversitiSains Malaysia  
(External Examiner)

**Mokhtar bin Abu Bakar, PhD**  
Professor  
Faculty of Medicine  
Universiti Kebangsaan Malaysia  
(External Examiner)

\[Signature\]

**SEOW HENG FONG, PhD**  
Professor and Deputy Dean  
School of Graduate Studies  
Universiti Putra Malaysia

Date: 28 June 2012
This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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

**Abdul Manaf Ali, PhD**  
Professor  
Faculty of Agriculture and Biotechnology  
Universiti Sultan Zainal Abidin Malaysia  
(Member)  

**Zubaidah Zakaria, M.D**  
Ketua Pusat Penyelidikan Kanser  
Ketua Unit Hematologi  
Institut Penyelidikan Perubatan  
(Member)  

---

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

Date:
DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

_____________________________
ALIZA BINTI MOHD YACOB
Date: 18 October 2011
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td></td>
<td>ii</td>
</tr>
<tr>
<td>ABSTRAK</td>
<td></td>
<td>v</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td></td>
<td>viii</td>
</tr>
<tr>
<td>APPROVAL</td>
<td></td>
<td>ix</td>
</tr>
<tr>
<td>DECLARATION</td>
<td></td>
<td>xi</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td></td>
<td>xii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td></td>
<td>xiii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS AND SYMBOLS</td>
<td></td>
<td>xvi</td>
</tr>
<tr>
<td>1</td>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>LITERATURE REVIEW</td>
<td></td>
</tr>
<tr>
<td>2.1</td>
<td>The haemoglobin molecule</td>
<td>8</td>
</tr>
<tr>
<td>2.2</td>
<td>Molecular genetics of the human $\alpha$-globin gene cluster</td>
<td>12</td>
</tr>
<tr>
<td>2.3</td>
<td>Epidemiology of $\alpha$-thalassaemia</td>
<td>16</td>
</tr>
<tr>
<td>2.4</td>
<td>Clinical description and laboratory detection of $\alpha$-thalassaemia</td>
<td>18</td>
</tr>
</tbody>
</table>
3 MATERIALS AND METHODS

3.1 Blood sample for analysis
   3.1.1 Subgroup samples
   3.1.2 Comparison samples
   3.1.3 Control samples
   3.1.4 Inclusion and Exclusion Criteria

3.2 BHES protocol, DNA testing and Statistical analysis

3.3 BHES protocol
   3.3.1 Blood cell count
   3.3.2 Blood film preparation
   3.3.3 Blood film staining
   3.3.4 H-inclusion test
   3.3.5 VARIANT™ hemoglobin test
   3.3.6 Haemoglobin electrophoresis

3.4 DNA extraction
   3.4.1 DNA yield and purity estimation

3.5 Multiplex PCR
   3.5.1 Preparation of primers
   3.5.2 Preparation of multiplex PCR reactions
   3.5.3 Optimization and development of multiplex assays
   3.5.4 Separation and identification of multiplex PCR products

3.6 Conventional routine method

3.7 Real-Time Quantitative PCR
   3.7.1 Preparation of primers and TaqMan® probes
   3.7.2 Preparation of human β-actin
   3.7.3 Preparation of standards
   3.7.4 Preparation of real time Q-PCR reactions
3.7.5 Minimizing DNA contaminants 70
3.7.6 Analysis of Data 71
3.8 DNA Sequencing 73
3.9 Statistical Analysis 74

4 RESULTS

4.1 Identification of rare and common α-thalassaemia deletions and α–globin gene triplication 77

4.1.1 Heterogeneity of α-thalassaemia 77
4.1.2 Genotypes of α-thalassaemia 79
4.1.3 Sensitive detection for rare mutations 81
4.1.4 Correct detection 81

4.2 Haematology phenotypic characteristics 82

4.3 Comparison between test method and routine method 83

4.3.1 Performance of test method 83
4.3.2 Evaluation of test method 84

4.4 Detection of five common α-thalassaemia deletions in blood donors 85

4.4.1 Presumptive identification of thalassaemia trait 85
4.4.2 Double and single α–thalassaemia deletions 87
4.4.3 Reliability of α–deletions detected 89
4.4.4 Correlating conventional findings to DNA findings 92

4.5 Prevalence of common α–thalassemia deletions in blood donors 94

4.5.1 Ethnicity 94
4.5.2 Allele frequency 97
4.5.3 The predicted future health burden 99
4.6 Quantitative analysis of $\alpha^{SEA}$ by real-time PCR for prenatal diagnosis

4.6.1 Raw Data Plot

4.6.2 Multicomponent Plot

4.6.3 Quality of Standard Curve

4.6.4 Absolute quantification

4.6.5 Normalization of $\alpha^{SEA}$-BR and $\alpha$-GCR

4.6.6 Local DNA Sequence Alignment

5 DISCUSSION

5.1 Correct approach in the detection of alleles of 5 common deletional $\alpha$-thalassaeemia in Malaysia

5.2 Screening for common deletional $\alpha$-thalassaemia carriers in blood donors

5.3 Prevalence of common $\alpha$-thalassemia deletions in blood donors

5.4 Quantitative analysis of deletional $\alpha$-thalassaemia for prenatal diagnosis of Hb Barts hydrops foetalis by real-time PCR

6 SUMMARY, CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH

REFERENCES

APPENDICES

BIODATA OF STUDENT

LIST OF PUBLICATIONS