UNIVERSITI PUTRA MALAYSIA

OPTIMIZATION OF POLYMERASE CHAIN REACTION-BASED SCREENING TECHNIQUES FOR THE DETECTION OF FRAGILE X SYNDROME

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FPSK(M) 2004 13

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By

SHARIZAH BINTI ALIMAT

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

September 2004

DEDICATION

This thesis is dedicated to my husband, Yusri Sabri and lovely children, Nur Aqilah, Muhammad Hafiz and Muhammad Danial.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

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Chairman: Associate Professor Rozita Rosli, PhD

Faculty : Medicine and Health Sciences

Fragile X syndrome is the second most common genetic cause of mental retardation. It is caused by a large expansion of a CGG repeat (full mutation) that leads to silencing of the FMR1 gene and the absence of the FMR1 gene product, a 70 - 80 kDa protein (FMRP). The aim of this study is to establish a rapid and non-radioactive molecular screening technique using PCR and Southern blotting and to qualify as well as quantify the expression level of proteins from the sera of normal and affected individuals using two-dimensional analysis. In order to carry out the investigation, a number of techniques were used in this study including Polymerase chain reaction (PCR), Southern blotting, DNA sequencing and two-dimensional polyacrylamide gel electrophoresis (2D PAGE). In this study, PCR and Southern blotting techniques were optimized and utilized in the amplification of the FMR1 gene in the subjects. The

results demonstrated that almost all the suspected individuals show the fully mutated fragments with more than 600 bp and for the premutation stage, higher than 500 bp fragments were amplified. Only a few suspected individuals showed the normal fragment amplification (152 bp to 265 bp). This may be caused by an alternative DNA structure formed from the triplet repeats and also other types of mutation such as point mutation and deletion, or other mentally dysregulation disorder, which block the amplification of the FMR1 region. The PCR products were also subjected to DNA sequencing to confirm the sequence data and size. The fragment from normal individual showed the expected sequence (~ 130 bp) but the positive individual yielded an unreadable pattern. A template with high GC content template always has the tendency to build secondary structures that block the amplification of the FMR1 gene and this might be one of the causes of the incidence. In the 2D PAGE study, the protein expression maps for sera from healthy individuals were compared with maps from patients with suspected fragile X syndrome using PDQuest™ software analysis. PDQuest™ analysis detected 75 % of proteins being conserved between the normal and fragile X serum which most likely are the housekeeping proteins of the samples. Two protein spots of interest (FMRP complex) and another four protein spots (Ig α light chain, Haptoglobin cleaved β chain, IgG γ intermediate chain and IgG heavy chain) were found to be 5 fold down-regulated in the serum from a suspected fragile X patient. In addition, there are also two protein spots (albumin proteins) that were over expressed. The results presented here point out that fragile X syndrome may induce changes at the protein level (indirectly rather than directly) that do not occur under all circumstances but nevertheless represent an important feature of this disorder. However, this study

relied on measures in the blood, which may not always reflect the situation in other tissues, especially the brain. The expansion sizes or degree of methylation in non-blood tissues could be very different compared to the blood cells. The expression of the FMRP also could be dissimilar between the blood cells and brain cells. Therefore, further studies should be carried out using a larger sample size, which would give better view of the structural, functional and interaction between FMRP protein with other proteins in serum towards gene therapy and drug development purposes.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGHASILAN KAEDAH-KAEDAH PENYARINGAN BERASASKAN TEKNIK TINDAKAN RANTAI POLYMERASE (PCR) BAGI PENENTUAN SINDROM FRAGILE X

Oleh

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Sindrom fragile X merupakan penyakit genetik kedua yang paling biasa berlaku dalam menyebabkan penyakit terencat akal. Ini adalah disebabkan oleh pemanjangan nukleotida CGG (sitosina-guanina-guanina) secara berterusan (mutasi sepenuhnya) yang menyebabkan ketidakaktifan gen FMR1 (Fragile X Mental Retardation 1) dan produk protein FMRP (Fragile X Mental Retardation Protein) berberat molekul 70-80 kDa tidak dihasilkan. Objektif-objektif utama kajian ini adalah untuk menghasilkan teknik-teknik penyaringan molekul yang cepat dan bukan radioaktif seperti Tindakan Rantai Polymerase (Polymerase Chain Reaction/ PCR) dan Southern blotting, dan juga untuk menyukat tahap pengekpresan protein-protein dalam serum individu normal dan saspek secara kualitif dan kuantitatif dengan menggunakan teknik analisis dua dimensi. Untuk menjalankan kajian ini, beberapa teknik telah digunakan termasuk PCR, Southern blotting, penjujukan DNA dan gel poliakrilamida dua dimensi (2D PAGE). Dalam kajian

ini, teknik-teknik PCR dan Southern blotting telah optimumkan dan digunakan semasa amplifikasi gen FMR1 subjek-subjek yang terlibat. Hampir kesemua individu-individu saspek menunjukkan fragmen-fragmen mutasi sepenuhnya bersaiz lebih dari 600 bp dan untuk peringkat pre-mutasi, fragmen-fragmen berberat molekul lebih dari 500 bp diamplifikasikan. Hanya beberapa individu saspek yang menunjukkan amplifikasi fragmen normal (152 bp hingga 265 bp). Ini mungkin disebabkan oleh struktur DNA alternatif terbentuk dari pemanjangan nukleotida CGG atau jenis-jenis mutasi lain seperti titik mutasi, delesi atau penyakit-penyakit disregulasi yang lain, di mana ianya menghalang amplifikasi gen daripada berlaku. Produk PCR juga dijujukkan bagi mempastikan data jujukan dan saiznya. Fragmen daripada individu normal menunjukkan saiz yang diperolehi adalah seperti dijangkakan (~ 130 bp) manakala individu positif menghasilkan paten jujukan yang tidak jelas. Templat berGC tinggi selalunya akan membentuk struktur-struktur kedua yang akan menghalang gen FMR1 diamplifikasikan dan ini mungkin merupakan salah satu daripada sebab-sebab mengapa produk PCR tidak dapat dihasilkan dengan baik dan menjejaskan eksperimen ini. Dalam kajian 2D PAGE, peta-peta pengekspresan protein daripada inidividu-individu sihat telah dibandingkan dengan peta-peta daripada saspek sindrom fragile X menggunakan analisis perisian lembut PDQuest™ . Analisis PDQuest™ telah mengesan 75% protein-protein yang sama antara serum individu normal dan saspek yang mungkin merupakan protein-protein yang terpelihara secara abadi dalam setiap individu manusia. Terdapat dua spot protein sasaran (kompleks FMRP) dan empat lagi spot-spot protein (Ig α light chain, Haptoglobin cleaved β chain, IgG γ intermediate chain dan IgG heavy chain) ditemui dalam sukatan 5 kali ganda regulasi rendah dalam saspek sindrom fragile X. Terdapat juga dua spot

protein (protein albumin) yang terlebih pengekspresannya dalam saspek sindrom fragile X. Keputusan-keputusan yang diperolehi ini menunjukkan bahawa kejadian sindrom fragile X mungkin mempengaruhi perubahan-perubahan pada tahap protein (samada secara langsung atau tidak langsung) yang akan berlaku pada keadaan tertentu sahaja. Walaubagaimanapun, kajian ini bergantung kepada pengukuran di dalam darah, yang mungkin tidak selalu refleks keadaan sebenar di dalam tisu-tisu, terutamanya tisu otak. Pemanjangan saiz-saiz atau tahap pengmetilan dalam tisu-tisu selain darah mungkin berbeza jika dibandingkan dengan sel-sel darah. Begitu juga pengekspresan FMRP mungkin berlainan di antara sel-sel darah dan sel-sel otak. Justeru itu, kajian-kajian selanjutnya harus dilaksanakan dengan menggunakan saiz sampel yang lebih besar, di mana akan memberikan pemerhatian yanng lebih baik terhadap struktur, fungsi dan interaksi di antara protein FMRP dengan protein-protein lain di dalam serum bagi tujuan penghasilan ubat-ubatan dan terapi gen.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to my main supervisor Associate Professor Dr. Rozita Rosli, co-supervisors, Professor Sazaly Abu Bakar and Associate Professor Dr. Mohamed Saifulaman Mohamed Said for their invaluable support and effort towards the success of my thesis.

I would also like to thank all the lab members and friends; Kak Shariza, Syahrilnizam, En. Roslaini, Kak Nurma, Suhaili, Wong, Lama, Nasir, Fei Ling, Michael, Pike See, Shaban, Patrick, Rohayu, Kak Wan, Ruzainah and Mohd. Farouk who always encouraged me, contributed their ideas and helped me to complete this research study successfully.

My deepest appreciation and thanks goes to my beloved family; my parents, my husband Yusri, sisters, brother and the most unforgettable my children, Nur Aqilah, Muhammad Hafiz and Muhammad Danial for their patience and sacrifices during my undertakings of completing this research.

Last but not least, loving thanks to my partners and good friends, Thilakavathy and Nazefah, who are always with me whenever I needed them.

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LIST OF ABBREVIATIONS

AGG	A nucleotide triplet of adenine – guanine – guanine
A_{260}	Absorbance at 260 nanometer
A_{280}	Absorbance at 280 nanometer
BD	Becton Dickinson
bp	base pair
CpG island	The region characterized by a high density of cytidine phosphate Guanosine dinucleotides and belongs to the class of regulatory sequences.
CNS	Central Nervous System
CGG	A nucleotide triplet of cytosine – guanine – guanine
CVS	Chorionic villus sampling
dGTP	Deoxyguanine triphosphate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
FMR1	Symbol for 'fragile X syndrome' gene (Fragile Mental Retardation 1)
FMR2	Symbol for gene associated with FRAXE (Fragile Mental Retardation 2)
FMRP	Protein from FMR1 gene (Fragile Mental Retardation Protein)
FRAXA	Chromosomal fragile site at Xq 27.3 that corresponds to the CGG repeat expansion of the FMR1 gene
GC	A nucleotide duplet of guanine – cytosine
G-G	Guanine-guanine
C-C	Cytosine-cytosine
HKL	Hospital Kuala Lumpur
IQ	Intelligent quotient

IEF	An electrophoretic method for the separation of proteins
kb	kilobase pair
kDa	kiloDalton unit
KH	A sequences of amino acids found in proteins involved in RNA binding
mM	millimolar unit
mg/ml	milligram per millilitter
MR	Mental retardation
Mg^{2+}	Magnesium ion
MW	Molecular weight
mRNA	messenger ribonucleic acid
NES	A nuclear export signal
NLS	A nuclear localization signal
NTM	Normal transmitting male
ng/µl	nanogram per microlitter
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
POF	Premature Ovarian Failure
pI	Isoelectric point
PCRx	PCR enhancer solution (INVITROGEN, USA)
RGG	A sequence of three amino acids (arginine-arginine- glycine)
RFU	Relative fluorescent unit
RNA	Ribonucleic acid
RNP	A ribonucleotide particle
RM	Ringgit Malaysia
SDS	Sodium dodecyl sulphate

TBP	Tributylphosphine
2DE	Two – dimensional protein electrophoresis
UK	United Kingdom
USA	United States of America
UTR	Untranslated region
µl	microlitter
Xq	Region of the long arm of the X chromosome
X	Chromosome X
2D PAGE	Two-dimensional polyacrylamide gel electrophoresis