UNIVERSITI PUTRA MALAYSIA

SCREENING OF ARX, CDKL5 AND STXBP1 GENE MUTATIONS IN MALAYSIAN PAEDIATRIC PATIENTS WITH EARLY-ONSET EPILEPTIC ENCEPHALOPATHY BY HRM TECHNIQUE

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By

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SCREENING OF *ARX*, *CDKL5* AND *STXBP1* GENE MUTATIONS IN MALAYSIAN PAEDIATRIC PATIENTS WITH EARLY-ONSET EPILEPTIC ENCEPHALOPATHY BY THE HRM TECHNIQUE

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Gene mutation is one of the etiologies of early-onset infantile Epileptic Encephalopathy (EE), an age-dependant seizure in infants which leads to brain defects. Previous studies have shown that several genes namely, Aristaless-related homeobox (*ARX*), Cyclin-dependent kinase-like 5 (*CDKL5*) and Syntaxin-binding protein 1 (*STXBP1*) are responsible for the pathophysiology of the syndrome. This study aims to investigate the clinical association of Malaysian subjects with known and novel mutation as well as developing an efficient and effective genetic screening method. In this study, 3 mL blood was extracted from the consented patients and control. The DNA was extracted (QiAamp blood mini kit, Qiagen), quantified on purity (ND-1000 spectrophotometer, NanoDrop Technologies, Inc.) and integrity (0.8% gel electrophoresis) and screened using High Resolution Melting Analysis (QiAamp Type-It HRM kit, Qiagen).Peripheral blood was obtained and genomic DNA was purified (DNA purification Kit, Intron Biotechnology). A total of 11 primer pairs were designed flanking 13 known mutations in all genes of interest. *ARX* exonic region was also screened for known and novel mutation. PCR specificity and efficiency were optimized using conventional PCR, Quantitative Polymerase Chain Reaction (qPCR) and High Resolution Melting Analysis (HRMA). Different melting profiles which distinguished homozygotes and heterozygotes based on the shape and temperature shifting were generated. Profiles were clustered and compared with homozygous wild type reference control. Samples from varying clusters were purified and subjected to direct DNA sequencing. All assays were successfully established. All known mutations reported previously were not found in Malaysian patients with 100% confirmation by sequencing results. A variant could be screened in duplicates of all samples within 3 hours. Subsequently, *ARX* exonic regions were also sequenced using published primers. No mutation was found in all patients samples. Nevertheless, HRMA is a robust, simple, rapid, accurate, efficient and cost-effective tool of screening for early onset infantile EE patients gene mutation.

Abstrak tesis yang di kemukakan kepada Senat Universiti Putra Malaysia Sebagai memenuhi keperluan untuk Ijazah Sarjana Sains

PENYARINGAN GEN MUTASI *ARX, CDKL5* AND *STXBP1* PADA PESAKIT PEDIATRIK 'EARLY-ONSET EPILEPTIC ENCEPHALOPATHY' DI MALAYSIA MENGGUNAKAN TEKNIK HRM

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Mutasi gen merupakan salah satu etiologi '*early-onset Epileptic Encephalopathy*' (EE), sejenis penyakit sawan mengikut peringkat umur bayi yang boleh membawa kepada kecacatan otak. Kajian telah menunjukkan bahawa beberapa gen iaitu '*Aristaless-related homeobox*' (*ARX*), '*Cyclin-dependent kinase-like 5'* (*CDKL5*) dan '*Syntaxin-binding protein 1*' (*STXBP1*) adalah berkaitlangsung dengan patofisiologi sindrom tersebut. Kajian ini dijalankan untuk mengkaji hubungan antara faktor klinikal pesakit EE di Malaysia dengan mutasi yang telah dikenalpasti serta yang baharu selain mencipta kaedah diidentifikasi genetik yang cekap dan berkesan. Dalam kajian ini, 3 mL darah telah diambil daripada pesakit dan subjek normal. DNA diekstrak (QiAamp blood mini kit, Qiagen), disaring bagi tujuan pengaslian (ND-100 0 spectrophotometer, NanoDrop Technologies, Inc.) dan integriti (0.8 % gel elektroforesis) dan didentifikasi dengan menggunakan High Resolution Melting Analysis (QiAamp Type-It HRM kit, Qiagen). Darah peripheral diambil dan DNA genomic diidentifikasi (DNA purification Kit, Intron Biotechnology). 11 set primer telah dihasilkan merangkumi 13 mutasi dari gen-gen yang dikaji. Exon *ARX* juga dikaji untuk mutasi yang telah dikenalpasti dan juga yang baharu. PCR *'specficity'*dan*'efficiency'* telah dioptimumkan menggunakan kaedah konvensional PCR, '*Quantitative Polymerase Chain Reaction*' (qPCR) dan '*High Resolution Melting Analysis*' (HRMA). Profil yang pelbagai dapat membezakan homozygotes dan heterozigot berdasarkan bentuk dan suhu peralihan yang telah dijana. Kumpulan-kumpulan Profil telah dibandingkan dengan homozigot kawalan sebagai rujukan. Sampel dari kelompok yang berbeza-beza telah disaring dan dikenalpasti melalui kaedah '*DNA sequencing'*. Semua esei telah berjaya dioptimumkan. Mutasi-mutasi yang pernah dilaporkan sebelum ini tidak terdapat pada pesakit EE di Malaysia dibuktikan 100% oleh keputusan '*jujukan DNA*'. Varian A boleh diproses dalam semua sampel dalam masa 3 jam. Selain itu, exon *ARX* juga dikaji menggunakan primer yang telah dioptimumkan pada kajian yang lepas. Tiada mutasi didapati dalam semua sampel pesakit. Walaubagaimanapun, HRMA adalah alat identifikasi mutasi yang teguh, mudah, cepat, tepat, cekap dan kos efektif bagi mengesan mutasi gen pada pesakit EE.

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

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LIST OF ABBREVIATIONS

1X	One time
6X	Six times
AD	Autosomal dominant
ARX	Aristaless-related homeobox
bp	Base pair
CDKL5	Cyclin-dependent Kinase-Like 5
CI	Confidence interval
dbSNP	Single Nucleotide Polymorphism Database
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotidephosphates
EDTA	Ethylenediaminetetraacetic acid
EE	Epileptic encephalopathy
HRMA	High Resolution Melting Analysis
LOVD	Leiden Open Variants Database
m	Miligram
M	Molar
MOH	Ministry of Health
MREC	Medical Research Ethics Committee
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCBI	National Contre of Biotechnology Information
ng	Nanogram
OMIM	Online Mendelian Inheritance in Man
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RPM	Revolutions per minute
rs	Reference SNP number
SNP	Single Nucleotide Polymorphism
STXBP1	Syntaxin binding protein 1
Ta	Annealing temperature
TAE	Tris-acetate-EDTA
Tm	Melting temperature
UPM	Universiti Putra Malaysia
UTR	Untranslated region
UV	Ultraviolet
V	Volt
w/v	Weight/ volume
XLD	X-linked dominant
XLR	X-linked recessive
µL	Microlitre

Gene mutation is one of the etiologies of early-onset infantile Epileptic Encephalopathy (EE), an age-dependant seizure in infants which leads to brain defects. Previous studies have shown that several genes namely, Aristaless-related homeobox (*ARX*), Cyclin-dependent kinase-like 5 (*CDKL5*) and Syntaxin-binding protein 1 (*STXBP1*) are responsible for the pathophysiology of the syndrome. This study aims to investigate the clinical association of Malaysian subjects with known and novel mutation as well as developing an efficient and effective genetic screening method. In this study, 3 mL blood was extracted from the consented patients and control. The DNA was extracted (QiAamp blood mini kit, Qiagen), quantified on purity (ND-1000 spectrophotometer, NanoDrop Technologies, Inc.) and integrity (0.8% gel electrophoresis) and screened using High Resolution Melting Analysis (QiAamp Type-It HRM kit, Qiagen).Peripheral blood was obtained and genomic DNA was purified (DNA purification Kit, Intron Biotechnology). A total of 11 primer pairs were designed flanking 13 known mutations in all genes of interest. *ARX* exonic region was also screened for known and novel mutation. PCR specificity and efficiency were optimized using conventional PCR, Quantitative Polymerase Chain Reaction (qPCR) and High Resolution Melting Analysis (HRMA). Different melting profiles which distinguished homozygotes and heterozygotes based on the shape and temperature shifting were generated. Profiles were clustered and compared with homozygous wild type reference control. Samples from varying clusters were purified and subjected to direct DNA sequencing. All assays were successfully established. All known mutations reported previously were not found in Malaysian patients with 100% confirmation by sequencing results. A variant could be screened in duplicates of all samples within 3 hours. Subsequently, *ARX* exonic regions were also sequenced using published primers. No mutation was found in all patients samples. Nevertheless, HRMA is a robust, simple, rapid, accurate, efficient and cost-effective tool of screening for early onset infantile EE patients gene mutation.

CHAPTER 1

INTRODUCTION

Early onset infantile epileptic encephalopathy (EE) is a subgroup of infantile epilepsy affecting infants at the age of onset typically less than a year of life. The seizures are very frequent and severe leading to progressive loss of cerebral function (Noh *et al.*, 2012). The diagnosis of early onset infantile EE involves clinical examination and electroencephalogram (EEG) to determine the presence of the syndrome. Apart from electro-clinical data, molecular investigation has also been used recently. With the advancement of molecular tools, genetic abnormalities become a key determinant of idiopathic early onset EE, a type of infantile epilepsy arising from unknown causes (Tavyev *et al.*, 2012). Other than idiopathic, there is also symptomatic early onset infantile EE such as hypoxic ischemic encephalopathy, infection, metabolic abnormalities and brain structural defects (Alam *et al.*, 2012).

To improve the condition of Malaysian early onset infantile EE patients from genetic aspect, screening of known causative mutation is practically helpful and providing new insights to other novel approaches. In this study, three genes of interest namely Aristaless-related homeobox (*ARX*), Cyclin-dependent kinase-like 5 (*CDKL5*) and Syntaxin binding protein 1 (*STXBP1*) genes were selected among other genes which are frequently associated with early onset infantile EE patients. *ARX* and *CDKL5* are well-known X-linked genes beneficial for further familial studies while *STXBP1* are being widely investigated as a new gene on chromosome 9 increasingly affecting epileptic patients worldwide (Saitsu *et al.*, 2008). The association of early onset infantile EE with an underlying genetic background has drawn a great concern among health practitioners. Multiple genes were unveiled from genomic level up to the protein and biological function related to patient's clinical phenotype. Despite the increasing information on genetic impairment, low sample size for population-based study would still require the significant global involvement. Nevertheless, studies from different population will be an added value to the existing data.

It has been reported that specific causative mutation at specific site may produce a degree of severity thus assisting the health practitioner to make a correct diagnosis and come out with a proper treatment at different epileptic stage. The identification of the causative mutation may serve as a predictive or a diagnostic testing that increases the understanding of varying aetiologies, omits the unnecessary assessment, and reduces the need of invasive investigations. It may also decrease the expensive cost, provide early prediction of the outcome that assists the prescription of anti-epileptic drugs and enables effective patients counselling (Alliance, 2010). Furthermore, on the other side, it may ease the psychological burden of the relatives that may have attributed other non-evidence based as causes thus lead to poor management of their child. The presence of causative mutation indicates that the patients has positive early onset EE which gives a confirmation to the family members. Necessary precautions can also be taken while dealing with patients' serious conditions.

To delineate the role and mechanism of the causative mutation, in particular, single nucleotide polymorphism (SNPs), the genetic background of early onset infantile EE

patients has to be characterized. A small scale study is essential to test the effect size that determines the importance of large population-based study in the future. Using a small sample size of Malaysian idiopathic early onset infantile EE patients, the hypothesis on the presence of the known mutations among patients' samples shall be a great benefit in epilepsy research in Malaysia. The objective is to screen known causative mutations that have been reported previously in Malaysian early onset infantile EE patients using High Resolution Melting Analysis (HRMA) as well as assessing clinical manifestation of the patients.

Meanwhile, the development of screening method facilitates the detection of gene mutation worldwide. However, the current methods such as direct sequencing, Restriction Fragment Length Polymorphism (RFLP), single strand chain polymorphism (SSCP) to screen the genes are considered laborious, expensive and impractical for a routine test. High resolution melting analysis (HRMA) is rapid to effectively and efficiently discriminate patient genetic profiles. Based on the temperature, differences in amino acid sequence can be distinguished accurately (Reed *et al.*, 2007). Although sequencing is a gold standard in mutation detection, due to its simplicity and reasonable cost, HRMA is more practical as a routine diagnostic method in lab-based setting making the daily patient diagnosis possible in a small clinical setting.

In general, this study focuses in identifying the presence of known causative mutation reported previously in Malaysian early onset infantile EE patients while finding the novel mutation in one of the gene of interest, *ARX*. The genetic information was compared with clinical data to derive as possible association. A rapid genetic screening method using a simple approach was also developed to serve as a routine screening diagnostic tool in a clinical setting in the future.

The main objective is to investigate the association of the *ARX*, *CDKL5* and *STXBP1* gene mutation in paediatric patients with early onset infantile epileptic encephalopathy. In specific, this study aims:

1. To screen *ARX*, *CDKL5* and *STXBP1* known gene mutations in Malaysian early onset infantile EE patients.
2. To investigate the frequency of *ARX*, *CDKL5* and *STXBP1* gene mutations among Malaysian early onset infantile EE patients.
3. To assess the full clinical feature of patients with early onset infantile EE associated with *ARX*, *CDKL5* and *STXBP1* gene mutations.

It is expected that the current study will give a better understanding on the effect of pathogenic mutations in *ARX*, *CDKL5* and *STXBP1* genes towards Malaysian early onset infantile EE patients as well as establishing a simple, rapid and efficient genetic screening method. It is also hoped that the study may assist the patient's diagnosis more effectively and efficiently.

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