UNIVERSITI PUTRA MALAYSIA

GENETIC POLYMORPHISMS OF ENDOTHELIAL MARKER, CONNEXIN AND ACYL-COA GENES AMONG ETHNIC MALAY SUBJECTS WITH ESSENTIAL HYPERTENSION

ELNAZ SALIM

FPSK(m) 2020 2

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By

ELNAZ SALIM

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

January 2020

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DEDICATION

Dedicated to My Beloved Parents

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

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ELNAZ SALIM

January 2020

Chairman : Professor Patimah Ismail, PhD
Faculty : Medicine and Health Sciences

Hypertension is a complex multifactorial disorder that is thought to result from an interaction between an individual's genetic background and various lifestyle also environmental factors. Essential hypertension (EH) are majority of hypertensive cases and diagnosed where there is no clear evidence of medical condition predisposing to the high blood pressure. There have been variety of genetic studies is relation to hypertension and some of them showed association with occurrence of hypertension. Family of Endotheline1 (EDN-1) and Endothelin converting enzyme (ECE) vasoconstricting peptide produced by vascular endothelial. Recent studies proposed that there was significant difference between EDN-1 lys198Asn(rs5370) and ECE (rs212526) with essential hypertension. As well as, Connexin gene family (Cxs) belong to the large family of gap junction that are involved in many physiological disorders like that hypertension. Cx37(C1019T) and Cx40+71 A/G were candidate gene for this study. Moreover, Acyl-CoA synthetase medium-chain family member3(rs886433) also another gene was determined in this study. The main objective of this study was to determine the candidate gene polymorphism and gene expression involved in essential hypertension among Malay subjects. Since, there have been variety of genetic association studies of EDN1, ECE, Cx37-40 and ACMS3 conducted on different population, however no study was done on Malaysia populations and in relation to hypertension. Genetic polymorphism and gene expression are serve as molecular biomarkers for the detection of the individual at risk of developing the disease. This association study included 177 of subjects without EH as control and 97 of subjects with EH as case. Extraction genomic DNA was done all subjects. Cx40 and Cx37 gene polymorphism was detected using Polymerase Chain reaction (PCR) followed by Restriction Fragment Length Polymorphism (PCR-RFLP). The PCR products were digested with EcorI and DrdI restriction enzyme at 65°c for 20 min. the RFLP products were detected using 3% agarose gel electrophoresis. ACMS3, EDN1 and ECE gene were detected by real time PCR (RT-

qPCR) with Taq-Man probes. Genotype and allele frequencies in case and control samples were compared by using Chi-Square test while clinical characteristic parameters and social-demographic background was analyzed using descriptive static. Furthermore, in this study, the expression profile of Acyl CoA enzyme (ACMS3) which involved in human fatty acid biosynthesis was analyzed. Real time PCR was carried out using RNA extracted from 50 cases as well as 50 control subjects. Relative analyzed was used to determined level of gene expression. The demographic characteristic of the subjects including age, body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP),low density lipoprotein(LDL), triglyceride (TG) and Cholesterol level were shown to be differentially significant ($p<0.05$) in the case subject when compared to control groups. HDL did not show any significance. The results of this study were show that the genotypes and allele frequencies of C1019T Cx37, +71Cx40 A/G, ET-1 rs5370 G/T and ACMS3 A/G gene were highly significant in hypertensive subjects as compared to the healthy ($p<0.05$). while SNP of ECE gene C/T did not significantly ($P>0.05$) when compared to control group. In gene expression demonstrated significant differences between expression of ACMS3gene at the mRNA level in patients with hypertension compare with control group. Hence, this candidate genes as possible genetic biomarker and risk factor for EH in Malay subjects.

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

POLIMORFISME GEN CONNEXIN, ENDOTHELIUM, DAN ACYL-COA DALAM SUBJEK BERHIPERTENSI PRIMER DIKALANGAN ETNIK MELAYU

Oleh

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Januari 2020

Pengerusi : Profesor Patimah Ismail, PhD
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Hipertensi adalah kondisi kompleks yang disebabkan oleh pelbagai faktor termasuk juga kemungkinan interaksi antara genetik profil individual dengan gaya hidup dan faktor persekitaran. Hipertensi primer (EH) adalah penyebab majoriti kes hipertensi dan kebiasaannya di diagnosis apabila tidak menjumpai masalah perubatan yang terdedah kepada tekanan darah tinggi. Terdapat pelbagai kajian genetik berkaitan dengan hipertensi dan ada di antaranya yang menunjukkan kaitan dengan terjadinya hipertensi. Kumpulan *Endotheline-1* (EDN-1) dan enzim penukaran Endothelin (ECE) vasoconstricting peptida dihasilkan oleh endothelial vaskular. Kajian terkini mencadangkan terdapat perbezaan signifikan di antara EDN-1 lys198Asn (rs5370) dan ECE (rs212526) dengan hipertensi primer. Selain itu, gen Connexin (Csx) tergolong dalam persimpangan jurang yang terlibat dalam pelbagai gangguan fisiologi seperti hipertensi. Cx37 (C1019T) dan Cx40 + 71 A / G adalah calon gen untuk kajian ini. Tambahan lagi, gen *Acyl-CoA synthetase medium-chain family member3* (rs886433) juga ditentukan dalam kajian ini. Objektif kajian ini adalah untuk mengenal pasti polimorfisme gen dan pengekspresan gen yang berkaitan dengan hipertensi primer di kalangan subjek Melayu. Terdapat pelbagai kajian berkaitan genetik EDN1, ECE, Cx37-40 dan ACMS3 yang dijalankan dalam populasi lain, namun tiada kajian yang berkaitan dengan hipertensi dilakukan terhadap populasi Malaysia. Polimorfisme genetik dan ekspresi gen berfungsi sebagai biomarker molekul untuk mengesan individu yang berisiko tinggi terhadap sesuatu penyakit. Kajian ini melibatkan 177 subjek tanpa EH sebagai kawalan dan 97 subjek dengan EH sebagai kes. Pengekstrekan DNA genom dilakukan pada semua subjek. Polimorfisme gen Cx40 dan Cx37 dikesan menggunakan tindak balas Rantaian Polimerase (PCR) diikuti dengan Pembatasan Panjang Fragmen Polimorfisme (PCR-RFLP). Kemudian, produk PCR dicerna dengan enzim EkorI dan DrdI pada suhu 65 ° c selama 20 minit. Produk RFLP seterusnya dianalisis menggunakan elektroforesis 3% gel agarose. Gen ACMS3, EDN1 dan ECE dianalisis menggunakan PCR masa sebenar (RT-qPCR) dan prob Taq-Man. Frekuensi genotip dan alel dalam sampel kes dan kawalan

dibandingkan dengan menggunakan ujian Chi-Square manakala parameter klinikal dan latar belakang sosio-demografi dianalisis dengan menggunakan statik deskriptif. Tambahan pula, dalam kajian ini, pengekpresan profil enzim Acyl CoA (ACMS3) yang terlibat dalam biosintesis asid lemak manusia juga dianalisis. PCR masa sebenar dijalankan menggunakan RNA yang diekstrak daripada 50 kes serta 50 subjek kawalan. Analisis relatif digunakan untuk menentukan tahap ekspresi gen. Terdapat perbezaan signifikan ($p<0.05$) terhadap ciri-ciri demografi subjek termasuk umur, indeks jisim badan (BMI), tekanan darah sistolik (SBP), tekanan darah diastolik (DBP), lipoprotein ketumpatan rendah (LDL), trigliserida (TG) dan tahap Kolesterol apabila dibandingkan antara subjek kes dengan kawalan. HDL tidak menunjukkan sebarang signifikan. Keputusan kajian ini menunjukkan bahawa frekuensi genotip dan alel C1019T Cx37, + 71Cx40 A / G, ET-1 rs5370 G / T, ACMS3 A / G sangat signifikan dalam subjek hipertensi berbanding dengan subjek yang sihat ($p < 0.05$). Manakala polimorfisme gen ECE C / T tidak signifikan ($P> 0.05$) berbanding dengan kumpulan kawalan. Ekspresi gen pada tahap mRNA menunjukkan perbezaan yang signifikan antara ekspresi gen ACMS3 dikalangan pesakit hipertensi berbanding dengan kumpulan kawalan. Oleh itu, terdapat kemungkinan calon-calon gen dalam kajian ini boleh menjadi biomarker genetik dan faktor risiko untuk EH dikalangan subjek Melayu.

ACKNOWLEDGEMENTS

"In the name of Allah S.W.T, the most Benevolent and Most Merciful"

Beauty and Admire to God, The Omnipotent, Omniscient and Omnipresent, for opening doors of opportunity for me throughout my life as well as giving me the strength and health to achieve my goal in this stage of life.

In the preparation of this project, I gained a lot of help from various people. Initially, I would like to express my sincere gratitude and appreciations to my mother and father who advised and supported me emotionally, mentally also financially to the pursuit of higher education and academic and compassionate friend throughout my life and I believed that the journey of life would have been impossible without your love and kindness. Words cannot express my gratitude for their love, support and patience during my life and study. Thank you and I shall never forget your sacrifice and support.

I would like to extend my heartfelt gratitude to my honoured supervisor, Prof. Dr. Patimah Ismail as the chairman of my supervisory committee, for her precious advices and invaluable guidance toward the period of study. She is one of admirable person in my life. Her immense knowledge, dedication and integrity has motivated all of students.

In addition, I would like to express my deepest gratefulness to my co-supervisor, Dr.Ramachandran Vasudevan, who patiently supported also encouraged me with his invaluable guidance during the research.

Besides that, I want to express my appreciation to all staffs of Molecular lab for kindness, cooperation and understanding during my lab work.
Nevertheless, I would like to extend my appreciation and gratefulness to my friend's meisam parsapour, also my partner neda Ansari for being supportive friends. Last but not least, I would like to thanks to my sister in low and brother for making the house environment peaceful and organized.

I certify that a Thesis Examination Committee has met on 20 January 2020 to conduct the final examination of Elnaz Salim on her thesis entitled "Genetic Polymorphisms of Endothelial Marker, Connexin and Acyl-CoA Genes among Ethnic Malay Subjects with Essential Hypertension" 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 Master of Science.

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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 ommittee are as follows:

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- the research conducted and the writing of this thesis was under our supervision;
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Name of Member of Supervisory Committee: Dr. R.Vasudevan

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LIST OF ABBREVIATIONS

ACMS	acetyl-Co-enzyme metabolism synthase
BMI	Body Mass Index
BP	Blood Pressure
bp	base pair
Chol	Cholesterol
C_t	Cycle Threshold for Real-Time PCR Analysis
DBP	Diastolic Blood Pressure
ECE	endothelin converting enzyme
ET-1	endothelin
EH	Essential Hypertension
FPG	Fasting Plasma Glucose
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
HDL	High Density Lipoprotein
HET	Heterozygous
HOM	Homozygous
LDL	Low Density Lipoprotein
Mm	Hg millimetre of mercury
NCBI	National Centre for Biotechnology Information
OR	Odd Ratio
PCR	Polymerase Chain Reaction
PCR-RFLP	PCR-Restriction Fragment Length Polymorphism
RT- qPCR	Reverse Transcript- quantitative PCR -Real-Time PCR
RE	Restriction Enzyme

RNA	Ribonucleic Acid
SBP	Systolic Blood Pressure
SNP	Single Nucleotide Polymorphism
TG	Triglyceride
Tm	Melting Temperature
WT	Wild Type
WHR	Waist Hip Ratio

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Hypertension is a major public health problem throughout the world as well as it is one of the most common risk factors for cardiovascular disease (CVD), stroke and renal disease due to the elevated levels of both systolic and diastolic blood pressure (Kjeldsen, 2018). The movement of blood against the walls of the arteries causes what is called blood pressure. If that force exceeds 140mm/90 mm Hg, hypertension is specified (The Joint National Committee 7, 2004). Hypertension is an asymptomatic disease of the circulatory system with levels above agreed normal values of blood pressure (BP). Variability of blood pressure is seen as a strong risk factor for cardiovascular problems and strokes (Webb *et al*., 2010; Muntner *et al*., 2015).

Hypertension can be classified as either essential hypertension (EH) or secondary hypertension. Essential hypertension makes up to 95% of the cases of hypertension and is diagnosed where there is no clear evidence of medical conditions predisposing to the high BP (Chern and Chiang *et al*., 2004). Furthermore, EH is referred to as a complex genetic trait caused by multiple genes and the polygenic effects are controlled by gene-gene and gene-environment interactions (Chern and Chiang *et al*., 2004). Whereas secondary hypertension can be caused by the medical conditions such as renal parenchymal disease, renal artery stenosis, hyperaldosteronism, or pheochromocytoma (Grossman *et al*., 2012).

Hypertension is a multifactorial disease involving interactions among genetic, environmental factors such as dietary, obesity, salt intake, alcohol consumption, and stress, demographic, vascular and neuroendocrine factors. Even though it is simple to diagnose and usually can be controlled by healthy diet, regular exercise, medication prescribed by doctors or a combination of these, untreated hypertension will cause serious condition (Campbell *et al*., 2006) as hypertension is does not usually cause any symptoms or signs and hence it named as 'silent killer' (Stamler *et al*., 1989).

Although there are several risk factors were noticed for the development of hypertension, genetically the cause of hypertension is quite complex as there is with no known single gene playing a major role, but rather many genes each with mild effects reacting to different environmental stimuli contribute to blood pressure (Kraja *et al*., 2011). There are so many pathways and genes were involved in the BP variation, vascular endothelial dysfunction (Widlansky et al., 2003). Vascular endothelial dysfunction is characterized by a pro-thrombotic, pro-inflammatory, and pro-constrictive phenotype are involved in altering the endovascular lining of blood vessels (Dharmashankar and Widlansky, 2010).

Endothelin is a chemical that occurs naturally in the human body which is produced by the endothelial cells. Normally, endothelin involved in regular blood flow. High level of endothelin than normal lead to vasoconstriction of blood vessels (Goraca, 2002). The changes can cause the difficulty for blood to flow through the blood vessels. Endothelin-1 (ET1) and endothelin-converting enzyme (ECE) genes which acts as potent vasoconstrictor peptide. The ET1 and ECE have been implicated in the development of hypertension due to vasoconstrictive and hypertrophic actions (Buhler *et al*., 2007).

Acyl-CoA synthetases form a large family of enzymes that catalyse the activation of fatty acids by coenzyme A to produce acyl-CoA, the first step in fatty acid metabolism. Therefore, acyl-CoA plays an important role in the intracellular signalling and contribute to the regulation of cell metabolism (Iwai *et al*., 2002).

Connexins (Cx) are a family of gap junction-forming proteins, which are widely expressed throughout the body. Hexamer of Cx protein subunits, forms a gap junction on the cell membrane and acts as a communication channel for rapid exchange of intercellular metabolites, small water-soluble molecules, inorganic ions, and electrical signals. Up-regulation of Cxs expression in vascular tissues may result in an increase in vascular activity that is linked to hypertension (Severs *et al*., 2001).

There are several genome-wide association studies (GWAS) were published on putative genes related to hypertension (Levy *et al*., 2009). The GWAS approach has been exceptionally successful in identifying the common genetic variants that are predisposed to a variety of complex human diseases, biochemical and anthropometric traits (Corvin *et al*., 2009). The most stable variation of the genome occurs in the form of SNPs which makes up to 90% of the common variations in the genome. Analysing SNPs for the identification of loci associated with complex diseases are common in susceptibility to hypertension and other disorders (Levy *et al*., 2009). The SNPs association and candidate gene studies have revealed promising results in the genetic studies of complex diseases particularly in hypertension (Yagil 2009).

Gene expression analysis is important in transcribing synthesize functional gene products such as RNA species or protein. This method can be broadly divided into four areas: RNA expression, promoter analysis, protein expression, and post-translational modification. Levels of mRNA are quantitated by reverse transcription of the RNA to cDNA followed by quantitative PCR (qPCR) on the cDNA. The amount of each specific target is determined by measuring the increase in fluorescence signal from DNA-binding dyes or probes during successive rounds of enzyme-mediated amplification. This precise, versatile tool is used to investigate mutations (including insertions, deletions, and SNPs) (Alberts *et al*., 2002) related to various disorders.

1.2 Problem Statement

Hypertension is a global public health issue. It contributes to the burden of heart disease, stroke, kidney failure, premature mortality and disability. It is considered as a polygenic disease and results from multiple gene-gene and gene environment interaction. Hypertension is affecting almost 40% of adult aged more than 25 years old and the number of people with the condition is approximately 1 billion. It is estimated that the number will increase to 1.56 billion adults by 2025 (Omar *et al*., 2016).

Malaysia, a developing country in the Southeast Asian Region with an upper income level has a multiracial and multi-ethnic population of 30.07 million spread over 13 states and 3 federal territories. A national health morbidity survey (NHMS) conducted in 2011 among adults aged ≥18 years reported that the overall prevalence of hypertension was 32.7 % and the treatment rate of those who were aware was 78.4 % (Abdul-Razak *et al*., 2016).

Genetic predisposition may play a role in the earlier progression of EH. The most common genes that were associated in EH susceptibility is EDN1, ECE, CX37, 40 and ACMS3 genes by different pathway. Recent evidence indicates that gap junctions may play a major role in the initial pathogenesis and subsequent clinical manifestations of human cardiovascular disease, including BP (Haefiger *et al*.,2004). Gap junctions in vascular wall cells are thought to play a critical role in coordinating vasomotor responses and in regulating vascular tone (Hill *et al*.,2002). In addition, gap junctions were involved in regulating renin release in the kidney (Ryan *et al*.,2003). The endothelin (EDN) system consists of 4 active EDNs, with EDN1 being the predominant cardiovascular isoform (Yanagisawa *et al*.,1988). Several studies have described that this polymorphism missense mutation Lys198Asn has been identified in preproEDN1 and showed a positive association elevation with blood pressure in obese people (Tiret *et al*.,1999). The gene ACMS3 (SAH) has been identified as a candidate gene that could induce EH by differential screening from a genetically hypertensive rat strain. It has recently been documented that the SA protein is highly homologous to bovine xenobiotic – metabolizing medium-chain fatty acid: CoA ligase (Iwai *et al*., 2002). Study of several F2 rat cohorts then development of several congenic rat strains verified that the locus of the ACMS3 gene contributes to the regulation of blood pressure in rats. This is therefore a candidate gene for human development that is important for hypertension (Frantz *et al*., 1998). There is genetic diversity between different ethnic groups, as well as the fact that the relationship of one population could not be extrapolated to another. Hence, determining the frequencies of genetic variants of EDN1, ECE, CX37, 40 and ACMS3 genes among Malay ethnics are much important to know the genetic susceptibility of hypertension.

1.3 Significant of study

The candidate gene analysis would provide a better approach to determining the genotypical and phenotypical frequencies and their possible associations (Tabor *et al*., 2002). The use of intermediate phenotypes and the detailed mapping of candidate genes would provide a better approach to identifying associations between genotype and phenotype, which could enable the use of genotypes to identify more precise therapeutic and preventive measures for hypertensives (Agarwal *et al*., 2005).

BP is a complex genetic disorder arising from the interplay of numerous risk genes and environmental factors. Around 30 percent of genetic heritage was registered. Several studies have suggested that, in various populations, the candidate genes are vulnerable to hypertension. However, there are controversies in the results obtained from those genetic studies because, majority of those studies did not conclude any possible interactions between candidate genes, association or phenotype. Hence, more studies using different population are much needed to provide more information on genetic susceptibility of EH (Agarwal *et al*., 2005). Additionally, to our knowledge there are lack of studies on association of ET1, ECE, Cx37, Cx40 and ACMS3 gene polymorphism on hypertension in Malay ethnics among Malaysians. This study was initiated to identify the genetic make-up differences between case and control subjects. Besides that, this study can look at the possible gene-gene interactions that may provide the information and understanding on the pathophysiology of the hypertension among Malay ethnics.

Study of genetic association is to check whether an allele or genotype frequency varies between case and control groups, as well as to analyse the statistical correlation between the genotype of an individual and their phenotype or disease. Genetic association studies are the most common method for determining the genotypical frequencies of the genetic variants in case-control studies for the different disorders (Lewis, 2002). Gene expression analysis can be used as a useful approach to find out interaction between gene and organism. In this study, gene expression analysis was applied using RT-qPCR to determine the level of ACMS3 gene expressed in kindly.

1.4 Hypothesis

Gene polymorphisms of the Cx37, Cx40, ET1, ECE and ACMS3 may be associated with the development of hypertension among hypertensive subjects in Malay ethnics.

1.5 Main objective

To elucidate the association between the gene variants of EDN1, ECE, Cx37, Cx40 genes involved in hypertension susceptibility among Malay hypertensive subjects

1.6 Specific objective

- To elucidate the association the genotypic and allelic frequency for Cx37, C1019T and Cx40 -44G/A polymorphisms of Connexien gene in Malay ethnics.
- To compare the genotypic and allelic frequency for END1 Lys198Asn and ECE C/T polymorphisms of Endothelin gene in Malay ethnics.
- To elucidate the correlation the genotypic and allelic frequency for ACMS3 A/G polymorphisms of Acyl-CoA gene in Malay ethnics.
- To compare the association between genotypic, phenotypic and biochemical parameters among Malay ethnics.
- To determine the level of ACMS3 gene expression in Malaysian subjects versus healthy individuals.

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