UNIVERSITI PUTRA MALAYSIA

ANALYSIS OF GENETIC POLYMORPHISMS AS RISK FACTORS IN ESSENTIAL HYPERTENSION AND TYPE 2 DIABETES MELLITUS AMONG MALAYSIANS

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By

R.VASUDEVAN

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

May 2009

DEDICATION

Dedicated to My Family, Friends and Researchers

Abstract of thesis presented to the Senate of University Putra Malaysia
in fulfilment of the requirement for the degree of Doctor of Philosophy

ANALYSIS OF GENETIC POLYMORPHISMS AS RISK FACTORS IN ESSENTIAL HYPERTENSION AND TYPE 2 DIABETES MELLITUS AMONG MALAYSIANS

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May 2009

Chairman: **Professor Patimah Ismail, PhD**

Faculty: **Medicine and Health Sciences**

Genetic Polymorphisms are the variations found in the DNA sequences and they are integral to the development of genetic markers to identify individuals at risk. The genotypic distribution of various genetic polymorphisms involved in essential hypertension (EHT) and type 2 diabetes mellitus subjects (T2DM) has been established in many populations with conflicting results but in Malaysian subjects it has not been well characterized. The main objective of this study was to determine the association of various polymorphisms involved in the renin angiotensin-aldosterone system (RAAS), insulin receptor, lipoprotein lipase, interleukin gene and G protein ß 3 subunit genes in EHT and T2DM of Malaysian subjects. This cross-sectional study includes 70 EHT without T2DM, 60 T2DM, 65 EHT with T2DM and 75 unrelated healthy control subjects. Genomic DNA was extracted from the peripheral blood and the plasma was separated and analyzed for the biochemical analyses. The genotypes of the various genetic polymorphisms were determined using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), mutagenically PCR and the hot-start PCR methods. All PCR products

and the restricted fragments were resolved electrophoretically on agarose and polyacrylamide gels. Statistical analyses was done using SPPS version 14.0. The baseline characteristics such as age, body mass index, diastolic blood pressure, fasting plasma glucose, high density lipoprotein cholesterol and sodium differed significantly ($p<0.05$) in case subjects compared to control subjects but not in low density lipoprotein cholesterol, total cholesterol and potassium. The insertion/deletion (I/D) and G2350A polymorphism of the angiotensin converting enzyme (ACE) gene and the I/D polymorphism of the alpha adrenoceptor gene differed significantly in EHT and T2DM subjects, whereas the A6G variant of the angiotensinogen (AGT) gene differed significantly ($p<0.05$) in the EHT and EHT+T2DM subjects when compared to the control subjects but not in the T2DM subjects. However, the *BglI* and *MboI* polymorphisms of the renin gene, the A6244G polymorphism of the insulin receptor gene, the S477X polymorphism of the lipoprotein lipase gene, the C511T polymorphism of the interleukin gene and the C825T polymorphism of the G protein ß3 subunit genes did not differ significantly ($p>0.05$) when compared to the control subjects. Therefore, the polymorphisms of ACE, AGT and alpha adrenoceptor genes involved in RAAS were significantly associated with EHT and T2DM in Malaysian subjects. The genotypes and alleles of those polymorphisms can be considered as possible genetic markers or predisposing risk factors for EHT and T2DM in Malaysian subjects.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
Sebagai memenuhi keperluan untuk Doktor Falsafah

ANALISIS POLIMORFISME GENETIK SEBAGAI FAKTOR-FAKTOR BERISIKO DALAM HIPERTENSI PRIMER DAN KENCING MANIS JENIS KEDUA DI KALANGAN PENDUDUK MALAYSIA

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Polimorfisme genetik adalah variasi yang terdapat di dalam jujukan DNA dan penting bagi pembentukan penanda-penanda genetik untuk mengenalpasti individu-individu yang berisiko. Taburan genotip bagi pelbagai polimorfisme genetik yang terlibat dalam hipertensi primer dan kencing manis jenis kedua telah ditubuhkan melalui kajian ke atas pelbagai populasi dengan keputusan yang bertentangan tetapi pesakit-pesakit di Malaysia belum dicirikan sepenuhnya lagi. Objektif utama kajian ini adalah untuk menentukan hubungan pelbagai polimorfisme yang terlibat dalam sistem renin angiotensi-aldosteron (RAAS), reseptor insulin, lipoprotein lipase, gen interleukin dan gen protein G beta 3 sub-unit di dalam hipertensi primer dan kencing manis jenis kedua dalam pesakit di Malaysia. Kajian keratan-lintang ini melibatkan 70 orang pesakit hipertensi primer, 60 orang pesakit kencing manis jenis kedua, 65 orang pesakit hipertensi primer dengan kencing manis jenis kedua dan 75 orang yang sihat sebagai kawalan. DNA genomik telah diekstrak daripada darah dan plasma telah diasingkan bagi analisis biokimia. Genotip pelbagai polimorfisme genetik telah

ditentukan menggunakan kaedah tindak balas rantaian polimerase polimorfisme panjang jalur terpotong (PCR-RFLP), tindak balas rantaian polimerase mutagenic (MS-PCR) dan tindak balas rantaian polimerase Hot-Start (Hot-Start PCR). Semua produk tindak balas rantaian polimerase (PCR) dan fragmen potongan (RFLP) telah ditentukan menggunakan elektroforesis gel agarosa dan gel poliakrilamida. Semua analisa statistik telah dilakukan menggunakan SPSS versi 14.0. Ciri-ciri asas seperti umur, indeks jisim tubuh, tekanan darah diastol, plasma glukos puasa, kolesterol lipoprotein berketumpatan tinggi dan natrium adalah signifikan ($p<0.05$) manakala kolesterol lipoprotein berketumpatan rendah, jumlah kolesterol dan kalium adalah tidak signifikan ($p>0.05$). Polimorfisme penambahan/pengurangan G2350A bagi gen enzim penukaran angiotensin (ACE), polimorfisme penambahan/pengurangan bagi gen Alfa Adrenoreseptor dan polimorfisme A6G bagi gen Angiotensinogen adalah signifikan ($p<0.05$). Walau bagaimanapun, polimorfisme *BgII* dan *MboI* bagi gen renin, polimorfisme A6244G bagi gen insulin reseptor, polimorfisme S477X bagi gen lipoprotein lipase, polimorfisme C511T bagi gen interleukin dan polimorfisme C825T bagi gen protein G beta 3 sub-unit adalah tidak signifikan ($p>0.05$) apabila dibandingkan dengan normal. Oleh yang demikian , polimorfisme bagi gen ACE, AGT, dan Alfa Adrenoreseptor yang terlibat dalam sistem renin angiotensin-aldosteron mempunyai hubungan dengan pesakit hipertensi primer dan kencing manis jenis kedua di Malaysia.

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I certify that an Examination Committee has met on 08-05-2009 to conduct the final examination of R.VASUDEVAN on his Doctor of Philosophy thesis entitled "**Analysis of Genetic Polymorphisms as Risk Factors in Essential Hypertension and Type 2 Diabetes Mellitus Among Malaysians**" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the Doctor of Philosophy.

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DECLARATION

I declare that the thesis is my original work except for quotation 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.

_____

R.VASUDEVAN

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LIST OF ABBREVIATIONS

ACE	Angiotensin Converting Enzyme
AGT	Angiotensinogen
BMI	Body Mass Index
BP	Blood Pressure
bp	Base pair(s)
C.I	Confidence Interval
DBP	Diastolic Blood Pressure
DNA	Deoxyribonucleic acid
EHT	Essential Hypertension
FPG	Fasting Plasma Glucose
HDL-C	High Density Lipoprotein Cholesterol
I/D	Insertion/Deletion
K	Potassium
LDL-C	Low Density Lipoprotein Cholesterol
MS-PCR	Mutagenic Separated Polymerase Chain Reaction
Na	Sodium
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
RAAS	Renin Angiotensin –Aldosterone System
REN	Renin
RFLP	Restriction Fragment Length Polymorphism
SBP	Systolic Blood Pressure
SNPs	Single Nucleotide Polymorphism
S.D	Standard Deviation
T2DM	Type 2 Diabetes Mellitus
TC	Total Cholesterol
TG	Triglycerides
UV	Ultraviolet light

CHAPTER 1

INTRODUCTION

Background of the Study

Genetic polymorphism is defined as the inheritance of a trait controlled by a single genetic locus with two alleles, in which the least common allele has a frequency of about 1% or greater (Gelehrler and Collins, 1990). Several studies have been done in understanding the possible role of genetic variation in the human genome. Identifying the relationship between the ancestral variants in genes are common in polygenic diseases become increasingly feasible with improved methods for finding DNA sequence variants on a genome-wide scale (Collins *et al*., 1998; Landegren *et al*., 1998).

Most of the variations found in DNA sequence are stable and occur in the form of single nucleotide polymorphism (SNPs), insertion/deletion (I/D) and variable tandem repeats. SNPs are stable, they are di-allelic, the two alleles representing the "wild-type" and the "mutant type" form (Doris *et al.,* 2002). Variation occurs when a single nucleotide (A, T, C, or G) alters in the genome sequence. Common sequence variants occur in about 1 in every 1000 bases of coding/ regulatory sequence. In every gene there is an average of 4-8 SNPs occurring either in the exons or coding regions or in the nearby exons and introns boundaries in the upstream regulatory regions. Each person would be heterozygous for 24,000-40,000 non-synonymous (amino acid

altering) substitutions (Lele *et al.,* 2003). The genetic polymorphisms are integral to the development of genetic markers to identify the individuals at risk of developing the diseases. To identify the common disease susceptibility loci, many studies encompassing genome-wide association mapping, linkage (Krushkal *et al.,* 1999), candidate gene association (Yamada *et al.,* 2002) have been utilized. It has been realized that genetic susceptibility to the common complex disorders probably involves many genes, most of which have small effects (Cordell and Clayton, 2005).

Various genetic polymorphisms in the candidate genes encoding proteins with known biochemical or physiologic function for blood pressure regulation have been identified in various populations with contradictory results (Agarwal *et al.,* 2005). Nearly 30-50% variations in genetic elements contribute to the dysregulation of blood pressure in human essential hypertension (Garcia *et al.,* 2003). Essential hypertension (EHT) is a multifactorial disease, and a number of risk factors underlying hypertension including age, sex, family history of hypertension, demographic factors, weight, diabetes mellitus, excess consumption of sodium, physical inactivity, smoking, and excess coffee and alcohol consumption have been identified (Chalmers *et al.,* 1999).

Type 2 diabetes mellitus (T2DM) is a complex heterogeneous group of conditions characterized by the elevated levels of plasma glucose, caused by impairment in both insulin secretion and resistance to insulin action (Radha *et al.,* 2007). It is a very common metabolic disorder with a substantial inherited component.

Undiagnosed or poorly control diabetes will lead to multitude of complications such as diabetic retinopathy, nephropathy, neuropathy and increased cardiovascular morbidity and mortality (Klein**,** 1995). A number of other risk factors underlying EHT and T2DM have also been identified including age, sex, genetics, demographic factors and others.

The genetics of the common variety of hypertension (HPT) and T2DM also called polygenic or multifactorial disease is a result of the interaction between the environment and multiple genes. The susceptibility is associated with frequent polymorphisms that create variation in exons or influence the expression of genes in the regulatory parts (McCarthy *et al.,* 2002). These sequence variants are associated with a limited increase in the risk of developing the disease. They can be considered as susceptibility variants, but by themselves are not causative factors that are unambiguous to determine the disease.

Several research strategies have been utilized to dissect the genetic variants of both EHT and T2DM predisposition genetic loci, including investigations of specific candidate genes, genome-wide searches, and the use of intermediate phenotypes, comparative genomics, and a combination of these methods (Timberlake *et al.,* 2001; Tanira *et al.,* 2005). One such research strategy is the 'candidate gene' approach and one of the most extensively studied in EHT and T2DM pathogenesis. The molecular events in diabetes pathogenesis have been examined directly by testing the role of sequence variants of specific candidate genes (McCarthy *et al.,* 2002). The candidate gene approach focuses on the search for an association between the disease and sequence variants in or near biologically defined candidate genes which have been

chosen based on their known physiological function. The candidate gene approach is useful for quickly determining the association of a genetic variant with a disorder and for identifying genes of modest effect. This approach has certain advantages over traditional linkage mapping or positional cloning approaches (Kwon *et al.,* 2000).

The association studies, most of which use the cross-sectional and case-control designs with groups of unrelated individuals, are designed to detect associations between disease and specific alleles. Alleles of these polymorphisms are present in both healthy individuals and patients, although with different frequencies (Timberlake *et al.,* 2001). Consistent with the very large numbers of studies, many have shown modest associations in single population, but these associations have not yet been replicated in other populations (Radha *et al.,* 2007). Unfortunately, to date, there is no clear picture as to the genetic determinants of EHT and T2DM, in part, due to the conflicting results even in the same gene polymorphism.

A number of laboratory techniques have been developed in recent years to analyze DNA rapidly with the advent of Polymerase Chain Reaction (PCR) and reliably for the purpose of mutation detection (Grompe*, et al., 1993***)**. One of the methods involves in detecting the mutation is Restriction Fragment Length Polymorphism (RFLP). RFLP is a method that uses specific restriction endonucleases to detect the differences in homologous DNA sequences by the presence or absence of different length of restriction fragments. RFLP method can be used to determine the disease status of an individual. PCR is a method of amplifying and enriching small amounts of DNA within a targeted region of a DNA sequence. When the two procedures are combined, the method is referred to as PCR-RFLP.

Many successful studies have been done to detect the genetic polymorphism in various populations in association with EHT and T2DM using PCR-RFLP method (Fabris *et al.*, 2005). This cross-sectional study mainly focuses on determining the association between genetic polymorphism of susceptibility genes and predisposition to EHT and T2DM subjects.

Problem Statement

Hypertension and T2DM is a major contributor to cardiovascular disease which is the leading cause of death in Malaysia. According to the Second National Health Morbidity Survey 1996 (NHMSII, 1996) in Malaysia, the prevalence of HPT among adults was found to be 33%, 8.3% for T2DM and for impaired glucose tolerance it was 4.3%. But a recent survey shows that, the current prevalence of hypertension registered a rise of 30% (33% vs 43%) and for known and newly diagnosed diabetes among adults above 30 years rose from 8.3% to 14.9% (NHMS III, 2006). In future it faces the daunting prospect of even higher prevalence of HPT as well as T2DM. While environmental factors also certainly play a major role in the hypertension and diabetes epidemic, this usually occurs on a background of genetic susceptibility. Several studies have been carried out to determine the candidate genes in predisposition to EHT and T2DM in various populations with conflicting results. Since genetic diversity exists among different ethnic populations and realizing the fact that the association in one population could not be extrapolated to another population. In Malaysia, there is a lack of data is available on the association of the genetic polymorphisms with EHT and T2DM. This stimulated us to study the association of genetic polymorphisms in relation to EHT and T2DM.

Significance of the Study

Identifying EHT and T2DM susceptibility genes will help us in understanding the pathophysiology of the disease. In addition to the potential impact of genomic information in selecting suitable anti-hypertensive and the anti-diabetic drug therapy, it may also help in recognizing those at the risk of developing the disease, which may lead to new preventive approaches. This cross-sectional study attempts to determine the presence of known genetic variations of candidate genes that may be implicated in the pathogenesis of EHT and T2DM. It also highlights some of the opportunities and challenges, which may be encountered in interpreting the value of these genetic variations to improve the management of EHT and T2DM. Association analyses are performed by comparing cases and controls for differences in presumed risk factors, like alleles. The association could be attributable to chance, artifact, or selection bias. Population stratification (namely the separation of a study population into subgroups) is often a confounding factor. Most genetic studies of hypertension-predisposing genetic loci have used this strategy to study the candidate genes. This systematic approach assumes that a gene or a set of genes involving a specific physiological or cellular function contribute to blood pressure variation. Most of the studies utilized genetic linkage and association methods and enrolled unrelated individuals**.** The cross-sectional study design also renders candidate gene studies less likely to be reproducible and less expected to include all causative genes and polymorphisms. The candidate gene analysis would provide a better approach for identifying the genotype–phenotype correlations. The candidate gene study is more effective than linkage analysis in studying EHT and T2DM since it has greater statistical power to detect several genes for small effect. Identification of the

contributing genes will allow us to recognize those vulnerable individuals, and to classify the patients in subgroups with definite genetic and pathogenic mechanisms, which might enable the use of genotypes to identify more-specific therapeutic and preventative measures for EHT and T2DM. The current study is conducted to determine the association of genetic polymorphisms of candidate genes involved in EHT and T2DM and the distribution of risk factors among Malaysian subjects.

Hypothesis

Genetic polymorphism of certain susceptibility genes is a risk factor or not for EHT and T2DM in Malaysian subjects.

Main Objective

To determine the association of genetic polymorphisms of susceptibility genes involved in Malaysian essential hypertensive and type 2 diabetic subjects.

Specific Objectives

1. To determine the genotypic and allelic frequencies of genetic polymorphisms of candidate genes in essential hypertensive subjects.
2. To determine the genotypic and allelic frequencies of genetic polymorphisms of candidate genes in type 2 diabetic subjects.
3. To determine the association of genetic polymorphisms between the case and control subjects.