UNIVERSITI PUTRA MALAYSIA

HOMOCYSTEINE METABOLISM ENZYME GENE POLYMORPHISMS IN NON-SYNDROMIC MALAYSIAN CONGENITAL HEART DISEASE PATIENTS

NUR AFIQAH BINTI MOHAMAD

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By

NUR AFIQAH BINTI MOHAMAD

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

October 2013

DEDICATIONS

This thesis is dedicated to my beloved parents who motivate me to have a higher education, my whole family and friends for their patience and extreme encouragement for me to accomplish my study.

Abstract of the thesis presented to the School of Graduate Studies of University Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

HOMOCYSTEINE METABOLISM ENZYME GENE POLYMORPHISMS IN NON-SYNDROMIC MALAYSIAN CONGENITAL HEART DISEASE PATIENTS

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Chair: Prof. Patimah Ismail, PhD
Faculty: Medicine and Health Sciences

Congenital heart disease (CHD) mainly is caused by the incomplete development of the heart during the first 6 weeks of pregnancy. Chromosomal and genetic abnormalities in the child and high levels of homocysteine in the blood are some of the risk factors related to CHD. Several studies in various populations have been done to determine the candidate genes in the predisposition to CHD with contradictory results, but there have been no studies that had been found in Malaysian CHD patients. Hence, this study was conducted to determine the allelic and genotypic analysis of the polymorphisms in candidate genes of the homocysteine enzymes which are the *Methylenetetrahydrofolate Reductase* (MTHFR), *Cystathionine-b-synthase* (CBS), *Methionine Synthase* (MTR) and *Methionine Synthase Reductase* (MTRR) genes. We conducted an unmatched cross-sectional study between CHD patients and healthy subjects to determine the association of these polymorphisms with CHD. Based on the inclusion and exclusion criteria, buccal or blood samples were collected from 150 Malaysian non-syndromic CHD patients and 150 samples from healthy subjects as controls with no matching of age, genders and race between cases and controls. Genomic DNA was extracted from the samples using commercially available kits and the genotyping analysis for C677T MTHFR, A1298C MTHFR, A66G MTRR, A2756G MTR and 844ins68 CBS gene polymorphisms are analyzed using the PCR-RFLP analysis. This study showed that there was a significant difference observed in the MTHFR A1298C gene polymorphisms between cases and controls with a significance value of P=0.008. When compared between genders and races, there was also a significant difference observed between males (P=0.003) and females (P=0.037) and among the Malay ethnics (P=0.023) of both CHD and control groups. On the other hand, no significant difference was observed for genotype frequencies between cases and controls of the MTHFR C677T, MTRR A66G, MTR A2756G and CBS 844ins68 gene polymorphism. The association of MTHFR A1298C with the development of CHD in this study emphasis the role of MTHFR gene in the pathogenesis of non-syndromic CHD. The other selected polymorphisms of MTHFR, MTRR, MTR And

CBS gene were not associated with the development of CHD in Malaysian subjects. However, investigating these genes in a bigger samples size for different variants might reveal an association of those genes polymorphisms with the development of CHD in Malaysian subjects.

Abstrak tesis yang dikemukakan kepada Sekolah Pengajian Siswazah Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

GEN POLIMORFISME TERHADAP GEN HOMOSISTIN METABOLISMA ENZIM DI KALANGAN PESAKIT JANTUNG KONGENITAL BUKAN SINDROMIK DI MALAYSIA

Oleh

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Penyakit jantung kongenital terutamanya disebabkan oleh pembentukan jantung yang tidak lengkap semasa 6 minggu pertama kehamilan. Kromosom dan keabnormalan genetik dalam kanak-kanak dan tahap homocysteine dalam darah adalah sebahagian daripada faktor-faktor risiko yang berkaitan dengan penyakit jantung kongenital. Beberapa kajian dalam populasi yang berlainan telah dilakukan untuk menentukan gen yang boleh membawa risiko penyakit jantung kongenital, tetapi dengan keputusan yang bercanggah. Setakat ini, di Malaysia tiada lagi kajian yang dilakukan untuk polimorfisme dalam gen calon enzim homosistein dalam risiko penyakit jantung kongenital. Oleh itu , kajian ini dijalankan untuk menentukan analisis alel dan genotip daripada polimorfisme dalam gen calon enzim homosistein iaitu *Methylenetetrahydrofolate Reductase* (MTHFR), *Cystathionine -b- synthase* (CBS), *Methionine sintase* (MTR) dan *Methionine sintase Reductase* (MTRR). Kami telah menjalankan satu kajian keratan rentas antara pesakit jantung kongenital dan kawalan yang sihat untuk menentukan hubungan polimorfisme ini dengan penyakit jantung kongenital. Berdasarkan kriteria pemilihan sampel, sampel pipi atau darah telah dikumpulkan daripada 150 pesakit jantung kongenital bukan sindromik dan 150 sampel dari orang yang sihat sebagai kawalan dengan perbezaan antara umur, jantina dan bangsa antara kes dan kawalan. Genomik DNA diekstrak daripada sampel menggunakan kit pengekstrakan komersialboleh dan analisis genotip untuk C677T MTHFR , A1298C MTHFR , A66G MTRR , A2756G MTR dan 844ins68 CBS polimorfisme gen adalah menggunakan analisis PCR-RFLP. Kajian ini menunjukkan bahawa terdapat perbezaan signifikan dalam polimorfisme gen MTHFR A1298C antara kes dan kawalan dengan nilai signifikan P=0.008. Apabila dibandingkan antara jantina dan bangsa di antara kes dan kawalan, terdapat juga perbezaan yang signifikan antara lelaki (P=0.003) dan perempuan (P=0.037) dan antara etnik Melayu (P=0.023). Sebaliknya , tidak ada perbezaan signifikan didapati untuk frekuensi genotip antara kes dan kawalan daripada MTHFR C677T , MTRR A66G , MTR A2756G dan CBS 844ins68 gen polymorfisme. Hubungan MTHFR A1298C dengan pembentukan penyakit kongenital dalam kajian ini menunjukkan kemungkinan terdapat peranan MTHFR gen dalam patogenesis penyakit jantung kongenital bukan

sindromik. Manakala polimorfisme lain idak dikaitkan dengan pembentukan jantung kongenital di kalangan pesakit di Malaysia. Walau bagaimanapun, ini perlu dikaji dengan saiz sampel yang lebih besar untuk membuktikan lagi risiko gen-gen polimorfisme ini dengan pembentukan jantung kongenital di kalangan pesakit di Malaysia.

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I Certify that a Thesis Examination Committee has met on 2 October 2013 to conduct the final examination of Nur Afiqah Binti Mohamad on her thesis entitled **"Homocysteine Metabolism Enzyme Gene Polymorphisms in Non-Syndromic Malaysian Congenital Heart Disease Patients"** 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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LIST OF ABBREVIATIONS

ASD	Atrial septal defect
AVSD	Atrioventricular septal defect
CCTGA	Congenitally corrected transposition of the great arteries
CHD	Congenital heart disease
CoA	Coarctation of aorta
DCSA	Doubly committed subarterial ventricular septal defects
DILV	Double inlet left ventricle
DORV	Double outlet right ventricle
MS	mitral stenosis
MR	mitral valve regurgitation
PAPVD	partial anomalous pulmonary venous drainage
PDA	Patent ductus arteriosus
SNP	single nucleotide polymorphism
TAPVD	Total anomalous pulmonary venous drainage
TOF	Tetralogy of Fallot
VSD	Ventricular septal defect

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Congenital heart disease or CHD is a common disorder or defect among newborn infants and children (Goldmuntz, 2001). It is a defect in the heart structure and blood vessels that are present at birth and occurs due to abnormal or improper development of the fetus' heart during fetal development (American Heart Association, 2011). In Malaysia, the number of children that requires surgery due to heart defects is about 2000-3000 per year and it is estimated that the number of children born with CHD is about 5,000 a year with an average of 500,000 deliveries a year (Ministry of Health, Malaysia).

CHD is known as a multifactorial disease where it can be caused by environmental or genetic factors. Some of the genetic factors may include chromosomal or genetic abnormalities of the infant or single gene defect. Maternal viral infection, certain medication taken during pregnancy and drug abuse are also some of the risk factors in the development of CHD (Deeparani *et al.*, 2009). A few types of CHD are induced by single gene mutation and chromosomal aberration but most of CHDs are polygenic diseases caused by both genetic and environmental factors (Zhu *et al.*, 2004). CHD has been featured in many genetic syndromes, while the genetic associations of non-syndromic CHD are starting to be increasingly recognized and being studied by many researchers. Both genetic causes of syndromic and non-syndromic CHD are important since this will provide an opportunity to develop new diagnostic and therapeutic strategies and decrease the economic burden, mortality and morbidity of CHD (Hinton *et al.*, 2005).

Genetic approaches for discovering monogenetic factors involved in the development of CHD include pedigree studies and candidate screens. Pedigree studies involve a large family study for the inheritance for CHD using the Mendelian pattern while candidate screens are used to identify SNPs in candidate genes. The latter is more advantage since genetic variants can be identified relatively rapidly and in single individuals or small pedigrees (Lagendijk *et al.*, 2010). These candidate genes have been chosen based on their physiological function that relate to the risk factors leading to the development of CHD. Nowadays, the current genetic techniques being used for evaluation of CHD include cytogenetic techniques, fluorescence in situ hybridization, and DNA mutation analysis (Huang *et al.*, 2010).

Genetic polymorphism is a variation in DNA sequence among individuals, groups or populations. There are a variety of genetic polymorphisms which include single nucleotide polymorphisms (SNPs), sequence repeats, insertions, deletions and recombination (Doris, 2002). This variety in the DNA sequence enables us to investigate disease processes and clinical variations such as; what determines the response of the individual to a drug, the different rate of disease progression between patients and even to determine a patient's susceptibility to a disease (Hanchard,

2005). Single Nucleotide Polymorphism is a single base mutation in DNA. SNPs are the most common form of genetic polymorphism accounted for about 90% of all human DNA polymorphisms in the human genome (Smith, 2002). SNP detection includes two functions where they are used to scan for new polymorphisms and also to determine the allele(s) of a known polymorphism in the target sequences (Kwok & Chen, 2003).

Homocysteine is an amino acid present in the blood and an elevated level of homocysteine known as hyperhomocysteinemia has been extensively studied in its relation to the development of CHD. Hyperhomocysteinemia is considered as multifactorial since it can be caused by genetic abnormalities, lifestyle and nutritional deficiencies such as Vitamin B12, Vitamin B6 and folate deficiency (Kluijtmans *et al.*, 2003). Elevated level of homocysteine may impair endothelial vasomotor function or damage coronary arteries causing abnormal blood flows.

The common enzymes involved in homocysteine or folate metabolism are the Methylenetetrahydrofolate Reductase (MTHFR), the Methionine Synthase Reductase (MTRR), Methionine Synthase (MTR) and Cystathionine-b-synthase (CBS) enzymes. Deficiencies or polymorphism in any of these enzymes is suspected to lead to an inborn error in the metabolism of homocysteine and also causing homocystinuria. There is evidence that this deficiency could alter the susceptibility to CHD (Garcia-Fragoso *et al.*, 2010). Previous studies in various populations have been done in identifying the association of the homocysteine enzyme gene polymorphisms with CHD and there were contradictory results (Junker *et al.*, 2001; Song *et al.*, 2006; van Beynum *et al.*, 2007).

In detecting the genetic polymorphism of genes where individual polymorphisms may play an important role in the disease process, recent advances made in Polymerase Chain Reaction (PCR) technology have significantly simplified the analysis of these polymorphisms. The most common method used in detecting these mutations is by using the Restriction Fragment Length Polymorphism (RFLP) method. RFLP is a method which refers to differentiating two or more homologous DNA molecules using specific restriction enzymes from differing locations of restriction sites, and by using a related laboratory technique, these segments can be distinguished (Smith, 2002).

1.2 Problem Statement

Congenital heart disease is a common disorder that causes mortality and morbidity among infants. According to a study done by the Malaysia's Ministry of Health in 2006, the mortality rate of children under five years old was 7.2 per thousand still births with congenital malformations, deformations and chromosomal abnormalities being the most common causes of death by a percentage of 25.1%. In Malaysia, 100 infants are born with an abnormal heart and it is estimated that at least 5000 children could be at risk of suffering from CHD with two-thirds will require surgical intervention (Lan and Ismail, 2006). Genetic factors are one of the risk factors of CHD. CHD has been featured in many genetic syndromes, while the genetic associations of non-syndromic CHD are starting to be increasingly recognized and been studied by many researchers. Recently, due to the huge information that is available from sequencing of the human genome , this had led to identifying of a

number of a single gene defect as a cause of CHD which led to establish the monogenic theory behind the etiology of isolated CHD (Bajolle *et al.*, 2009). Conducting genetic association study by comparing the genetic variations of a DNA between cases and control subject is considered to be a powerful theme to determine if the variant is disease associated (Little *et al.*, 2009). There have been several studies done on the association of gene polymorphisms of homocysteine metabolism with CHD. The candidate genes related to homocysteine enzymes that are most extensively studied are the MTHFR, CBS, MTR and MTRR genes which is mostly related to development of CHD. In Malaysia, there is a lack of data available on the association of the homocysteine enzymes gene polymorphism with non-syndromic CHD. This interested us in doing a research on the association of the genetic polymorphisms with development of non-syndromic CHD.

1.3 Study Justification

Genetic association analysis is to test whether an allele or genotype frequency differs between the two groups and examines the statistical correlation between a person's genotype with his phenotype or disease. Most commonly the genetic association involves a study of the Single Nucleotide Polymorphisms (SNPs) genotype frequency in a group of cases and controls. According to this, association analysis can be used as a useful approach in studying the role of candidate genes in the development of multifactorial diseases (Cordell & Clayton, 2005). The genetic polymorphisms of the homocysteine metabolism genes can provide a basis for studying the relationship between genetic variants and the development or progression of CHD. There have been various studies done to study this relationship, but still with conflicting results (Bruneau, 2008). So far in Malaysia, there has been no study conducted on the association of the homocysteine enzyme gene polymorphisms with the development of CHD. The aim of this study is to explore the gene polymorphisms of the enzymes involved in the homocysteine metabolism among the Malaysian population and performing a genetic analysis of the association of these gene polymorphisms with the development of CHD. Thus, the result from this study could be useful in future research and could also enrich our knowledge on the pathogenesis of non-syndromic CHD. This will be useful in improving the diagnosis and provide better information on the prognosis of non-syndromic CHD patients in which in the future could be applied to prevent this birth defect.

1.4 Hypothesis

Polymorphisms in the genes (*methylenetetrahydrofolate reductase, cystathionine-b-synthase, methionine synthase* and *methionine synthase reductase)* involved in homocysteine metabolism may be associated with the development of non-syndromic CHD among Malaysian patients.

1.5 Objectives

1.5.1 Main Objective

To identify polymorphisms in the genes of four enzymes involved in homocysteine metabolism that may be associated with the development of non-syndromic CHD in Malaysian patients.

1.5.2 Specific Objectives

1) To determine the genotype frequency for C677T and A1298C polymorphism of the MTHFR gene
2) To determine the genotype frequency for A2756G polymorphism of the MTR gene
3) To determine the genotype frequency for A66G polymorphism of the MTRR gene
4) To determine the genotype frequency for 844ins68 polymorphism of the CBS gene
5) To determine if there is an association between polymorphisms in the genes of four enzymes involved in homocysteine metabolism with the development of non-syndromic CHD in Malaysian patients

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