SINGLE NUCLEOTIDE POLYMORPHISM OF SELECTED GENES ASSOCIATED WITH STROKE

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ABSTRACT

Stroke is the second leading cause of death and the third leading cause of disability worldwide. Generally, higher stroke mortality rate was observed in the Asian than Western Europe and America. Stroke is the third leading cause of death in Malaysia with 5876 stroke death cases in year 2016 and poses a great economic burden to the country where the estimated lifetime economic burden for stroke patients was MYR 5.5 billions. Recently, studies have reported that single nucleotide polymorphisms (SNPs) within genes may alter an individual's susceptibility towards the development of stroke incidence. In present study, SNPs from six different genes namely *MTHFR* rs1801133, *eNOS* rs1799983, *ANRIL* rs10757278, *LIPG* rs9958947, *BDNF* rs6265 and *ApoE* had been studied to identify the associations of these SNPs with stroke risk. Blood samples from 113 stroke patients and 138 healthy controls were collected and DNA was extracted from the blood samples. The Taqman® SNP genotyping assay was used to identify the gene polymorphism in the subjects and *ApoE* polymorphism is identified using restriction fragment length polymorphism (RFLP) method. Odds Ratio (OR) test with 95% confidence interval (CI) was used to identify the association of the SNPs with stroke risk. Overall, the *MTHFR* rs1801133-T allele and *BDNF* rs6265-A allele conferred a reduced stroke risk in this study. After gender and lifestyle stratification, the protective effects against stroke of the *MTHFR* rs1801133-T allele was significant in male, non-smokers, non-drinkers and fast food goers group while female, non-smoker, non-drinkers and fast food goer group for the *BDNF* rs6265-A allele. The *eNOS* rs1799983-T allele increased stroke risk among non-smokers and fast food goers. Finally, the E4/E3 genotype of the *ApoE* polymorphism was determined to be associated with increased stroke risk among drinkers. The results corroborate previous studies that discovered the association of *eNOS* rs1799983-T allele and *ApoE*-E4 allele with increased stroke risk. The results also suggested that the *MTHFR* rs1801133-T allele and *BDNF* rs6265-A allele may be linked to decresed stroke risk and hence, protective effects against stroke.

ABSTRAK

POLIMORFISME NUKLEOTIDA TUNGGAL DALAM GEN-GEN TERPILIH YANG BERKAITAN DENGAN STROK

Strok ialah punca kedua kematian dan punca ketiga kecacatan di dunia. Secara umumnya, kadar kematian strok adalah lebih tinggi di Asia berbanding dengan Eropah dan Amerika. Secara statistiknya, strok adalah punca ketiga kematian di Malaysia dengan 5876 kes kematian strok pada tahun 2016 dan mengakibatkan beban ekonomi yang besar di mana hidup beban ekonomi yang dianggarkan adalah RM 5.5 bilion. Baru-baru ini, terdapat kajian-kajian yang melaporkan bahawa polimorfisme nukleotida tunggal (SNP) dalam gen-gen tertentu dapat mempengaruhi risiko seseorang itu untuk menghidapi strok. Dalam kajian ini, SNP dari enam gen-gen yang berbeza iaitu MTHFR rs1801133, eNOS rs1799983, ANRIL rs10757278, LIPG rs9958947, BDNF rs6265 dan ApoE telah dikaji untuk mengenalpasti kaitan SNP-SNP tersebut dengan risiko stroke. Darah daripada 113 orang pesakit strok dan 138 orang kawalan sihat telah dikumpul dan DNA diekstrak daripada sampel-sampel darah tersebut. Taqman® SNP genotyping assay digunakan untuk mengenalpasti genotip SNP daripada subjek kecuali polimorfisme ApoE di mana kaedah polimorfisme panjang berkas restriksi (RFLP) digunakan. Ujian nisbah ganjil (OR) dengan selang keyakinan 95% (CI) telah digunakan untuk mengenalpasti kaitan SNP dengan risiko strok. Secara keseluruhannya, MTHFR rs1801133-T alel dan BDNF rs6265-A alel dikenalpasti dapat megurangkan risiko strok di dalam kajian ini. Selepas stratifikasi jantina dan gaya hidup, perlindungan terhadap strok daripada MTHFR rs1801133-T alel adalah signifikan dalam kalangan lelaki, bukan perokok, bukan peminum alcohol dan mereka yang sering makan makanan segera manakala perlindungan terhadap strok daripada BDNF rs6265-A alel adalah signifikan dalam kalangan wanita, bukan perokok,bukan peminum alkohol dan mereka yang sering makan makanan segera. Alel T daripada eNOS rs1799983 meningkatkan risiko strok di dalam kalangan bukan perokok dan mereka yang sering makan makanan segera. Akhirnya, genotip E4/E3 daripada polimorphisme ApoE dikaitkan dengan peningkatan risiko strok di dalam kalangan peminum alcohol. Keputusan kajian menyokong kajian terdahulu yang menemui perkaitan eNOS rs1799983-T alel dan ApoE-E4 alel dengan peningkatan risiko strok. Keputusan kajian ini mencadangkan MTHFR rs1801133-T alel dan BDNF rs6265-A alel mungkin terlibat dalam pengurangan risiko strok dan perlindungan daripada strok.

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LIST OF ABBREVIATIONS

AHA/ASA	American Heart Association/American Stroke Association
ANRIL	Antisense noncoding RNA in the INK4 locus
ApoE	*Apolipoprotein E*
BDNF	*Brain-derived neurotrophic factor*
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
CDC	Centers for Disease Control and Prevention
CDK	Cyclin-dependent kinase
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CDKN2B	Cyclin-dependent kinase inhibitor 2B
CDKN2B-AS1	*Cyclin-dependent kinase inhibitor 2B antisense RNA 1*
CI	Confidence interval
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
eNOS	*Endothelial nitric oxide synthase*
FAD	Flavin adenine dinucleotide
GABA	Gamma-aminobutyric acid
GBD	Global Burden of Disease
GWAS	Genome-wide association study
HDL-C	High-density lipoprotein cholesterol
HS	Haemorrhagic stroke
HWE	Hardy-Weinberg equilibrium
IA	Intracranial aneurysm
ICH	Intracerebral haemorrhage
IL	Interleukin
IS	Ischemic stroke

LDL-C	Low-density lipoprotein cholesterol
LIPG	*Lipase G*
LncRNA	long non-coding ribonucleic acid
$MgCl_2$	Magnesium chloride
MTHFR	*Methylenetetrahydrofolate reductase*
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
NINDS	National Institute of Neurological Disorders and Stroke
NINJ2	*Ninjurin 2*
NO	Nitric oxide
NOS3	*Nitric oxide synthase 3*
NTC	No template control
OR	Odd ratio
***p*-value**	Probability value
PCR	Polymerase chain reaction
RCLB	Red Cell Lysis Buffer
RE	Restriction enzyme
rs	Reference SNP ID number
SAH	Subarachnoid haemorrhage
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SNP	Single nucleotide polymorphism
STAT1	Signal transducer and activator of transcription 1
TIA	Transient ischemic attack
Trk	Tropomyosin-related kinase
UV	Ultraviolet
VLDL	Very low-density lipoprotein
WHO	World Health Organization

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

A stroke is a damage to the brain that occurs when blood flow to an area of brain is cut off, causing brain cells death due to the lack of oxygen and nutrients. When brain cells die during a stroke, abilities controlled by that area of the brain such as memory and muscle control are lost. Stroke remains a major health burden worldwide. Stroke is the second leading cause of death and the third leading cause of disability worldwide (World Health Organization (WHO), 2012) and it is also the leading cause of dementia and depression (Owolabi *et al.*, 2015). Globally, 70% of strokes and 87% of both stroke-related deaths and disability-adjusted life years occur in low- and middle-income countries (Feigin *et al.*, 2009). Asia, which represents the largest continent that consisted of more than 60% of the world population had been reported to have a higher stroke mortality rate than Western Europe and America, except for some countries such as Japan (Feigin *et al.*, 2014).

Stroke deaths in Malaysia has reached 15,497 or 12.19% of total deaths in year 2014. The age adjusted death rate is 80.59 per 100,000 of population which ranks Malaysia no.97th in the world and stroke incidence in Malaysia is estimated to increase annually by 29.5% for ischemic stroke and 18.7% for haemorrhagic stroke (Aziz *et al.*, 2015). According to the data from the Department of Statistics Malaysia, stroke accounts for 7.1% of total death in year 2014, ranking stroke the third cause of deaths in Malaysia after ischemic heart diseases and pneumonia (Department of Statistics Malaysia, 2016). Hence, stroke poses great economic burden to the country.

In general, stroke can be divided into three main type namely ischemic stroke (IS), haemorrhagic stroke (HS) and transient ischemic attack (TIA). An ischemic stroke occurs when blood flow through the artery in the brain is obstructed, usually blocked by blood clots. About 87% of strokes are ischemic strokes (Mozzafarian *et*

al., 2016). A haemorrhagic stroke occurs when a weakened blood vessel in the brain ruptured, mostly due to brain aneurysm or high blood pressure (CDC, 2017). TIA or "mini stroke" is a warning sign of a future stroke that caused by temporary blood clot in the brain's blood vessels for a short period of time which is usually no more than 5 minutes (CDC, 2017).

Single nucleotide polymorphisms (SNPs) are variations of one single DNA base pair where a nucleotide has been exchanged for another. Recently, SNPs had received a lot of attentions from researchers worldwide because these slight difference in gene sequence will affect the structure and functions of the translated products (e.g., an enzyme) that may involves in the pathogenesis of some serious diseases such as cardiovascular diseases, cancer and stroke. There are many SNPs on different genes that had been proposed to be associated with stroke risk such as various SNPs on *MTHFR, eNOS, ApoE, NINJ, ANRIL,* etc. In this study, six single nucleotide polymorphisms (SNPs) of six different genes (*eNOS, MTHFR, ANRIL, LIPG, BDNF* and *ApoE*) were selected to determine their association with stroke risk in the Malaysian population.

The SNP rs1799983 of the *endothelial nitric oxide synthase (eNOS)* gene has been associated with abnormalities in the expression level and activity of the eNOS enzyme in the body (Tesauro *et al.*, 2000) which is responsible for the production of nitric oxide (NO) that is important in maintaining resting cerebral blood flow, promotes blood clotting and regulates neuronal activity (Faraci and Brian, 1994). Elevated homocysteine level is also a risk factor for ischemic stroke, thrombotic and cardiovascular diseases (Alluri *et al.*, 2005). Methylene tetrahydrofolate reductase (MTHFR) that encoded by the by the *MTHFR* gene is an important folate-dependant regulatory enzyme in the metabolism of homocysteine (Matthews *et al.*, 1998) and its enzymatic activity has been shown to influenced by the *MTHFR* rs1801133 SNP (Goyette and Rozen, 2000). A brain aneurysm is a bulging, weak area in the wall of an artery in the brain and is more likely to burst, causing a haemorrhagic stroke. The SNP rs10757278 that located in the *ANRIL* (Antisense Noncoding RNA in the INK4 Locus) (Holdt and Teupser, 2013) on chromosome 9p21.3 has been associated with brain aneurysm (Yasuno *et al.*, 2010; Olsson et al., 2011) that can indirectly leads to haemorrhagic stroke through the development of weak brain arteries.

Besides that, a decrease in the high-density lipoprotein cholesterol (HDL-C) level is also a precise and controllable risk factor for atherosclerotic diseases such as ischemic stroke (Linsel-Nitsehke and Tall, 2005). Human genome-wide association studies (GWAS) had found that the level of HDL-C is associated with the genetic variation of *LIPG* gene (Ma *et al.*, 2010) such as the SNP rs9958947 that responsible in synthesizing the endothelial lipase G (LIPG) (NCBI, 2017). Brain-derived neurotrophic factor (BDNF) synthesized by the *BDNF* gene function in stimulating the growth and survival of variety of neuronal cells (Acheson *et al.*, 1995; Huang and Reichardt, 2001). The rs6265 SNP of the *BDNF* gene may influence the neuro-protective effects of the BDNF protein, which aids in the pathogenesis of stroke. The Apolipoprotein E (ApoE), encoded by the *ApoE* gene is a lipoprotein involved in lipid transport by removing cholesterol from the bloodstream and hence, influencing the blood low-density lipoprotein (LDL) cholesterol level (Volcik *et al.*, 2006) which is also a risk factors of stroke.

Stroke had been known to be a heterogeneous multifactorial disorder (Dichgans, 2007) that is contributed by various health conditions and risk factors. The SNPs mentioned above are selected in this study because of their individual relations with specific risk factors of stroke where *eNOS* rs1799983 SNP involves in the regulation of vascular stiffness, *MTHFR* rs1801133 SNP in the regulation of homocysteine level, *ANRIL* rs10757278 SNP in the development of weak brain vessels, *BDNF* rs6265 SNP in neuroprotection, *LIPG* rs9958947 SNP in regulation of HDL-C level and *ApoE* in regulation of LDL, VLDL level in the body. Furthermore, although these 6 SNPs are widely studied, but their associations with stroke risk remained controversial and underreported in the Malaysian population. Hence, this study aims to investigate the association of these 6 SNPs with stroke risk in a Malaysian population.

In Malaysia, stroke possessed a huge burden on the economy where the estimated lifetime economic burden for stroke patients was USD$1,359,953,353 (MYR 5.5 billions) in year 2016 and this figure is most likely to rise (Lee *et al.*, 2017). This study will allow better understanding of genetic influences on the individual risk and susceptibility towards stroke. Identifying the specific genes that contribute to stroke will enable the designing of more effective gene-targeted medication, professional genetic counselling and possibly, gene therapies for the rehabilitation of

stroke patients and thus, reducing the economic burden exerted by stroke to the country.

1.2 Problem Statement

Stroke is a heterogeneous multifactorial disorder that is caused by various health conditions and environmental factors. One of the major factor of developing stroke is the difference in genetic-makeup between individual. The DNA sequence differences contribute to the variation in activity level of some crucial enzymes, inflammation and metabolic response involved in maintaining healthy cerebral blood flow. SNPs from *eNOS*, *MTHFR, ANRIL, LIPG, BDNF* and *ApoE* have been proposed to be associated with stroke risk but conflicting results have been obtained. Furthermore, most of the stroke related genetic polymorphisms are underreported in the Malaysian population.

1.3 Research Question

1.) Does the genetic polymorphisms predict a person's susceptibility towards stroke?

2.) What are the genetic polymorphisms that can increases/decreases individual's risk towards developing stroke?

1.4 Hypothesis

Single nucleotide polymorphisms (SNPs) associated with *eNOS, MTHFR, ANRIL, LIPG, BDNF* and *ApoE* can be linked to stroke risk.

1.5 Objectives of Study

1.) To determine the genotypic distribution of the selected SNPs among stroke patients.

2.) To determine the association of selected gene polymorphisms of *eNOS* (rs1799983), *MTHFR* (rs1801133), *ANRIL* (rs10757278), *LIPG* (rs9958947), *BDNF* (rs6265) and *ApoE* (rs429358 & rs7412) with individual's stroke risk.

3.) To establish the association of gender towards stroke risk.

4.) To establish the association of lifestyle (smoking status, alcohol consumption status and fast food consumption status) with stroke risk.

CHAPTER 2

LITERATURE REVIEW

2.1 Stroke Statistics

Stroke is one of the major cause of death and disability in many countries. In year 2013, it had been reported that there were approximately 6.5 million deaths due to stroke, 25.7 million stroke survivors and 10.3 million new cases of stroke globally (Feigin *et al.*, 2015). In high-income country such as United States, every year nearly 800,000 people experience a new or recurrent stroke and this had become the fifth leading cause of death in the country (What is stroke, 2016). For low to middle income countries, the prevalence of stroke cases are increasing in parallel with their rising prevalence of stroke risk factors such as hypertension, diabetes and atrial fibrillation (Zhou and Hu, 2008; Gao *et al.*, 2013; Gunarathne *et al.*, 2009).

While Western countries experienced a 42% decrease in stroke incidence in the past 20 years (GBD 2013 Mortality and Causes of Death Collaborators, 2015), Asia countries had been reported to have an increased and higher stroke mortality rate than Western Europe and America, except for some countries such as Japan (Feigin *et al.*, 2014). Interestingly, the number of stroke incidences have been found to be varied among and even within Asian countries. In China, stroke incidence is higher in the northern regions compared to the southern regions (Sun *et al.*, 2013). In India, the stroke incidence is higher in rural areas (Pandian and Sudhan, 2013). On the contrary, the stroke incidence is higher in urban area compared with rural area in Thailand (Suwanwela, 2014) and Indonesia (Kusuma *et al.*, 2009). It had also been documented that the types of stroke incidences differed greatly by ethnicity. White (Caucasian) population was found to have higher ischemic stroke incidence and Chinese population have higher haemorrhagic stroke incidence compared to other populations (Khan *et al.*, 2017).

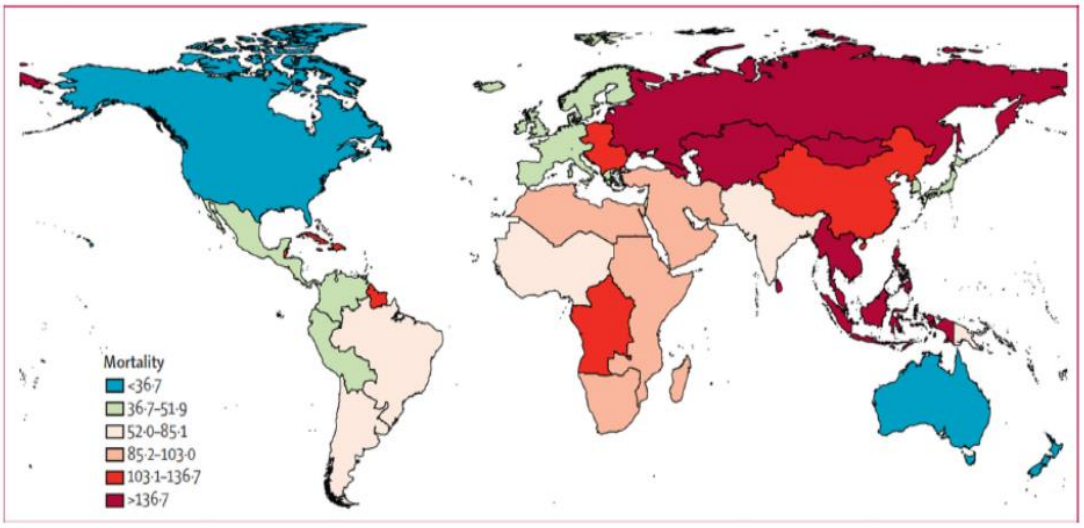

Figure 2.1: Age-standardised stroke mortality per 100 000 people in year 2010.

Source : Feigin *et al.*, 2014.

In Malaysia, stroke has been listed as the forth cause of death for male (after heart diseases, pneumonia and transport accidents) and third cause of death for female (after pneumonia and heart diseases) in year 2014 (Department of Statistics Malaysia, 2016). Furthermore, stroke is one of the top 10 leading causes of hospitalization in Malaysia and top five diseases with the greatest burden based on disability-adjusted life years (Aziz *et al.*, 2015). When stratified according to ethnicity, stroke is the third leading cause of death in the Bumiputeras and Chinese while stroke is the fifth cause of death for the Indian. In age stratification, stroke is the forth cause of death in the population aged 15-64 years old and the third cause of death in the elder population aged 65 years old and above (Department of Statistics Malaysia, 2016).

Besides, the estimated lifetime treatment cost for each ischemic and haemorrhagic stroke patient in Malaysia was USD$8,607 and USD$8,928, respectively where the major total cost driver was acute stroke management for both stroke type; ischaemic (68.7%) and haemorrhagic (80.3%). The projected lifetime economic burden for stroke patients diagnosed in 2016 was USD$1,359,953,353 in Malaysia (Lee *et al.*, 2017).

2.2 Types of Stroke

2.2.1 Ischemic Stroke

Ischemic stroke is a complex health problem with multiple etiologies and variable clinical manifestations. Approximately 45% of ischemic strokes are caused by small or large artery thrombus, 20% are caused by embolism, and others have an unknown cause (Hickey, 2003). Hence, a large amount of genes regarding thrombosis and embolisms such as SNPs on *IL-6, IL-10, IL-18, CCL2, CCL11,* etc. had been studied to investigate their associations with IS risk.

As for IS that occurs because of thrombus, thrombosis can form in the extracranial and intracranial arteries when the intima is roughened and plaque forms along the injured vessel. The endothelial injury (roughing) allows platelets to adhere and aggregate, causing coagulation and thrombus develops at site of plaque. Because of that, blood flow through the extracranial and intracranial systems decreases, however, the collateral circulation maintains its function. When the compensatory mechanism of collateral circulation fails, perfusion is compromised, leading to decreased perfusion and cell death (Hinkle and Guanci, 2007).

While for IS caused by embolism, a clot formed in other place of the body (a source) travels in the blood vessels and eventually reaches and lodges in cerebral vessels. Microemboli can break away from a sclerosed plaque in the carotid artery or from cardiac sources such as atrial fibrillation (Hickey, 2003). Emboli that are in the form of blood, fat, or air can occur during surgical procedures, commonly during cardiac surgery, but also after long bone surgeries (Warlow et al., 2001).

There are also some less common cause of stroke which included carotid dissection (Bader and Littlejohns, 2004), arteritis, infections, drug abuse such as cocaine (Hickey, 2003). Interestingly, the presense of periodontal disease and tooth loss is also an associated risk for ischemic stroke (Joshipura *et al.*, 2003).

2.2.2 Haemorrhagic Stroke

Haemorrhagic stroke (HS) or intracerebral haemorrhage (ICH) occurs from the rupture of cerebral vessels, stopping the delivery of oxygen and nutrients to brain cells. ICH encompasses 10% to 15% of all strokes (Labovitz and Sacco, 2001). ICH