ASSOCIATION OF MTHFR AND 9p21 WITH ISCHEMIC STROKE IN THE CHINESE HAN POPULATION

SHUO LI

Association of *MTHFR* and 9p21 with Ischemic Stroke in the Chinese Han Population

by

© Shuo Li

A Thesis submitted to the

School of Graduate Studies

in partial fulfillment of the requirements for the degree of

Master of Science

Discipline of Human Genetics, Faculty of Medicine

Memorial University of Newfoundland

August 2012

St. John's Newfoundland

ABSTRACT

Previous studies have revealed inconsistent results in examining the association of *MTHFR* c.677C>T, c.1298A>C variants and 9p21 locus with ischemic stroke. This thesis includes two case control studies by enrolling 1,429 ischemic stroke patients and 1,197 control individuals from the Chinese Han population. One study validates the association between the candidate 9p21 locus and ischemic stroke both at the SNP and Haplotype levels by genotyping four common variants encompassing a 44kb candidate risk region on 9p21. Moreover, the haplotype analysis reduces the candidate risk region on 9p21 locus to 28 kb. The other study indicates that the *MTHFR* locus is associated with ischemic stroke in the Chinese Han population. The *MTHFR* c. 677C>T variant is a risk factor, and its effect could be modified by genetic or environmental factors which vary among different ethnic populations. The *MTHFR* c. 1298A>C variant is more likely to play the role of a genetic marker than a causative variant in association with ischemic stroke. Combined analysis suggests the 9p21 locus and *MTHFR* risk alleles confer an additive co-effect for increased risk in ischemic stroke.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my gratitude to my supervisors, Drs. Yagang Xie and Feiyu Han, for providing the precious opportunity to accept my application as a graduate student. This offer opened a whole new chapter in my life, from China to Canada, from clinical work to biomedical research, from a spoiled girl to an independent individual. No words can express my gratitude for your support, patience and guidance both in my academic and personal life.

I would like to extend thanks to my committee members, Drs. Edward Randell and Rodger Green, for providing generous help in my research and thesis writing. I would also like to extend thanks for course instructors, Drs. Sevtap Savas, and Michael Woods, for helping me in course study, presentation skill and proposal writing. Your help gave me confidence to complete the course requirements in genetics.

I would like to extend thanks for the Keith Griffiths family, for announcing me the award of the Keith Griffiths Memorial heart and stroke foundation scholarship in 2011. The award enlightened my life in lab work, especially after a series of unexpected difficulties in the experiment.

In the deep of my heart, there is a warm home in Dr. Yuming Xu' team in neurology department of the first affiliated hospital of ZhengZhou University. I would like to

express my gratitude to Drs. Yuming Xu, Hong Zheng, Song Tan, Bo Song, and Haizhen Wang. Thank you for your help in my two-year period in clinical neurology, which is a warm memory I cannot forget.

To all my friends, both in China and Canada, thank you for your accompany. I cherished all the moments we shared together.

To Mom and Dad, thank you for your understanding, patience and support ever since I was born. Thank you for always being there for me. I love you.

Table of Contents

List of Tables

List of Figures

Chapter 1

Stroke Introduction

1.1 Stroke Definition and Clinical Features

Stroke, according to the World Health Organization (WHO), is rapidly developed focal or global neurological deficits, persisting more than 24 hours or interrupted by intervention or death within 24 hours, with a presumed vascular cause (WHO 1990). The clinical symptoms of stroke vary depending on lesion size and location in the brain. The common symptoms include hemiplegia, hemianesthesia, aphasia, vision field defects, dysarthria, headache, conscious impairments, dysphonia and spatial neglects (Adams and Victoria Principles of Neurology, 8th edition, 2005).

Advances in neuroimaging techniques have helped in facilitating the diagnosis of stroke. X-ray computed tomography (CT) scans can differentiate hemorrhagic from ischemic stroke immediately after onset. Magnetic resonance imaging (MRI) perfusion weighted imaging (PWI) and diffusion weighted imaging (DWI) mismatch scans can identify the penumbra area where the affected brain tissue can be treated by thrombolysis in cases of early ischemic stroke. MRI scans can identify the lesion location and size in the brain. Magnetic resonance angiography (MRA) scans or computed tomography angiography (CTA) scans can reveal the morphological changes in cerebral vasculature (Warach et al. 1995; Baird et al. 1998; Peter et al. 2003).

1.2 Stroke Subtypes

Based on current neuroimaging examinations, the etiology of stroke is categorized into four subtypes: ischemic, intracranial hemorrhagic, subarachnoid hemorrhagic and

undetermined. Ischemic stroke results from the occlusion of cerebral arteries by thrombi or emboli generated in the cardiovascular system, mainly due to atherosclerosis and atrial fibrillation. Intracranial hemorrhage refers to situations in which blood leaks from cerebral arteries into brain tissue. Subarachnoid hemorrhage is caused by rupture of an aneurysm in the primary branches from the circle of Willis, and blood bursts into the subarachnoid space. The undetermined type of stroke occurs when a neuroimaging examination is unavailable (Adams and Victoria Principle of Neurology, 8th edition, 2005).

The frequencies of stroke subtypes have been investigated worldwide. Ischemic stroke is the leading subtype according to most studies. Feigin et al. (2003) pooled ten population-based studies across European, Australian and Caribbean areas. Ischemic stroke was the most frequent in these studies, which constituted 67.3% – 80.5% of all stroke events. Intracranial hemorrhage, subarachnoid hemorrhage and unknown types accounted for 6.5% – 19.6%, 0.8% – 7.0%, and 2.0% – 14.5%, respectively. A similar frequency was also observed in the Chinese population. A large comprehensive study was conducted by the China Multicenter Collaborative Study of Cardiovascular Epidemiology (Zhang et al. 2003). A total of 16,031 first-time stroke patients (≥25 years old) were registered in 17 communities from 1991 to 2000. A comparison of the frequency distribution of stroke subtypes in 1991 and 2000 are shown in Figure 1.1. The ischemic stroke rate increased from the second (30.2%) in 1991 to the most common subtype (61.9%) in 2000. Intracranial hemorrhage accounted for 28.5% of cases in 2000, which was much higher

than 16.8%, as recorded in 1991. The reason for this difference lies in the implementation of neuroimaging techniques during the study period. In 2000, 91.4% of patients received CT scans, compared to that of 49.4% in 1991.

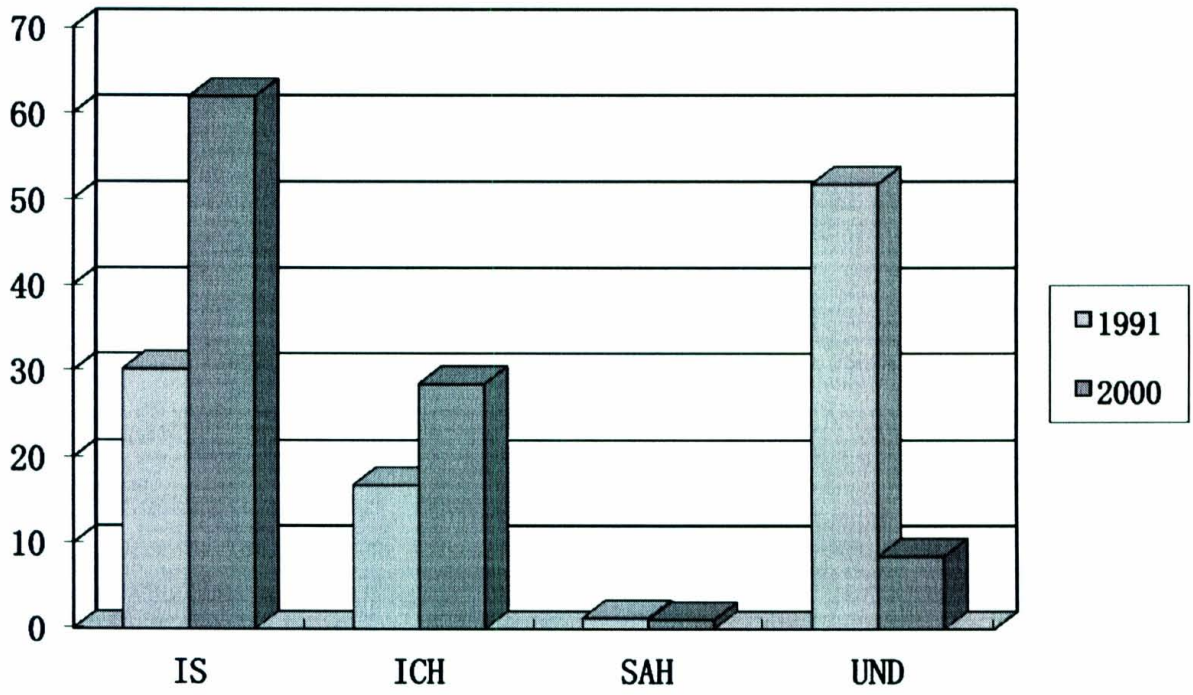

Figure 1.1 Comparison of the frequency of four subtypes of stroke in China during 1991 and 2000 (Adapted from Zhang et al. 2003) (IS: ischemic stroke, ICH: intracranial hemorrhage, SAH: subarachnoid hemorrhage; UND: undetermined)

1.3 Stroke Epidemiology

Prevalence: 9.0 million individuals are estimated to suffer from stroke worldwide each year, while approximately 1.5 to 2.0 million patients reside in China (Liu et al. 2007; Meretoja 2011). Stroke occurs in individuals from all age groups, with an increasing trend after 30 years old. About 95% of stroke events affect individuals older than 45 years old, and two thirds occur in individuals over 65 years old (Senelick et al. 1994). In China, the mean onset for stroke is 63.9±8.4 years old for males and 64.9±7.0 years old for females (Zhao et al. 2008).

Incidence: The incidence of stroke showed an increasing trend in the past three decades in China. Zhao et al. (2008) observed 14,585 consecutive acute stroke events in Beijing from 1984 to 2004, confined to patients aged 25 through 74 years old. The annual incidence increased by 6.7% and 8.7% for total and ischemic stroke events, respectively. The increasing incidence was associated with rapid development during the three decades, which has led to a growing number of senior individuals with prolonged life expectancy. Also, a shift to a westernized lifestyle in Chinese society, especially in cities, may contribute to increased atherosclerotic risk factors. This study also recorded the increasing intake of fat and cholesterol during the study period. Fat intake increased from 88.1 g/d to 97.4 g/d, while cholesterol intake increased from 334.5 g/d to 488.4 g/d from 1983 to 2002. The prevalence of type 2 diabetes mellitus and obesity also increased by 97% and 85% in rural areas, respectively (Liu et al. 2007; Zhao et al. 2008).

Mortality: Stroke is the second most common cause of adult death worldwide. Stroke-related deaths showed an upward trend during the two decades, increasing from 4.4 million in 1990 to 5.7 million in 2004, which approximately constituted 9.7% of total deaths worldwide (Murray et al. 1997; Bonita et al. 2004; Geneva: World Health Organization. 2000, 2004; Donnan et al. 2008; Mathers et al. 2009). The World Health Organization (2000) estimates that stroke and coronary artery disease will rank as the top cause for lost healthy life-years by the year 2020. According to a report from the World Health Organization, 85% of stroke-related deaths occur in low and middle-income countries (Mathers et al. 2009). Vascular disease, including myocardial infarction and stroke, is the predominant cause for adult death in China. The stroke mortality rate varied from 116.63 per 100,000 per year for urban areas to 111.74 per 100,000 per year in suburban areas in China. It constitutes 40% of all stroke-related deaths in developing countries (Reddy et al. 1998; Wu et al. 2001; Zhao et al. 2008).

Impact on health care: Stroke remains a substantial financial challenge for public health care services. Stroke is the main cause of long-term disability worldwide, and 50% - 75% of survivors live with stroke-related disabilities (Foulkes et al. 1988; Geddes et al. 1996; Bonita et al. 1997; O'Mahony et al. 1999). Garo et al. (2000) performed a meta-analysis, enrolling 1,446 ischemic stroke patients from 13 developed countries in the 1990s. This study showed that the mean total cost of healthcare was US$14,000 per individual in the first three months after a stroke, and the estimated lifelong cost per survivor ranged from

US$59,800 to US$230,000 in developed countries. A total of US$65.5 billion is estimated to cover the total stroke-related costs in the United States for the year 2008 (Rosamond et al. 2008). The European Heart Network (2008) reported that a total of €27 billion was devoted to stroke-related fields in 27 European countries each year.

Geographical variation of stroke in China: One interesting phenomenon is the geographical variation of stroke incidence in China. The SINO-MONICA project registered stroke events in individuals aged between 25 to 64 years old in 16 provinces in China from 1987 to 1993. The highest incidence was reported in the northern province of Heilongjiang (337.7 and 553.3 per 100,000 per year for male and female, respectively), while the lowest was in southern provinces such as Anhui (33.0 per 100,000 per year for male) and Fujian (29.7 per 100,000 per year for female) (Wu et al. 2001). Other epidemiology studies also documented the north - south gradient (Li et al. 1985; Wang et al. 1985; Chen et al. 1993; Cheng et al. 1995).

The reason for the geographical differentiation remains unknown, but genetic factors might play a role. The analysis of genetic markers on the Y chromosome and mitochondrial DNAs reveal that the Chinese Han population can be differentiated into northern Han and southern Han, geographically separated by the Yangtze River (Du et al. 1997; Wen et al. 2004). Recent genome-wide studies subdivided the Chinese Han into northern, central and southern Han subgroups (Xu et al. 2009; Chen et al. 2009). The Chinese Han population occupied Northern China area approximately 4,700 years ago,

the area from which the Han language and culture originated. Three massive migrations from Northern to Southern China occurred during the western Jin Dynasty (AD 265-316), the Tang Dynasty (AD 618-907) and the Southern Song Dynasty (AD 1127-1279), respectively (Wen et al. 2004). The Han culture expanded into southern China, and merged with southern natives (Fei et al. 1999, Ge et al. 1997). The southern Chinese Han population subsequently developed (Wen et al. 2004). The genetic differentiation of the Chinese Han population will be further described in Chapter 2.

1.4 Cascade of Ischemic Stroke

The availability of glucose and oxygen is crucial for maintaining normal neurological function. In ischemic stroke, the occlusion of cerebral arteries leads to a loss of blood supply. The depletion of glucose and oxygen causes functional and metabolic impairment in the affected tissue. In cases where oxygenated blood is entirely absent, the neurons will undergo necrosis within eight minutes; however, in most ischemic strokes, collateral vessels will complementarily supply blood to the lesion tissue, and the neuron cells in marginal lesion locations can survive for up to five or six hours before irreversible damage occurs (Adams and Victoria Neurology 8th).

Animal experiments (gerbil and rat penumbra models) indicate that the normal and critical cerebral blood flow for brain metabolism and function are 0.55 L/g/min and 0.23 L/g/min, respectively (Hossmann et al. 1994). Once cerebral blood flow falls below 0.23

L/g/min in the ischemic area, a cascade of multiple functional and metabolic changes will occur at the molecular and cellular levels. These include ATP depletion, efflux of potassium, influx of sodium and calcium, release of glutamate, accumulation of free fatty acids, prostaglandins, leukotrienes and free radicals, degeneration of proteins and membrane disruption. All these changes finally trigger cell edema, necrosis and apoptosis after several hours if effective intervention is not provided (Dirnagl et al. 1999). When cerebral blood flow is reduced below 0.12 L/g/min, the histological infarction lesion can be identified (Hossmann et al. 1994).

1.5 Risk factors for Ischemic Stroke

According to the primary prevention guidelines established by the American Heart Association/American Stroke Association Stroke Council (Goldsteine et al. 2006), risk factors for ischemic stroke can be divided into modifiable and non-modifiable categories. Modifiable risk factors can be controlled by a healthy life style or medication, while non-modifiable risk factors include age, sex, genetic predisposition and ethnicity. Currently, known risk factors account for only 60% of stroke, and the etiology of the remaining 40% of cases remains undetermined (Whisnat et al. 1997; Donnan et al. 2008).

1.5.1 Modifiable Risk Factors

Hypertension

Hypertension, defined as systolic blood pressure (SBP) $\geq$ 140 mmHg or diastolic BP $\geq$ 90 mmHg, is an independent risk factor for stroke (Rosamond et al. 2007). The number of adults with hypertension was approximately 972 million worldwide in 2000, and is estimated to reach 1.56 billion by the year 2025 (Kearney et al. 2005). Persistent hypertension can lead to vascular endothelial damage, oxidative stress, altered vascular tone and permeability, and the degeneration of vascular smooth muscle cells, all of which contribute to stroke by aggravating atherosclerosis (Johansson et al. 1999). The severity of hypertension is directly correlated with stroke risk (Lewington et al. 2002). One epidemiology survey estimated that 18.8% of adult Chinese (160 million individuals) are hypertensive (Wang et al. 2005; Fang et al. 2006). A prospective observational survey followed up on 5,092 male Chinese steelworkers for an average of 13.5 years. About 31% of these participants were hypertensive, and a total of 87 (1.7%) individuals developed ischemic stroke. Logistic regression analysis revealed that, for every ten mmHg rise in systolic or diastolic blood pressure, there was an associated 1.4- or 1.8-fold higher risk for stroke, respectively (Zhang et al. 2004).

Diabetes mellitus

Diabetes mellitus refers to systematic metabolic syndromes characterized by persistently increased plasma glucose levels due to a lack of insulin secretion or increased resistance to insulin. According to the WHO (2006), a diagnosis of diabetes mellitus is made if any

of the following four situations is met: 1) fasting plasma glucose ≥ 7.0 mmol/L; 2) plasma glucose ≥ 11.1 mmol/L two hours after a 75g oral glucose load; 3) symptoms of hyperglycemia and casual plasma glucose level ≥11.1 mmol/L; and 4) glycoglated hemoglobin ≥ 6.5%. A meta-analysis including 39 countries estimated that 171 million individuals have been diagnosed with diabetes mellitus worldwide. This number is expected to rise to 366 million by the year 2030 (Wild et al. 2004).

Diabetes mellitus is an independent risk factor for first-ever stroke and accounts for 9.1% of recurrent stroke (Stamler et al. 1993; Burchfiel et al. 1994; Petty et al. 1998). Chronic diabetes mellitus facilitates atherosclerosis by inducing endothelial dysfunction, inhibiting nitric oxide production and enhancing the proliferation of vascular smooth muscle cells (Brownlee et al. 2001). About 15% to 33% of stroke patients have diabetes mellitus (Hier et al. 1991; Woo et al. 1999; Hillen et al. 2003; Megherbi et al. 2003; Karapanayiotides et al. 2004). A meta-analysis of seven studies identified that diabetes mellitus conferred a 1.7-fold higher risk for ischemic stroke in patients with atrial fibrillation (95%CI: 1.4 – 2.0). The annual stroke incidence varied from 2.0% to 3.5% in individuals with diabetes mellitus and atrial fibrillation (Stroke Risk in Atrial Fibrillation Working Group, 2007). Diabetes mellitus is also associated with increased stroke mortality and morbidity. Jia et al. (2011) registered 14,526 hospitalized patients with acute ischemic stroke and followed up on them for six months in China. They found that 27.0% of stroke patients had diabetes mellitus. A multivariate logistic regression analysis illustrated that the prevalence of diabetes mellitus independently predicted higher risk for

death or living dependency after stroke onset in the Chinese population (OR: 1.23; 95% CI: 1.10 to 1.37).

Atrial Fibrillation

Atrial fibrillation denotes cardiac arrhythmia with chaotic electrical signals generated from the atria. Atrial fibrillation, which can enhance emboli formation via abnormal hemostasis, endothelial dysfunction and increased platelet activation, accounts for approximately 20% of ischemic stroke (Savelieva et al. 2007; Atrial Fibrillation Investigators 1999). A cross-sectional study of 1.89 million adults in California screened 17,974 adults age ≥ 20 years old with atrial fibrillation. It estimated that 2.3 million adults have atrial fibrillation in the US, and 60,000 of these patients develop ischemic stroke annually (Go et al. 2001) The Framingham study followed up on 5,070 individuals for vascular events for 34 years. A total of 572 stroke events were recorded. Compared to individuals without atrial fibrillation, individuals with this condition possessed a 2.6- 4.5 fold higher risk for stroke after adjustment for other cardiovascular risk factors, such as coronary heart disease, heart failure and hypertension ($P<0.001$)(Wolf et al. 1991).

Dyslipidemia

Dyslipidemia denotes derangement in lipid components, such as elevated total cholesterol and triglycerides, and imbalance in lipoproteins, i.e., increased low-density lipoprotein

cholesterol (LDL-C) or reduced high-density lipoprotein cholesterol (HDL-C). Dyslipidemia triggers the atherosclerotic processes by inducing release of inflammatory factors, proliferation of smooth muscle cells, and dysfunction of endothelium and plaque formation (Gau et al. 2006). Elevated total cholesterol, LDL-C, and triglycerides have been reported to be associated with increased risk for ischemic stroke, especially for the large artery atherosclerotic stroke subtype (Iso et al. 1989; Leppala et al. 1999; Ebrahim et al. 2006; Bansal et al. 2007; Freiberg et al. 2008; Bang et al. 2008). The associations of fat and cholesterol intake with ischemic stroke were assessed in the Chinese Han population. Zhao et al. (2008) reported that ischemic stroke events increased by 8.7% annually in the Beijing area from 1984 to 2004. Dyslipidemia was suggested to contribute to this upward trend. During the study period, fat intake increased from 88.1 g/d in 1983 to 97.4 g/d in 2002, and cholesterol intake from 334.5 g/d in 1983 to 488.4 g/d in 2002. Studies in other populations also confirmed this association. Koren-Morag et al. (2002) followed up on 11,177 patients in Israel with coronary artery disease for eight years. A total of 487 individuals developed ischemic stroke or transient ischemic attacks (TIA). Logistic regression analysis showed that reduced HDL cholesterol (OR: 0.89, 95%CI: 0.81 – 0.98), elevated total cholesterol level (OR: 1.12, 95%CI: 1.03 – 1.23) and LDL cholesterol (OR: 1.14, 95%CI: 1.00 – 1.26) were associated with ischemic stroke or transient ischemic attacks (Koren-Morag et al. 2002).

Asymptomatic Carotid Stenosis

Asymptomatic carotid stenosis (ACS) is the presence of atherosclerotic plaque without clinical manifestations. Emboli can break off from the plaque and block cerebral blood. Stroke develops in such cases when persistent ischemia occurs in the affected region. In a prospective observational study, Norris et al. (1991) followed 696 patients with asymptomatic carotid stenosis. Annual stroke rates were 1.3% and 3.3% for patients with carotid occlusion < 75% and ≥ 75%, respectively. A cohort study in Toronto followed 106 patients diagnosed with asymptomatic carotid stenosis for ten years. Forty-eight patients showed moderate carotid stenosis <50%, while 58 patients showed severe stenosis (≥50%). The ten-year risk of stroke was 5.7% in patients with moderate stenosis, and 9.3% in patients with severe stenosis (Nadareishvili et al. 2002).

Hyperhomocysteinemia

Homocysteine is an intermediate product formed in methionine metabolism. Methionine first forms *S*-adenosylmethionine (SAM) by receiving adenosine from adenosine triphosphate (ATP), then SAM demethylates to *S*-adenosylhomocysteine (SAH) , which donates adenosine to generate homocysteine. Homocystiene has two destinies in humans: a) reversible remethylation to methione via folate and vitamin B12, or b) irreversible transsulfuration to cystathionine by Vitamin B6 (Selhub et al. 1999). The normal plasma homocysteine concentration in humans is < 15 μmol/L. (Handy and Loscalzo 2003).

The contribution of hyperhomocysteinemia to vascular disease was first proposed by McCully in 1969, who reported a death from homocystinemia, cystathioninemia and methylmalonic acidemia due to a defect in cobalamin metabolism (McCully 1969). Arteriosclerosis was found at autopsy, which suggested that elevated homocysteine could induce vascular endothelium damage. Elevated plasma homocysteine levels can induce vascular damage through the following pathways: 1) facilitation of the atherosclerostic process by increasing vascular inflammatory mediators (Hofmann et al. 2001; Zhou et al. 2001); 2) impaired endothelial vasomotor function（Eberhardt et al. 2000; Lentz et al. 2000; Dayal et al. 2001）; 3) reduced bioavailability of nitric oxide (NO) by inducing vascular oxidative stress and elevating plasma asymmetric dimethylarginine (ADMA) levels (Vallance et al. 2001; Weiss et al. 2001; Weiss et al. 2002; Ungvari et al. 2003; Boger et al. 2003) ; and 4) dysregulation of lipid metabolism, inflammation and apoptosis by inducing protein modification and endoplasmic reticulum stress (Lentz et al. 1991; Lentz et al. 1993; Jakubowski et al. 2000; Austin et al. 2004).

Mild to moderate hyperhomocysteinemia (Hcy = 15 - 100 μmol/L) has been recognized as an independent risk factor for vascular disease, including ischemic stroke (Graham et al. 1997; Welch et al. 1998; Rozen et al. 2000; Bersano et al. 2008). The Framinghan study measured the total plasma homocysteine levels of 1,947 US participants (mean age ±SD: 70±7 years) and followed up for 9.9 years. One hundred atherothrombotic stroke events occurred. Logistic regression analysis documented the association between

hyperhomocysteinemia (greater than 14.2 μmol/L) and antherothrombotic stroke (OR: 1.90, 95%CI: 1.02 – 3.51, P< 0.001) after adjustment for conventional vascular risk factors (Bostom et al. 2003). Other studies also confirmed the contribution of hyperhomocysteinemia to ischemic stroke. Eikerlboom et al. (2000) compared the plasma homocysteine level between 219 first-ever ischemic stroke patients and 205 age and gender matched healthy individuals. The mean plasma homocysteine levels were 14.1 μmol/L in patients with large-artery ischemic stroke and 12.7 μmol/L for those with small-artery ischemic stroke, both of which were significantly higher than that found in the control group (10.5 μmol/L). Logistic regression analysis revealed that the upper quartile of the plasma homocysteine level (greater than 13.8 μmol/L) was associated with increased risk for ischemic stroke (OR: 2.2, 95%CI: 1.1 to 4.2) compared to the lowest quartile group (homocysteine level less than 9.0 μmol/L) after adjustment for conventional vascular risk factors. The association of homocysteine levels with the large artery subtype of ischemic stroke exhibited a dose-dependent pattern. Compared with the lowest quartile of homocysteine (less than 9.0 μmol/L), the ORs for large artery stroke risk of the other three quartile groups were 3.0 (95%CI: 0.8 to 10.8), 5.6 (95%CI: 1.6 to 20) and 8.7 (95%CI: 2.4 to 32), corresponding to the second quartile (9.0 μmol/L to 11.2 μmol/L), the third quartile (11.3 μmol/L to 13.8 μmol/L) and the fourth quartile (beyond 13.8 μmol/L), respectively. A similar trend was observed in the Chinese Han population. Li et al. (2003) measured the plasma homocysteine level in 807 cerebral thrombosis patients, 513 small artery stroke cases and 1,832 age and sex-matched controls. The median total plasma homocysteine levels were 14.7 μmol/L in atherothrombotic stroke

and 14.8 μmol/L in small artery stroke, respectively. Both of these were significantly higher than the median value of 12.8 μmol/L found in the controls. Logistic regression analysis indicated that the elevated homocysteine level was independently associated with atherothrombotic stroke (OR: 1.72, 95%CI: 1.39 to 2.12, P<0.001) and small artery disease after adjusting for conventional vascular risk factors (OR: 1.89, 95%CI: 1.50 to 2.40, P<0.001) (Li et al. 2003).

Life style: cigarette smoking, nutrition, obesity and physical inactivity

Unhealthy modern life styles, including smoking, poor diet, obesity and physical inactivity, can impair the cardiovascular system and increase stroke risk.

Smoking, including exposure to a smoking environment and active cigarette smoking, has been recognized as a significant risk factor for ischemic stroke (Wolf et al. 1988; Mast et al. 1998; Bonita et al. 1999; Heuschmann et al. 2007). Cigarette smoking can enhance vascular inflammation, thrombosis and oxidation of LDL cholesterol (Ambrose and Barua 2004). In a large, population-based cohort study, Mannami et al. (2004) observed 19,782 males and 21,500 females aged from 40 to 59 years old, all of whom had no history of vascular disease. Their smoking status and incidence of stroke were recorded from 1990 to 2001. A total of 327 and 176 ischemic stroke events occurred in male and female individuals, respectively. Compared to stroke patients without a history of smoking, logistic regression analysis revealed that current smokers showed a 1.66-fold

(95%CI: 1.25 to 2.20) higher risk for ischemic stroke independent of conventional risk factors. A similar trend was observed in other studies. Another prospective study enrolled 118,539 US female participants aged from 30 to 55 years old and without a history of vascular disease, and were followed up on for eight years. A total of 122 thromboembolic stroke events were recorded. Data analysis indicated that current smokers consuming more than 15 cigarettes per day conferred a 2.7-fold higher risk for ischemic stroke compared to non-smokers (Colditz et al. 1988).

Excess intake of sodium can induce water retention in the cardiovascular system which increases the risk for hypertension (He and MacGregor 2004). Recommended intake for sodium and potassium are ≤2.3 g/d and ≥4.7 g/d, respectively (Goldstein et al. 2006). A 19-year cohort study, including 2, 688 overweight individuals aged 25 to 74 years old, revealed that a daily intake of extra 100 mmol predicted a 1.32- fold higher risk for stroke (95%CI: 1.07 – 1.64, $P < 0.01$) (He et al. 1999). Another population-based study enrolling 43,783 middle-aged US male individuals reported a positive association between potassium supplements and a reduced risk for stroke after eight years follow-up (RR: 0.36, 95%CI: 0.18 – 0.72) (Ascherio et al. 1998).

Obesity, defined as body mass index (BMI) >30 kg/m^2, contributes to ischemic stroke by inducing endothelial damage and the proliferation of inflammatory factors in vessels (Poirier et al. 2006; Strazzullo et al. 2010). Kurth et al. (2002) enrolled 21,414 US male physicians free of cardiovascular disease in 1982. A total of 631 (5.5%) ischemic stroke

events were recorded in the subsequent 12.5 years. Compared to those with normal weight (BMI <23), obese individuals with a BMI > 30 showed a 1.95-fold higher risk (95%CI: 1.39 to 2.73, P < 0.001) for ischemic stroke. In addition to males, obesity also confers a risk for ischemic stroke in females. A prospective cohort study enrolled 116,759 US female nurses aged 30 to 55 years old, who were free of cardiovascular diseases, and followed up for 16 years. Compared to normal weighted participants (BMI< 27 kg/m^2), the relative risk ratios for ischemic stroke in overweight individuals were 1.75 (95%CI: 1.17 – 2.59), 1.90 (95%CI: 1.28 – 2.82) and 2.37 (95%CI: 1.60 – 3.50) for individuals with 27 $kg/m^2 \leq BMI \leq 28.9 kg/m^2$, $29 kg/m^2 \leq BMI \leq 31.9 kg/m^2$, and $BMI \geq 32 kg/m^2$, respectively (P < 0.001) (Rexrode et al. 1997).

Physical inactivity contributes to ischemic stroke by affecting vascular risk factors (Lee et al. 2003). In a large population-based cohort, 39,315 US healthy individuals over 45 years old were recruited. During 11.9 years follow-up, 473 ischemic stroke events occurred. Compared to individuals with little physical activity (<200 kcal/week), participants with moderate (600 – 1,499 kcal/week) and high physical activity (≥1,500 kcal/week) showed a lower risk for ischemic stroke, OR ratios were 0.66 (95%CI: 0.52 to 0.84) and 0.61 (95%CI: 0.47 to 0.80) for moderate and high physical activity, respectively. Other studies also provided evidence that regular physical activity has beneficial effects in reducing the incidence of ischemic stroke. Lee et al. (2003) analyzed 23 studies from 1966 to 2002, and revealed that physical activity was associated with a lower risk of stroke, showing a

20% reduction for moderate activity (RR = 0.80) and a 27% reduction for high strength activity (RR: 0.73, 95%CI: 0.67 – 0.79), respectively, compared to low levels of activity.

1.5.2 Non-modifiable Risk Factors

Age

Advanced age is a significant risk factor for stroke, resulting in a 2-fold increased risk for every ten years after the age of 55 years old, with two thirds of stroke patients being greater than 65 years old (Wolf et al. 1992; Senelick et al. 1994; Brown et al. 1996;). Feigin et al. (2003), after performing a meta-analysis involving a total of 3,266,366 individuals from 13 countries worldwide, revealed the trend toward increased stroke incidence with advanced age. The age-standardized stroke incidence rate ranged from 0.1 to 0.3 per 1,000 person-years for individuals with an age less than 45 years old; 4.2 – 6.5 per 1,000 person-years for individuals aged greater than or equal to 55 years old; and incidence increased to 12.0 – 20.0 per 1,000 person-years for elderly people aged greater than 75 years old. Another meta-analysis reported that the stroke incidence was 30 times higher in individuals beyond 75 years old, compared to individuals aged 35 to 44 years old in the Chinese Han population (Liu et al. 2007).

Sex

Males are more vulnerable to stroke than females. The China Multicenter Collaborative Study of Cardiovascular Epidemiology in 17 communities across China from 1991 to 2000 showed that men accounted for 59.0% of total stroke events among all 16,031 registered first-ever stroke patients (≥ 25 years old) (Zhang et al. 2003). An eight-year national registry in Denmark aimed at hospitalized individuals with first-ever ischemic stroke reported that males accounted for 52.1% of 40,102 individuals, and the incidence rate was obviously higher in males than females among middle-aged individuals (Andersen et al. 2010).

Race-Ethnicity

African Americans show higher stroke incidence than European Caucasians (Broderick et al. 1998). The reason for this might include higher prevalence of vascular risk factors such as hypertension and diabetes in African Americans compared to Caucasian and Hispanic populations (Schcamm et al. 2010). The prospective population-based Northern Manhattan Stroke Study (NOMASS) examined 210,000 residents in northern Manhattan area. The study revealed that African Americans showed a 2-3 fold higher risk for developing ischemic stroke per year compared to European descendants (Sacco et al. 1998). A more recent Greater Cincinnati/Northern Kentucky population project ascertained stroke events among 1.3 million residents in the study area in 1993, 1999 and 2005. The race-adjusted ischemic stroke incidence rates were 303, 291 and 294 per 100,000 persons for African Americans for the same periods, respectively. The incidence rates for their European descendants were 206, 241 and 179 per 100,000 persons for the

year 1993, 1999 and 2005, respectively. Comparison between the two ethnic groups revealed that African Americans have a higher chance of developing ischemic stroke than Caucasian populations despite the declining trend in incidence within each ethnic group (Kleindorfer et al. 2010).

Genetic predisposition

The genetic predisposition for stroke was identified in animal models. Rubattu et al. (1996) created a stroke-prone hypertension-independent rat model by hybridizing male stroke-prone spontaneously hypertensive rats and female stroke-resistant spontaneously hypertensive rats. The researchers observed stroke incidence among the 220 normotensive rats and performed a genome-wide linkage analysis by genotyping a total of 1,038 genetic markers. Three quantitative loci on Chromosomes 1 and 5 showed LOD scores > 3.0. This study indicated that genetic components contribute to stroke, independent of hypertension.

Population studies suggest that genetic component play a substantial role in ischemic stroke in humans. The nationwide twin study in Denmark recruited 351 monozygotic and 639 dizygotic twins born from 1870 to 1952. At the initial screening, one twin from each pair was verified for stroke death, the cause of death in the other twin was then recorded from the Register of Causes of Death or the Danish National Discharge Register. It showed that monozygotic twins had a higher concordance rate (35 pairs, 10%) of stroke

death compared to dizygotic twins (34 pairs, 5%) (OR: 2.1, 95%CI: 1.3 – 3.3) (Bak et al. 2002). This study provides evidence for genetic predisposition to stroke-related death. The Sahlgrenska Academy study on the European Caucasian population in Sweden registered 600 consecutive ischemic stroke patients with onset age < 70 years old and 600 age- and sex- matched controls. Comparison between cases and controls showed that the first-degree family history of stroke was associated with increased risk of ischemic stroke (OR: 1.75, 95%CI: 1.26 – 2.43). Statistical significance remained when performing similar analysis in three subtypes of ischemic stroke. The odd ratios were 1.88, 1.79 and 1.70 for large-vessel stroke (95%CI: 1.02 – 3.44), small-vessel stroke (95%CI: 1.13 – 2.84) and cryptogenic stroke (95%CI: 1.13 – 2.56), respectively (Jood et al. 2003).

The mechanisms involved in genetic predisposition for stroke are proposed to involve the following pathways: 1) genetic components facilitate the development of conventional vascular risk factors, such as hypertension and hyperhomocysteinemia, which increased stroke risk in vulnerable individuals; 2) genetic components interact with environmental factors to enhance stroke risk; and 3) genetic components contribute to intermediate phenotypes, such as atherosclerosis, which can lead to stroke (Dichgans et al. 2007; Stankovic et al. 2010). During the past two decades, dozens of candidate genes have been reported to be associated with stroke; however, few of these show consistent results in the studied populations. The candidate gene study of ischemic stroke will be discussed in chapter 2.

Chapter 2

Candidate Genes for Ischemic Stroke

2.1 Single Nucleotide Polymorphism, Haplotype and Linkage Disequilibrium

Single nucleotide polymorphisms (SNP) refer to variation in DNA sequence caused by a single nucleotide (adenine, thymine, cytosine and guanine) variation at specific loci. The human genome is estimated to contain 6.0 million common SNPs (minor allele frequency > 5%) (Dana et al. 2005). Approximately 2.3 million SNPs differ between two unrelated individuals, i.e., 1 nucleotide variation per 1,000 - 2,000 base pairs (The International SNP Map Working Group 2001). When SNPs occur in the protein-coding region, they are categorized into two types, depending on their effects on the resulting polypeptide sequences: (1) synonymous variation, in which the nucleotide variation does not change amino acid sequence; and (2) non-synonymous variation, which results in amino acid change or premature termination. SNPs are usually biallelic. The frequency of alleles can vary geographically between different ethnic populations: the major allele at a certain locus in a population can be the minor one in another (Taras et al. 2010).

In genetic association studies, haplotype refers to SNPs on different loci on the same chromosome statistically associated with each other (The International HapMap Consortium 2003, 2005). Linkage disequilibrium describes the non-random association of alleles on different loci (Falconer et al. 1996). Genetic linkage disequilibrium can help identify candidate loci contributing to diseases in case that the tested genetic marker and the disease causing variant are closely linked (Briscoe et al. 1994 Plomin et al. 1994; Jorde et al. 1995; Kaplan et al.1995; Chapman and Wijsman 1998). For two loci not

tightly linked, linkage disequilibrium will gradually decay over generations for recombination occurs between the two loci. For those closely linked loci, linkage disequilibrium between disease and marker loci will remain strong through generations. These assumptions provide the basis for genetic association studies identifying candidate genetic variants for diseases.

The International HapMap Project categorizes the human genome into linkage disequilibrium (LD) blocks, i.e., certain chromosome regions where allelic association between SNPs shows few historical recombination events (Tailon-Miller et al. 2000; Daly et al. 2001). The identification of LD blocks provides an efficient tool in genetic association studies. Testing tagSNPs, which represent these LD blocks, can avoid the need to genotype all SNPs within the candidate region (Risch et al. 2000; Zhang et al. 2002). The LD patterns of more than 4.0 million SNPs across the whole genome have been characterized in different ethnic populations following the completion of the International HapMap Project phase I and II in 2005 and 2007 (Chee et al. 2010).

2.2 Candidate Gene Approach Study of Ischemic Stroke

The candidate gene approach study has been widely used in investigating genetic risk factors for complex disease (Matarin et al. 2009). SNPs are often used as genetic variants in these association studies. These SNPs are genotyped in subjects with the target phenotype (cases) and age- and sex- matched healthy individuals (controls). The

association between the candidate variants and targeted disease can be assessed by the χ^2 test analyzing the frequency difference between case and control groups. Odds ratios are used to indicate the relative risk of the tested variants on disease (Sanja 2010). A reliable genetic association study requires a large sample size (>1,000) with adjustment for age and sex (Li et al. 2003).

2.3 Candidate Genetic Variants in Ischemic Stroke

Genetic association studies have been widely applied to map common genetic variants (minor allele frequency > 5%) conferring risk for stroke (Matarin et al. 2009). To date, studies have revealed pathophysiological process of ischemic stroke are involved in the following pathways: 1. haemostasis system: factor V, factor VII, factor XII, factor XIII, factor II (prothrombin), fibrinogen, plasminogen activator inhibitor-1, platelet glycoprotein receptors, von Willebrand factor, tissue plasminogen activator, thrombin activable fibrinolysis inhibitor, thrombomodulin, protein Z, and annexin A5 ; 2. homocysteine metabolism: methylene tetrahydrofolate reductase, cystathione beta-synthase, and methionine synthase; 3. lipid metabolism: apolipoproteins, lipoprotein receptors, and key enzymes of plasma lipoprotein metabolism (Bersano et al. 2008, Stankovic et al. 2010). The candidate genetic variants for ischemic stroke are summarized in table 2.1. The association between these candidate genetic variants and stroke remains inconsistent in different ethnic populations (Matarin et al. 2009). The reason accounting

for this phenomenon may lie in selection bias, small sample size, and heterogeneity of stroke. Therefore, further studies are needed to help resolve this debate.

Table 2.1 Candidate genes for ischemic stroke (Adapted from Bernaso et al. 2008 and Stankovic et al. 2010)

Pathogenetic systems	Candidate genes
Haemostasis	
	F2, F5, FGA/FGB, F7, F12A1, VWF, F12, SERPINE1, ITGB3, ITGA2B, ITGA2, GP1BA, ACE, ACT
Homocysteine metabolism	
	MTHFR, CBS, MTR
Lipid metabolism	
	APO ε2, ε3, ε4, LPL, PON 1/2/3, CETP, ABCA1

2.4 Genomewide Association Study of Stroke

A genome-wide association study (GWAS) is a method by which large numbers of SNPs evenly scattered throughout the whole genome are genotyped. It is expected to discover novel genetic variants by providing a hypothesis-free method to investigate common genetic variants with moderate effects on complex disease (Kul et al. 2010). So far, GWAS have identified several novel candidate genetic loci for ischemic stroke, including 4q25 (*PITX2* gene) (Gretarsdottir et al. 2008), 16q22 (*ZFHX3* gene) (Gudbjartsson et al. 2008) and 12p13 (*NINJ2* gene) (Ikram et al. 2009). However, subsequent independent validation studies have failed to reproduce these GWAS findings (Hegele et al. 2010). In addition, genetic variants for ischemic stroke identified by the candidate gene approach did not achieve the statistical significance level in genome-wide association studies (Ikram et al. 2009). Up to now, GWAS have not revolutionized the genetic study of stroke as expected.

2.5 Candidate Genetic Variants in the Present Study

2.5.1 *MTHFR* c.677C>T and c.1298 A> C variants

The association between hyperhomocysteinemia and stroke is described in chapter 1. 5,10-Methyleneterahydrofolate reductase (MTHFR), the key enzyme regulating homocysteine metabolism, remethylates homocysteine back to methionine by catalyzing

the reduction of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (Ueland et al. 2000, 2001; Champe et al. 2008). Impaired MTHFR activity interrupts the remethylation process of homocysteine to methionine, and the plasma level of homocysteine subsequently accumulates leading to hyperhomocysteinemia.

The *MTHFR* gene, which is located on chromosome 1p36.3 and encodes the MTHFR enzyme, has been widely investigated for its contribution to cardiovascular disease (Goyette et al. 1994; Bersano et al. 2008; Stankovic et al. 2010). The most studied variant is the *MTHFR* c.677C>T (p.Ala222Val) vairant. *In vitro* experiments indicated that heterozygous CT and homozygous TT genotypes reduce MTHFR activity to 65% and 35% compared to the homozygous CC genotype. The homozygous TT genotype leads to enzyme thermoliability and increases plasma homocysteine levels by 25% (Frosst et al. 1995). Van der Put et al. (1998) confirmed this phenomenon by comparing the homocysteine level among different genotypes of the *MTHFR* c.677C>T variant in 186 individuals. The number of individuals with *MTHFR* c.677C>T genotypes was 44.0% (n=82), 41.9% (n=78) and 14.0% (n=26) for CC, CT and TT genotypes, respectively. The mean plasma homocysteine level was 18.4 μmol/L in the TT genotype, which was significantly higher than that of the CC genotype (13.1μmol/L). This suggests that the *MTHFR* c.677C>T variant may confer risk to stroke via influence on plasma homocysteine levels.

The association between *MTHFR* c.677C>T and ischemic stroke remains inconsistent. Cronin et al. (2005) analyzed 22 studies, recruiting 4,740 ischemic stroke cases and 7,486 controls, and revealed a dose-dependent association between the *MTHFR* c.677C>T variant and ischemic stroke. Compared to CC genotype, odd ratios were 1.18 for heterozygous CT genotype (95%CI: 1.09 to 1.29, P<0.001) and 1.48 for homozygous TT genotype (95%CI: 1.22 to 1.8, P<0.001). Li et al. (2003) recruited 807 ischemic stroke patients and 1,832 controls. The median plasma homocysteine level was significantly higher in cases (14.7 μmol/L) compared to controls (12.8 μmol/L), and increased risk for ischemic stroke in the Chinese Han population (OR: 1.72, 95%CI: 1.39 – 2.12, P < 0.001). In addition, the homozygous TT genotype was found in 24.5% of cases, versus 21.7% in controls (OR: 1.37, 95%CI: 1.06 to 1.78). This indicates that the TT genotype is associated with increased risk for ischemic stroke. However, another association study in the Singapore Chinese population did not confirm this association (Low et al. 2011). A recent meta-analysis, including six meta-analyses and ten case-control studies, failed to confirm the association between the *MTHFR* c.677C>T variant and ischemic stroke (Bersano et al. 2009).

Another common variant, *MTHFR* c.1298A>C (p.Glu429Ala), was identified by sequencing the coding region of *MTHFR* in 86 individuals with an open neural-tube defect and 403 control subjects (Van der Put et al. 1998). They established an association between AC/CC genotypes and reduced MTHFR activity in isolated human lymphocytes. However, the effect of *MTHFR* c.1298A>C variant on plasma homocysteine levels

remains uncertain. Studies reported that this variant either did not affect the homocysteine levels, or was associated with elevated or even reduced homocysteine levels (van der Put et al. 1998, Friedman et al. 1999, Lievers et al. 2001, Castro et al. 2003). Sazci et al. (2006) genotyped the *MTHFR* c.1298A>C variant in 92 ischemic stroke patients and 259 healthy controls from a Turkish Caucasian population. The CC genotype distributed to 20.7% of cases, which was significantly higher than 8.1% in the control group (OR: 2.950, 95%CI: 1.504 – 5.786, P = 0.001). Han et al. (2010) performed a similar study on 264 silent brain infarction patients and 234 controls in the Korean population. Heterozygous AC genotype possessed 30.3% of cases compared to 21.8% of control individuals, and increased risk (OR: 1.734, 95%CI: 1.13 – 2.66, $P < 0.05$) for silent brain infarction.

2.5.2 Chromosome 9p21 Locus

The chromosome 9p21 locus has been identified to be associated with a broad range of vascular diseases. Rs1333049, an SNP within the chromosome 9p21 locus, was reported to be associated with increased risk of coronary heart disease in the large Wellcome Trust Case Control Consortium (WTCCC) study (1,926 cases and 2,938 controls, OR = 1.37, 95%CI: 1.26 to 1.48, $P = 1.80\times10^{-14}$), and was validated subsequently in the German Myocardial Infarction Family Study (875 cases and 1,644 controls, OR=1.33, 95%CI: 1.18 to 1.51, $P = 3.40\times10^{-6}$) (Samani et al. 2007). A meta-analysis involving 12,004 patients with coronary heart disease and 28,949 controls confirmed the association between rs1333049 and coronary heart disease in the Caucasian population (OR: 1.14,

95%CI: 1.20 – 1.29, P=6.04 $\times 10^{-10}$) (Schunkert et al. 2008). Saxena et al. (2007) genotyped 386,731 SNPs in 1,464 patients with Type 2 diabetes mellitus and 1,467 age and gender-matched controls, and found that the T allele of rs10811661 on chromosome 9p21 locus distributed significantly higher in cases than in controls. (OR: 1.37, 95%CI: 1.18 - 1.59, P = 3.6×10^{-5}). A subsequent cohort of 3,167 Chinese Han individuals replicated the enhanced risk of the variant rs10811661 conferring to Type 2 diabetes mellitus (OR: 1.23, 95%CI: 1.03 to 1.47, P = 0.02) (Cheng et al. 2011). A large case control study suggested that the G allele of rs10757278 on chromosome 9p21 locus is associated with abdominal aortic aneurysm (2,836 patients and 16,732 controls, OR: 1.31, 95%CI: 1.22 - 1.42, P=1.2 $\times 10^{-12}$) and intracranial aneurysm (1,134 patients and 15,481 control individuals, OR: 1.29, 95%CI: 1.16 - 1.43, P= 2.5×10^{-6}) (Helgadottir et al. 2008).

The association between 9p21 locus and ischemic stroke represents one of the most intriguing discoveries in the genetic study of ischemic stroke for the year 2010 (Meschia 2011). Matarin et al. (2008) recruited 249 ischemic stroke patients and 268 controls from a Caucasian population. They identified that SNPs rs10116277, rs1333040, rs1333042 and rs2383207, which encompassed a 44-kb region on 9p21, were associated with increased risk for ischemic stroke (all $P < 0.05$). The association between this locus and ischemic stroke remains weak in the Chinese Han population. Hu et al. (2009) registered 355 ischemic stroke patients and 430 healthy controls in the Beijing area. By genotyping SNPs rs2383206 and rs10757278, this study identified that the AG/GG genotypes of rs2383206 conferred a 1.51-fold higher risk for ischemic stroke (95%CI: 1.11 to 2.05, P =

0.009), especially significant in large vessel stroke (OR: 2.09, 95%CI: 1.30 to 3.37, P = 0.002). However, the haplotype analysis did not reach significance level (P = 0.067). This suggested that 9p21 confers a genetic risk for ischemic stroke in the Chinese Han population. Ding et al. (2009) genotyped 17 tagSNPs on chromosome 9p21 in 558 patients with ischemic stroke, 510 patients with coronary artery disease and 557 control individuals free of these conditions in the Chinese Han population. Their study showed that rs2383206 (OR: 1.35, 95%CI: 1.11 to 1.64, P = 0.006), rs1004638 (OR: 0.65, 95%CI: 0.53 to 0.81, P = 0.001) and rs10757278 (OR: 1.39, 95%CI: 1.15 to 1.69, P= 0.002) distributed differently between coronary artery disease patients and control individuals. However, none of the tested SNPs reached statistical significance level for ischemic stroke in the study. Haplotype analysis revealed that the ATTA haplotype of tag SNPs rs2383206, rs1004638, rs17761442 and rs10757278 distributed 0.7% in ischemic stroke patients, significantly lower than in control subjects (4.1%) (OR: 0.78, 95%CI: 0.67 to 0.87, P = 4.1×10^{-4}). Subsequent validation studies by the same research group recruited 442 ischemic stroke patients and 502 control individuals. The above four SNPs on 9p21 candidate locus were genotyped. Statistical analysis confirmed the above association at the haplotype level (OR: 0.85, 95%CI: 0.77-0.95, P=0.003). However, no statistical significance was reached at the SNP level (Ding et al. 2009). More investigations are needed on the association between chromosome 9p21 locus and ischemic stroke in the Chinese Han population.

The 9p21 locus also conferred risk for several non-vascular diseases, such as open-angle glaucoma (Ramdams et al. 2010), melanoma (Bishop et al.2010), childhood acute lymphoblastic leukemia (Sherborne et al. 2010), glioma (Shete et al. 2009), basal cell carcinoma (Stacey et al. 2009) and breast cancer (Turnbull et al. 2010).

The candidate locus on 9p21 is devoid of any known gene. Since these associated SNPs identified in previous studies were common SNPs (minor allele frequency > 5% among the studied population) from the initial GWAS SNP panel, scientists proposed that inter-frequency variants (minor allele frequency varies at 2% - 5%), or those common SNPs not included in the published data, might confer a higher risk for these phenotypes. Shea et al. (2011) then sequenced a 240-kb region on 9p21 in 47 individuals and established 536 novel SNPs including both common and inter-frequency variants. Two association studies were performed on diabetes mellitus (1,000 type 2 diabetes patients and 1,048 controls) and myocardial infarction (1,274 cases and 1,407 controls) by genotyping these variants. However, none of these newly established SNPs showed higher odds ratios compared with the published GWAS SNP signals.

Function studies have examined the effect of this locus on gene expression. Two tumor suppressor genes, cyclin-dependent kinase inhibitor 2A (*CDKN2A*) and *CDKN2B* reside approximately 100 kb away from the core vascular disease risk region. They are involved in the regulation of cell cycle, aging, senescence, and apoptosis (Kim and Sharpless 2006; Gil and Peters 2006). *ANRIL* is a non-coding RNA with its exons 13-19 overlapping the

core vascular risk region on 9p21, and is transcribed in the opposite direction to the *CDKN2B* gene. *ANRIL* is expressed in vascular tissues, such as coronary smooth muscle, vascular endothelial cells, human monocyte-derived macrophages, carotid endarterectomy specimens, and abdominal aneurysm (Cunnington and Keavney 2011). To date, function studies have revealed inconsistent results. Liu et al. (2009) identified the association between rs10757278 on 9p21 and reduced expression of *CDKN2A*, *CDKN2B* and *ANRIL* in peripheral blood T lymphocytes from 170 healthy volunteers. Cunnington et al. (2010) validated a similar association between chromosome 9p21 locus and decreased *ANRIL* transcription in peripheral blood extracted from 487 healthy individuals. These studies suggested that chromosome 9p21 locus may contribute to vascular disease by regulating expression of *CDNKN2A*, *CDKN2B* and *ANRIL* in vascular tissues. However, other studies failed to confirm this association (Michael et al. 2011). Hold et al. (2010) revealed that chromosome 9p21 was associated with reduced *ANRIL* and *CDKN2B* expression in mononuclear cells from 1,098 coronary artery patients. However, this correlation diminished when testing the association in atherosclerotic plaque specimens. An animal study knocked out a 70kb on chromosome 4 in mice, which was estimated to be orthologous to 9p21 in humans. These mice showed reduced expression of *CDKN2A* and *CDKN2B* and proliferation of aortic smooth muscle cells. However, they did not show a predisposition to vascular disease (Visel et al. 2010). Another study suggested that 9p21 risk alleles contribute to vascular disease by increasing platelet reactivity (Musunuru et al 2010). Musunuru et al (2010) identified that 12 SNPs in the 9p21 region showed an association with increased platelet reactivity among 1,402

asymptomatic participants (P<0.001). They also validated this association in the 2,363 participants from the Framingham heart study cohort. The exact mechanism underlying the association between 9p21 and vascular diseases needs further elucidation.

2.6 The Genetic Complexity of the Chinese Han Population

China has the largest population worldwide, with 1.3 billion individuals from 56 ethnic groups. About 92% Chinese belong to the Han Chinese ethnic group, which constitutes 20% of the world's entire population. Genetic studies analyzing Y chromosome and mitochondrial DNA variations suggest that the Chinese Han population could be sub-categorized into Northern Han and Southern Han, geographically divided by the Yangtze River, the largest river running from west to east across China (Xiao et al. 2000; Wen et al. 2004). A recent genomewide study, which mapped 160,000 SNPs in 1,700 individuals from 22 regions across China indicated that the Chinese Han population could be further differentiated into three subgroups: the Northern, Central and Southern Han (Xu et al. 2009). Another larger study, genotyping 350,000 SNPs in over 6,000 participants sampled from ten provinces, confirmed these genetic subgroups within the Chinese Han population. They further indicated that the Chinese Han Beijing (CHB) population in the international HapMap database could represent the Chinese Han from northern and central areas (Chen et al. 2009). Our sample was collected solely from Henan province, which is located in central China among 34 administrative regions in China.

Chapter 3

Materials and Methods

3.1 Rationale

Stroke is the second most common cause of adult death and is the leading reason for long-term adult disability worldwide (Donnan et al.2008; Bonita et al. 2004). Assessment of genetic predisposition will facilitate prevention and individualized treatment of stroke. The association of *MTHFR* c.677C>T, c.1298A>C variants and 9p21 locus with ischemic stroke is inconsistent in published data. The reasons for these inconsistent reports might lie in the heterogeneity of stroke, genetic complexity between ethnic groups, or limits of the study design.

The Chinese Han population, a seemingly homogenous ethnic population, has been classified into three geographic subregions: the North Han, Central Han and South Han (Chen et al. 2009, Xu et al. 2009). Genetic variants associated with complex disease might be different in these subpopulations; therefore, sample selection should be area specific. The present study focuses on the Chinese Han population from Henan province, the central China.

3.2 Objectives

In the present study, we performed two case control studies, with the following aims:

To assess the association between two common variants, *MTHFR* c.677 C>T and

c.1298 A>C, and ischemic stroke in the Chinese Han population from Henan province;

To investigate the genetic association between the candidate chromosome 9p21 locus and ischemic stroke in the Chinese Han population from Henan province.

3.3 Materials and Methods

From February 2006 to March 2007, 1,429 consecutive patients (Mean age ± SD: 62.71 ± 11.80, male: n=868, 60.7%), diagnosed with ischemic stroke from 18 hospitals in Henan province, were enrolled in the present study. The inclusion criteria were: 1) stroke diagnosis according to WHO criteria, and 2) an MRI or CT scan indicating the ischemic lesion corresponding to the neurological deficits. Patients with hemorrhagic stroke, subarachnoid hemorrhage, transient ischemic attack and severe systemic diseases were excluded from this study. The control group consisted of 1,197 (Mean age ± SD: 58.51 ± 9.237, male: n=760, 63.5%) ethnic matched individuals without a history of myocardial infarction or stroke who were admitted to these hospitals for routine health examinations. Informed consent was obtained from all participants. Samples were collected by the Genetics department of Zhengzhou University. The study protocol on the collaboration between Zhengzhou University and Department of Laboratory medicine in Health Sciences Centers, Newfoundland and Labrador, was approved by institutional boards.

SNP Genotyping

Genomic DNA samples were obtained by salt extraction protocol (Miller et al 1988). In the *MTHFR* study, two variants were tested, including *MTHFR* c.677C>T (rs1801133, C__1202883_20) and *MTHFR* c.1298A>C (rs1801131, C__850486_20) variants. For the 9p21 study, four SNPs were selected from Chromosome 9p21.3 locus: including rs1333049 (C__1754666_10), rs2383207 (C__15789010_10), rs10757274 (C__26505812_10), and rs10116277 (C__29991625_20), which encompassed the 44-kb candidate region on chromosome 9p21 (the position mapped to chr9.22071397 and chr9.22115503). All tested SNPs were genotyped using TaqMan SNP genotyping kit (ABI; Foster City, CA) on real-time PCR platform (ABI Prism 7000 sequence detection system). The reaction volume included: 2.0 µl genomic DNA (100 ng/µl), 2.5µl TaqMan universal PCR master mix, 0.125 µl primer and probes, 0.375 µl ddH2O. The standard reaction condition included activation of uracil-N-glycolase (UNG) (2 min, 50°C), polymerase activation (10min; 95°C), 40 cycles of denaturation (15s; 95°C), annealing and extension (1min; 60°C).

Statistical Analysis

The genotype frequency data for each variant in the control group was tested for deviation from the Hardy-Weinberg equilibrium using online software (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). Haplotype frequencies were estimated using the PowerMarker V3.25

Software. Genotype and haplotype distribution between case and control groups were tested by the χ^2 test under recessive, dominant and co-dominant models by SPSS 16.0 software package (SPSS Inc.). Statistical power was calculated using QUANTO V1.2.3 software. Differences with $P< 0.05$ (two-tailed) were considered statistically significant. Bonferroni correction was not performed in the two studies due to the following reasons: 1) for the *MTHFR* study, two tested variants of the *MTHFR* gene are in linkage disequilibrium; 2) for the 9p21 study, the four variants are from one candidate risk region, where rs10757274 and rs2383207 are in one LD block, and two other variants adjacent to this block; 3) Bonferroni correction is too conservative.

Chapter 4

Reduction of Chromosome 9p21 Locus Interval Associated with Risk for Ischemic Stroke in the Chinese Han Population

Shuo Li[1,2], Yu-Ming Xu[5], Edward Randell[1], Hong Zheng[6], Hai-Zheng Wang[5], Guang Sun[4], Fei-Yu Han[1,2], and Ya-Gang Xie[1,2,3,4]

Disciplines of [1]Laboratory Medicine, [2]Genetics, [3]Pediatrics, and [4]Medicine, Memorial University of Newfoundland, St. John's, NL, Canada.

Departments of [5]Neurology of the First Affiliated hospital, and [6]Genetics, Zhengzhou University, Henan, P.R. China.

This manuscript has not yet been submitted for publication.

Abstract

Background: Chromosome 9p21 common variants are strongly associated with coronary arterial disease (CAD). This association has been expended to other vascular disorders including ischemic stroke in Caucasians. However, the association between 9p21 common variants and ischemic stroke in the Chinese Han population lacks strong evidence. **Method:** Four common variants, rs1333049, rs2383207, rs10757274, and rs10116277 were selected from the 44 kb candidate region on chromosome 9p21 locus. We genotyped these SNPs in 1,429 ischemic stroke patients and 1,191 controls from the Chinese Han population by using the TaqMan SNP genotyping technology on a real-time PCR platform. **Results**: Heterozyous AG and homozygous GG genotypes of rs2383207 conferred a increased risk for ischemic stroke in the present study ($P<0.05$). Haplotype analysis of rs10757274 and rs2383207 showed that the G-A haplotype was associated with significantly lower risk with ischemic stroke (OR: 0.359, 95%CI: 0.256 to 0.504, $P<0.001$). **Conclusion**: A candidate region on 9p21 locus is associated with ischemic stroke in the Chinese Han population both at SNP and Haplotype levels. Our study reduces the candidate region to 28 kb in the Chinese Han population.

Introduction

Stroke is defined as acute focal or global neurological deficits persisting more than 24 hours, or interrupted by intervention or death within 24 hours, with a presumed vascular cause (WHO 1990). 9 million individuals are estimated to suffer from stroke each year, and approximately 1.5- 2 million of these patients reside in China (Liu et al 2007, Zhao et al 2008, Meretoja 2011). Stroke is the second most common cause of adult death worldwide, and about 5.4 million individuals are estimated to die of stroke-related events each year, accounting for approximately 10% of total deaths worldwide (Murray et al 1997, Donnan et al, 2008 Bonita et al, 2004). Two-thirds of stroke related deaths occur in developing countries, with approximately 40% occurring in China (Geneva: World Health Organization. 1998, Reddy et al 1998, Feigin et al 2003,).

Stroke is a complex disease to which both genetic and environmental factors contribute. Common variants in a candidate chromosome 9p21 locus have recently been associated with increased risk for coronary arterial disease (CAD). The 9p21 locus was first identified as a genetic risk factor in CAD by four parallel genome-wide association studies (The Wellcome Trust Case Control Consortium 2007; Helgadottir et al. 2007; McPherson et al. 2007; Samani et al. 2007). Subsequently, genome-wide association studies have also demonstrated the association between this locus and other vascular diseases, including diabetes mellitus, abdominal aortic aneurysm, and intracranial aneurism (Saxena et al 2008, Helgadottir et al. 2008). Matarin et al. (2008) documented that common variants from a 44-kb core candidate region on 9p21 were associated with ischemic stroke in a Caucasian population. However, replication studies in the Chinese

Han population have revealed weak association between 9p21 and ischemic stroke (Hu et al. 2009, Ding et al. 2009). This may be due to a relatively small sample size and genetic heterogeneity among the Chinese Han population. In the present study, we performed a large case control study to replicate the association between the core candidate 9p21 region and ischemic stroke in the Chinese Han population.

Materials and Methods

Subjects

From February 2006 to March 2007, 1,429 consecutive patients (Mean age ± SD: 62.7 ± 11.8, male: n=868, 60.7%), diagnosed with ischemic stroke from 18 hospitals in Henan province, were enrolled in the present study. The inclusion criteria were: 1) stroke diagnosis according to WHO criteria, and 2) MRI or CT scan indicating the ischemic lesion corresponding to the neurological deficits. Patients with hemorrhagic stroke, subarachnoid hemorrhage, transient ischemic attack and severe systemic diseases were excluded from this study. The control group consisted of 1,197 (Mean age ± SD: 58.5 ± 9.2 male: n=760, 63.5%) ethnically-matched individuals without history of myocardial infarction or stroke who were admitted to these hospitals for routine health examinations. Informed consent was obtained from all participants. Peripheral blood samples were collected from patients and controls by the Genetics Department of Zhengzhou University. The study protocol on the collaboration between Zhengzhou University and Department of laboratory medicine, Health Sciences Centers, Newfoundland and Labrador was approved by institutional boards.

SNP Genotyping

Genomic DNA was extracted from peripheral blood samples by using a standard salt precipitation method (Miller et al. 1988). Four SNPs, rs1333049 (C__1754666_10), rs2383207 (C__15789010_10), rs10757274 (C__26505812_10) and rs10116277 (C__29991625_20) were selected from a 44-kb candidate region on chromosome 9p21 (chr 9.22071397-chr 9.22115503). SNP Genotyping was performed using the TaqMan SNP genotyping technology on a real-time PCR platform (ABI Prism 700 sequence detection system). The reaction volume included: 2.0 µl genomic DNA (100ng/µl), 2.5 µl TaqMan universal PCR master mix, 0.125 µl primer and probes, and 0.375 µl ddH2O. The standard reaction condition included the activation of uracil-N-glycolase (UNG) (2 min; 50 °C), polymerase activation (10min; 95°C), 40 cycles of denaturation (15s; 95 °C), annealing and extension (1 min; 60 °C).

Statistical Analysis

The genotype frequency data for each variant in the control group was tested for deviation from the Hardy-Weinberg equilibrium using online software (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). Haplotype frequencies and association of haplotypes for case and control status were estimated using the PowerMarker V3.25 Software. Genotype frequencies and haplotype distribution between case and control groups were tested by the χ^2 test under recessive, dominant and co-dominant models by the SPSS 16.0 software

package (SPSS Inc.). Statistical power was calculated using QUANTO V1.2.3 software. Differences with P< 0.05 (two-tailed) were considered statistically significant.

The minor allele frequencies (MAF) of the four tested SNPs ranged from 0.31 to 0.49, according to HapMap CHB (Chinese Han Beijing) data, with a mean MAF of 0.40 for the four SNPs. Given the disease prevalence at 0.5%, 1,429 patients and 1,197 controls yielded a statistical power > 0.8 to detect an OR of 1.50 for the MAF of 0.40 for the tested SNPs at a significance level of 0.05 (two-tailed) under recessive, dominant and additive models. The software QUANTO version 1.2.3 was used for the calculation of statistical power.

Results

The physical locations of the four studied genetic variants (rs1333049, rs2383207, rs10757274, and rs10116277) within the 44 kb core candidate region for stroke are given in Figure 4.1. Four selected genetic variants were genotyped in 1,429 patients and 1,191 controls. The distributions of all possible genotypes for each variant are given in Table 4.1. The genotype frequencies of all four genetic variants in the control subjects were all under the Hardy–Weinberg equilibrium (all P>0.05).

The result showed that the AG / GG genotypes of SNP rs2383207 were associated with increased risk for ischemic stroke (OR: 1.417, 95%CI: 1.123 to 1.786, P= 0.003), with

OR at 1.382 (95%CI: 1.081 – 1.766) and 1.417 (95%CI: 1.123 – 1.786) for heterozygous AG and homozygous GG genotypes, respectively. According to the data from HapMap CHB (Chinese Han Beijing), the rs10757274 and the rs2383207 are within one LD block ($r^2 > 0.8$), and the rs1333049 and the rs10116277 are scattered into two flanking LD blocks, respectively. None of the other three SNPs achieved a significance level in the present study, which indicates a smaller interval of the candidate region (<28 kb) in the Chinese Han population compared with the one (44 kb) previously reported in Caucasians (Matarin et al. 2008).

In the haplotype frequency analysis, only rs10757274 and rs2383207 were included because these two variants are in the same LD blocks. The distributions of all four possible haplotype frequencies for rs10757274 and rs2383207 are given in Table 4.2. Among the four possible haplotypes, the G-A haplotype was associated with significantly lower risk for ischemic stroke (OR: 0.359, 95%CI: 0.256 to 0.504, P<0.001), which suggests a protective haplotype in the studied population.

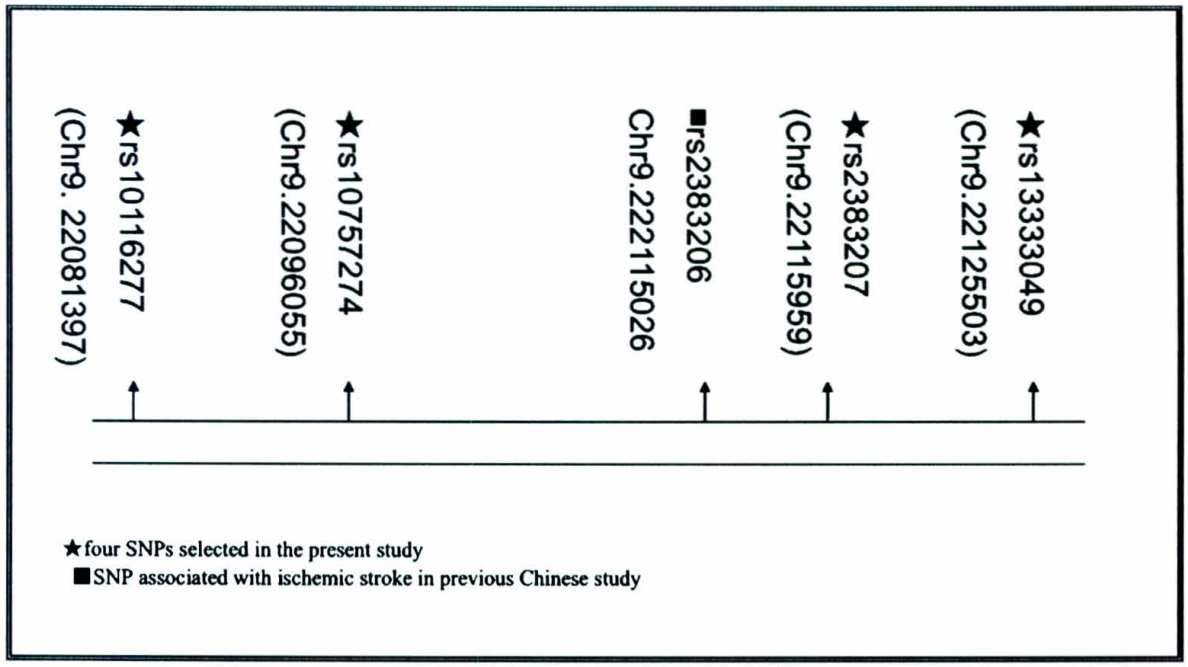

Figure 4.1 Candidate region on 9p21 for ischemic stroke

Table 4.1 Genotype distributions of rs10116277, rs10757274, rs2383207, and rs1333049 in stroke and control subjects in the Chinese Han population

Genotype	Stroke (n=1,429)	NC (n=1,191)	OR (95%CI)	P-value
Rs10116277				
GG	137(9.6%)	137(11.5%)		
GT	606(42.4%)	501(42.1%)	1.210 (0.928-1.576)	0.159
TT	686(48.0%)	553(46.4%)	1.241 (0.955-1.612)	0.106
GT+TT	1292(90.4%)	1054(88.5%)	1.226 (0.954-1.575)	0.111
Allele F.				
G	30.8%	32.5%		
T	69.2%	67.5%	1.084 (0.964 – 1.218)	0.176
Rs10757274				
AA	453(31.7%)	354(29.7%)		
AG	676(47.3%)	600(50.4%)	0.880 (0.737-1.051)	0.159
GG	300(21.0%)	237(19.9%)	0.989 (0.794-1.232)	0.923
AG+GG	976(68.3%)	837(70.3%)	0.911 (0.771-1.077)	0.275
Allele F.				
A	55.4%	54.9%		
G	44.6%	45.1%	0.982 (0.881 – 1.096)	0.749
Rs2383207				
AA	154(10.8%)	174(14.6%)		
AG	642(44.9%)	525(44.1%)	1.382 (1.081-1.766)	0.010
GG	633(44.3%)	492(41.3%)	1.454 (1.136-1.861)	0.003
AG+GG	1275(89.2%)	1017(85.4%)	1.417(1.123-1.786)	0.003

Genotype	Stroke (n=1,429)	NC (n=1,191)	OR (95%CI)	P-value
Allele F.				
A	33.2%	36.6%		
G	66.8%	63.4%	1.162 (1.037 – 1.302)	0.010
Rs1333049				
GG	381(26.7%)	334(28.0%)		
GC	733(51.3%)	571(47.9%)	1.125 (0.937-1.352)	0.206
CC	315(22.0%)	286(24.0%)	0.966 (0.777-1.200)	0.752
GC+CC	1048(73.3%)	857(72.0%)	1.072 (0.902-1.274)	0.429
Allele F.				
G	52.3%	52.0%		
C	47.7%	48.0%	0.988(0.886 – 1.102)	0.832

Table 4.2 Distribution of haplotype frequency estimation for rs10757274 and rs2383207 in ischemic stroke and control subjects

Haplotype Frequencies	**Stroke (2n=2,858)**	**NC (2n=2,382)**	**OR (95%CI)**	**P-value**
rs10757274 - rs2383207				
A-A	898	750		
A-G	683	555	1.028 (0.886-1.192)	0.717
G-A	52	121	0.359 (0.256-0.504)	<0.001
G-G	1225	953	1.074 (0.944-1.221)	0.280

Discussion

In the present study, four variants, rs1333049, rs2383207, rs10757274, and rs10116277 selected from the core candidate locus on chromosome 9p21, have been genotyped in 1,429 ischmic stroke patients and 1,191 controls from the Chinese Han population. The present study has shown that the G allele of rs2383207 on chromosome 9p21 locus is associated with ischemic stroke in the Chinese Han population. Interestedly, none of the other three SNPs selected from the flanking LD blocks and the LD block encompassing the rs2383207 showed an association with ischemic stroke in the present study which further narrowed down the interval of the candidate region from the previously reported 44 kb to an interval less than 28kb. This finding will help further characterize the candidate 9p21 region and finally identify the critical DNA variation associated with cardiovascular diseases. Haplotype frequency analysis pinpoints the G-A (rs10757274-rs2383207) haplotype as a protective haplotype for ischemic stroke in the Chinese Han population.

The association between 9p21 candidate region and cardiovascular diseases, i.e., coronary artery disease and type 2 diabetes mellitus, was validated in the Chinese Han population (Ding et al. 2009, Chang et al. 2011). However, the evidence for the association between 9p21 locus and ischemic stroke is rather weak in the Chinese Han population. Hu et al. (2009) genotyped rs2383206 and rs10757278 from the core risk region on 9p21 in 355 patients with ischemic stroke and 430 controls. In this study, the G allele (AG/GG

genotypes) of rs2383206, a SNP 933 bp away from rs2383207, conferred a 1.51-fold higher risk for ischemic stroke in the Chinese Han population (95%CI: 1.11 to 2.05, P = 0.009). The variant rs2383206 is only 933 bp away from rs2383207, and these two variants are known to be in a same LD block in Chinese Han population. Another association study between 9p21 locus and ischemic stroke was conducted using the Chinese Han population in which 17 tagSNPs across 58 kb on the 9p21 region, including the rs2383206, were genotyped in 558 patients and 557 controls (Xu et al. 2009). This study did not validate the association between 9p21 locus and ischemic stroke at the SNP level. The inconsistent results may be due to either the relatively small sample size or genetic heterogeneity in the studied Chinese Han population. The Chinese Han population has been suggested to be genetically differentiated into three subgroups: the Northern, Central and Southern Han (Xu et al. 2009; Chen et al. 2009).

So far, there is no coding sequence identified within the core candidate region on the chromosome 9p21 locus. Two tumor suppressor genes, cyclin-dependent kinase inhibitor 2A (*CDKN2A*) and *CDKN2B* reside approximately 100 kb away from the core vascular disease risk region. *ANRIL* is a non-coding RNA with its exons 13-19 overlapping the core vascular risk region on 9p21, and is transcribed in the opposite direction to the *CDKN2B* gene. Function studies revealed inconsistent results regarding the association between the risk alleles on the core candidate region and expression of *CDKN2A*, *CDKN2B* and *ANRIL* (Liu et al. 2009; Cunnington et al. 2010; Hold et al. 2010; Michael et al. 2011). An animal study knocked out a 70kb sequence of chromosome 4 in mice,

which was estimated to be orthologous to 9p21 in humans. These mice showed reduced expression of *CDKN2A* and *CDKN2B* and proliferation of aortic smooth muscle cells. However, they did not show a predisposition to vascular disease (Visel et al. 2010). Another study suggested that 9p21 risk alleles contribute to vascular disease by increasing platelet reactivity (Musunuru et al 2010).

Conclusion

The present study validates the association between 9p21 and ischemic stroke in the Chinese Han population. The exact mechanism underlying the association of 9p21 with vascular diseases needs further elucidation.

Conflicts of interests

No conflicts of interest occurred in this study.

Chapter 5

The Methylenetetrahydrofolate Reductase *(MTHFR)* is associated with ischemic stroke in the Chinese Han Population

Shuo Li[1,2], Yu-Ming Xu[5], Edward Randell[1], Fei-Yu Han[1,2], Hong Zheng[6], Hai-Zheng Wang[5], Guang Sun[4] and Ya-Gang Xie[1,2,3,4]

Disciplines of [1]Laboratory Medicine, [2]Genetics, [3]Pediatrics, and [4]Medicine, Memorial University of Newfoundland, St. John's, NL, Canada

Departments of [5]Neurology of the First Affiliated hospital, and [6]Genetics, Zhengzhou University, Henan, P.R. China

This manuscript has not yet been submitted for publication.

Abstract

Background: Two common variants, *MTHFR* c.677C>T and c.1298A>C, have been extensively studied for association with ischemic stroke. Previous studies revealed discordant results. **Method:** Genotyping of *MTHFR* c.677C>T and c.1298A>C was performed on 1,429 consecutive patients with ischemic stroke and 1,171 ethnically and gender matched healthy controls by using TaqMan SNP genotyping technology on real-time PCR. **Results**: The *MTHFR* c.677T allele was interestingly found to be the major allele in the studied Chinese Han population with an allele frequency of 59.5%. Moreover, the *MTHFR* c.677T allele showed a significantly higher allele frequency (OR: 1.245, 95%CI: 1.112 to 1.393, P<0.001), and higher heterozygote (OR: 1.474, 95%CI: 1.170 – 1.858, P=0.001) and homozygote prevalence (OR: 1.642, 95%CI: 1.296 to 2.080, P<0.001) in ischemic stroke patients. For the *MTHFR* c.1298A>C variant, the heterozygous AC genotype exhibited a lower risk for ischemic stroke (OR=0.803, 95%CI: 0.664 to 0.970, P=0.023). Permutated haplotype analysis showed that the 677T-1298A haplotype was associated with a 1.138-fold increased risk for ischemic stroke (OR; 1.138, 95%CI: 1.003 to 1.292, P = 0.045). **Conclusion**: The results of the present study suggest that *MTHFR* is associated with ischemic stroke in the Chinese Han population. *MTHFR* c. 677C>T is a risk variant, and its effect could be modified by genetic or environmental factor(s) which vary among different ethnic populations. The *MTHFR* c.1298A>C variant_is more likely to play the role of a genetic marker than

causative variant in association with ischemic stroke. The *MTHFR* c. 677T-1298A is a risk haplotype for ischemic stroke in the Chinese Han population.

Introduction

Stroke is the second most common cause of adult death and the main cause for adult disability worldwide (Bonita et al. 2004). The incidence of stroke in Chinese is 1.5 – 2.0 million per year (National 8.5 collaborative Group 1985, Shi et al 1989), with an obvious geographical trend increasing from south to north, approximately divided by the Yangtze River (Li et al 1985, Chen et al 1993). Among cardiovascular disorders, stroke accounts for the highest mortality rate in the Chinese Han population, three times higher than myocardial infarction (Li et al. 2003).

Methylenetetrahydrofolate reductase (MTHFR) is essential for homocysteine metabolism (Frosst et al. 1995) . Mild to moderate hyperhomocysteinemia (15 − 100 μmol/L) has been considered as an independent risk factor for ischemic stroke (Graham et al. 1997, Rozen et al. 2000, Bersano et al. 2008). Two common variants in the coding region of the *MTHFR* gene, *MTHFR* c.677C>T (p. Ala222Val) and c.1298A>C (p. Glu429Ala), have been reported to be prevalent in North American, European and Asian populations (Botto et al. 2000; Guillen et al. 2001; Castro et al. 2003; Kohara et al 2003; Li et al. 2003; Kim et al 2007). The *MTHFR* c.677 C>T variant was reported to be associated with impaired

MTHFR enzyme activity and elevated plasma homocysteine levels (Frosst et al. 1995, Van der Put et al. 1998). The *MTHFR* c.1298 A>C variant was shown to be associated with reduced MTHFR activity in *in vitro* experiments (Van der Put et al.1998). However, studies on the effect of this variant on plasma homocysteine levels remain controversial (Weisberg et al. 1998, Yamada et al. 2001). The association between *MTHFR* c.677C>T, c.1298A>C variants and ischemic stroke has revealed inconsistent results in different ethnic populations. (Casas et al. 2001, Pezzini et al 2002, Li et al. 2003, Sazci et al. 2006, Kim et al. 2007, Dichgans 2007). Therefore, the association between the two variants and ischemic stroke has not been clearly established and continues to be the subject of debate. Etiologies of complex disease usually involve genetic and environmental factors. The genetic mixture within a population could complicate results due to heterogeneity among genetic modifiers. To minimize these effects, we undertook a case control study by enrolling 1,429 ischemic stroke patients and 1,197 controls from the Chinese Han population to investigate the genetic effects of the *MTHFR* c.677C>T and c.1298A>C variants on stroke.

Materials and Methods

Subjects

From February 2006 to March 2007, 1,429 consecutive patients (Mean age ± SD: 62.7 ± 11.8, male: n=868, 60.7%), diagnosed with ischemic stroke from 18 hospitals in Henan province, were enrolled in the present study. The inclusion criteria were: 1) stroke diagnosis according to WHO criteria, and 2) an MRI or CT scan indicating an ischemic

lesion corresponding to the neurological deficits. Patients with hemorrhagic stroke, subarachnoid hemorrhage, transient ischemic attack and severe systemic diseases were excluded from this study. The control group consisted of 1,197 (Mean age ± SD: 58.5 ± 9.2 male: n=760, 63.5%) ethnically matched individuals without a history of myocardial infarction or stroke who came to these hospitals for routine health examinations. Informed consent was obtained from all participants. Samples were collected by the genetics department of Zhengzhou University. The study protocol on the collaboration between Zhengzhou University and Department of laboratory medicine, Health Sciences Centers, Newfoundland and Labrador, was approved by institutional boards.

Genotyping of the *MTHFR* c. 677C>T and c.1298A>C variants

Genomic DNA samples were obtained by salt extraction protocol (Miller et al 1988). The two variants, *MTHFR* c.677C>T (rs1801133, C__1202883_20) and MTHFR c.1298A>C (rs1801131, C__850486_20) were genotyped using a TaqMan SNP genotyping kit (ABI; Foster City, CA) on a real-time PCR platform (ABI Prism 7000 sequence detection system). The reaction volume included: 2.0 µl genomic DNA (100 ng/µl), 2.5µl TaqMan universal PCR master mix, 0.125 µl primer and probs, 0.375 µl ddH2O. The standard reaction condition included: activation of uracil-N-glycolase (UNG) (2 min, 50°C), polymerase activation (10min; 95°C), 40 cycles of denaturation (15s; 95°C), annealing and extension (1min; 60°C).

Statistical Analysis

The genotype frequency data for each variant in the control group was tested for deviation from the Hardy-Weinberg equilibrium using online software (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). Haplotype frequencies were estimated using the PowerMarker V3.25 Software. Genotype frequencies and haplotype distribution between case and control groups were tested by the χ^2 test under recessive, dominant and co-dominant models by the SPSS 16.0 software package (SPSS Inc.). Statistical power was calculated using QUANTO V1.2.3 software. Differences with $P< 0.05$ (two-tailed) were considered statistically significant.

Results

A total of 1,429 patients and 1,171 controls were successfully genotyped for both variants. The genotype frequencies for the *MTHFR* c.677C>T and c.1298A>C variants in the control group did not deviate from the Hardy-Weinberg equilibrium ($P>0.05$).

MTHFR c.677C>T and c.1298A>C variants frequency distribution

The distribution of genotype and allele frequencies for each variant between patients and controls are shown in Table 5.1. In terms of the *MTHFR* c. 677C>T variant, the T allele has been found to be the major allele in the studied Chinese population with a frequency of 59.5%. In comparison with the controls, the T allele in ischemic stroke patients was significantly higher (OR: 1.245, 95%CI: 1.112 to 1.393, $P<0.001$). Heterozygous CT and

homozygous TT genotypes showed association with increase risk for ischemic stroke, with OR of 1.474 (95%CI: 1.170 - 1.858, P = 0.001) and 1.642 (95%CI: 1.296 – 2.080, P<0.001) for heterozygous CT and homozygous TT, respectively. For the *MTHFR* c. 1298A>C variant, the heterozygous AC genotype associated with lower risk for ischemic stroke (OR: 0.815, 95%CI: 0.664 – 0.970, P=0.023).

Analyses of estimated haplotype frequency

The *MTHFR* c.677C>T and c.1298A>C variants are in linkage disequilibrium (r^2>0.8) based on the data from the HapMap project (CHB population) by using Haploview software version 4.1. To further validate the results from the genotype analysis, the haplotype between these two variants were permuted, as shown in table 5.2. The haplotype analyses showed that the 677T- 1298A haplotype was associated with a marginal 1.138-fold increased risk for ischemic stroke (95%CI: 1.003 to 1.292, P = 0.045).

Table 5.1 Genotype distributions of MTHFR c.677C>T and c.1298A>C variants in ischemic stroke patients and control subjects

Genotype	Stroke (n=1429)	NC (n=1171)	OR (95%CI)	P-value
677C>T				
CC	174	207		
CT	663	535	1.474 (1.170 – 1.858)	0.001
TT	592	429	1.642 (1.296 – 2.080)	< 0.001
CT/TT	1255	964	1.549 (1.245-1.927)	< 0.001
Allele Frequency.				
C	35.4%	40.5%		
T	64.6%	59.5%	1.245 (1.112-1.393)	<0.001
1298A>C				
AA	1129	880		
AC	277	269	0.803 (0.664 – 0.970)	0.023
CC	23	22	0.815 (0.451 – 1.472)	0.497
AC/CC	300	291	0.840 (0.669 – 0.966)	0.020
Allele Frequency.				
A	88.7%	86.6%		
C	11.3%	13.4%	0.826(0.700– 0.975)	0.024

Table 5.2 Distribution of haplotype frequencies for *MTHFR* c.677C>T and c.1298A>C variants in ischemic stroke and control subjects

Haplotype Frequencies	**Stroke (2n=2,858)**	**NC (2n=2,342)**	**OR (95%CI)**	**P-value**
MTFHR c.677-c.1298				
C-A	737	653		
C-C	340	297	1.014 (0.841 – 1.224)	0.882
T-A	1769	1377	1.138 (1.003 – 1.292)	0.045
T-C	11	16	0.609 (0.281 – 1.322)	0.206

Discussion

In the present study, two common variants, *MTHFR* c.677C>T and c.1298A>C, were genotyped in 1,429 ischemic stroke patients and 1,171 controls from Chinese Han population in Henan province. The results suggest that: 1) the *MTHFR* is associated with ischemic stroke in the Chinese Han population, and *MTHFR* c. 677C>T variant is a risk factor; 2) the *MTHFR* c. 1298A>C variant_is more likely to play the role of a genetic marker than a causative variant in association with ischemic stroke; and 3) the *MTHFR* c. 677T-1298A is a risk haplotype for ischemic stroke in the Chinese Han population.

The *MTHFR* c.677C>T variant is an extensively studied genetic variant in ischemic stroke. A recent meta-analysis pooled data from six previous meta-analyses and twelve case-control studies on the association between *MTHFR* c.677C>T variant and stroke. Two of the case-control studies and four of the meta-analyses associated the *MTHFR* c.677T allele with stroke, while the other ten case-control studies and four meta-analyses failed to validate any association (Bersano et al. 2009). These inconsistent results may result from stroke heterogeneity, population admixture and sample bias.

The T allele frequency of *MTHFR* c.677C>T variant varies among different ethnic populations. According to the HapMap database, the T allele varies from 0.10 in the YRI (Yoruba in Ibadan) population, 0.24 in the CEU (Caucasian of European descent) population to 0.48 in the CHB (Chinese Han Beijing) population. The T allele frequency

also showed differences within the same ethnic population. In Caucasians, the T allele frequency was reported as 0.33 in Portuguese (Castro et al. 2003), 0.42 in Spanish (Guillen et al. 2001), and 0.44 in Italian (Botto et al. 2000) populations. In the Asian populations, the T allele frequency was shown to be 0.40 in Japanese (Kohara et al 2003), and 0.44 in Korean (Kim et al. 2007) and Chinese Han populations (Li et al. 2003). The T allele of the *MTHFR* c.677C>T variant in our study presented a much higher frequency compared with that in a previous reported Chinese study (0.60 vs.0.44) (Li et al. 2003), of which the studied subjects were collected from several provinces across China. A recent study also reported significant ethnic and regional variations concerning the frequency of *MTHFR* c.677C>T variant in Chinese ($P<0.01$) (Mao et al. 2008). In the published studies, which showed an association between the *MTHFR* c. 677C>T variant and ischemic stroke, the T allele usually was a minor allele. Interestingly in the present study, the T allele, as the major allele, was also associated with increased risk for ischemic stroke. This indicates that the *MTHFR* c.677C>T is a risk variant for stroke, but its effect may be modified by genetic or environmental effects which vary among different ethnic populations..

The *MTHFR* c.1298A>C variant can reduce MTHFR enzyme activity in *in vivo* (van der Put et al. 1998, Weisberg et al. 1998) and *in vitro* (Weisberg et al. 2001) experiments, although to a lesser extent than the MTHFR c.677C>T variant. However, the impact of the MTHFR c.1298A>C variant on plasma homocysteine levels remain unclear. Previous studies reported that this variant either did not affect the homocysteine levels, or was

associated with elevated or even reduced homocysteine levels (van der Put et al. 1998, Friedman et al. 1999, Lievers et al. 2001, Castro et al. 2003). Our result suggests that the heterozygous AC genotype associates with lower risk for ischemic stroke, which contradicts previous studies (Sazci et al 2006, Kim et al 2010). A Korean study indicated that *MTHFR* 1298AC (OR: 1.734, 95%CI: 1.13 to 2.66) and AC/CC genotypes (OR; 1.825, 95%CI: 1.20 to 2.78) were associated with moderately increased risk for silent brain infarction in the Korean population (Kim et al. 2010). Another study indicated that the homozygous CC genotype was strongly associated with ischemic stroke in a Turkish population based on genotyping of 92 cases and 259 controls (OR: 2.950, 95%CI: 1.504 to 5.786, P=0.001) (Sazci et al. 2006). Combining our findings with previous studies, we consider the *MTHFR* c.1298A>C variant to be a marker in linkage disequilibrium with a causative variant rather than a functional variant responsible for ischemic stroke. Further studies are needed to elucidate the relationship between the *MTHFR* c.1298A>C variant and homocysteine metabolism.

The *MTHFR* c.677C>T and c.1298A>C variants are known to occur in linkage disequilibrium. The haplotype of 677T-1298A, in the present study, was present at significantly increased prevalence in ischemic stroke patients, and it, therefore, could be regarded as a risk haplotype for ischemic stroke in the Chinese Han population.

Conclusion

Our study demonstrated that the *MTHFR* is associated with ischemic stroke in the Chinese Han population. The *MTHFR* c. 677C>T variant is a risk factor in this association. The *MTHFR* c.1298A>C variant is a genetic marker rather than a causative variant in ischemic stroke. Target sequencing the *MTHFR* locus with next-generation technology may help identify the causative variants.

Conflicts of interest

No conflicts of interest occurred in this study.

Chapter 6

Conclusion

6.1 Combined Analysis on Association of *MTHFR* and 9p21 Variants with Ischemic Stroke

A total of 1,429 patients and 1,166 control subjects were successfully genotyped for all six variants examined in the two studies described in chapter 4 and 5. To assess if any increased risk occurs for coexistence of risk alleles in 9p21 locus and *MTHFR* gene, we selected two variants showed association with ischemic stroke in chapter 4 and 5, rs2308207 from 9p21 locus and *MTHFR* c.677C>T (rs1801133), respectively. We performed the χ^2 analysis and the results were shown in Table 6.1. With the presence of both risk allele carriers from the two variants, the ORs exceed 2.0, which are greater than ORs of the two risk alleles, respectively. The ORs for the combined risk genotypes are in compliance with an additive model when considering their co-effect on ischemic stroke in the Chinese Han population. This suggests that coexistence of both risk genotypes for these two variants increased risk for ischemic stroke.

Table 6.1 Distribution of combined genotype frequency of rs2383207 and *MTHFR* c.677C>T (rs1801133) in stroke patients and control subjects

Combined genotypes	**Stroke (n=1429)**	**NC (n=1166)**	**OR (95%CI)**	**P-value**
Rs2383207/ rs1801133				
AA/CC	13	24		
AA/CT	78	70	2.057 (0.974 – 4.347)	0.056
AA/TT	63	73	1.593 (0.749 – 3.388)	0.224
AG/CC	79	95	1.535 (0.734 – 3.211)	0.253
AG/CT	278	240	2.138 (1.065 -4.292)	0.029
AG/TT	285	181	2.907 (1.443-5.855)	0.002
GG/CC	82	86	1.760 (0.840 – 3.688)	0.131
GG/CT	307	225	2.519 (1.255-5.055)	0.007
GG/TT	244	172	2.619 (1.297 – 5.288)	0.006
AG+GG/CT+TT	1114(79.8%)	818(70.1%)	1.505 (1.260 – 1.796)	<0.001

6.2 Conclusion

Stroke is a complex disease with multiple risk factors. Animal, familial and twin studies indicate genetic involvement in stroke predisposition. However, the causative variants remain undetermined. Our study confirms the genetic association of variants within the *MTHFR* and the chromosome 9p21 locus with ischemic stroke in the Chinese Han population. With our results, we conclude:

1. The chromosome 9p21 locus is associated with ischemic stroke in the Chinese Han population at SNP and Haplotype levels. Our result reduces the candidate region to 28 kb in the Chinese Han population;

2. The *MTHFR* is associated with ischemic stroke in the Chinese Han population: a) The *MTHFR* c. 677T variant is a risk allele, and its effect could be modified by genetic or environmental factor(s) which vary among different ethnic population; b) The *MTHFR* c. 1298A>C variant is more likely to play the role of a genetic marker than causative variant in association with ischemic stroke; c) The *MTHFR* c. 677T-1298A is a risk haplotype for ischemic stroke in the Chinese Han population;

3. The presence of risk alleles from both the 9p21 candidate locus and *MTHFR* (*MTHFR* c. 677C>T) confer an additive effect for increased risk of ischemic stroke in the Chinese Han populatio

References

Adams HP, Jr,, Bendixen BH, Kappelle LJ, Biller J, Love BB, Gordon DL, Marsh EE, 3d. Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of Org 10,172 in Acute Stroke Treatment. *Stroke* 1993, 24:35-41.

Ambrose JA, Barua RS. The pathophysiology of cigarette smoking and cardiovascular disease: An update. *Journal of the American College of Cardiology* 2004; 43(10): 1731 - 1737.

American Heart Association. Heart and Stroke Facts Statistics: Dallas: American Heart Association 1997.

American Heart Association. Heart Disease and Stroke Statistics—2004 Update. *Dallas, Tex: American Heart Association;* 2003.

Andersen KK, Andersen ZJ, Olsen TS. Age- and Gender-Specific Prevalence of Cardiovascular Risk Factors in 40 102 Patients With First-Ever Ischemic Stroke: A Nationwide Danish Study. *Stroke* 2010; 41: 2768 – 2774.

Ascherio A, Rimm EB, Hernan MA, Giovannucci EL, Kawachi I, Stampfer MJ, Willett WC. Intake of potassium, magnesium, calcium, and fiber and risk of stroke among US men. *Circulation* 1998; 98: 1198 – 1204.

Atrial Fibrillation Investigators: Atrial Fibrillation, Aspirin, Anticoagulation Study; Boston Area Anticoagulation Trial for Atrial Fibrillation Study; Canadian Atrial Fibrillation Anticoagulation Study; Stroke Prevention in Atrial Fibrillation Study; Veterans Affairs Stroke Prevention in Nonrheumatic Atrial Fibrillation Study. Risk factors for stroke and efficacy of antithrombotic therapy in atrial fibrillation: analysis of pooled data from five randomized controlled trials. *Archives of Internal Medicine*. 1994;154: 1449-1457.

Austin RC, Lentz SR, Werstuck GH. Role of hyperhomocysteinemia in endothelial dysfunction and atherothrombotic disease. *Cell Death and Differentiation.* 2004; 11: S56–S64.

Ay H, Benner T, Arsava EM, Furie KL, Singhal AB, Jensen MB, Ayata C, Towfighi A, Smith EE, Chong JY, Koroshetz WJ, Sorensen GA. A Computerized Algorithm for Etiologic Classification of Ischemic Stroke: The Causative Classification of Stroke System. *Stroke* 2007; 38; 2979-2984.

Baird AE, Warach S. Magnetic resonance imaging of acute stroke. *Journal of Cerebral Blood Flow Metabolism* 1998; 18: 583 – 609.

Bak S, Gaist D, Sindrup SH, Skytthe A, Christensen K. Genetic liability in stroke: a long-term follow-up study of Danish twins. *Stroke* 2002; 33: 769 -774.

Bang OY, Saver JL, Liebeskind DS, Pineda S, Ovbiagele B. Association of serum lipid indices with large artery atherosclerotic stroke. *Neurology* 2008; 70: 841 – 847.

Bansal S, Buring JE, Rifai N, Mora S, Sacks FM and Ridker PM, Fasting compared with nonfasting triglycerides and risk of cardiovascular events in women. *JAMA* 2007; 298: 309 – 316.

Berger K, Ajani UA, Kase CS, Gaziano JM, Buring JE, Glynn RJ, Hennekens CH. Light-to-moderate alcohol consumption and risk of stroke among U.S. male physicians. *New England Journal of Medicine* 1999; 341: 1557 –1564.

Bersano A, Ballabio E, Bresolin N, Candelise L. Genetic Polymorphisms for the Study of Multifactorial Stroke. *Human Mutation* 2008; 29(6): 776 - 795.

Bishop DT, Demenais F, Iles MM, Harland M, Taylor JC, Corda E, Randerson-Moor J, Aitken JF, Avril MF, Azizi E, Bakker B, Bianchi-Scarrà G, Bressac-de Paillerets B, Calista D, Cannon-Albright LA, Chin-A-Woeng T, Debniak T, Galore-Haskel G, Ghiorzo P, Gut I, Hansson J, Hocevar M, Höiom V, Hopper JL, Ingvar C, Kanetsky PA, Kefford RF, Landi MT, Lang J, Lubiński J, Mackie R, Malvehy J, Mann GJ, Martin NG, Montgomery GW, van Nieuwpoort FA, Novakovic S, Olsson H, Puig S, Weiss M, van Workum W, Zelenika D, Brown KM, Goldstein AM, Gillanders EM, Boland A, Galan P, Elder DE, Gruis NA, Hayward NK, Lathrop GM, Barrett JH, Bishop JA. Genome-wide association study identifies three loci associated with melanoma risk. *Nature Genetics* 2009; 41: 920–925.

Bo¨ger RH. The emerging role of asymmetric dimethylarginine as a novel cardiovascular risk factor. *Cardiovascular Research* 2003; 59: 824 – 833.

Bonita R, Duncan J, Truelsen T, Jackson RT, and Beaglehole R. Passive smoking as well as active smoking increases the risk of acute stroke. *Tobacco Control*. 1999; 8:156 – 160.

Bonita R, Mendis S, Truelsen T, Bogousslavsky J, Toole J, Yatsu F. The global stroke initiative. *Lancet Neurology* 2004; 3: 391–393.

Bonita R, Solomon N, Broad JB. Prevalence of stroke and stroke-related disability: estimates from the Auckland stroke studies. *Stroke* 1997; 28: 1898 –1902.

Bostom AG, Rosenberg IH, Silbershatz H, Jacques PF, Selhub J, D'Agostino RB, Wilson PW, Wolf PA. Nonfasting Plasma Total Homocysteine Levels and Stroke Incidence in Elderly Persons: The Framingham Study. *Annual Internal Medicine* 1999; 131: 352 – 355.

Briscoe D, Stephens JC, O'Brien SJ. Linkage disequilibrium in admixed populations: applications in gene mapping. *Journal of Heredity* 1994; 8: 59 - 63.

Broderick J, Brott T, Kothari R, Miller R, Khoury J, Pancioli A, Gebel J, Mills D, Minneci L, Shukla R. The Greater Cincinnati/Northern Kentucky Stroke Study: preliminary first-ever and total incidence rates of stroke among blacks. *Stroke* 1998; 29: 415 – 421.

Brown RD, Whisnant JP, Sicks JD, O'Fallon WM, Wiebers DO. Stroke incidence, prevalence, and survival: secular trends in Rochester, Minnesota, through 1989. *Stroke* 1996; 27(3):373 –380.

Brownlee M. Biochemistry and molecular cell biology of diabetic complications. *Nature* 2001; 414(13): 813 - 820.

Burchfiel CM, Curb JD, Rodriguez BL, Abbott RD, Chiu D, Yano K. Glucose intolerance and 22-year stroke incidence: the Honolulu Heart Program. *Stroke* 1994; 25: 951–957.

Caro JJ, Huybrechts KF, Duchesne I. Management Patterns and Costs of Acute Ischemic Stroke: An International Study. *Stroke* 2000; 31: 582 -590.

Chapman NH, Wijsman EM. Genome screens using linkage disequilibrium tests: optimal marker characteristics and feasibility. American Journal of Human Genetics 1998; 63: 1872 -1885.

Champe PC and Harvey RA. "Biochemistry. Lippincott's Illustrated Reviews" 4th ed. Lippincott Williams and Wilkins, 2008

Chen JM, Zheng HF, Bei JX, Sun LD, Jia WH, Li T, Zhang FR, Seielstad M, Zeng YX, Zhang XJ, Liu JJ. Genetic Structure of the Han Chinese Population Revealed by Genome-wide SNP Variation. *The American Journal of Human Genetics* 2009; 85: 775 – 785.

Chen XM. Xinde W, Mingxun T, Yupu G, Yonglian Z. Epidemiology of cerebrovascular diseases. *Cerebrovascular Diseases*. Chinese Science and Technology Publishing House 1993.

Cheng X, Shi LS, Nie SF, Wang F, Li XC, Xu CQ, Wang PY, Yang BF, Li QX, Pan ZW, Li Y, Xia H, Zheng CH, Ke YH, Wu YX, Yan XX, Yang Y, Xia N, Yao R, Wang BB, Ma X, Zeng QT, Tu X, Liao YH, Wang QK. The Same Chromosome 9p21.3 Locus Is Associated With Type 2 Diabetes mellitus and Coronary Artery Disease in a Chinese Han Population. *Diabetes mellitus* 2011; 60(2): 680 - 684.

Cheng XM, Ziegler DK, Lai YC, Li SC, Jiang GX, Du XL, Wang WZ, Wu SP, Bao SG, Bao QJ. Stroke in China, 1986 through 1990. *Stroke* 1995; 26: 1990 – 1994.

Colditz GA, Bonita R, Stampfer MJ, Willett WC, Rosner B, Speizer FE, and Hennekens CH. Cigarette smoking and risk of stroke in middle-aged women. *New England Journal of medicine* 1988; 318(15): 937 - 941.

Crawford DC, Nickerson DA. Definition and Clinical importance of haplotypes. *Annual Reviews of Medicine* 2005; 56: 303 – 320.

Cronin S, Furie KL, Kelly PJ. Dose-related association of MTHFR 677T allele with risk of ischemic stroke: evidence from a cumulative meta-analysis. *Stroke* 2005; 36: 1581–1587.

Cunnington MS, Keavney B. Genetic mechanisms mediating atherosclerosis susceptibility at the chromosome 9p21 locus. *Current Atherosclerosis Reports* 2011; 13: 193 - 201.

Daly MJ, Rioux JD, Schaffner SF, Hudson TJ, Lander ES. High-resolution haplotype structure in the human genome. *Nature Genetics* 2001; 29: 229 – 232.

Dayal S, Bottiglieri T, Arning E, Maeda N, Malinow MR, Sigmund CD, Heistad DD, Faraci FM, Lentz SR. Endothelial dysfunction and elevation of S-adenosylhomocysteine in cystathionine b-synthasedeficient mice. *Circulation Research* 2001; 88: 1203 – 1209.

Dewey HM, Thrift AG, Mihalopoulos C, Carter R, Macdonell RAL, McNeil JJ Donnan GA. Cost of stroke in Australia from a societal perspective: results from the North East Melbourne Stroke Incidence Study (NEMESIS). *Stroke* 2001; 32: 2409 – 2416.

Dichgans M. Genetics of ischaemic stroke. *Lancet Neurology* 2007; 6: 149 – 161.

Ding H, Xu YJ, Wang XJ, Wang Q, Zhang L, Tu TC, Yan JT, Wang W, Hui RT, Wang CY and Wang DW. 9p21 is a Shared Susceptibility Locus Strongly for Coronary Artery Disease and Weakly for Ischemic Stroke in Chinese Han Population *Cardiovascular Genetics* 2009; 2; 338 – 346.

Dirnagl U, Iadecola C, Moskowitz MA. Pathobiology of ischaemic stroke: an integrated view. *Trends in Neurosciences* 1999; 22: 391 – 397.

Djoussé L, R. Ellison C, Beiser A, Scaramucci A, D'Agostino RB and Gorelick PB, Rodin MB, Langenberg P, Hier DB, Costigan J. Weekly alcohol consumption, cigarette smoking, and the risk of ischemic stroke: results of a case-control study at three urban medical centers in Chicago, Illinois. *Neurology*. 1989;39: 339 –343.

Donnan GA, Fisher M, Macleod M, Davis SM. Stroke. *Lancet* 2008; 371 (9624): 1612–1623.

Du RF, Xiao CJ, Cavalli-Sforza LL. Genetic distances calculated on gene frequencies of 38 loci. Sciences China 1997; 40: 613 -614.

Eberhardt RT, Forgione MA, Cap A, Leopold JA, Rudd MA, Trolliet M, Heydrick S, Stark R, Klings ES, Moldovan NI, Yaghoubi M, Goldschmidt-Clermont PJ, Farber HW, Cohen R, Loscalzo J. Endothelial dysfunction in a murine model of mild hyperhomocyst(e)inemia. *Journal of Clinical Investigation* 2000; 106: 483 – 491.

Ebrahim S, Sung J, Song YM, Ferrer R, Lawlor DA, Smith GD. Serum cholesterol, haemorrhagic stroke, ischaemic stroke, and myocardial infarction: Korean national health system prospective cohort study. BMJ doi:10.1136/bmj.38855.610324.80.

Eikelboom JW, Hankey GJ, Anand SS, Lofthouse E, Staples N, Baker RI. Association Between High Homocyst(e)ine and Ischemic Stroke due to Large and Small-Artery Disease but Not Other Etiologic Subtypes of Ischemic Stroke. *Stroke* 2000; 31: 1069 - 1075.

European cardiovascular disease statistics 2008. European Heart Network, Brussels, 2008.

Falchi M, Bataille V, Hayward NK, Duffy DL, Bishop JA, Pastinen T, Cervino A, Zhao ZZ, Deloukas P, Soranzo N, Elder DE, Barrett JH, Martin NG, Bishop DT, Montgomery GW and Spector TD. Genome-wide association study identifies variants at 9p21 and 22q13 associated with development of cutaneous nevi. *Nature Genetics* 2009; 41: 915–919 .

Falconer DS, Mackay TFC. Introduction to Quantitative Genetics 4th edition (1996). Harlow, Essex, UK: Addison Wesley Longman.

Fang XH. Epidemiology trend, risk factor study and preventive model of stroke. In: Theory and practice for cerebrovascular diseases. Ling F, ed. Beijing: People's Health Publishing House 2006: 23–32.

Fei XT. The Pattern of Diversity in Unity of the Chinese Nation. Central Univ. for Nationalities Press, Beijing, 1999.

Feigin VL, Lawes CM, Bennett DA, Anderson CS. Stroke epidemiology: a review of population-based studies of incidence, prevalence, and case-fatality in the late 20th century. *Lancet Neurology* 2003; 2: 43–53.

Flossmann E, Schulz UG, Rothwell PM. Systematic review of methods and results of studies of the genetic epidemiology of ischemic stroke. *Stroke* 2004; 35: 212 – 227.

Foulkes MA, Wolf PA, Price TR, Mohr JP, Hier DB. The stroke data bank: design, methods, and baseline characteristics. *Stroke* 1988; 19: 547 –554.

Freiberg JJ, Tybjærg-Hansen A, Jensen JS, Nordestgaard BG. Non-fasting triglycerides and risk of ischemic stroke in the general population. *JAMA* 2008; 300: 2142 – 2152.

Friedman G, Goldschmidt N, Friedlander Y, Ben-Yehuda A, Selhub J, Babaey S, Mendel M, Kidron M, Bar-On H. A common mutation A1298C in human methylenetetrahydrofolate reductase gene: association with plasma total homocysteine and folate concentrations. *Journal of Nutrition* 1999; 129:1656–61.

Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJH , den Heijer M, Kluijtmans, LAJ, van den Heuvel LP and Rocen R. A candidate genetic risk factor for vascular disease: A common mutation in methylenetetrahydrofolate reductase. *Nature Genetics* 1995; 10:111-113.

Furie KL, Kasner SE, Adams RJ, Albers GW, Bush RL, Fagan SC, Halperin JL, Johnston SC, Katzan I, Kernan WN, Mitchell PH, Ovbiagele B, Palesch YY, Sacco RL, Schwamm LH, Wassertheil-Smoller S, Turan TN, Wentworth D. Guidelines for the Prevention of Stroke in Patients With Stroke or Transient Ischemic Attack: A Guideline for Healthcare Professionals From the American Heart Association/American Stroke Association. *Stroke* 2011; 42: 227 - 276.

Gau GT, Wright RS. Pathophysiology, Diagnosis, and Management of Dyslipidemia. Current Problems in Cardiology 2006; 31: 445 - 486.

Ge JX, Wu SD, Chao SJ. The Migration History of China. Fujian People's Publishing House, Fuzhou, China, 1997.

Geddes JM, Fear J, Tennant A, Pickering A, Hillman M, Chamberlain MA. Prevalence of self reported stroke in a population in northern England. *Journal of Epidemiology of Community Health* 1996; 50: 140 –143.

Geschwendtner A, Bevan S, Cole JW, Plourde A, Matarin M, Ross-Adams H, Meitinger T, Wichmann E, Mitchell BD, Furie K, Slowik A, Rich SS, Syme PD, MacLeod MJ, Meschia JF, Rosand J, Kittner SJ, Markus HS, Muller-Myhsok B, Dichgans M. Sequence variants on chromosome 9p21.3 confer risk for atherosclerotic stroke. *Annals of Neurology* 2009;65: 531–539.

Gil J, Peters G. Regulation of the INK4b-ARF-INK4a tumour suppressor locus: all for one or one for all. *Nature Reviews Molecular Cell Biology* 2006; 7(9): 667 – 677.

Giles WH, Croft JB, Greenlund KJ, Ford ES, Kittner SJ. Total homocyst(e)ine concentration and the likelihood of nonfatal stroke: results from the Third National Health and Nutrition Examination Survey, 1988–1994. *Stroke* 1998; 29: 2473 – 2477.

Giles WH, Kittner SJ, Hebel JR, Losonczy KG, Sherwin RW. Determinants of black-white differences in the risk of cerebral infarction: the National Health and Nutrition Examination Survey Epidemiologic Follow-up Study. *Archives of Internal Medicine* 1995; 155: 1319 –1324.

Gill JS, Zezulka AV, Shipley MJ, Gill SK, Beevers DG. Stroke and alcohol consumption. *New England Journal of Medicine* 1986; 315: 1041–1046.

Gillum RF. Risk factors for stroke in blacks: a critical review. *American Journal* of *Epidemiology* 1999; 150: 1266 – 1274.

Go AS, Hylek EM, Phillips KA, Chang YC, Henault LE, Selby JV, Singer DE. Prevalence of Diagnosed Atrial Fibrillation in Adults: National Implications for Rhythm Management and Stroke Prevention: the AnTicoagulation and Risk Factors In Atrial Fibrillation (ATRIA) Study. *JAMA* 2001; 285: 2370 - 2375

Goldstein LB, Adams R, Mark J. Alberts MJ, Appel LJ, Brass LM, Bushnell CD, Culebras A, DeGraba TJ, Gorelick PB, Guyton JR, Hart RG, Howard G, Kelly-Hayes M, Nixon JV, Sacco RL. Primary prevention of ischemic stroke: a guideline from the American Heart Association/American Stroke Association Stroke Council: cosponsored by the Atherosclerotic Peripheral Vascular Disease Interdisciplinary Working Group; Cardiovascular Nursing Council; Clinical Cardiology Council; Nutrition, Physical Activity, and Metabolism Council; and the Quality of Care and Outcomes Research Interdisciplinary Working Group. *Stroke* 2006; 37: 1583 – 1633.

Gretarsdottir S, Thorleifsson G, Manolescu A, Styrkarsdottir U, Helgadottir A, Gschwendtner A, Kostulas K, Kuhlenbumer G, Bevan S, Jonsdottir T, Bjarnason H, Saemundsdottir J, Palsson S, Arnar DO, Holm H, Thorgeirsson G, Valdimarsson EM, Sveinbjo¨rnsdottir S, Gieger C, Berger K, Wichmann HE, Hillert J, Markus H, Gulcher JR, Ringelstein EB, Kong A, Dichgans M, Gudbjartsson DF, Thorsteinsdottir U,

Stefansson K. Risk variants for atrial fibrillation on chromosome 4q25 associate with ischemic stroke. *Annals of Neurology* 2008; 64: 402 – 409.

Gu D, Reynolds K, Wu X, Chen J, Duan X, Muntner P, Huang G, Reynolds RF, Su S, Whelton PK, He J. Prevalence, awareness, treatment, and control of hypertension in China. *Hypertension* 2002; 40: 920 – 927.

Gudbjartsson DF, Holm H, Gretarsdottir S, Thorleifsson G, Walters GB, Thorgeirsson G, Gulcher J, Mathiesen EB, Njolstad I, Nyrnes A, Wilsgaard T, Hald EM, Hveem K, Stoltenberg C, Kucera G, Stubblefield T, Carter S, Roden D, Ng MC, Baum L, So WY, Wong KS, Chan JC, Gieger C, Wichmann HE, Gschwendtner A, Dichgans M, Kuhlenbaumer G, Berger K, Ringelstein EB, Bevan S, Markus HS, Kostulas K, Hillert J, Sveinbjornsdottir S, Valdimarsson EM, Lochen ML, Ma RC, Darbar D,
Kong A, Arnar DO, Thorsteinsdottir U, Stefansson K. A sequence variant in zfhx3 on 16q22 associates with atrial fibrillation and ischemic stroke. *Nature Genetics* 2009; 41: 876 – 878.

Handy DE, Loscalzo J. Homocysteine and Atherothrombosis: Diagnosis and Treatment. *Current Atherosclerosis Reports* 2003; 5: 276 – 283.

Hart RG, Benavente O, McBride R, Pearce LA. Antithrombotic therapy to prevent stroke in patients with atrial fibrillation: a meta-analysis. *Annals of Internal Medicine* 1999; 131: 492 – 501.

Hassan A, Markus HS. Genetics and ischaemic stroke. *Brain* 2000; 123: 1784–1812.

He J, Ogden LG, Vupputuri S, Bazzano LA, Loria C, Whelton PK. Dietary sodium intake and subsequent risk of cardiovascular disease in overweight adults. *JAMA* 1999; 282: 2027 – 2034.

Helgadottir A, Thorleifsson G, Magnusson KP, Gretarsdottir S, Steinthorsdottir V, Manolescu A, Jones GT, Rinkel GJ, Blankensteijn JD, Ronkainen A, Jaaskelainen JE, Kyo Y, Lenk GM, Sakalihasan N, Kostulas K, Gottsater A, Flex A, Stefansson H, Hansen T, Andersen G, Weinsheimer S, Borch-Johnsen K, Jorgensen T, Shah SH, Quyyumi AA, Granger CB, Reilly MP, Austin H, Levey AI, Vaccarino V, Palsdottir
E, Walters GB, Jonsdottir T, Snorradottir S, Magnusdottir D, Gudmundsson G, Ferrell RE, Sveinbjornsdottir S, Hernesniemi J, Niemela M, Limet R, Andersen K, Sigurdsson G, Benediktsson R, Verhoeven EL, Teijink JA, Grobbee DE, Rader DJ, Collier DA, Pedersen O, Pola R, Hillert J, Lindblad B, Valdimarsson EM, Magnadottir HB, Wijmenga C, Tromp G, Baas AF, Ruigrok YM, van Rij AM, Kuivaniemi H, Powell JT, Matthiasson SE, Gulcher JR, Thorgeirsson G, Kong A, Thorsteinsdottir U, Stefansson K. The same sequence variant on 9p21 associates

with myocardial infarction, abdominal aortic aneurysm and intracranial aneurysm. *Nature Genetics* 2008; 40: 217–224.

He FJ, MacGregor GA. Effect of longer-term modest salt reduction on blood pressure. *Cochrane Database Systematic Reviews* 2004; (3): CD004937.

Heuschmann PU, Heidrich J, Wellmann J, Kraywinkel K, Kei U. Stroke mortality and morbidity attributable to passive smoking in Germany. *European Journal of Cardiovascular Prevention and Rehabilitation* 2007; 14: 793 – 795.

Hier DB, Foulkes MA, Swiontoniowski M, Sacco RL, Gorelick PB, Mohr JP, Price TR, Wolf PA. Stroke recurrence within 2 years after ischemic infarction. *Stroke* 1991; 22: 155 – 161.

Hill WG, Robertson A. Linkage disequilibrium in finite populations. Theoretical Application in Genetics 1968; 38: 226 – 231.

Hillbom M, Numminen H, Juvela S. Recent heavy drinking of alcohol and embolic stroke. *Stroke* 1999; 30: 2307–2312.

Hillen T, Coshall C, Tilling K, Rudd AG, McGovern R, Wolfe CDA. Cause of stroke recurrence is multifactorial: patterns, risk factors, and outcomes of stroke recurrence in the South London Stroke Register. *Stroke* 2003; 34: 1457 – 1463.

Hofmann MA, Lall E, Lu Y, Gleason MR, Wolf BM, Tanji N, Ferran LJ, Jr., Kohl B, Rao V, Kisiel W, Stern DM, Schmidt AM. Hyperhomocysteinemia enhances vascular inflammation and accelerates atherosclerosis in a murine model. *Journal of Clinical Investigation* 2001; 107: 675 – 683.

Homocysteine Studies Collaboration. Homocysteine and risk of ischemic heart disease and stroke: a meta-analysis. *JAMA* 2002; 288: 2015 – 2022.

Hossmann KA. Viability thresholds and the penumbra of focal ischemia. *Annals of Neurology* 1994; 36: 557-565.

Hu WL, Li SJ, Liu DT, Wang Y, Niu SQ, Xinchun Yang, Qi Zhang, Yu SZ, Jin L, Wang XF. Genetic variants on chromosome 9p21 and ischemic stroke in Chinese . *Brain Research Bulletin* 2009; 79(6): 431- 435.

Ikram MA, Seshadri S, Bis JC, Fornage M, DeStefano AL, Aulchenko YS, Debette S, Lumley T, Folsom AR, van den Herik EG, Bos MJ, Beiser A, Cushman M, Launer LJ, Shahar E, Struchalin M, Du Y, Glazer NL, Rosamond WD, Rivadeneira F, Kelly-Hayes

M, Lopez OL, Coresh J, Hofman A, DeCarli C, Heckbert SR, Koudstaal PJ, Yang Q, Smith NL, Kase CS, Rice K, Haritunians T, Roks G, de Kort PL, Taylor KD, de Lau LM, Oostra BA, Uitterlinden AG, Rotter JI, Boerwinkle E, Psaty BM,
Mosley TH, van Duijn CM, Breteler MM, Longstreth WT Jr, Wolf PA. Genomewide association studies of stroke. *New England Journal of Medicine* 2009; 360: 1718 – 1728.

Iso H, Jacobs DR, Jr., Wentworth D, Neaton JD, Cohen JD. Serum cholesterol levels and six-year mortality from stroke in 350,977 men screened for the Multiple Risk Factor Intervention Trial. *New England Journal of Medicine* 1989; 320: 904 – 910.

Jakubowski H, Zhang L, Bardeguez A, Aviv A. Homocysteine thiolactone and protein homocysteinylation in human endothelial cells: implications for atherosclerosis. Circulation Research 2000; 87: 45–51.

Jamrozik K, Broadhurst RJ, Anderson CS, Stewart-Wynne EG. The role of lifestyle factors in the etiology of stroke: a population-based case-control study in Perth, Western Australia. *Stroke* 1994; 25: 51–59.

Jarinova O, Stewart AF, Roberts R, Wells G, Lau P, Naing T, Buerki C, McLean BW, Cook RC, Parker JS, McPherson R. Functional analysis of the chromosome 9p21.3 coronary artery disease risk locus. *Arteriosclerosis Thrombosis and Vascular Biology* 2009; 29: 1671 – 1677.

Johansson BB. Hypertension mechanisms causing stroke. *Clinical and Experimental Pharmacology and Physiology* 1999; 26: 563–565

Jood K, Ladenvall C, Rosengren A, Blomstrand C, Jern C. Family History in Ischemic Stroke Before 70 Years of Age: The Sahlgrenska Academy Study on Ischemic Stroke. *Stroke* 2005; 36: 1383 - 1387.

Jorde LB. Linkage disequilibrium as a gene-mapping tool. American Journal of Human Genetics 1995; 56: 11-14.

Jousilahti P, Rastenyte D, Tuomilehto J, Sarti C, Vartiainen E. Parental history of cardiovascular disease and risk of stroke: a prospective follow-up of 14,371 middle aged men and women in Finland. *Stroke* 1997; 28: 1361-1366.

Kannel WB, McGee DL. Diabetes mellitus and cardiovascular disease: the Framingham study. *JAMA* 1979; 241: 2035–2038.

Kaplan NL, Martin ER, Weir BS. Power studies for transmission/disequilibrium tests with multiple alleles. American Journal of Human Genetics 1997; 60: 691-702.

Karapanayiotides T, Piechowski-Jozwiak B, van Melle G, Bogousslavsky J, Devuyst G. Stroke patterns, etiology, and prognosis in patients with diabetes mellitus mellitus. *Neurology*. 2004; 62: 1558 –1562.

Kearney PM, Whelton M, Reynolds K, Muntner P, Whelton PK, He J. Global burden of hypertension: analysis of worldwide data. *Lancet* 2005; 365: 217 – 223.

Kiely DK, Wolf PA, Cupples LA, Beiser AS, Myers RH. Familial aggregation of stroke: the Framingham Study. *Stroke* 1993; 24: 1366 – 1371.

Kim WY, Sharpless NE. The regulation of INK4/ARF in cancer and aging. *Cell* 2006; 127(2): 265 – 275.

Kleindorfer DO, Khoury J, Moomaw CJ, Alwell K, Woo D, Flaherty ML, Khatri P, Adeoye O, Ferioli S, Broderick JP, Kissela BM. Stroke Incidence Is Decreasing in Whites But Not in Blacks: A Population-Based Estimate of Temporal Trends in Stroke Incidence From the Greater Cincinnati/Northern Kentucky Stroke Study. *Stroke* 2010; 41: 1326 - 1331.

Koren-Morag N, Tanne D, Graff E, Goldbourt U. Low- and high-density lipoprotein cholesterol and ischemic cerebrovascular disease: the bezafibrate infarction prevention registry. *Archives of Internal Medicine* 2002; 162: 993 – 999.

Kul CS, Loyl EY, Pawitan Y, Chia KS. The pursuit of genome-wide association studies: where are we now? *Journal of Human Genetics* 2010; 55: 195 – 206.

Kurth T, Gaziano JM, Berger K, Kase CS, Rexrode KM, Cook NR, Buring JE, Manson JE. Body mass index and the risk of stroke in men. *Archives of Internal Medicine* 2002; 162: 2557 – 2562

Kwon, JM, Goate AM. The Candidate Gene Approach. Alcohol Research and Health 2000; 24: 164 - 168.

Lawes CMM, Bennett DA, Feigin VL, Rodgers A. Blood pressure and stroke: an overview of published reviews. *Stroke* 2004; 35: 776 –785.

Lee CD, Folsom AR, Blair SN. Physical activity and stroke risk: a meta-analysis. *Stroke* 2003; 34: 2475 – 2481.

LENTZ SR. Mechanisms of homocysteine-induced atherothrombosis. *Journal of Thrombosis and Haemostasis* 2005; 3: 1646–1654

Lentz SR, Erger RA, Dayal S, Maeda N, Donald MR, Heistad D and Faraci FM. Folate dependence of hyperhomocysteinemia and endothelial dysfunction in cystathionine b-synthase-deficient mice. *American Journal of Physiology* 2000; 278: 970 – 975.

Lentzt SR, Sadler JE. Inhibition of thrombomodulin surface expression and protein C activation by the thrombogenic agent homocysteine. *Journal of Clinical Investigation* 1991; 88: 1906 – 1914.

Leppälä JM, Virtamo J, Fogelholm R, Albanes D, Heinonen OP. Different risk factors for different stroke subtypes: association of blood pressure, cholesterol, and antioxidants. *Stroke* 1999; 30: 2535 – 2540.

Lewington S, Clarke R, Qizilbash N, Peto R, Collins R. Age-specific relevance of usual blood pressure to vascular mortality: a meta-analysis of individual data for one million adults in 61 prospective studies *Lancet* 2002; 360: 1903 –1913.

Li SC, Schoenberg BS, Wang CC, Cheng XM, Bolis CL, Wang KJ. Cerebrovascular disease in the People's Republic of China: epidemiologic and clinical features. *Neurology* 1985; 35: 1708–1713.

Li ZH, Sun L, Zhang HY, Liao YH, Wang DW, and Zhao BR. Elevated Plasma Homocysteine Was Associated With Hemorrhagic and Ischemic Stroke, but

Methylenetetrahydrofolate Reductase Gene C677T Polymorphism Was a Risk Factor for Thrombotic Stroke : A Multicenter Case-Control Study in China. *Stroke* 2003; 34: 2085 - 2209

Liao DP, Myers R, Hunt S, Shahar E, Paton C, Burke G, Province M, Heiss G. Familial history of stroke and stroke risk: the Family Heart Study. *Stroke* 1997; 28: 1908-1912.

Lievers KJ, Boers GH, Verhoef P, den Heijer M, Kluijtmans LA, van der Put NM, Trijbels FJ, Blom HJ. A second common variant in the methylenetetrahydrofolate reductase (MTHFR) gene and its relationship to MTHFR enzyme activity, homocysteine, and cardiovascular disease risk. *Journal of Molecular Medicine* 2001; 79:522–8.

Liu M, Wu B, Wang WZ, Lee LM, Zhang SH, Kong LZ. Stroke in China: epidemiology, prevention, and management strategies. *Lancet Neurology* 2007; 6: 456 – 464.

Low HQ, Chen CPLH, Kasiman K, Thalamuthu A, Ng S-S. A Comprehensive Association Analysis of Homocysteine Metabolic Pathway Genes in Singaporean Chinese with Ischemic Stroke. PLoS ONE 2011; 6(9): e24757. doi:10.1371/journal.pone.0024757

Mannami T, Iso H, Baba S, Sasaki S, Okada K, Konishi M, Tsugane S. Prospective Study on Cancer and Cardiovascular Disease Group. Cigarette Smoking and Risk of Stroke and

Matarin M, Brown WM, Singleton A, Hardy JA, Meschia JF. Whole genome analyses suggest ischemic stroke and heart disease share an association with polymorphisms on chromosome 9p21. *Stroke* 2008; 39: 1586 – 1589.

Mathers CD, Boerma T, Fat DM. Global and regional causes of death. *British Medical Bulletin.* 2009; 92: 7 – 32.

McCully KS. Vascular pathology of homocysteinemia: implications for the pathogenesis of arteriosclerosis. *American Journal of Pathology* 1969; 56: 111–128.

Megherbi SE, Milan C, Minier D, Couvreur G, Osseby GV, Tilling K, Carlo AD, Inzitari D, Wolfe CD, Moreau T, Giroud M. Association between diabetes mellitus and stroke subtype on survival and functional outcome 3 months after stroke: data from the European BIOMED Stroke Project. *Stroke* 2003; 34: 688 – 694.

Meretoja A. Perfect Stroke: performance, effectiveness, and costs of treatment episodes in stroke. Helsinki University print 2011, ISBN 978-952-10-6835-5.

Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Research 1988; 16 (3): 1215.

Minsitry of Health. Chinese Health Statistical Digest 2006. Ministry of Health, People's Republic of China 2006; 45: 1989 – 2005.

Mueller JC, Lõhmussaar E, Mägi R, Remm M, Bettecken T, Lichtner P, Biskup S, Thomas Illig T, Pfeufer A, Luedemann J, Schreiber S, Pramstaller P, Pichler I, Romeo G, Gaddi A, Testa A, Wichmann HE, Andres Metspalu, and Thomas Meitinger. Linkage Disequilibrium Patterns and tagSNP Transferability among European Populations. *American Journal of Human Genetics* 2005; 76(3): 387 – 398.

Murray CJL, Lopez AD. Mortality by cause for eight regions of the world: global burden of disease study. *Lancet* 1997; 349: 1269 –1276.

Musunuru K, Post WS, Herzog W, Shen H, O'Connell JR, McArdle PF, Ryan KA, Gibson Q, Cheng YC, Clearfield E, Johnson AD, Tofler G, Yang Q, O'Donnell CJ, Becker DM, Yanek LR, Becker LC, Faraday N, Bielak LF, Peyser PA, Shuldiner AR, Mitchell BD. Association of single nucleotide polymorphisms on chromosome 9p21.3 with platelet reactivity: a potential mechanism for increased vascular disease. *Circulation Cardiovascular Genetics* 2010; 3: 445 – 453.

Nadareishvili ZG, Rothwell PM, Beletsky V, Pagniello A, Norris JW. Long-term risk of stroke and other vascular events in patients with asymptomatic carotid artery stenosis. *Archives of Neurology* 2002; 59: 1162 – 1166.

National 8.5 CVD collaborative Group. Community comprehensive preventive study on cardial and cerebral vascular diseases. *Chinese Journal of Prevention Medicine* 1998; 32: 3–4.

National Institute of Neurological Disorders and Stroke (NINDS) (1999). "Stroke: Hope Through Research". National Institutes of Health. http://www.ninds.nih.gov/disorders/stroke/detail_stroke.htm.

Norris JW, Zhu CZ, Bornstein NM and Chambers BR. Vascular risks of asymptomatic carotid stenosis. *Stroke* 1991; 22: 1485 - 1490.

O'Mahony PG, Thomson RG, Dobson R, Rodgers H, James OF. The prevalence of stroke and associated disability. *Journal of Public Health Medicine* 1999; 21: 166 –171.

Petty GW, Brown RD Jr, Whisnant JP, Sicks JD, O'Fallon WM, Wiebers DO. Survival and recurrence after first cerebral infarction: a population-based study in Rochester, Minnesota, 1975 through 1989. *Neurology* 1998; 50: 208 – 216.

Phillips SJ. Pathophysiology and management of hypertension in acute ischemic stroke. *Hypertension* 1994; 23:131-136.

Plomin R, Owen MJ, McGuffin P. The genetic basis of complex human behaviors. *Science* 1994; 264: 1733 - 1739.

Poirier P, Giles TD, Bray GA, Hong Y, Stern JS, Pi-Sunyer FX, Eckel RH. Obesity and cardiovascular disease: pathophysiology, evaluation, and effect of weight loss. *Arteriosclerosis Thrombosis and Vascular Biology* 2006; 26: 968–976.

Qian Jia, Xingquan Zhao, Chunxue Wang, Yilong Wang, Yu Yan, Hao Li, Liyong Zhong, Liping Liu, Huaguang Zheng, Yong Zhou, and Yongjun Wang. Diabetes mellitus and Poor Outcomes Within 6 Months After Acute Ischemic Stroke The China National Stroke Registry. *Stroke* 2011; 42: 2758 - 2762.

Ramdas WD, van Koolwijk LM, Ikram MK, Jansonius NM, de Jong PT, Bergen AA, Isaacs A, Amin N, Aulchenko YS, Wolfs RC, Hofman A, Rivadeneira F, Oostra BA, Uitterlinden AG, Hysi P, Hammond CJ, Lemij HG, Vingerling JR, Klaver CC, van Duijn CM.. A genome-wide association study of optic disc parameters. *PLoS Genetics 2010;* 6, e1000978.

Reddy KS, Yusuf S. Emerging epidemic of cardiovascular disease in developing countries. *Circulation* 1998; 97: 596 – 601.

Rexrode KM, Hennekens CH, Willett WC, Colditz GA, Stampfer MJ, Rich-Edwards JW, Speizer FE, Manson JE. A prospective study of body mass index, weight change, and risk of stroke in women. *JAMA* 1997; 277: 1539 – 1545.

Risch N. Searching for genes in complex diseases: lessons from systemic lupus erythematosus. Journal of Clinical Investigation 2000; 105: 1503 – 1506.

Robert A. Hegele, Martin Dichgans. Advances in Stroke 2009 : Update on the Genetics of Stroke and Cerebrovascular Disease 2009. *Stroke.* 2010; 41:e63-e66:

Rodriguez BL, D'Agostino R, Abbott RD, Kagan A, Burchfiel CM, Yano K, Ross GW, Silbershatz H, Higgins MW, Popper J, Wolf PA, Curb JD. Risk of hospitalized stroke in men enrolled in the Honolulu Heart Program and the Framingham Study: a comparison of incidence and risk factor effects. *Stroke* 2002; 33: 230 – 236.

Ropper AH, Brown RH. Adams and Victor's Principles of Neurology 8th edition (2005). *The McGraw-Hill Companies, Inc.* 1234567890 DOW/DOW 098765 ISBN: 0-07-141620-X

Rosamond W, Flegal K, Friday G, Furie K, Go A, Greenlund K, Haase N, Ho M, Howard V, Kissela B, Kittner S, Lloyd-Jones D, McDermott M, Meigs J, Moy C, Nichol G,

O'Donnell CJ, Roger V, Rumsfeld J, Sorlie P, Steinberger J, Thom T, Wasserthiel-Smoller S, Hong YL. Heart disease and stroke statistics–2007 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* 2007; 115: 69 – 171.

Rosamond W, Flegal K, Furie K, Go A, Greenlund K, Haase N, Hailpern SM, Ho M, Howard V, Kissela B, Kittner S, Lloyd-Jones D, McDermott M, Meigs J, Moy C, Nichol G, O'Donnell C, Roger V, Sorlie P, Steinberger J, Thom T, Wilson M and Hong Y. Heart disease and stroke statistics—2008 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation 2008; 117: 25–146.

Rubattu S, Volpe M, Kreutz R, Ganten U, Ganten D, Lindpaintner K. Chromosomal mapping of quantitative trait loci contributing to stroke in a rat model of complex human disease. *Nature Genetics* 1996; 13: 429 – 434.

Sacco RL, Boden-Albala B, Gan R, Chen X, Kargman DE, Shea S, Paik MC, and Hauser WA. Stroke incidence among white, black, and Hispanic residents of an urban community: the Northern Manhattan Stroke Study. *American Journal of Epidemiology* 1998; 147: 259 – 268.

Samani NJ, Erdmann J, Hall AS, Hengstenberg C, Mangino M, Mayer B,

Dixon RJ, Meitinger T, Braund P, Wichmann HE, Barrett JH, Konig IR, Stevens SE, Szymczak S, Tregouet DA, Iles MM, Pahlke F, Pollard H, Lieb W, Cambien F, Fischer M, Ouwehand W, Blankenberg S, Balmforth AJ, Baessler A, Ball SG, Strom TM, Braenne I, Gieger C, Deloukas P, Tobin MD, Ziegler A, Thompson JR, Schunkert H. Genomewide association analysis of coronary artery disease. *New England Journal of Medicine* 2007; 357: 443 – 453.

Sattelmair JR, Kurth T, Buring JE, Lee IM. Physical Activity and Risk of Stroke in Women. *Stroke* 2010; 41:1243 - 1250.

Sanja Stankovic, Nada Majkic-Singh. Genetic Aspects of Ischemic Stroke: Coagulation, Homocysteine, and Lipoprotein Metabolism as Potential Risk Factors. *Critical Review in Clinical Laboratory Sciences* 2010; 47(2): 72-123.

Savelieva I, Bajpai A, Camm AJ. Stroke in atrial fibrillation: Update on pathophysiology, new antithrombotic therapies, and evolution of procedures and devices. *Annals of Medicine* 2007; 39: 371–391.

Saxena R, Voight BF, Lyssenko V, Burtt NP, de Bakker PI, Chen H, Roix JJ, Kathiresan S, Hirschhorn JN, Daly MJ, Hughes TE, Groop L, Altshuler D, Almgren P, Florez JC, Meyer J, Ardlie K, Bengtsson Boström K, Isomaa B, Lettre G, Lindblad U, Lyon HN, Melander O, Newton-Cheh C, Nilsson P, Orho-Melander M, Råstam L, Speliotes EK,

Taskinen MR, Tuomi T, Guiducci C, Berglund A, Carlson J, Gianniny L, Hackett R, Hall L, Holmkvist J, Laurila E, Sjögren M, Sterner M, Surti A, Svensson M, Svensson M, Tewhey R, Blumenstiel B, Parkin M, Defelice M, Barry R, Brodeur W, Camarata J, Chia N, Fava M, Gibbons J, Handsaker B, Healy C, Nguyen K, Gates C, Sougnez C, Gage D, Nizzari M, Gabriel SB, Chirn GW, Ma Q, Parikh H, Richardson D, Ricke D, and Purcell S. Genome-wide association analysis identifies loci for type 2 diabetes mellitus and triglyceride levels. *Science* 2007; 316,:1331 –1336.

Sazci A, Ergul E, Nese Tuncer N, Akpinar G and Kara I. Methylenetetrahydrofolate reductase gene polymorphisms are associated with ischemic and hemorrhagic stroke: Dual effect of MTHFR polymorphisms C677T and A1298C. *Brain Research Bulletin.* 2006;71: 45–50.

Schellinger PD, Fiebach JB, Hacke W. Imaging-Based Decision Making in Thrombolytic Therapy for Ischemic Stroke : Present Status. *Stroke* 2003; 34: 575 – 583.

Schunkert H, Gotz A, Braund P, McGinnis R, Tregouet DA, Mangino M, Linsel-Nitschke P, Cambien F, Hengstenberg C, Stark K, Blankenberg S, Tiret L, Ducimetiere P, Keniry A, Ghori MJ, Schreiber S, El Mokhtari NE, Hall AS, Dixon RJ, Goodall AH, Liptau H, Pollard H, Schwarz DF, Hothorn LA, Wichmann HE, Konig IR, Fischer M, Meisinger C, Ouwehand W, Deloukas P, Thompson JR, Erdmann J, Ziegler A, Samani NJ. Repeated

replication and a prospective meta-analysis of the association between chromosome 9p21.3 and coronary artery disease. *Circulation*. 2008; 117: 1675 – 1684.

Schwamm LH, Reeves MJ, Pan WQ, Smith EE, Frankel MR, Olson DW, Zhao X, Peterson E, Fonarow GC. Race/Ethnicity, Quality of Care, and Outcomes in Ischemic Stroke. *Circulation* 2010; 121: 1492 - 1501.

Selhub J. Homocysteine metabolism. *Annual Review of Nutrituion* 1999; 19: 217 – 246.

Senelick RC, Rossi PW and Dougherty K. (1994). Living with Stroke: A Guide for Families. Contemporary Books, Chicago. ISBN 0809226073. OCLC 42835161 40856888 42835161.

Shea J, Agarwala V, Philippakis AA, Maguire J, Banks E, DePristo M, Thomson B, Guiducci C and Onofrio RC. The Myocardial Infarction Genetics Consortium, Sekar Kathiresan, Stacey Gabriel, Noël P Burtt, Mark J Daly, Leif Groop & David Altshuler. Comparing strategies to fine-map the association of common SNPs at chromosome 9p21 with type 2 diabetes mellitus and myocardial infarction. *Nature Genetics* 2011; 43(8): 801-806.

Sherborne AL, Hosking FJ, Prasad RB, Kumar R, Koehler R, Vijayakrishnan J, Papaemmanuil E, Bartram CR, Stanulla M, Schrappe M, Gast A, Dobbins SE, Ma Y,

Sheridan E, Taylor M, Kinsey SE, Lightfoot T, Roman E, Irving JA, Allan JM, Moorman AV, Harrison CJ, Tomlinson IP, Richards S, Zimmermann M, Szalai C, Semsei AF, Erdelyi DJ, Krajinovic M, Sinnett D, Healy J, Gonzalez Neira A, Kawamata N, Ogawa S, Koeffler HP, Hemminki K, Greaves M, Houlston RS. Variation in *CDKN2A* at 9p21.3 influences childhood acute lymphoblastic leukemia risk. *Nature Genetics* 2010; 42: 492–494.

Shete S, Hosking FJ, Robertson LB, Dobbins SE, Sanson M, Malmer B, Simon M, Marie Y, Boisselier B, Delattre JY, Hoang-Xuan K, El Hallani S, Idbaih A, Zelenika D, Andersson U, Henriksson R, Bergenheim AT, Feychting M, Lönn S, Ahlbom A, Schramm J, Linnebank M, Hemminki K, Kumar R, Hepworth SJ, Price A, Armstrong G, Liu Y, Gu X, Yu R, Lau C, Schoemaker M, Muir K, Swerdlow A, Lathrop M, Bondy M, Houlston RS. Genome-wide association study identifies five susceptibility loci for glioma. *Nature Genetics* 2009; 41: 899–904.

Shi FL, Hart RG, Sherman DG, Tegeler CH. Stroke in the People's Republic of China. *Stroke* 1989; 20: 1581–1585.

Stacey SN, Sulem P, Masson G, Gudjonsson SA, Thorleifsson G, Jakobsdottir M, Sigurdsson A, Gudbjartsson DF, Sigurgeirsson B, Benediktsdottir KR, Thorisdottir K, Ragnarsson R, Scherer D, Hemminki K, Rudnai P, Gurzau E, Koppova K, Botella-

Estrada R, Soriano V, Juberias P, Saez B, Gilaberte Y, Fuentelsaz V, Corredera C, Grasa M, Höiom V, Lindblom A, Bonenkamp JJ, van Rossum MM, Aben KK, de Vries E, Santinami M, Di Mauro MG, Maurichi A, Wendt J, Hochleitner P, Pehamberger H, Gudmundsson J, Magnusdottir DN, Gretarsdottir S, Holm H, Steinthorsdottir V, Frigge ML, Blondal T, Saemundsdottir J, Bjarnason H, Kristjansson K, Bjornsdottir G, Okamoto I, Rivoltini L, Rodolfo M, Kiemeney LA, Hansson J, Nagore E, Mayordomo JI, Kumar R, Karagas MR, Nelson HH, Gulcher JR, Rafnar T, Thorsteinsdottir U, Olafsson JH, Kong A, Stefansson K. New common variants affecting susceptibility to basal cell carcinoma. *Nature Genetics* 2009; 41: 909–914.

Stamler, J Vaccaro O, Neaton JD, Wentworth D. Diabetes mellitus, other risk factors, and 12-yr cardiovascular mortality for men screened in the Multiple Risk Factor Intervention Trial. *Diabetes mellitus Care* 1993; 16: 434 – 444.

Stankovic S, Majkic-Singh N. Genetic Aspects of Ischemic Stroke: Coagulation, Homocysteine, and Lipoprotein Metabolism as Potential Risk Factors. *Critical Review in Clinical Laboratory Sciences* 2010; 47(2):72-123.

Strazzullo P, D'Elia L, Cairella G, Garbagnati F, Cappuccio FP, Scalfi L. Excess Body Weight and Incidence of Stroke: Meta-Analysis of Prospective Studies With 2 Million Participants. *Stroke* 2010; 41: 418-426.

Steven R. Lentzt and J. Evan Sadler. Homocysteine inhibits von Willebrand factor processing and secretion by preventing transport from the endoplasmic reticulum. *Blood* 1993; 81: 683 – 689.

Stroke Risk in Atrial Fibrillation Working Group. Independent predictors of stroke in patients with atrial fibrillation: a systematic review. Neurology 2007; 69(6): 546 - 554.

Taillon-Miller P, Bauer-Sardina I., Saccone NL, Putzel J, Laitinen, T, Cao A, Kere, J., Pilia, G., Rice, J.P., Kwok, P.Y. Juxtaposed regions of extensive and minimal linkage disequilibrium in human Xq25 and Xq28. *Nature Genetics* 2000; 25: 324 – 328.

The Advisory Council for the National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health. A Classification and Outline of Cerebrovascular Diseases II. *Stroke* 1975; 6: 564 -616

The International SNP Map Working Group. A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. *Nature* 2001; 409: 928 - 933.

The International HapMap Consortium. The International HapMap Project. *Nature.* 2003; 426(6968): 789-796.

The International HapMap Consortium. A haplotype map of the human genome. *Nature.* 2005; 437(7063): 1299-1320.

Turnbull C, Ahmed S, Morrison J, Pernet D, Renwick A, Maranian M, Seal S, Ghoussaini M, Hines S, Healey CS, Hughes D, Warren-Perry M, Tapper W, Eccles D, Evans DG; Breast Cancer Susceptibility Collaboration (UK), Hooning M, Schutte M, van den Ouweland A, Houlston R, Ross G, Langford C, Pharoah PD, Stratton MR, Dunning AM, Rahman N and Easton DF. Genome-wide association study identifies five new breast cancer susceptibility loci. *Nature Genetics* 2010; 42: 504–507.

Turnbull F. Effects of different blood-pressure-lowering regimens on major cardiovascular events: results of prospectively-designed overviews of randomized trials. *Lancet* 2003; 362: 1527–1535.

Ueland PM, Hustad S, Schneede J, Refsum H, Vollset SE. Biological and clinical implications of the MTHFR C677T polymorphism. *Trends in Pharmacological Science* 2001;22:195 – 201.

Ueland M, Refsum H, Beresford SAA, and Vollset SE. The controversy over homocysteine and cardiovascular risk. *American Journal of Clinical Nutrition* 2000; 72: 324 – 332.

Ungvari Z, Csiszar A, Edwards JG, Kaminski PM, Wolin MS, Kaley G, Koller A. Increased superoxide production in coronary arteries in hyperhomocysteinemia: role of tumor necrosis factor-alpha, NAD(P)H oxidase, and inducible nitric oxide synthase. Arteriosclerosis Thrombosis Vascular Biology 2003; 23: 418 – 424.

Vallance P. The asymmetrical dimethylarginine/dimethylarginine dimethylaminohydrolase pathway in the regulation of nitric oxide production. *Clinical Science* 2001; 100: 159 – 160.

van der Put NMJ, Gabree¨ls F, Stevens EMB, Smeitink JAM, Trijbels FJM, Eskes TKAB, van den Heuvel LP, Blom HJ. A Second Common Mutation in the Methylenetetrahydrofolate Reductase Gene: An Additional Risk Factor for Neural-Tube Defects? *American Journal of Human Genetics* 1998; 62:1044 – 1051.

Villarosa L, Singleton LF, Johnson KA. Black Health Library Guide to Stroke. Henry Holt and Company (1993) New York.

Wang CC, Cheng XM, Li SC. Epidemiological survey of neurological disorders in six urban areas of People's Republic of China. *Chinese Neurosurgery Journal* 1985;1: 2–7.

Wang LD, Kong LZ, Wu F, Bai YM, Burton R. Preventing chronic diseases in China. *Lancet* 2005; 366: 1821 – 1824.

Warach S, Gaa J, Siewert B, Wielopolski P, Edelman RR. Acute human stroke studied by whole brain echo planar diffusion-weighted magnetic resonance imaging. *Annals of Neurology* 1995; 37: 231 – 241.

Weisberg L, Tran P, Christensen B, Sibani S and Rozen R. A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity, *Molecular Genetics and Metabolism* 1998; 64 (3):169 –172.

Weiss N, Heydrick S, Zhang YY, Bierl C, Cap A, Loscalzo J. Cellular redox state and endothelial dysfunction in mildly hyperhomocysteinemic cystathionine beta-synthase-deficient mice. Arteriosclerosis, Thrombosis and Vascular Biology 2002; 22: 34–41.

Weiss N, Zhang YY, Heydrick S, Bierl C, Loscalzo J. Overexpression of cellular glutathione peroxidase rescues homocyst(e)ine-induced endothelial dysfunction. Procedings of the National Academy of Sciences of the United States of America 2001; 98: 12503 – 12508.

Wen B, Li H, Lu DR, Song XF, Zhang F, He YG, Li F, Gao Y, Mao XY, Zhang L, Qian J, Tan JZ, Jin JZ, Huang W, Deka RJ, Su B, Chakraborty R, Jin L. Genetic evidence supports demic diffusion of Han culture. *Nature* 2004; 431: 302 - 305.

Whisnant JP. Modeling of risk factors for ischemic stroke. The Willis lecture. *Stroke* 1997; 28: 1840–1844.

WHO. *The world health report*. WHO Geneva (1998).

WHO. *The world health report*. WHO Geneva (2000).

WHO. *The world health report*. WHO Geneva (2004).

WHO MONICA project. MONICA Manual. Available at: URL: http:// www.ktl.fi/publications/monica. URNLNBN:fi-fe 19981147, 1990.

Wild S, Roglic G, Green A, Sicree R, King H. Global Prevalence of Diabetes mellitus: Estimates for the year 2000 and projections for 2030. *Diabetes mellitus Care* 2004; 27:1047 – 1053.

Wolf PA, Abbott RD, Kannel WB. Atrial fibrillation as an independent risk factor for stroke: the Framingham Study. *Stroke* 1991; 22: 983 – 988.

Wolf PA, D'Agostino RB, Kannel WB, Bonita R, Belanger AJ. Cigarette smoking as a risk factor for stroke: the Framingham Study. *JAMA* 1988; 259: 1025–1029.

Wolf PA, D'Agostino RB, O'Neal MA, Sytkowski P, Kase CS, Belanger AJ, Kannel WB. Secular trends in stroke incidence and mortality: the Framingham Study. *Stroke* 1992;23:1551 – 1555.

Woo D, Gebel J, Miller R, Kothari R, Brott T, Khoury J, Salisbury S, Shukla R, Pancioli A, Jauch E and Broderick J. Incidence rates of first-ever ischemic stroke subtypes among blacks: a population-based study. *Stroke* 1999; 30: 2517–2522.

World Health Organization. Definition and diagnosis of diabetes mellitus mellitus and intermediate hyperglycemia: report of a WHO/IDF consultation. WHO Document Production Services, Geneva, Switzerland. 2006. ISBN 92 4 159493 4.

Wu ZS, Yao CH, Zhao D, Wu GX, Wang W, Liu J, Zeng ZC, Wu YK. Sino-MONICA project: A collaborative study on trends and determinants in cardiovascular diseases in China, part I: morbidity and mortality monitoring. *Circulation* 2001; 103: 462–468.

Xu SH, Yin XY, Li SL, Jin WF, Lou HY, Yang L, Gong XH, Wang HY, Shen YP, Pan XD, He YG, Yang YJ, Wang Y, Fu WQ, An Y, Wang JC, Tan JZ, Qian J, Chen XL, Zhang X, Yangfei Sun YF, Xuejun Zhang XJ, Wu BL and Jin L. Genomic Dissection of Population Substructure of Han Chinese and Its Implication in Association Studies. *The American Journal of Human Genetics* 2009; 85: 762 – 774.

Xu X, Li J, Sheng W, Liu L. Meta-analysis of genetic studies from journals published in China of ischemic stroke in the Han Chinese population. *Cerebrovascular Diseases* 2008; 26: 48–62.

Xue GB. Yu BX, Wang XZ, Wang GQ, Wang ZY. Epidemiological survey: stroke in urban and rural areas of China. *Chinese Medicine Journal* 1991; 104: 697 – 704.

Yamada K, Chen Z, Rozen R, Matthews RG. "Effects of common polymorphisms on the properties of recombinant human methylenetetrahydrofolate reductase". *Proceedings of National Academy of Sciences of the United States of America.* 2001; 98 (26): 14853–14858.

Yusuf S , Sleight P, Pogue J, Bosch J, Davies R, Dagenais G. The Heart Outcomes Prevention Evaluation Study Investigators. Effects of an angiotensin- converting-enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. *New England Journal of Medicine* 2000; 342: 145 – 153.

Zhang K, Calabrese P, Nordborg M, Sun F. Haplotype block structure and its applications to association studies: power and study designs. *American Journal of Human Genetics* 2002; 71: 1386 – 1394.

Zhang LF, Yang J, Hong Z, Yuan GG, Zhou BF, Zhao LC, Huang YN, Chen J, Wu YF. Proportion of diff erent subtypes of stroke in China. *Stroke* 2003; 34: 2091 – 2096.

Zhang X, Patel A, Horibe H, Wu Z, Barzi F, Rodgers A, MacMahon S, Woodward M, Asia Pacific Cohort Studies Collaboration. Cholesterol, coronary heart disease, and stroke in the Asia Pacific region. *Inteternational Journal of Epidemiology* 2003; 32: 563 – 572.

Zhang XF, Attia J, D'Este C, Yu XH. Prevalence and magnitude of classical risk factors for stroke in a Cohort of 5092 Chinese steelworkers over 13.5 years of follow up. *Stroke* 2004; 35: 1052 – 1056.

Zhao D, Liu J, Wang W, Zeng ZC, Cheng J, Liu J, Sun JY, Wu ZS. Epidemiological Transition of Stroke in China: Twenty-One-Year Observational Study From the Sino-MONICA-Beijing Project *Stroke* 2008; 39: 1668 -1674.

Zhou J, Møller J, Danielsen CC, Bentzon J, Ravn HB, Austin RC and Falk E. Dietary supplementation with methionine and homocysteine promotes early atherosclerosis but not plaque rupture in apoE-deficient mice. *Arteriosclerosis, Thrombosis and Vascular Biology* 2001; 21: 1470 – 1476.