POLYMORPHISMS OF EPOXIDE HYDROLASE GENES AND ISCHEMIC STROKE RISK IN TURKISH POPULATION

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POLYMORPHISMS OF EPOXIDE HYDROLASE GENES AND ISCHEMIC STROKE RISK IN TURKISH POPULATION

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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ABSTRACT

POLYMORPHISMS OF EPOXIDE HYDROLASE GENES AND ISCHEMIC STROKE RISK IN TURKISH POPULATION

Miçooğulları,Yağmur

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Stroke is characterized with loss of one or more functions of the body resulted by inadequate blood supply to the brain. Most of the cases result from a blood clot forms on an atherosclerotic plaque in the brain which is called as ischemic stroke. Structure of the arteries and vascular tone are listed in major determinants in the development of the disorder. Soluble epoxide hydrolase (sEH, *EPHX2*) catalyzes conversion of epoxyeicosatrienoic acids to inactive diol metabolites. EETs are potent vasodilators that participate in the regulation of vascular tone and cerebral blood flow. Microsomal epoxide hydrolase (mEH, *EPHX1*) is a critical phase I enzyme that catalyzes the conversion of various xenobiotic epoxide substrates and polycyclic aromatic hydrocarbons (PAHs). Animal studies show that tobacco smoke mutagens such as PAHs and heterocyclic amines directly increase the development of atherosclerotic lesions. The main purpose of this study is evaluation of effect of Arg287Gln single nucleotide polymorphism of *EPHX2* gene and Tyr113His and His139Arg single nucleotide polymorphisms of *EPHX1* gene as a risk factor for ischemic stroke in Turkish population.

Blood samples of 237 ischemic stroke patients and 120 controls were collected and all polymorphisms were determined by PCR-RFLP method. Mutant allele

frequencies in terms of Arg287Gln polymorphism of *EPHX2* gene (A) were found as 0.08 for patient group and 0.09 for controls. Tyr113His polymorphism of *EPHX1* gene (C) were found as 0.27 for patient group and 0.31 for controls when, His139Arg polymorphism of *EPHX1* gene (G) were 0.820 and 0.814 for patient and control groups, respectively. The differences between mutant allele frequencies of patients and controls were not found to be statistically significant.

Subgroup analysis was used to investigate the effects of conventional vascular factors according to the genotypes in the stroke susceptibility. Smoking, diabetes, obesity and hypertension were found to significantly increase the risk of having stroke. More detailed analysis on these factors with respect to genotypes showed that the risk of hypertensive individuals having ischemic stroke was higher in wild type homozygous genotype groups of Tyr113His (TT) and His139Arg (AA) polymorphisms and heterozygous and mutant homozygous genotypes of Arg287Gln (GA+AA) polymorphism than their counterparts (OR= 3.21, 3.15 and 4.69, respectively). Smoker people within the heterozygous and mutant homozygous genotypes group of Arg287His (GA+AA) polymorphism and wild type homozygous group of His139Arg (AA) polymorphism were found to be more susceptible to have stroke (OR= 11.81 and 4.78 respectively). Finally, diabetes mellitus was found to double the risk of having stroke regardless of the genetic background.

Logistic regression analyses were used to ascertain the effects of vascular factors, lipid parameters and genotypes in the stroke susceptibility. LDL-cholesterol (OR=1.46; 95%CI, 1.12-1.89, P=0.00), smoking (OR=3.46; 95%CI, 1.66-7.21, P=0.00) and hypertension (OR=3.19; 95%CI, 1.92-5.30, P=0.00) were found to be significant risk factors for ischemic stroke, whereas HDL (OR=0.27; 95%CI, 0.12-0.65, P=0.02) was found to be a protective factor in general population.

In this study, the relation of Tyr113His and His139Arg polymorphisms of *EPHX1* gene and risk of ischemic stroke is investigated for the first time in literature

while, Arg287Gln polymorphism and ischemic stroke risk in Turkish population was studied for the first time

Keywords: Ischemic stroke, single nucleotide polymorphism, epoxide hyrolase, Turkish population

ÖZ

EPOKSİD HİDROLAZ GEN POLİMORFİZMLERİ VE TÜRK POPULASYONUNDA İSKEMİK İNME RİSKİ

Miçooğulları, Yağmur

Yüksek Lisans, Biyoloji Bölümü

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Temmuz 2011, 144 sayfa

İnme beyine yetersiz kan akışından kaynaklanan bir ya da daha fazla vücut fonksiyonunun kaybıyla tanımlanan nörolojik bir hastalıktır. Çoğu vaka beyinde aterosiklerotik plaktan kaynaklanan kan pıhtılarının oluşmasıyla gelişir ve bu durum iskemik inme olarak adlandırılır. Damar yapısı ve vasküler tonus hastalığın gelişmesinde önemli rol oynayan faktörlerdir. Çözünebilir epoksid hidrolaz enzimi araşidonik asitten üretilen epoksiekosatrienoik asitlerin dönüşümünü gerçekleştirmektedir. Epoksiekosatrienoik asitler vasküler tonus ve serbral kan akışını düzenlemede etkili olan potansiyel vazodilatörlerdir. Mikrozomal epoksid hidrolaz enzimi ise faz I enzimi olup, çeşitli zenobiyotik epoksid substratlarının ve polisikik aromatik hidrokarbonların daha polar diol metabolitlere dönüşümünü gerçekleştirir. Hayvanlar üzerinde yapılan araştırmalar polisiklik aromatik hidrokarbon ve heterosiklik aminler içeren sigara dumanının aterosiklerotik lezyon oluşumunu doğrudan arttırdığını göstermiştir. Bu çalışmanın esas amacı Türk populasyonunda *EPHX2* geni üzerindeki Arg287Gln tek nükleotid polimorfizmi ile *EPHX1* geni üzerindeki Tyr113His ve His139Arg tek nükleotid polimorfizmlerinin iskemik inme üzerindeki etkilerinin araştırılmasıdır.

Çalışma grubu 237 iskemik inme hastası ve 120 kontrolden oluşturulmuştur ve bu grubun kanları toplanılarak PCR-RFLP tekniği ile genotip tayini yapılmıştır. Mutant alel frekansı istatistiksel olarak anlamlı olmamakla birlikte *EPHX2* geni üzerinde bulunan Arg287Gln polimorfizmi (A) için hastalarda 0.084, kontrollerde 0.092 olarak bulunmuştur. Mutant alel frekansları *EPHX1* geni üzerinde bulunan Tyr113His polimorfizmi (C) için hastalarda 0.274, kontrollerde 0.308, His139Arg polimorfizmi (G) için hastalarda 0.175 ve kontrollerde 0.192 olarak bulunmuştur.

Vasküler risk faktörlerinin etkisini araştırmak için yapılan alt grup analizlerinde sigara kullanımı, diyabet, obezite ve yüksek tansyonun istatistiksel olarak anlamlı bir biçimde inme riskini arttırdığı bulunmuştur. Daha detaylandırılmış istatistiksel analizler yüksek tansiyonun Tyr113His ve His139Arg polimorfizmleri yabanıl genotip gruplarında (sırasıyla TT ve AA) ve Arg287Gln polimorfizmi heterozigot ve mutant homozigot genotip grubunda (GA+AA) iskemik inme riskini arttırdığı sonucunu göstermiştir (Eşitsizlik oranı sırasıyla 3.21, 3.15 ve 4.69). Sigara kullanımına bağlı iskemik inme riskinde Arg287Gln (GA+AA) heterozigot ve mutant homozigot genotip grubunda 12 kata yakın, His139Arg risksiz genotip grubunda (AA) ise 5 kata yakın artış gözlenmiştir. Diyabetin genotip dağılımından bağımsız olarak iskemik inme riskini ikiye katladığı bulunmuştur.

Lojistik regresyon analizi ile vasküler faktörler, lipit parametreleri ve gentiplerin hastalık riski üzerine etkileri araştırılmıştır. Bu değerlendirmede çalışma grubunda düşük dansiteli lipoprotein kolesterolün, sigara kullanımının ve yüksek tansiyonun anlamlı risk faktörleri olduğu sonucuna varılırken, yüksek dansiteli lipoprotein kolesterolün hastalığa karşı koruyu etki gösterdiği tespit edilmiştir.

Bu çalışma sonucunda, *EPHX1* geni üzerindeki Tyr113His ve His139Arg tek nükleotid polimorfizmlerinin iskemik inme üzerindeki etkileri ilk defa araştırılmıştır ve literatürde buna benzer bir çalışmaya rastlanmamıştır. Aynı çalışma içerisinde, Türk populasyonunda *EPHX2* geni üzerindeki Arg287Gln tek

nükleotid polimorfizminin iskemik inme riski ile olan bağlantısı ilk defa incelenmiştir.

Anahtar Kelimeler: İskemik inme, tek nükleotid polimorfizmi, epoksid hidrolaz, Türk populasyonu.

To My Beloved Family...

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CI	Confidence Interval
CT	Computated Tomography
CAD	Coronary Artery Disease
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetra Acetic Acid
HDL	High Density Lipoprotein
LDL	Low Density Lipoprotein
mEH	Microsomal Epoxide Hydrolase
PCR	Polymerase Chain Reaction
RE	Restriction Endonuclease
OR	Odds Ratio
sEH	Soluble Epoxide Hydrolase
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SNP	Single Nucleotide Polymorphism
TIA	Transient Ischemic Attack

CHAPTER 1

INTRODUCTION

1.1 Stroke

Stroke or cerebrovascular accident is defined as sudden loss of brain function or functions due to shortage of the blood supply to the brain. This shortage may be caused by loss of blood which known as hemorrhagic stroke or blockage that disturbs blood flow to the brain that called as ischemic stroke. Consequently, brains cells destitute of nutrient and oxygen supplies are unable to function, leading to loss of one or more abilities.

Symptoms of a stroke depend on which part of the brain is affected. Abrupt numbness of the face, leg or arm on one side of the body is the most common symptom while loss of balance, coordination and walking, difficulty in speaking, understanding or seeing and unconsciousness are listed as other symptoms.

Globally, stroke is the second leading cause of death behind the cancer according to World Health Organization, 2011. It is reported that annually 15 million people suffer stroke; 33% of them die while others are left permanently disabled (http://www.who.int).

1.1.1 Ischemic Stroke

Ischemic stroke is caused by blockage of arteries which supplies blood in other words energy to the brain and accounts for almost 87% of all cases according to statistics of Stroke Association, USA, while rest of the cases classed as

hemorrhagic stroke. Obstruction within an artery is generally originated from disruption of atherosclerotic lesions which are asymmetrical focal thickenings of the intima composed of lipids, cells, debris and connective tissue elements (Figure 1.1). Three conditions are resulting in blockage;

1. Thrombosis, formation of a thrombus in other words blood clot within a blood vessel,
2. Embolism, blockage of the artery by immigrant thrombus,
3. Stenosis, abnormal narrowing of blood vessels

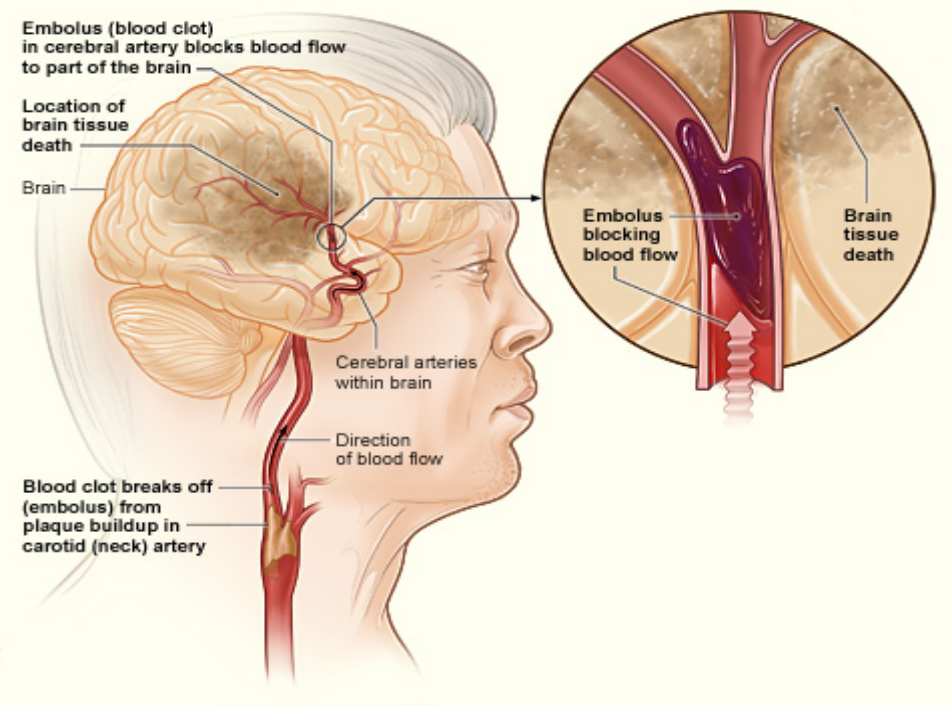

Figure 1.1 Development of ischemic stroke (taken from http://www.doctortipster.com).

1.1.1.1 Risk Factors for Ischemic Stroke

Ischemic stroke is a multifactorial complex neurological disorder and several risk factors have been associated with disease susceptibility. More than 300 risk factors have been associated with stroke. Major ones were established according to three criteria; an independent significant effect on the risk of stroke, reduction in the risk when it is treated or controlled and a high prevalence in many populations (http://www.americanheart.org, Table 1.1).

Table 1.1 Risk factors of ischemic stroke

Unalterable Risk Factors
•Genetic Background •Age •Gender •Prior Stroke, Heart Attack and Transient Ischemic Attack
Alterable Risk Factors
•High Blood Pressure •Diabetes Mellitus •Smoking •Obesity •Unabalanced Diet and Physical Inactivity • High Blood Cholestrol •Carotid or Other Artery Disease •Atrial Fibrillation •Sickle Cell Disease
Others
•Geographic Location •Socioeconomic Factors •Alcohol Abuse •Drug Abuse

1.1.1.1.1 Unalterable Risk Factors

Studies reveal that stroke is highly associated with genetic background, age, gender and prior stroke, heart attack and transient ischemic attack which are unchangeable factors (Stegmayr et al. 1997).

Genetic material determines development of the circulatory system and structure of the blood vessels as well as metabolism of a person. World Health Organization MONICA Project (Multinational MONItoring of Trends and Determinants in CArdiovascular Disease) related incidence and mortality of stroke and myocardial infarction to risk factors in eighteen populations in eleven countries over a 10-year period. Results showed that ethnic background explains variations in incidence of stroke and rate of mortality among populations. In addition, studies within the populations revealed existence of familial genetic factors (Stegmayr et al. 1997).

Age is one of the strongest determinants of the stroke. Although ischemic stroke might be seen in children, it is classified as a late onset disorder. It is estimated that probability of having stroke doubles for every ten years after age 55 (http://www.americanheart.org).

Gender is known to be one of the strongest determinants of the stroke as well. Results of the MONICA project was also analyzed in terms of subgroups and it was shown that women are less prone to having stroke than men (Stegmayr et al. 1997).

Prior stroke, heart attack and transient ischemic attack are accepted as unmodifiable risk factors of stroke. Transient ischemic attack (TIA) is redefined as “a brief episode of neurological dysfunction caused by focal brain or retinal ischemia, with clinical symptoms typically lasting less than one hour, and without evidence of acute infarction” instead of having a focal cerebral ischemic event with symptoms lasting less than 24 hours (Albers et al. 2002). Recent evidences

show that transient ischemic attack is generally followed by stroke; ten to fifteen percent of the TIA patients have a stroke within three months while half occurring within 48 hours (Easton et al. 2009). Having a prior heart attack or stroke story increases the incidence of stroke too.

1.1.1.1.2 Alterable Risk Factors

High blood pressure is one of the major factors in the development of stroke and stroke incidence is proportional to the level of blood pressure (Hebert et al. 1988). It is known that effective treatment of hypertension reduces the mortality rate of stroke.

Diabetes mellitus is a distinct risk factor for stroke. Multiple studies reveal that people with diabetes are at greater risk for stroke compared to ones without diabetes regardless of other risk factors. Many diabetes patients also suffer from high blood pressure, high blood cholesterol and obesity which increase likelihood of having stroke (http://www.diabetes.org).

Smoking is a well established risk factor for stroke development. It was reported that the risk of stroke is proportional to the number of cigarettes smoked. The relative risk of stroke in light smokers was half that of heavy smokers and lapsed smokers were found to develop stroke at the same level as nonsmokers (Wolf P.A. et al. 1988).

Body mass index (kg/m^2) was found to be associated with ischemic stroke risk as well. The association was found to be mediated by elevated blood cholesterol, hypertension and diabetes (Kurth et al. 2005, Song et al. 2004).

Studies have shown that life style is one of the determinants of stroke risk (Fung et al. 2009, Joshipura et al. 1999). Diet and physical activity affect level of blood

pressure, blood cholesterol and body mass index of a person (Renaud 2001). It is reported that a diet containing alpha-linolenic acid, fruit, vegetables and folic acid have a protective effect on stroke (Fung et al. 2009, Joshipura et al. 1999).

Possible associations between blood cholesterol level and stroke were investigated in several studies. It is known that high blood cholesterol level promotes plaque built up within the arteries so, storke risk is elevated. Lipoproteins are the carriers of the cholesterol in blood stream and there are two main types of lipoproteins: low-density lipoprotein (LDL) and high-density lipoprotein (HDL). Elevated total cholesterol level was found to be weakly associated with nonfatal ischemic stroke risk (Tirschwell et al. 2004, Wannamethee et al. 2000).

Artery diseases caused by plaque buildups within the arteries result in narrowing of the arteries and thus, the risk of blockage of the circulation by a blood clot raises. The carotid arteries supply blood to brain and narrowing of these vessels (carotid artery stenosis) increases the risk of stroke (Inzitari et al. 2000).

Atrial fibrillation, or arrhythmia is highly associated with cardiovascular abnormalities and is prevalent among persons older than 70 years. Investigation of the relation between atrial fibrillation and stroke was conducted in the content of Framingham Heart Study, a joint project of the National Heart, Lung and Blood Institute and Boston University which was carried on since 1948. It was found that the elderly are vulnerable to stroke when atrial fibrillation is present (Wolf P. A. et al. 1991b).

Sickle cell anemia is a genetic disorder which results in production of abnormal red blood cells. Structural abnormality of these cells causes them tend to aggregate and stick to blood vessels (Hart and Halperin 2001, Wolf P.A. et al. 1991a). Childhood stroke is generally caused by sickle cell disease.

1.1.1.1.3 Other Risk Factors

The incidence of stroke shows an alteration in terms of the geographic location, socioeconomic factors and alcohol and drug abuse. Although these factors were associated with stroke, further studies should be carried on.

1.1.2 Atherosclerosis

Atherosclerosis is described as hardening of an artery due to an atheromatous plaque. This chronic disorder remains asymptomatic for several years. Atherosclerotic lesions, in other words atheromata are asymmetric focal thickenings of the intima, the innermost layer of the artery.

Atheroma is preceded by accumulation of lipid-laden cells under the endothelium which is called as fatty streak (Figure 1.2). The core region of the atheroma is composed of foam cells which evolved from macrophages by accumulation of cholesterol and lipid droplets into the cell. Collagen rich matrix and smooth muscle cells enclose the fatty streak. Macrophages, T cells and mast cells are found around the core as a result of inflammatory response of the body and produce inflammatory cytokines.

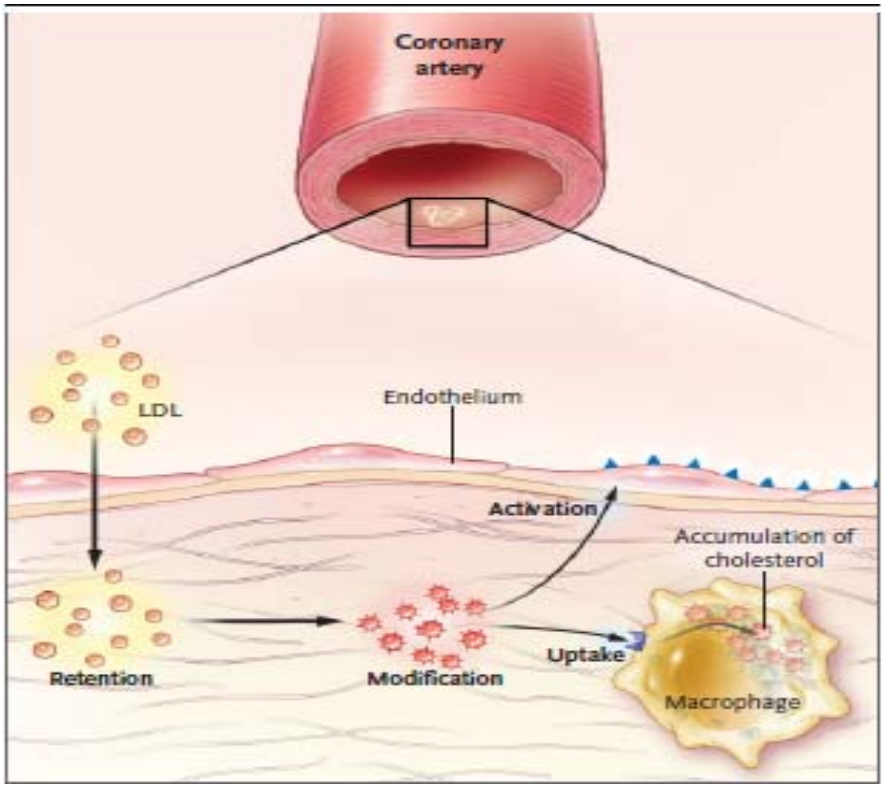

Figure 1.2 Atheromata formation (taken from Hansson, G.K. 2005).

Prevention of the blood flow through the carotid artery due to atheromatous process gives rise to development of ischemic stroke. Not only luminal narrowing caused by continued growth of the plaque, but also plaque rupture and/or endothelial erosion result in blockage of the artery. Plaque rupture is disruption of a fibrous cap by the activation of the immune cells followed by release of the debris into the blood. Released material form a thrombus and elicit acute coronary syndrome or ischemic stroke (Hansson, G.K. 2005).

It was reported that severe atherosclerotic plaques in aortic arch are the third leading cause of embolic stroke in 1990s (Amarenco et al. 1994, Jones et al.

1995). Recent data suggests that atherosclerotic plaques in aortic arch (14%-21%) ares more abundant in embolic stroke cases than carotid artery disease (10%-13%), while atrial fibrillation (18%-30%) is the major cause of the disorder (Kronzon and Tunick 2006, Ko et al. 2010).

1.1.3 Prevention of Stroke

Stroke is a complex neurological disorder and multiple factors play role in disease development. More than three hundred risk factors have been associated with this disorder and majority of them are related with life style. Consequently, it is possible to control stroke risk and to prevent recurrence.

Although several alternatives have been suggested for stroke prevention, three major strategies are followed. These are drug therapy (anticoagulants/antiplatelets, statin), carotid endarterectomy and angioplasty and/or stent implantation (http://www.stroke- association.org).

Use of statins, antiplatelets and anticoagulants and their preventive effects on stroke have been investigated for decades. Statins are 3-hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductase inhibitors with cholesterol lowering effect and are used to reduce incidence of stroke. Recently a meta-analysis involving more than 90,000 stroke patients was conducted to evaluate the relative risk reduction for stroke and it was found that statins reduce the risk by 21% (Amarenco et al. 2004). Antiplatelets are the drugs with platelet aggregation lowering effect and prevent thrombus formation while, anticoagulants are the substances with clotting preventive effect. Findings on use of aspirin (antiplatelet), extended-release dipyridamole (anticoagulant) and combination of both show that primary risk reduction is 13% with aspirin monotherapy, 15% with dipyridamole and 24% with the combination (Diener et al. 1996).

Recent review of The American Heart Association and American Stroke Association (AHA/ASA) Writing Committee for the Prevention of Stroke in Patients with Stroke and Transient Ischemic Attack (TIA) revisits outcome of the use of specific antiplatelet agents and statins for stroke prevention in patients. Results suggest that antiplatelet agent selection should be based on individual patient characteristics because of side effects and comorbid illnesses. Aspirin monotherapy, combination of aspirin with extended-release dipyridamole and clopidogrel monotherapy are suggested options for initial treatment of noncardioembolic ischemic stroke and transient ischemic attack, while they are still investigated in secondary prevention in acute ischemic stroke trials. On the other hand, statin usage for prevention of ischemic stroke in patients with coronary heart disease was questioned on the basis of the recurrent stroke prevention. It is reported that although a number of questions remains unanswered, use of statins with intensive lipid-lowering effect on patients with transient ischemic attack or atherosclerotic ischemic stroke and without coronary heart disease is recommended to reduce the risk of stroke (Adams R. J. et al. 2008).

Carotid endarterectomy (CEA) is a surgical operation that aims to revert narrowing of common carotid artery by removal of inside material. Successful operations for asymptomatic patients younger than 75 years old were found to lower the stroke risk from 12% to 6% and reduce ten years stroke risk (half this reduction benefit in disabling or fatal strokes) (Halliday et al. 2004, Halliday et al. 2010).

Angioplasty is widening of narrowed blood vessel with the help of a balloon catheter and stent implantation is insertion of an artificial tube into the natural passage in the body, including blood vessels. In spite of being feasible in 95% of patients with high risk of stroke and proposed to provide a long term prevention, angioplasty and stenting bring on procedural risk (risk of stroke is 5% to 10%

after implantation in 30 days) and restenosis (formation of thrombus into the stent or neointimal proliferation with subsequent endothelialization) (Fields et al. 2010).

1.2 Epoxide Hydrolase Enzymes

Epoxides are organic three membered oxygen compounds which arise from the oxidative metabolism of xenobiotic and endogenous compounds. Chemical and enzymatic oxidation processes for instance P450 monooxygenase system give rise to formation of epoxides which are chemically reactive and unstable in aqueous environment. Reactivity of the compound makes it a potential mutagenic and carcinogenic initiator therefore, levels of these compounds are strictly regulated (Adams J. D., Jr. et al. 1995, Sayer et al. 1985).

The epoxide hydrolases catalyze hydration of chemically active epoxides to their corresponding dihydrodiol products and classed as a sub-category in hydrolytic enzymes with proteases, esterases, lipases and dehalogenases (Beetham et al. 1995). Epoxide hydrolases are grouped into five in mammalian species with respect to their chemical and immunological nature; hepoxilin A_3 hydrolase, microsomal cholesterol 5,6-oxide hydrolase, leukotriene A_4 hydrolase, soluble and microsomal epoxide hydrolases (Seidegard and Ekstrom 1997, Fretland and Omiecinski 2000).

1.2.1 Soluble Epoxide Hydrolase

Human soluble epoxide hydrolase enzyme (sEH) coding gene *EPHX2* gene is localized to chromosomal region 8p21-p12 (Larsson et al. 1995) and consists of 19 exons encoding 555 amino acids (Sandberg and Meijer 1996).

Figure 1.3 represents three dimensional structure of the human soluble epoxide hydrolase enzyme that taken from protein data bank database. Crystal structure of the enzyme represents that soluble epoxide hydrolase is a homodimeric enzyme in which each monomer has an N-terminal domain with phosphatase activity and C-terminal domain with hydrolase activity. This data support the previous findings about structure-function model of the soluble epoxide hydrolase (Argiriadi et al. 1999).

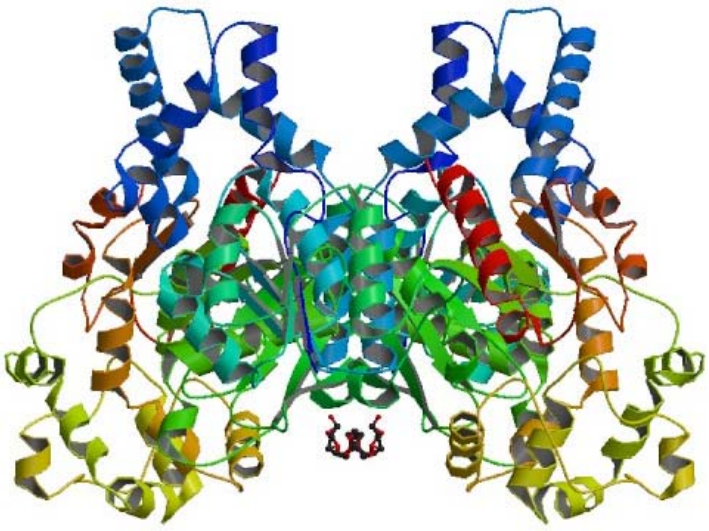

Figure 1.3 Crystal structure of human soluble epoxide hydrolase enzyme (taken from http://pdb.rcsb.org).

Oxidation of polyunsaturated fatty acids by microsomal P450 system yields epoxides which are excellent substrates for sEH (Zeldin 2001). One of these epoxides, epoxyeicosatrienoic acids (EETs) are lipid metabolites of arachidonic

acid, which are synthesized in vascular endothelial cells by the cytochrome P450 system (Capdevila et al. 1992, Zeldin 2001). EETs are potent vasodilators that participate in the regulation of vascular tone, cerebral blood flow, renal functions, cardiac function after ischemia, ionic transport, inflammation and pulmonary smooth muscle function (Alkayed et al. 1996, Node et al. 1999, Pascual et al. 1998, Rahman et al. 1997, Su et al. 1998, Wu et al. 1997). Hydrolysis of the EETs to their corresponding diols by epoxide hydrolases regulates EETs levels and represents a major mechanism by which the biological effects of EETs are attenuated (Figure 1.4). Consequently, they were suspected to play a role in predisposition to and/or recovery from cerebrovascular injury (Zhang L. et al. 2008a). Protective effect of the sEH inhibition against ischemic injury by non-vascular mechanisms was confirmed on rodent models (Zhang W. et al. 2008b, Zhang W. et al. 2007).

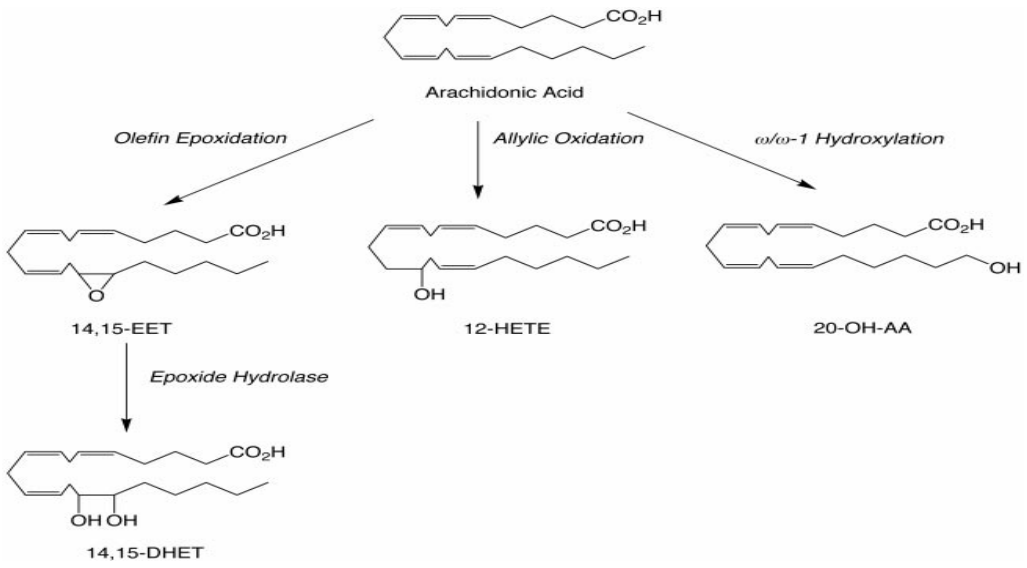

Figure 1.4 Endogenous metabolism of arachidonic acid by cytochrome P-450 and soluble epoxide hydrolase (Sinal et al. 2000).

1.2.2 Microsomal Epoxide Hydrolase

Human microsomal epoxide hydrolase enzyme (mEH) coding gene *EPHX1* is localized to chromosomal region 1q42.1 and consists of 12 exons encoding 455 amino acids (Hassett et al. 1994).

Microsomal epoxide hydrolase (mEH) is a crucial phase I enzyme that catalyzes the conversion of various xenobiotic epoxides and polycyclic aromatic hydrocarbons (PAHs) to more polar diol metabolites. Hydrolysis of epoxides through the action of *EPHX1* enzyme gives rise to detoxification after conjugation by phase II enzymes. In contrast, some epoxides that converted to diol metabolites are used by phase I; cytochrome P450s and reaction of diol metabolites by cytochrome P450s give rise to formation of carcinogenic and toxic compounds. Consequently, it is documented that microsomal epoxide hydrolase plays dual role in both detoxification and activation of epoxides (Oesch 1973).

Polycyclic aromatic hydrocarbons (PAHs) are a group of molecules composed of three or more fused aromatic rings. This group of molecules includes both toxic and non-toxic members. Benzo(a)pyrene is the firstly discovered extremely toxic PAH which is highly carcinogenic and found in cigarette smoke. Six other PAHs namely chrysene, benzo[k]fluoranthene, benzo[b]fluoranthene, benz[a]anthracene, dibenz(a,h)anthracene and indeno(1,2,3-cd)pyrene are classed with benzo(a)pyrene as highly carcinogenic compounds by EPA (Environmental Protection Agency of United States) and more are listed as mutagens (http://www.epa.gov/).

Ability of microsomal epoxide hydrolase to hydrolyse arene, alkene and aliphatic epoxides which are derivatives of polycyclic aromatic hydrocarbons (PAHs) and aromatic amines makes mEH a novel target against effect of carcinogens and mutagens (Figure 1.5). Studies suggest that mutagens found in tobacco smoke stimulate the formation of DNA adducts, which lead to genetic alterations in a

variety of organs and gives rise to development of cancer, chronic obstructive pulmonary disorder, myocardial infarction, atherosclerosis and many other disorders (Chappell et al. 2008, Murry et al. 1997, Ross et al. 2001, Varkonyi et al. 2006). Animal studies show that tobacco smoke mutagens such as PAHs and heterocyclic amines directly increase the development of atherosclerotic lesions (Izzotti et al. 1995, Zhang Y. J. et al. 1998). Consequently, role of mEH in promotion of atherosclerosis may increase the risk of ischemic stroke which should be investigated.

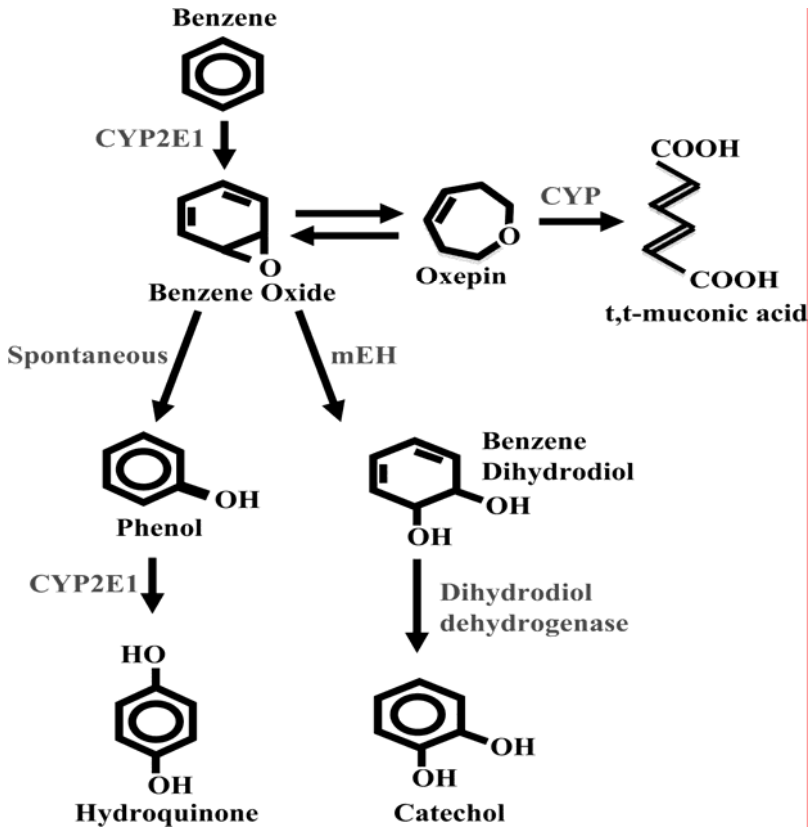

Figure 1.5 Simplified schematic representation of benzene metabolism (Badham et al. 2010)

1.3 Genetic Polymorphism

Genetic polymorphism is defined as a difference in DNA sequence among individuals of a population in other words existence of more than one phenotype within a species. These differences include insertions, deletions, inversions, sequence repeats and single nucleotide polymorphisms. Such alterations in genetic material may arise by chance, evolutionary forces or environmental factors. This term is limited to be used in variations present in more than 1% of the population (Ford 1966).

1.3.1 Single Nucleotide Polymorphism

Single nucleotide polymorphism means substitution of a single base pair in DNA with at least 1% frequency in population. According to cross reference between Celera Genomics and HapMap Project of National Center of Biotechnology Information (NCBI) single nucleotide polymorphism databases, human genome constains one million SNPs which are highly heterozygous with high validation rates. On the other hand, publication of The International SNP Map Working Group of NCBI reveals that there are almost twice as much SNPs within human genome than ones overlapping with findings of Celera Genomics (Sachidanandam et al. 2001).

Single nucleotide polymorphism is the most abundant genetic alteration in human genomic DNA. Discovery of large numbers of SNPs throughout the genome and improvements in genome wide scanning technology gave rise to research on importance of SNPs in components of biological pathways. Recent developments in gene analysis technology can now achieve high throughput assessment of SNPs in genomic DNA and several gene variants have been associated with disease risk or therapeutic effect. High throughput assessment of SNPs in genomic DNA is

used in the construction of human SNP maps and enable scientists to assess the prognostic and predictive power of a small number of candidate SNPs in large clinical trials (McCarthy and Hilfiker 2000).

1.3.2 Polymorphisms of Human Soluble Epoxide Hydrolase *(EPHX2)* Gene

Human soluble epoxide hydrolase enzyme (sEH) includes 44 SNPs; 31 of them are in intronic region while, 15 of them are in exonic ones. Six of the 15 exonic SNPs result in amino acid substitutions, whereas nine are silent (Figure 1.6).

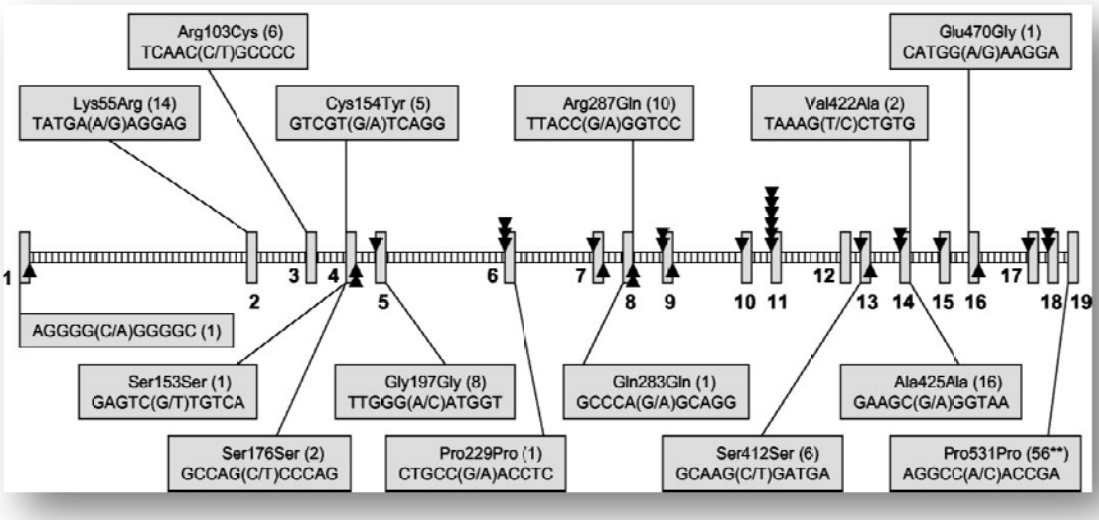

Figure 1.6 Single nucleotide polymorphisms on *EPHX2* gene (taken from Przybyla-Zawislak et al. 2003).

Evaluation of effect of amino acid substitutions on enzyme activity *in vitro* were performed by Przybyla-Zawislak et al.,2003 by using 14,15-EET as substrate. Result revealed that amino acid substitutions at Arg103Cys, Cys154Tyr and Arg287Gln result in significant changes in sEH activity *in vitro*. Arg287Gln polymorphism was the one of the mutations that cause reduce in enzyme activity which is 25-75% (Przybyla-Zawislak et al. 2003).

Figure 1.7 illustrates the structure of wild type enzyme and Figure 1.8 shows the changed confirmation resulted by amino acid substitution. These images were generated by using web tools of SwissProt database. Figure 1.9 represents the human soluble epoxide hydrolase gene in three dimensional structure which 287th amino acid of the enzyme is labelled with red bu using Chimera® web tool.

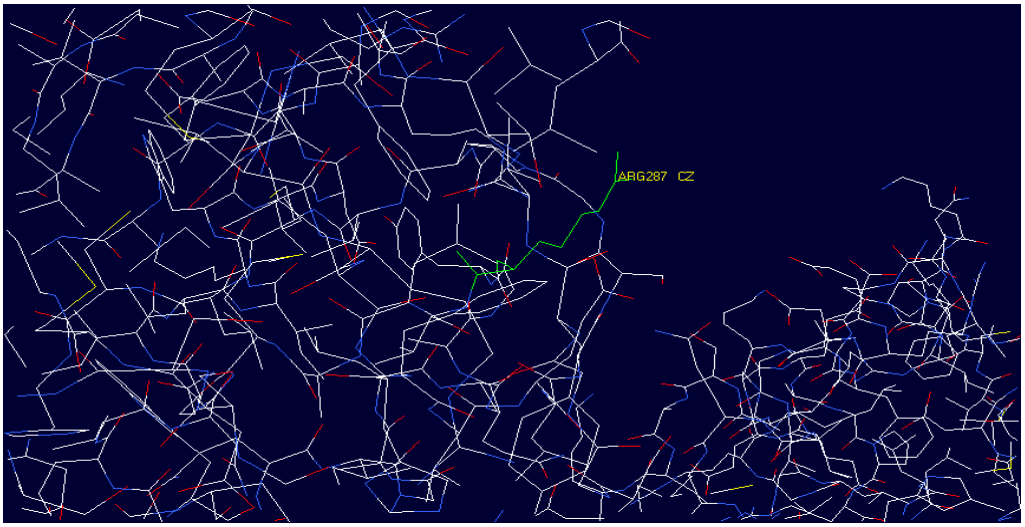

Figure 1.7 Three dimensional structure of wild type human soluble epoxide hydrolase, 287th amino acid (Arg) is labeled with green. This image was generated in our lab by using web tools of SwissProt database.

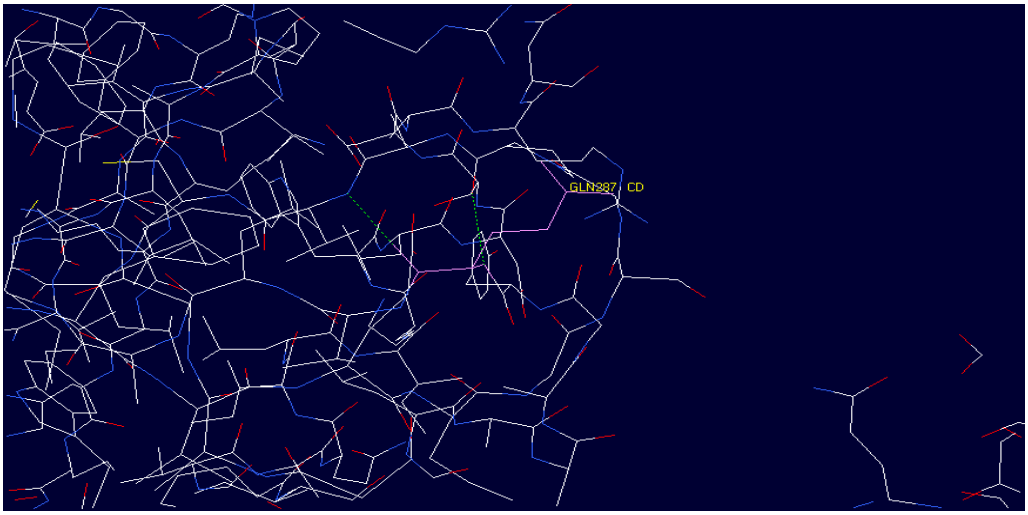

Figure 1.8 Three dimensional structure of mutated human soluble epoxide hydrolase, 287th amino acid (Gln) is labeled with purple. This image was generated in our lab by using web tools of SwissProt database.

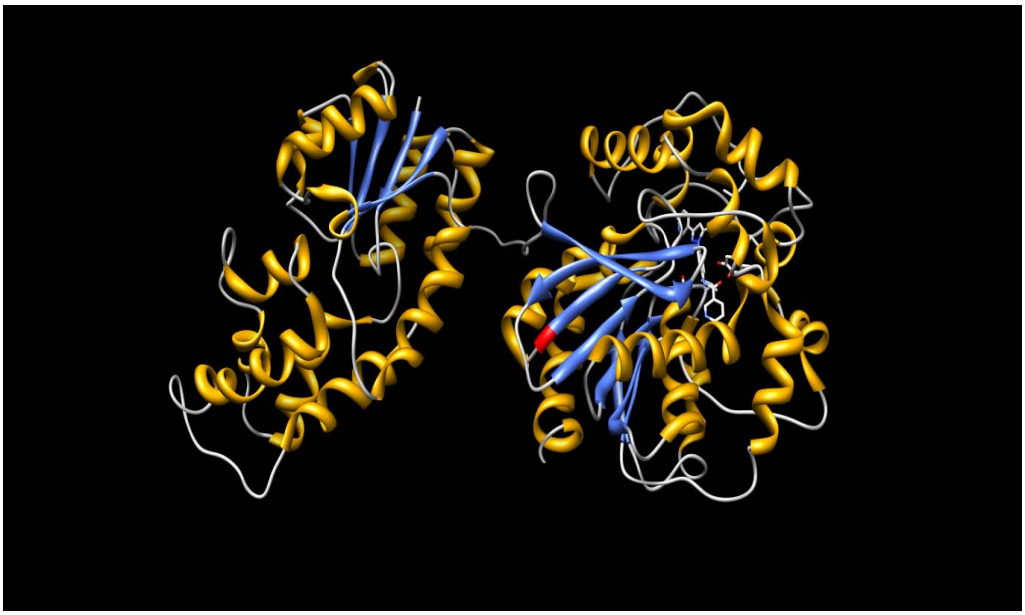

Figure 1.9 Three dimensional structure of human soluble epoxide hydrolase enzyme, 287th amino acid is labeled with red.

1.3.3 Polymorphisms of Human Microsomal Epoxide Hydrolase *(EPHX1)* Gene

Human microsomal epoxide hydrolase enzyme (mEH) includes 98 SNPs; 78 of them are in intronic region while, 20 of them are in exonic ones. Six of the 17 exonic SNPs result in amino acid substitutions, whereas the others are silent.

Enzymatic analyses to evaluate the phenotypic effect of amino acid substitutions were carried on by Hassett et al., 1994 by employing benzo[a]pyrene-4,5-oxide and *cis*-stilbene oxide as substrates. Results obtained using purified enzyme with amino acid substitutions at Tyr113His that takes place in 3rd exon revealed 40-50% reduction in enzyme activity. On the other hand, the other critical amino acid substitution at His139Arg, which resuts from a SNP in the 4th exon, results in 25% increase in enzyme activity (Hasset et al., 1994).

1.4 The Aim of the Study

Stroke is caused by abrupt blockage of the arteries. Most of the cases result from a blood clot that forms on an atherosclerotic plaque in an artery leading to the brain so, both elements that take part in development and progression of the atheromata and factors playing role in plaque rupture are worth to investigate.

Human soluble epoxide hydrolase enzyme is responsible for conversion of epoxides to less reactive diol compounds. Epoxyeicosatrienoic acids (EETs) are lipid metabolites of arachidonic acid and converted to dihydroxyeicosatrienoic acids (DiHETs) by soluble epoxide hydrolase. The roles of EETs in cardiovascular functions as a potent vasodilator which produced by vascular endothelium, a hyperpolarizing factor with signaling property and a regulator of blood flow in vascular beds including coronary and renal circulations stress the importance of EET levels in regulation of cerebral blood flow.

Although studies on direct administration of EETs as a therapeutic agent found them to be protective against cardiac and cerebral ischemia, practical use of these compounds on human is limited due to reduced availability of EETs, because of high endogenous sEH activity *in vivo*. Alternatively, inhibition of sEH activity was suggested to promote accumulation of endogenous EETs and it was shown that reduction in sEH activity is a promising therapeutic target for both treatment and prevention of ischemic stroke (Zhang L. et al. 2008a).

Previous research on association of sEH variants and ischemic stroke risk overlaps with the proposed effect on sEH activity on EET bioavailability. It was reported that gain of function variants of human sEH such as ones with Lys55Arg, Cys154Tyr and Glu470Gly substitutions had an increased sEH activity. As a result, they were found to increase the risk of ischemic stroke as well. On the contrary, loss of function variant of the enzyme, the one with Arg287Gln substitution had a decreased enzyme activity. Thus, it was found to reduce the risk of ischemic stroke (Przybyla-Zawislak et al. 2003). Protective effect of human sEH Arg287Gln variant from ischemic injury was confirmed with work of Koerner et al. in rat model (Koerner et al. 2007).

Microsomal epoxide hydrolase is one of the key enzymes in metabolism of carcinogenic and mutagenic compounds in tobacco smoke and variations in its activity have been associated with development atherosclerotic lesions caused by such compounds. Studies suggest that mutagens found in tobacco smoke stimulate the formation of DNA adducts, which lead to genetic alterations in blood vessels (De Flora et al. 1997). Animal studies show that tobacco smoke mutagens such as PAHs and heterocyclic amines directly increase the development of atherosclerotic lesions. Results suggested that not only being a smoker but also exposure to the environmental tobacco smoke affects platelet functions and promotes aortic and pulmonary atherosclerotic lesions (Zhu et al. 1993). Two of the SNPs in *EPHX1* gene result in critical amino acid substitutions; Tyr113His

causes reduction in enzyme activity, while His139Arg results in increase in enzyme activity (Hassett et al., 1994). Ability of microsomal epoxide hydrolase to convert mutagens to less reactive species makes mEH a novel target against effect of these hazardous chemical compounds in development of ischemic stroke.

The aim of this study was to investigate frequency of Arg287Gln polymorphism of *EPHX2* gene and Tyr113His and His139Arg polymorphisms of *EPHX1* gene and their association with the risk of ischemic stroke in Turkish population. It is well established that frequencies of mentioned polymorphisms vary among populations with different ethnicity. Therefore, investigation of Arg287Gln polymorphism of *EPHX2* gene and Tyr113His and His139Arg polymorphisms of *EPHX1* gene in different populations come into prominence.

Eventhough association between Arg287Gln polymorphism of *EPHX2* gene and ischemic stroke risk in various populations were carried out, evaluation of this polymorphism in Turkish Population with respect to stroke risk was conducted for the first time. In addition, the relation of Tyr113His and His139Arg polymorphisms of *EPHX1* gene and risk of ischemic stroke which have not been studied in any population before was investigated in the scope of this study. The listed steps were designed to achieve the goal:

- Acquirement of blood samples of ischemic stroke patients and healthy controls,
- Isolation of genomic DNA of the subjects in an intact form,
- Amplification of interested gene regions with polymerase chain reaction,
- Restriction of amplified products with suitable endonuclease to assess genotype of patients for SNPs of interest,
- Determination of genotype and allele frequencies of Arg287Gln polymorphism of *EPHX2* gene and Tyr113His and His139Arg polymorphisms of *EPHX1* gene in Turkish population,

- Evaluation of possible associations between allele and genotype frequencies with ischemic stroke risk in Turkish population via comparison of risky and control groups,
- Comparison of the results of this study with the others conducted on different ethnic groups, if any.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Population and Blood Sampling

This study was approved by Ethical Committee of Gülhane Military Medical Academy and carried out according to the principles of Declaration of Helsinki (APPENDIX A). All participating individuals were informed about the study and then informed consent forms (APPENDIX B and C) were signed by them.

Blood samples of ischemic stroke patients and controls who were unrelated, Caucasian people were collected from Central Anatolia, Turkey with the collaboration of Gülhane Military Medical Academy, Department of Neurology, Ankara, from October 2005 to April 2011. Ischemic stroke cases were diagnosed with neurological examination followed by computer tomography (CT) scan, transthoracic echocardiographic examination, Holter study and Transcranial Doppler emboli detection. Selection criteria for patient group were: being admitted to the neurology services of Gülhane Military Medical Academy within 24 h after onset, having anterior circulation stroke without other major illnesses such as autoimmune diseases, hepatic or renal failure, no known embolic source, no family history of hematological, autoimmune or chronic inflammatory diseases, no history of myocardial infarction within three weeks or of transient ischemic attack. Control group met all the criteria applied to the patient group, plus not having carotid stenosis, lumen narrowing, more than 50% or ulcerated carotid plaque. All subjects underwent bilateral carotid Doppler ultrasound

(CUSG) and transthoracic echocardiographic studies. The details of inclusion and exclusion criteria were as described before (Can Demirdöğen et al., 2008, Can Demirdöğen et al., 2009, Türkanoğlu et al., 2010).

Detailed medical records of patients and controls were collected by our collaborators from Gülhane Military Medical Academy, Department of Neurology. All participants were subjected to routine laboratory tests such as complete blood count, leukocyte differential, erythrocyte sedimentation rate, electrocardiogram, chest x-ray, biochemical laboratory tests such as lipid profile (LDL, VLDL-C, HDL, total cholesterol and triglycerides), fasting glucose, bilirubin, creatinine, sodium and potassium, routine urine tests, liver function tests and rheumatologic screening tests. Hypertension was assessed when systolic blood pressure was higher than 140 mm Hg and/or diastolic blood pressure was higher than 90 mm Hg and/or anti-hypertensive was used. Diabetes was assessed when fasting glucose level was higher or equal to 6.99 mmol/L and/or anti-diabetic drug was used. Obesity was assessed when body mass index of an individual was higher or equal to 30. Smoking status was evaluated as "yes", if individual was a current smoker or quitted smoking less than three months ago. These evaluations and all laboratory studies were performed blinded to medical conditions of the subjects.

2.1.2 Chemicals

Agarose (A5093), bromophenol blue (B8026), trizma base (T1503), ethidium bromide (E1510), triton X-100 (T1503), sodium dodecyl sulfate (SDS, for molecular biology, L4390) and sodium chloride (for molecular biology, S3014) were supplied from Sigma- Aldrich, Germany. Ethylenediaminetetraacetic acid **(**EDTA, A5097) and potassium chloride (molecular grade, A2939) were obtained from AppliChem GmbH, Germany; while magnesium chloride (molecular grade,

Art5833) and boric acid (molecular grade, A949265) were purchased from Merck KgaA, Germany. Ethanol (absolute, 32221) was supplied from Riedel de Haën, Honeywell International Inc., Germany.

dNTP mixture (R0192), 50 bp GeneRuler^{TM} DNA ladder (SM0371), restriction endonuclease enzymes namely *MspI* (*HpaII*, ER0541), *RsaI* (ER1121) and *PsyI* (*Tth111I*, ER1331) with suitable buffers and recombinant Taq Polymerase enzyme, supplied with amplification buffers and $MgCl_2$ (EP0402), were obtained from MBI Fermentas, USA.

2.1.3 Primers

Three pairs of the primers were purchased from Iontek (Iontek, İstanbul, Turkey) to amplify the gene fragments containing the single nucleotide polymorphisms of interest. The primer sequences of Arg287Gln polymorphism within *EPHX2* gene were designed in our lab by using Primer 3 0.4.0 web tool offered by Massachussetts Institute of Technology, USA (http://frodo.wi.mit.edu/primer3/) and PrimerBlast web tool, offered by National Center for Biotechnology Information, National Institute of Health, USA web page (http://www.ncbi.nlm.nih.gov /tools/primer-blast/). The primer pairs for Tyr113His and His139Arg polymorphisms of *EPHX1* gene were already described by Korhonen et al., 2003 and Smith and Harrison, 1997, respectively. Sequences of primer pairs are listed below.

Specific primer pair which was designed by us for analysis of Arg287Gln polymorphism was

F AGGAGGGTGACTCCAGACCT

R CCTTGGAGCATGAGCCTTAG

Specific primer pair for analysis of Tyr113His polymorphism was

F GGGGTCCTGAATTTTGCTCC

R CAATCTTAGTCTTGAAGTGACGGT

Specific primer pair for analysis of His139Arg polymorphism was

F ACATCCACTTCATCCACGT

R ATGCCTCTGAGAAGCCAT

2.2 Methods

2.2.1 Preparation of Human Genomic DNA Samples for Genotype Analysis

2.2.1.1 Human Genomic DNA Isolation from Human Whole Blood Samples

Principle

Whole blood samples of participants were collected into EDTA containing tubes to prevent blood clots by our collaborators at Gülhane Military Medical Academy. Salting-out DNA isolation protocol described by Lahiri and Schanabel, 1993, was preferred over the traditional DNA isolation protocols to eliminate harmful effects of isopropyl alcohol and chloroform on human health (Lahiri and Schanabel, 1993). Reagents used for human genomic DNA isolation from human whole blood samples were given in Appendix D.

Procedure

750 µL of total blood was added onto 750 µL of TKM buffer, pH 7.6 in micro centrifuge tube. 20 µL of Triton X-100 detergent was added to tube and solution is mixed with several inversions in order to get cellular components out of the cells. Centrifugation was done to separate cellular components in different layers by using Sigma 1-15 benchtop microcentrifuge (Sigma, Postfach 1713- D-37507, Osterode) for ten minutes. Two phases were observed after the centrifugation; DNA containing pellet and supernatant with eligible content. Two more washing with TKM buffer was done to eliminate other materials than DNA from the pellet. This step was followed by dissolution of pellet in 200 µL of TKM buffer, pH 7.6 and addition of 10 µL of 10% sodium dodecyl sulfate (SDS). Suspension was properly mixed and incubated at 58ºC for ten minutes. Addition of 75 µL cold saturated NaCl onto the suspension resulted in precipitation of proteins and centrifugation was done at 14,000 x g for ten minutes at 4ºC. DNA containing supernatant was taken into a clean micro centrifuge tube and 2X volume ice-cold absolute ethanol was added to precipitate DNA. Tube was inverted for several times and kept at -20ºC for at least thirty minutes. Centrifugation was done to obtain the entire DNA in the solution at 10,000 x g for ten minutes at 4ºC. Pellets were dried until ethanol was completely removed and dissolved in 100 µL of TE buffer, pH 8.0. Tubes were incubated at 37ºC for at least two hours.

2.2.1.2 Quantification of Human Genomic DNA Samples by Spectrometry

DNA concentration was determined by using NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, USA). Integrated software was used to output absorbance values and to calculate DNA concentration.

Theoretically, absorbance value of 50 ng genomic DNA in 1 µL solution corresponds to 1.0 at 260 nm. Then, concentration of DNA was determined with the Beer- Lambert Law stated below.

$$\text{Concentration (ng/µL)} = A_{260nm} \times 50 \text{ (ng/µL)}$$

2.2.1.3 Qualification of Human Genomic DNA Samples by Spectrometry

Purity of the nucleic acids was determined by measurements of the absorbance at 280 nm which proteins give maximum absorbance and at 260 nm which DNA gives maximum absorbance. The ratio of absorptions at 260 nm vs. 280 nm was used to assess protein and RNA contamination in DNA sample. $A_{260/280}$ is 1.8 for pure DNA samples and deviation indicates impurity, the higher and lower values show either RNA or protein contaminations, respectively.

2.2.1.4 Qualification of Human Genomic DNA Samples by Agarose Gel Electrophoresis

Quality of DNA samples was also determined by agarose gel electrophoresis to evaluate intactness. 0.5% agarose gel electrophoresis was performed by using Scie-Plas HU13W horizontal gel electrophoresis device. Reagents used for qualification of human genomic DNA samples by agarose gel electrophoresis were given in Appendix D.

Procedure

0.5 % (w/v) agarose gel was prepared by dissolving 1 g agarose powder into 200 mL 0.5X TBE buffer, pH 8.3 (stock solution was ten times diluted to achieve 1 mM EDTA and 45 mM Tris-borate). Suspension was heated in microwave oven until agarose powder completely melts and seems colorless.

The solution was cooled on a magnetic stirrer with continuous stirring to get a homogenous mixture. 10 µL of ethidium bromide (10 mg/mL) was added into solution when it cools approximately to 60ºC and stirred.

The warm gel solution was poured into gel tray on a flat surface which was enclosed by stoppers and combs were placed approximately 1 cm and 7 cm above the top of the gel tray. Air bubbles were removed carefully. Gel was allowed to harden almost for half an hour at room temperature and stoppers and combs were removed.

The gel tray was placed into the gel tank which was filled with 0.5X TBE buffer until gel was completely sunk. 5 µL of each DNA sample was mixed with 1 µL of bromophenol blue on a clean parafilm piece with the help of micropipette to prevent floating of the DNA into the buffer and to give color. Mixtures were loaded into the wells and lid of the tank was placed.

Gel tank is surrounded by electric wires to provide an electrical current to force negatively charged DNA to move towards positive pole, anode. Consequently, DNA containing wells heads towards the negative pole while cathode is at the opposite site. Wires on the gel tank were placed on power supply and 120 volts was given to the system. Gel was run for almost half an hour until the bromophenol blue reached at the bottom of the gel.

Picture of the gel was taken by using Bio-Capture (Version 99.03) integrated to Vilber Lourmat Gel Imaging System (Marre La Vallee, Cedex, France). First gel

was placed under visible light and then exposed to UV light to excite ethidium bromide. Ethidium bromide is an intercalating agent that interferes into the double helix and reflects the light only under this condition. Pure genomic DNA isolates were identified with the existence of single band while RNA contamination resulted in second band and degraded DNA was seen as a smear.

2.2.2 Genotyping for Arg287Gln Polymorphism of *EPHX2* Gene, Tyr113His and His139Arg Polymorphisms of *EPHX1* Gene

Genotyping of all polymorphisms were performed by taking guidelines of standard restriction fragment length polymorphism detection method. In this procedure, polymerase chain reaction is followed by restriction enzyme digestion. SNP involving genes, polymorphisms, regions of amplification, PCR product sizes and restriction endonucleases used for genotyping of polymorphisms in this study were given in Table 2.1.

Table 2.1 Genes of interest, polymorphisms, regions of amplification, PCR product sizes and restriction endonucleases used for genotyping of Arg287Gln polymorphism of *EPHX2* gene, Tyr113His and His139Arg polymorphisms of *EPHX1* gene.

Gene	Polymorphism	Region of amplification	PCR Product Size	Restriction Endonuclease
EPHX2	Arg287Gln	Coding Region	676 bp	*MspI*
EPHX1	Tyr113His	Coding Region	198 bp	*PsyI (Tth111)*
EPHX1	His139Arg	Coding Region	210 bp	*RsaI*

Techne TC-4000 thermal cycler (Techne Ltd., Duxford, Cambridge) was used to carry out PCR while Biosan TDB-120 Heat Block (Biosan Ltd., Latvia) was used to incubate the samples during the digestion.

2.2.2.1 Tyr113His Single Nucleotide Polymorphism

2.2.2.1.1 Polymerase Chain Reaction for Tyr113His SNP

Tyr113His single nucleotide polymorphism is involved in the coding region of *EPHX1* gene (exon 3). Gene fragment that involved Tyr113His was amplified using specific primer pair.

Reagents

1. Taq DNA Polymerase (10 U/µL)
2. PCR Amplification Buffer with KCl* (100 mM Tris-HCl, 500 mM KCl, 0.8% Nonidet P40; pH 8.8 at 25ºC)
3. $MgCl_2$ Solution* (25 mM)
4. dNTP Mixture (10 mM of each nucleotide)
5. Forward and Reverse Primers (10 pmol/ µL)

   *Supplied with Taq DNA polymerase enzyme

Procedure

Standard polymerase chain reaction protocol was optimized to gather a single specific product which involves target SNP. Twenty four conditions with different Taq polymerase concentrations (2.5 U, 2 U and 1.5 U), $MgCl_2$ concentrations (2.5 mM, 2.25 mM, 2 mM, 1.5 mM and 1.25 mM) and primer concentrations (20

pmol, 30 pmol and 40 pmol) were tested to get the best result. Optimized PCR mixture content is listed in Table 2.2.

Table 2.2 PCR mixture components for Tyr113His polymorphism.

Constituent	**Stock Concentration**	**Volume Added**	**Final Concentration**
Amplification Buffer with KCl	10X	5 µL	1X
$MgCl_2$	25 mM	2.5 µL	1.25 mM
dNTP Mixture	10 mM	1 µL	200 µM
Forward Primer	10 pmol/µL	4 µL	0.8 pmol/µL
Reverse Primer	10 pmol/µL	4 µL	0.8 pmol/µL
Taq DNA Polymerase	5 U/µL	0.5 µL	2.5 U
Template DNA	varies	varies	~200 ng
Ultra Pure H_2O		Up to 50 µL	

The optimized thermal cycling program to amplify Tyr113His single nucleotide polymorphism is given in Table 2.3.

Table 2.3 Thermal cycling conditions for Tyr113His polymorphism.

	Temperature	Time	
Initial Denaturation	95ºC	5 min	
Denaturation	95ºC	1 min	35 Cycles
Annealing	55ºC	1 min	
Extension	72ºC	1 min	

Amplified region of *EPHX1* gene including Tyr113His single nucleotide polymorphism is represented in Figure 2.1 with forward and reverse primers, recognition site of restriction endonuclease and single nucleotide substitution.

2% agarose gel was used to analyze the length of PCR products. 10 μL PCR product was mixed with 3 μL bromophenol blue and loaded to gel while DNA ladder was also loaded to determine length of the products. Gel was run for almost 30 min at 120 V.

Figure 2.1 Schematic representation of the amplified region of *EPHX1* gene containing the Tyr113His single nucleotide polymorphism. Forward and reverse primers are highlighted with yellow while restriction endonuclease recognition sites are with purple and single nucleotide polymorphism with pink. (http://www.ncbi.nlm.nih.gov)

2.1.1.2 Restriction Endonuclease Digestion for Tyr113His SNP

Genotyping in terms of Tyr113His single nucleotide polymorphism was done by restriction fragment length polymorphism technique. Each restriction endonuclease has a specific recognition pattern which is usually a hexamer or a tetramer. Any change in the specific recognition pattern ends up with inability of enzyme to cut the DNA. Nucleotide substitutions which may result in single nucleotide polymorphisms give rise to this event as well. Consequently, it is possible to determine genotypes of a population with this technique.

Wild type individuals for Tyr113His SNP have no recognition site for *PsyI (Tth111)* enzyme within the vicinity of the SNP, while base substitution (T/C) results in formation of the recognition site. This event can be detected by looking at the lengths of the DNA fragments after the digestion. In the wild type allele *PsyI* digestion yields 198 bp long single band while 175 bp and 23 bp long two bands indicates base substitution. Finally, individuals with three bands are heterozygous for Tyr113His polymorphism (Figure 2.1).

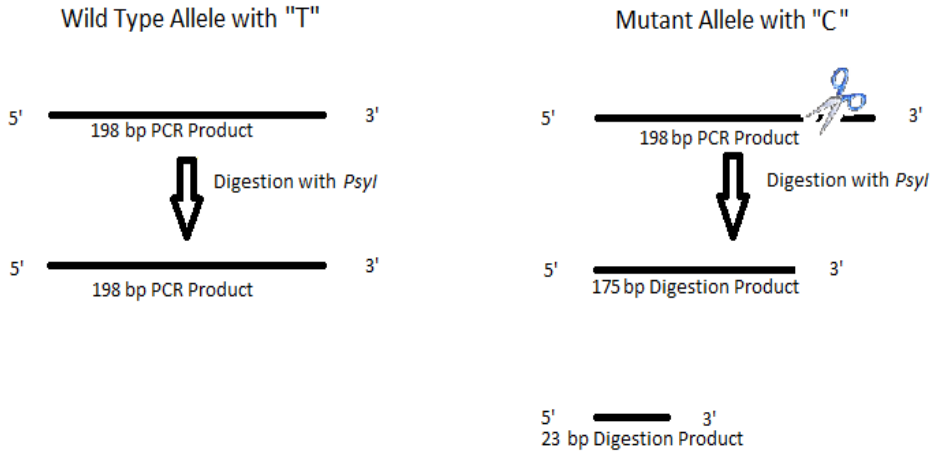

Figure 2.2 Representation of the banding patterns resulted by *PsyI* endonuclease digestion of the Tyr113His SNP containing PCR product for wild type and mutant allele.

Reagents

1. *PsyI (Tth111)* restriction enzyme
2. Buffer B* (10 mM Tris-HCl (pH 7.5 at 37°C), 10 mM $MgCl_2$ and 0.1 mg/ml BSA)

*Supplied with *PsyI (Tth111)* restriction enzyme

Procedure

Standard digestion protocol offered by the producer was modified to get clearer image on gel. Four conditions with different restriction enzyme concentrations (10

U, 7 U, 5 U and 3 U) were tested to get the best result. Optimized mixture content, is listed in Table 2.4.

Table 2.4 Restriction endonuclease digestion mixture components for Tyr113His polymorphism.

Constituent	Stock Concentration	Volume	Final Concentration
PCR Product		20 µL	
Buffer B	10X	3 µL	1X
PsyI	10 U/µL	0.3 µL	3 U
Ultra Pure dH_2O		6.7 µL	
Final Volume		30 µL	

20 µL of PCR product was incubated with 3U of *PsyI* at 37ºC for 18 hours. At the end of incubation, 3% agarose gel was used to analyze the length of digestion products. 20 µL of digestion product was mixed with 4 µL loading dye and loaded to gel, while DNA ladder was also loaded to determine length of the products. Gel was run for almost 30 min at 130 V.

2.2.2.2His139Arg Single Nucleotide Polymorphism

2.2.2.2.1 Polymerase Chain Reaction for His139Arg SNP

His139Arg single nucleotide polymorphism is found in the coding region of *EPHX1* gene (exon 4). Gene fragment that involved His139Arg was amplified using specific primer pair.

Reagents

1. Taq DNA Polymerase (10 U/µL)
2. PCR Amplification Buffer with KCl* (100 mM Tris-HCl, 500 mM KCl, 0.8% Nonidet P40; pH 8.8 at 25ºC)
3. $MgCl_2$ Solution* (25 mM)
4. dNTP Mixture (10 mM of each nucleotide)
5. Forward and Reverse Primers (10 pmol/ µL)

*Supplied with Taq DNA polymerase enzyme

Procedure

Standard polymerase chain reaction protocol was optimized to gather a single specific product which involves target SNP. Twenty four conditions with different Taq polymerase concentrations (2.5 U, 2 U and 1.5 U), $MgCl_2$ concentrations (2.5 mM, 2.25 mM, 2 mM, 1.5 mM and 1.25 mM) and primer concentrations (20 pmol, 30 pmol and 40 pmol) were tested to get the best result. Optimized mixture content is listed in Table 2.5.

Table 2.5 PCR mixture components for His139Arg polymorphism.

Constituent	Stock Concentration	Volume Added	Final Concentration
Amplification Buffer with KCl	10X	5 µL	1X
$MgCl_2$	25 mM	2.5 µL	1.25 mM
dNTP Mixture	10 mM	1 µL	200 µM
Forward Primer	10 pmol/µL	2 µL	0.4 pmol/µL
Reverse Primer	10 pmol/µL	2 µL	0.4 pmol/µL
Taq DNA Polymerase	5 U/µL	0.5 µL	2.5 U
Template DNA	varies	Varies	~200 ng
Ultra Pure H_2O		Up to 50 µL	

The optimized thermal cycling program to amplify His139Arg single nucleotide polymorphism is given in Table 2.6.

Table 2.6 Thermal cycling conditions for His139Arg polymorphism.

	Temperature	Time	
Initial Denaturation	95ºC	5 min	
Denaturation	95ºC	1 min	35 Cycles
Annealing	55ºC	1 min	
Extension	72ºC	1 min	

Amplified region of *EPHX1* gene including His139Arg single nucleotide polymorphism is represented in Figure 2.3 with forward and reverse primers, recognition site of restriction endonuclease and single nucleotide substitution.

2% agarose gel was used to analyze the length of PCR products. 10 µL of PCR product was mixed with 3 µL loading dye and loaded to gel while DNA ladder was also loaded to determine length of the products. Gel was run for almost 30 min at 120 V.

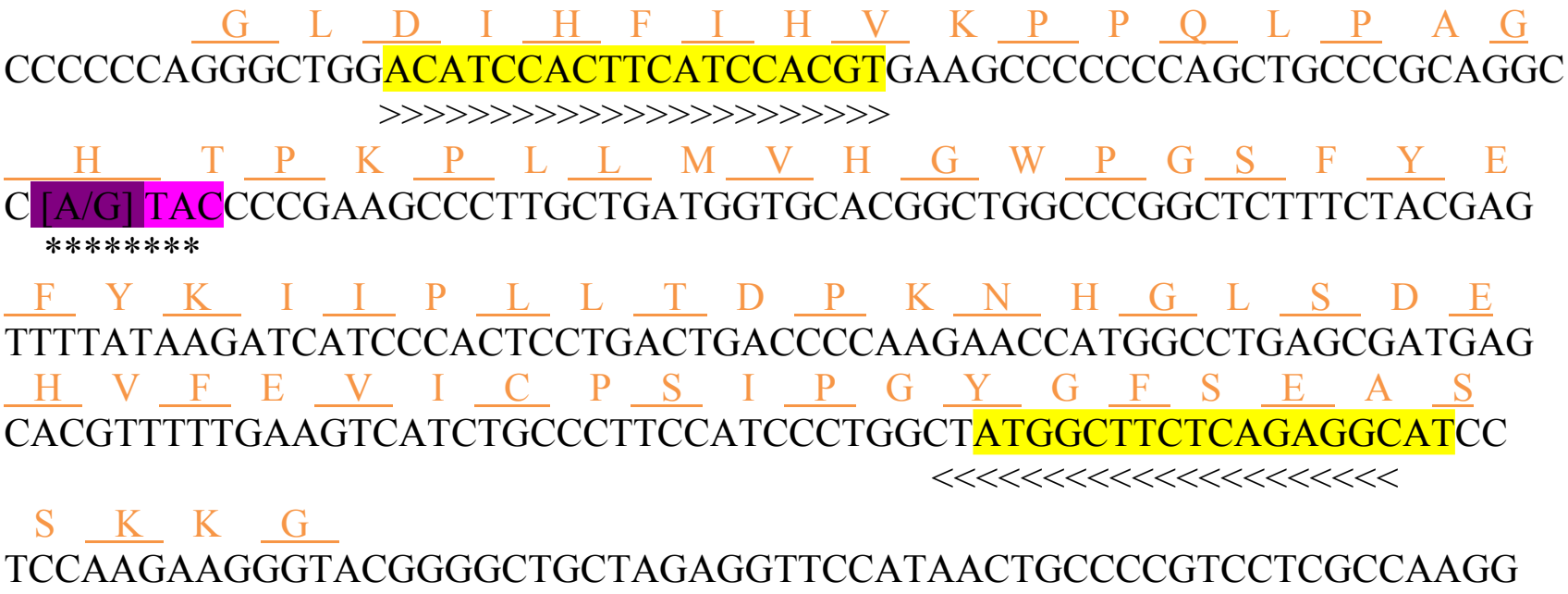

Figure 2.3 Schematic representation of the amplified region of *EPHX1* gene with His139Arg single nucleotide polymorphism. Forward and reverse primers are highlighted with yellow while restriction endonuclease recognition sites are with pink and single nucleotide polymorphism with purple. (http://www.ncbi.nlm.nih.gov).

2.2.2.2.2 Restriction Endonuclease Digestion for His139Arg SNP

Genotyping in terms of His139Arg single nucleotide polymorphism was done by restriction fragment length polymorphism technique.

Wild type individuals for His139Arg SNP have no recognition site for *RsaI* enzyme while base substitution (A/G) results in formation of it. This event can be detected by looking at the length of the DNA fragments after the digestion. In the wild type allele *RsaI* digestion yields 210 bp long single band while 164 bp and 46 bp long two bands indicates base substitution. Finally, individuals with three bands are heterozygous for His139Arg polymorphism (Figure 2.4).

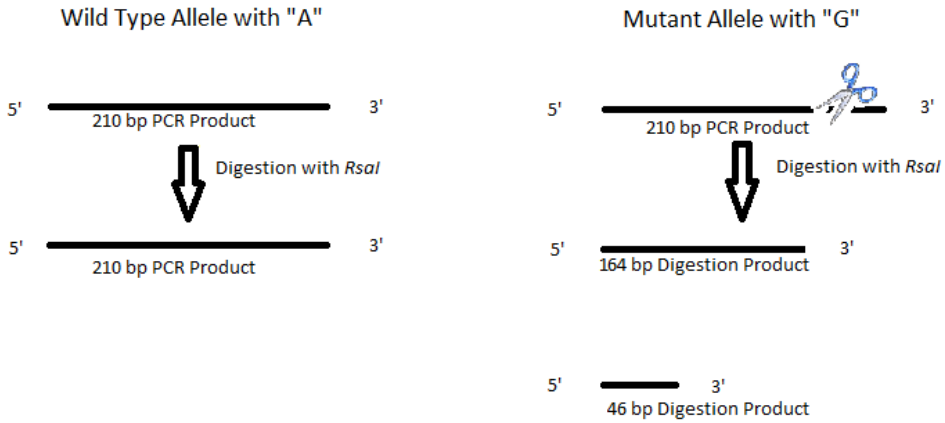

Figure 2.4 Representation of the banding patterns resulted by *RsaI* endonuclease digestion of the His139Arg SNP containing PCR product for wild type and mutant allele

Reagents

1. *RsaI* restriction enzyme
2. Buffer Tango* (33 mM Tris-Acetate (pH 7.9 at 37°C), 10 mM Mg-Acetate and 0.1 mg/ml BSA)

*Supplied with *RsaI* restriction enzyme

Procedure

Standard digestion protocol offered by producer was modified to get clearer image on gel. Four conditions with different restriction enzyme concentrations (20 U, 10 U, 7 U and 5 U) were tested to get the best result. Optimized mixture content, 20 µL of PCR product incubated with 10 U of *RsaI* at 37ºC for 18 hours, is listed in Table 2.7.

Table 2.7 Restriction endonuclease digestion mixture components for Tyr113His polymorphism.

Constituent	Stock Concentration	Volume	Final Concentration
PCR Product		20 µL	
Buffer Tango	10X	2 µL	~0.7X
RsaI	10 U/µL	1 µL	10 U
Ultra Pure dH_2O		7 µL	
Final Volume		30 µL	

3% agarose gel was used to analyze the length of digestion products. 20 µL of digestion product was mixed with 4 µL loading dye and loaded to gel while DNA ladder was also loaded to determine length of the products. Gel was run for almost 30 min at 130 V.

2.2.2.3 Arg287Gln Single Nucleotide Polymorphism

2.2.2.3.1 Polymerase Chain Reaction for Arg287Gln SNP

Arg287Gln single nucleotide polymorphism is involved in gene coding region of *EPHX2* gene (exon 8). Gene fragment that involved Arg287Gln was amplified using specific primer pair.

Reagents

1. Taq DNA Polymerase (10 U/µL)
2. PCR Amplification Buffer with KCl* (100 mM Tris-HCl, 500 mM KCl, 0.8% Nonidet P40; pH 8.8 at 25ºC)
3. $MgCl_2$ Solution* (25 mM)
4. dNTP Mixture (10 mM of each nucleotide)
5. Forward and Reverse Primers (10 pmol/ µL)

*Supplied with Taq DNA polymerase enzyme

Procedure

Standard polymerase chain reaction protocol was optimized to gather a single specific product which involves target SNP. Twenty four conditions with different Taq polymerase concentrations (2.5 U, 2 U and 1.5 U), $MgCl_2$ concentrations (2.5 mM, 2.25 mM, 2 mM and 1.5 mM) and primer concentrations (20 pmol, 30 pmol

and 40 pmol) were tested to get the best result. Optimized mixture content is listed in Table 2.8.

Table 2.8 PCR mixture components for Arg287Gln polymorphism.

Constituent	Stock Concentration	Volume Added	Final Concentration
Amplification Buffer with KCl	10X	5 µL	1X
$MgCl_2$	25 mM	4 µL	2 mM
dNTP Mixture	10 mM	1 µL	200 µM
Forward Primer	10 pmol/µL	2 µL	0.4 pmol/µL
Reverse Primer	10 pmol/µL	2 µL	0.4 pmol/µL
Taq DNA Polymerase	5 U/µL	0.3 µL	1.5 U
Template DNA	varies	varies	~200 ng
Ultra Pure H_2O		Up to 50 µL	

The optimized thermal cycling program to amplify Arg287Gln single nucleotide polymorphism is given in Table 2.9.

Table 2.9 Thermal cycling conditions for Arg287Gln polymorphism.

	Temperature	Time	
Initial Denaturation	95ºC	2 min	
Denaturation	95ºC	15 sec	32 Cycles
Annealing	55ºC	30 sec	
Extension	72ºC	30 sec	
Final Extension	72ºC	3 min	

Amplified region of *EPHX2* gene including Arg287Gln single nucleotide polymorphism is represented in Figure 2.5 with forward and reverse primers, recognition site of restriction endonuclease and single nucleotide substitution.

2% agarose gel was used to analyze the length of PCR products. 10 µL PCR product was mixed with 3 µL loading dye and loaded to gel while DNA ladder was also loaded to determine length of the products. Gel was run for almost 30 min at 120 V.

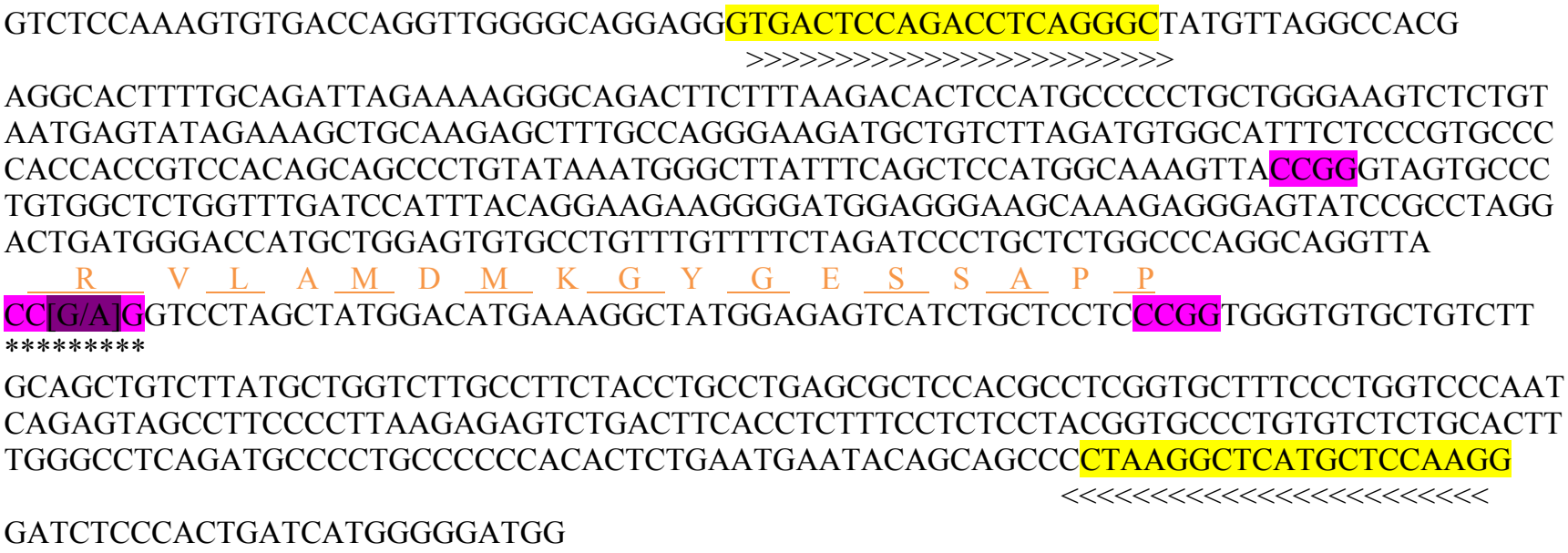

Figure 2.5 Schematic representation of the amplified region of *EPHX2* gene with Arg287Gln single nucleotide polymorphism. Forward and reverse primers which were designed in our lab are highlighted with yellow while restriction endonuclease recognition sites are with pink and single nucleotide polymorphism with purple. (http://www.ncbi.nlm.nih.gov)

2.2.2.3.2 Restriction Endonuclease Digestion for Arg287Gln SNP

Genotyping in terms of Arg287Gln single nucleotide polymorphism was done by restriction fragment length polymorphism technique. Each restriction endonuclease has a specific recognition pattern which is usually a hexamer or a tetramer. Any change in the specific recognition pattern ends up with inability of enzyme to cut the DNA. Nucleotide substitution resulted from single nucleotide polymorphism gives rise to this event as well. Consequently, it is possible to determine genotypes of a population with this technique.

Wild type individuals for Arg287Gln SNP has three recognition sites for *MspI (HpaII)* enzyme, while base substitution (G/A) results in disappearance of one of the three recognition sites. This event can be detected by looking at the length of the DNA fragments after the digestion. In the wild type allele *MspI (HpaII)* digestion yields 240 bp, 236 bp, 150 bp and 50 bp long four bands, while 240 bp, 236 bp and 200 bp long three bands indicates base substitution. Finally, individuals with five bands are heterozygous for Arg287Gln polymorphism (Figure 2.6).

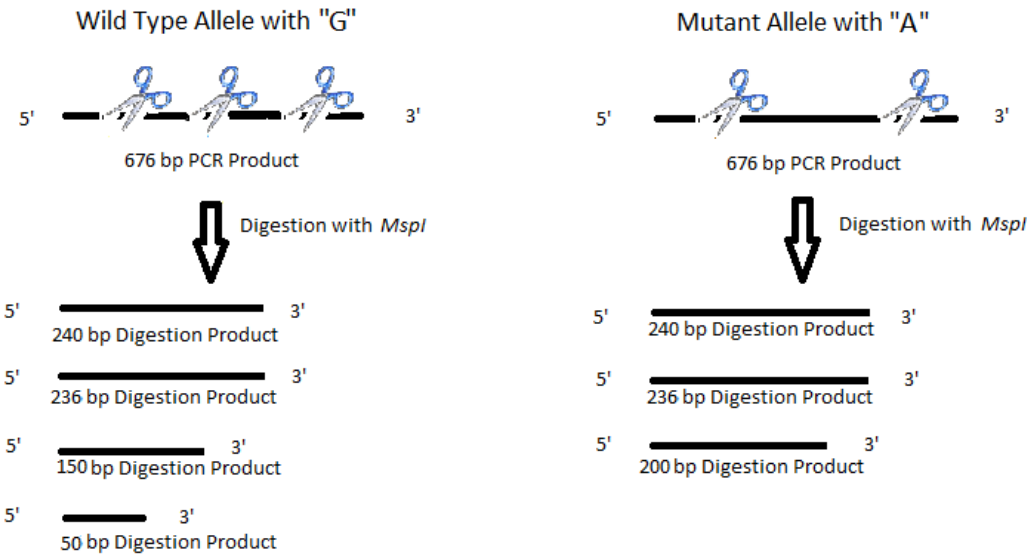

Figure 2.6 Representation of the banding patterns resulted by *MspI* endonuclease digestion of the Arg287Gln SNP containing PCR product for wild type and mutant allele.

Reagents

1. *MspI (HpaII)* restriction enzyme
2. Buffer Tango* (33 mM Tris-Acetate (pH 7.9 at 37°C), 10 mM Mg-Acetate and 0.1 mg/ml BSA)

*Supplied with *MspI (HpaII)* restriction enzyme

Procedure

Standard digestion protocol offered by producer was modified to get a clearer image on gel. Three conditions with different restriction enzyme concentrations (5

U, 4 U and 2 U) were tested to get the best result. Optimized mixture content is listed in Table 2.9.

Table 2.10 Restriction endonuclease digestion mixture components for Arg287Gln polymorphism.

Constituent	Stock Concentration	Volume	Final Concentration
PCR Product		10 µL	
Buffer Tango	10X	1 µL	0.5X
MspI (HpaII)	10 U/µL	0.4 µL	4 U
Ultra Pure dH_2O		8.6 µL	
Final Volume		20 µL	

20 µL of PCR product was incubated with 4 U of *MspI (HpaII)* at 37ºC for 16 hours.3% agarose gel was used to analyze the length of digestion products. 20 µL of digestion product was mixed with 4 µL bromophenol blue and loaded to gel while DNA ladder was also loaded to determine length of the products. Gel was run for almost 30 min at 130 V.

2.2.3 Statistical Analysis

Statistical analyses were performed by SPSS 18.0 software program package which developed by SPSS, Chicago, Illionis, USA. In these analyses continuous variables such as lipid parameters and age were expressed as mean plus/minus standard deviation. Kolmogorov-Smirnov test was used to test normality of the distribution of continuous variables that included in analyses while, Independent

Samples T-Test and/or Mann-Whitney U Test were employed to evaluate differences of continuous variables depending on the shape of the distribution curves. Categorical variables such as sex, statin usage, hypertension, smoking status, diabetes and obesity were expressed as proportions and Chi-square analysis were applied.

Logistic regression analysis with backward selection method was employed to investigate effect of the vascular risk factors, age, sex, lipid parameters and targeted polymorphisms on ischemic stroke in Turkish population. Two-tailed probability values with 95% confidence intervals were used in estimation of odds ratios. Calibration of the logistic regression analysis was done with Hosmer-Lemeshow goodness of fit test.

Significance of the results was determined with *P* value. Tests with a P value less than 0.05 were accepted as significant while, ones higher than this value were accepted as insignificant.

CHAPTER 3

RESULTS

3.1 Study Population

Blood samples of 237 ischemic stroke patients and 120 healthy controls who were unrelated, Caucasian people were collected from Central Anatolia, Turkey with the collaboration of Gülhane Military Medical Academy, Department of Neurology, Ankara, from October 2005 to April 2011. Patients who were younger than 82 years old, being admitted to the neurology services of Gülhane Military Medical Academy within 24 h after onset, having anterior circulation stroke without other major illnesses such as autoimmune diseases, hepatic or renal failure, no known embolic source, no family history of hematological, autoimmune or chronic inflammatory diseases, no history of myocardial infarction within three weeks or of transient ischemic attack were selected to carry out the study. On the other hand, healthy individuals who were older than 38 years old and met all the criteria applied to the patient group instead of suffering from stroke, plus not having carotid stenosis, lumen narrowing, more than 50% or ulcerated carotid plaque were considered as control. All subjects underwent bilateral carotid Doppler ultrasound (CUSG) and transthoracic echocardiographic studies.

Detailed medical records of patients and controls were collected by our collaborators from Gülhane Military Medical Academy, Department of Neurology. All participants were subjected to routine laboratory tests such as complete blood count, leukocyte differential, erythrocyte sedimentation rate,

electrocardiogram, chest x-ray, biochemical laboratory tests such as lipid profile (LDL, VLDL-C, HDL, total cholesterol and triglycerides), fasting glucose, bilirubin, creatinine, sodium and potassium, routine urine tests, liver function tests and rheumatologic screening tests. Hypertension was assessed when systolic blood pressure was higher than 140 mm Hg and/or diastolic blood pressure was higher than 90 mm Hg and/or anti-hypertensive was used. Diabetes was assessed when fasting glucose level was higher or equal to 6.99 mmol/L and/or anti-diabetic drug was used. Obesity was assessed when body mass index of an individual was higher or equal to 30. Smoking status was evaluated as "yes", if individual was a current smoker or quitted smoking less than three months ago. These evaluations and all laboratory studies were performed blinded to medical conditions of the subjects.

In this study, effects of demographic features, clinically determined levels of the biomolecules and conventional risk factors were evaluated regarding to ischemic stroke risk (Table 3.1). The age of study population was varied to 20 to 81 years in patient group, while it was 39 to 90 in control group and there was not any significant difference between mean ages of two groups which are 65.3 ± 11.8 and 65.3 ± 13.2, respectively ($P = 0.444$). Composition of patient group was 99 females and 138 males, while it was 64 females and 56 males for control population. Gender has been stated as a risk factor for stroke incidence for decades and it is known that males prone to develop stroke more than females. This statement was confirmed in our study as well, males had 1.5-fold relative risk in patients when compared to control group ($P = 0.038$).

The effect of the conventional risk factors were found to be significant on development of the ischemic stroke according to the comparison of the patient and control groups in terms of hypertension, diabetes, smoking status and obesity (Table 3.1).

Table 3.1 Effects of demographic features and conventional risk factors on ischemic stroke risk.

Parameter	Patients (n=237)	Controls (n=120)	*P*	OR (95% CI)
Age (years)[a]	65.3 ± 11.8	65.3 ± 13.2	0.444	
Male, n (%)[b]	138 (58.2)	56 (46.6)	0.038	1.593 (1.024-2.478)
Hypertension, n (%)[b]	161 (67.9)	52 (43.3)	0.000	2.770 (1.762-4.356)
Diabetes mellitus, n (%)[b]	80 (33.7)	23 (19.1)	0.003	2.149 (1.267-3.645)
Smokers, n (%)[b]	65 (27.4)	12 (10.0)	0.000	3.401 (1.756-6.588)
Obesity, n (%)[b]	53 (22.3)	9 (7.5)	0.000	3.553 (1.687-7.482)

Values are either number of subjects, percentage or mean ± SD
[a] Mann Whitney U test is applied
[b] Chi-square test is applied

Hypertension was seen in 67.9% of the patient group, when it is 43.3% in control group (*P*=0.000) and estimated relative risk of ischemic stroke according to the hypertension was found to be almost 3-fold based on comparison of patient group with the controls.

Diabetes was observed in 33.7% of the patients, while 19.1% in controls and the frequency of diabetes was significantly higher in patient group then controls (*P*=0.003). Estimated relative risk of ischemic stroke according to diabetes mellitus was found to be more then 2-fold based on comparison of patient group with the controls.

Smoking status was also found to be a significant conventional risk factor in terms of stroke development. Smoker patients (27.4%) were found to be in 3-fold relative risk while compared controls (10%) and the frequency of smoking was significantly higher in patient group then controls (*P*=0.000).

Obesity exhibited more then 3-fold relative risk in patients than in controls. The percentage of obesity in patients was reported as 22.3%, while it is 7.5% in controls. The frequency of the obesity in patients was found to be significantly higher when compared to the controls (P=0.000).

Clinical characteristics of patient group were also compared with the controls (Table 3.2).

Table 3.2 Clinical characteristics of ischemic stroke patients and controls.

Parameter	Patients (n=237)	Controls (n=120)	*P*
Total cholesterol (mmol/L)[a]	4.8 ± 1.3	4.6 ± 1.3	0.107
Triglycerides (mmol/L)[a]	1.4 ± 0.2	1.3 ± 0.2	0.138
HDL-cholesterol (mmol/L)[a]	1.1 ± 0.3	1.2 ± 0.3	0.003
LDL-cholesterol (mmol/L)[a]	2.9 ± 1.1	2.7 ± 1.0	0.009

Values are either number of subjects, percentage or mean ± SD
[a] Independent Samples T-test is applied

Clinical testes reveal that HDL-cholesterol level of the patient group (1.1 ± 0.3 mmol/L) is significantly lower than that of controls (1.2 ± 0.3 mmol/L, P=0.003), while LDL-cholesterol level of the patient group (2.9 ± 1.1 mmol/L) is significantly higher than that of controls (2.7 ± 1.0 mmol/L, P=0.009). On the contrary, total cholesterol (4.8 ± 1.3 vs. 4.6 ± 1.3) and triglycerides (1.4 ± 0.2 vs. 1.3 ± 0.2) were not found to significantly differ between two groups.

3.2 Genotyping for Tyr113His and His139Arg Polymorphisms of *EPHX1* Gene and Arg287Gln Polymorphism of *EPHX2* Gene

Genotyping of all polymorphisms were performed by taking guidelines of standard restriction fragment length polymorphism detection method. In this procedure, polymerase chain reaction is followed by restriction enzyme digestion. Results of PCR and endonuclease digestion were visualized on agarose gel.

3.2.1 Genotyping for Tyr113His Single Nucleotide Polymorphism

Tyr113His polymorphism takes place in 3rd exon of human microsomal epoxide hydrolase enzyme (mEH) coding gene *EPHX1* which localized to chromosomal region 1q42.1. Standard polymerase chain reaction protocol was optimized to gather a single specific product which involves target SNP. The best result was obtained by using 1X amplification buffer (100 mM Tris-HCl, 500 mM KCl, 0.8% Nonidet P40; pH 8.8), 1.25 mM $MgCl_2$, 200 µM dNTP mixture, 40 pmol primer concentrations, 2.5 U Taq polymerase enzyme and 200 ng DNA template. 2% agarose gel was used to analyze the length of PCR products which expected as 198 bp long single band on the gel (Figure 3.1).

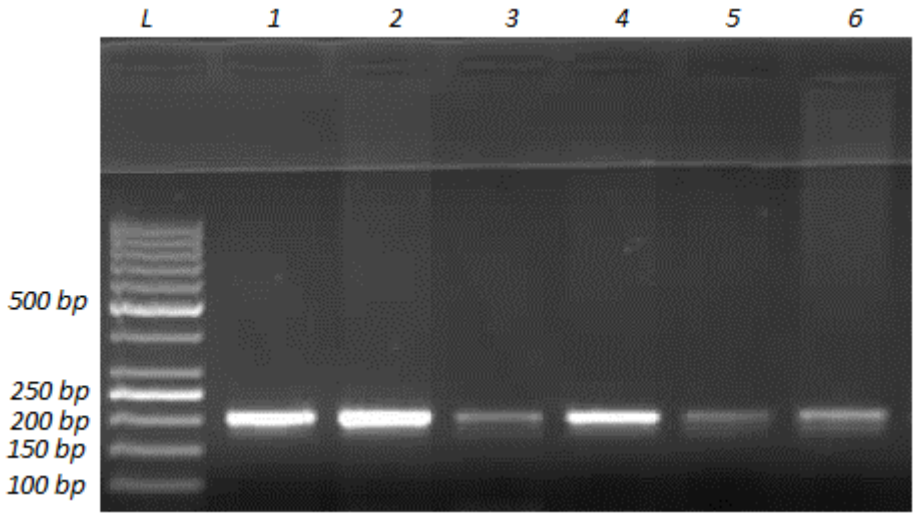

Figure 3.1 Agarose gel image for polymerase chain reaction product of *EPHX1* gene exon 3 region. First lane represented with L stands for DNA ladder (1000 bp-50 bp) which labeled on the left side, the other lanes represented with numbers show the banding pattern of PCR products which is a single band around 200 bp as expected.

Genotypes of the study population were determined with restriction length polymorphism method. Wild type individuals for Tyr113His have no recognition site for *PsyI (Tth111)* enzyme within the vicinity of the SNP, while base substitution (T/C) results in formation of the recognition site. This event can be detected by looking at the lengths of the DNA fragments after the digestion given in Figure 3.2. In the wild type allele *PsyI* digestion yields 198 bp long single band while 175 bp and 23 bp long two bands indicate base substitution (mutant). Finally, individuals with three bands are heterozygous for Tyr113His polymorphism (Figure 3.2).

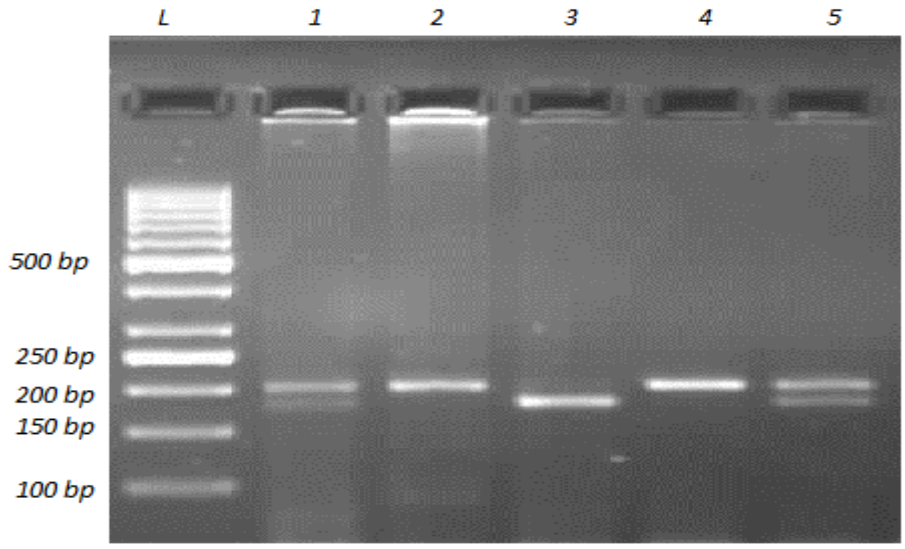

Figure 3.2 Agarose gel image for restriction endonuclease digestion with *PsyI (Tth111)* of amplified *EPHX1* gene exon 3 region. Lane represented with L stands for DNA ladder (1000 bp-50 bp) which labeled on the photo. Lanes 2 and 4 show the banding pattern of wild type homozygous individuals which is a band at 198 bp, while lane 3 shows the banding pattern of mutant homozygous individuals which is two bands at 175 and 23 bp. Lanes 1 and 5 show the banding pattern of heterozygous individuals which is three bands at 198, 175 and 23 bp as expected.

3.2.2 Genotyping for His139Arg Single Nucleotide Polymorphism

His139Arg polymorphism takes place in 4th exon of human microsomal epoxide hydrolase enzyme (mEH) coding gene *EPHX1* which localized to chromosomal region 1q42.1. Standard polymerase chain reaction protocol was optimized to gather a single specific product which involves target SNP. The best result was obtained by using 1X amplification buffer (100 mM Tris-HCl, 500 mM KCl, 0.8% Nonidet P40; pH 8.8), 1.25 mM $MgCl_2$, 200 µM dNTP mixture, 20 pmol

primer concentrations, 2.5 U Taq polymerase enzyme and 200 ng DNA template. 2% agarose gel was used to analyze the length of PCR products which expected as 210 bp long single band on the gel (Figure 3.3).

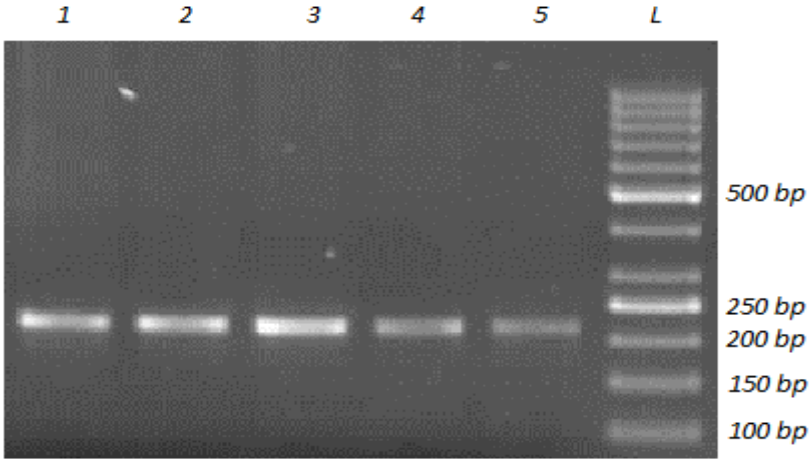

Figure 3.3 Agarose gel image for polymerase chain reaction product of *EPHX1* gene exon 4 region. The lane represented with L stands for DNA ladder (1000 bp-50 bp) which labeled on the photo, the other lanes represented with numbers show the banding pattern of PCR products which is a single band at 210 bp as expected.

Genotypes of the study population were determined with restriction length polymorphism method. Wild type individuals for His139Arg have no recognition site for *RsaI* enzyme within the vicinity of the SNP, while base substitution (A/G) results in formation of the recognition site. This event can be detected by looking at the lengths of the DNA fragments after the digestion shown in Figure 3.3. In the wild type allele *RsaI* digestion yields 210 bp long single band while 164 bp and 46 bp long two bands indicate base substitution (mutant). Finally, individuals with three bands are heterozygous for His139Arg polymorphism (Figure 3.4).

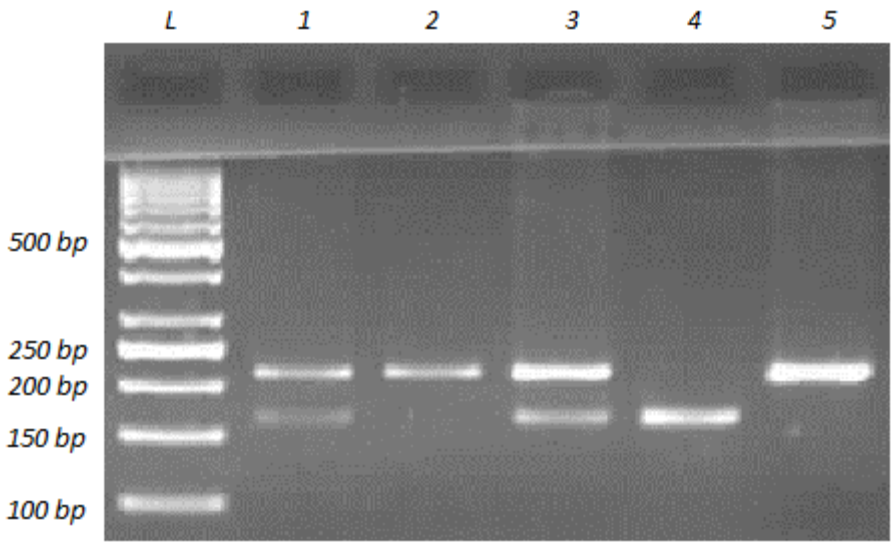

Figure 3.4 Agarose gel image for restriction endonuclease digestion with *RsaI* of amplified *EPHX1* gene exon 4 region. Lane represented with L stands for DNA ladder (1000 bp-50 bp) which labeled on the photo. Lanes 2 and 5 show the banding pattern of wild type homozygous individuals which is a band at 210 bp, while lane 4 shows the banding pattern of mutant homozygous individuals which is two bands at 164 and 46 bp. Lanes 1 and 3 show the banding pattern of heterozygous individuals which is three bands at 210, 164 and 46 bp as expected.

3.2.3 Genotyping for Arg287Gln Single Nucleotide Polymorphism

Arg287Gln polymorphism takes place in 8th exon of human soluble epoxide hydrolase enzyme (sEH) coding gene *EPHX2* which localized to chromosomal region 8p21-p12. Standard polymerase chain reaction protocol was optimized to gather a single specific product which involves target SNP. The best result was obtained by using 1X amplification buffer (100 mM Tris-HCl, 500 mM KCl, 0.8% Nonidet P40; pH 8.8), 2 mM $MgCl_2$, 200 µM dNTP mixture, 20 pmol primer concentrations, 1.5 U Taq polymerase enzyme and 200 ng DNA template.

2% agarose gel was used to analyze the length of PCR products which expected as 676 bp long single band on the gel (Figure 3.5).

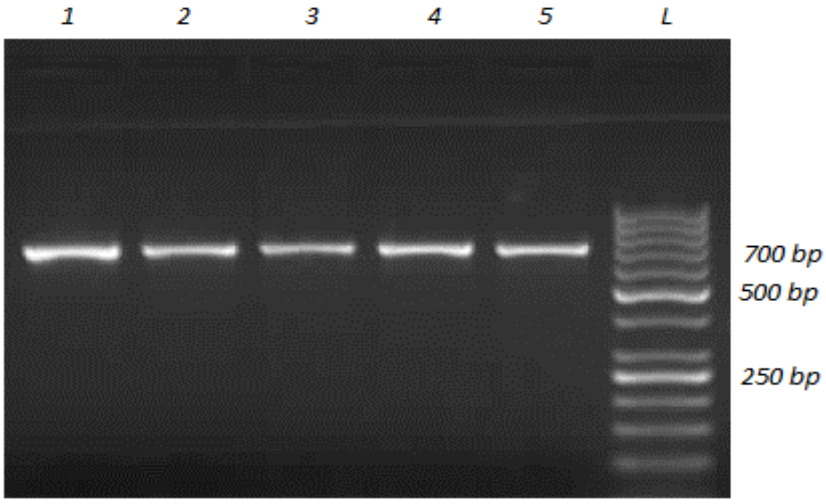

Figure 3.5 Agarose gel image for polymerase chain reaction product of *EPHX2* gene exon 8 region. The lane represented with L stands for DNA ladder (1000 bp-50 bp) which labeled on the photo, the other lanes represented with numbers show the banding pattern of PCR products which is a single band at 676 bp as expected.

Genotypes of the study population were determined with restriction length polymorphism method. Wild type individuals for Arg287Gln have three recognition site for *MspI* enzyme within the vicinity of the SNP, while base substitution (G/A) results in disappearance of one of the recognition site. This event can be detected by looking at the lengths of the DNA fragments after the digestion as shown in Figure 3.6. In the wild type allele *MspI* digestion yields 240 bp, 236 bp, 150 bp and 50 bp long four fragments, while 240 bp, 236 bp and 200

bp long three fragments indicate base substitution (mutant). The bands that represent 240 bp and 236 bp are overlapped so, they seem as one single band. These bands are seen in all individuals so this problem does not interfere with the evaluation of the genotypes. Finally, individuals with five fragments, seen as four bands in the gel are heterozygous for Arg287Gln polymorphism (Figure 3.6).

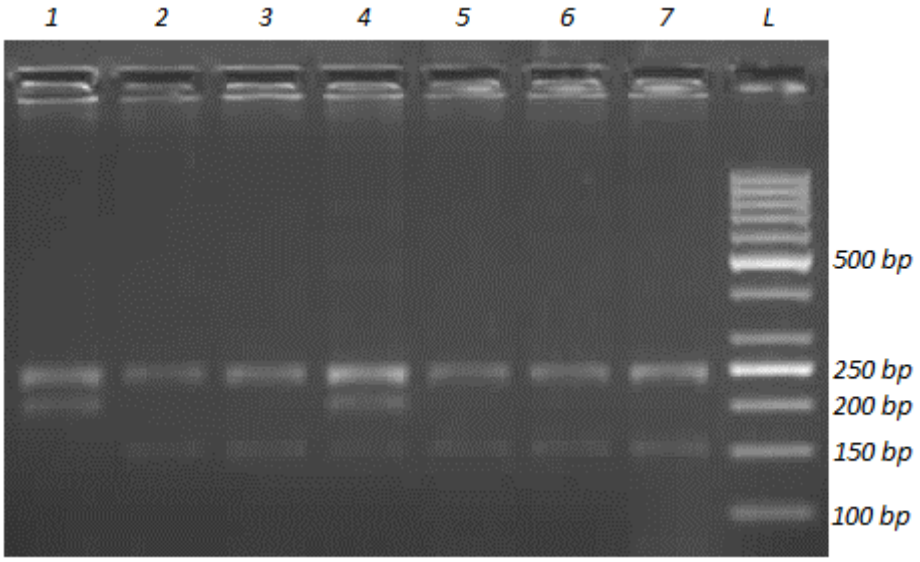

Figure 3.6 Agarose gel image for restriction endonuclease digestion with *MspI* of amplified *EPHX2* gene exon 8 region. Lane represented with L stands for DNA ladder (1000 bp-50 bp) which labeled on the photo. Lanes 2, 3, 5, 6 and 7 show the banding pattern of wild type homozygous individuals which is three bands at 240-236, 150 and 50 bp, while lane 1 shows the banding pattern of mutant homozygous individuals which is two bands at 240-236 and 200 bp. Lane 4 shows the banding pattern of heterozygous individuals which is four bands at 240-236, 200, 150 and 50 bp as expected.

3.3 Genotypes and Allele Frequencies of Tyr113His and His139Arg Polymorphisms of *EPHX1* Gene and Arg287Gln Polymorphism of *EPHX2* Gene

Genotypes and allele frequencies of 237 stroke patients and 120 healthy controls were determined for Tyr113His and His139Arg polymorphisms of *EPHX1* gene and Arg287Gln polymorphism of *EPHX2* gene in the scope of this study.

The case-control analysis of single nucleotide polymorphisms were carried out to evaluate the effect of single nucleotide polymorphism in terms of risk assessment. In this study, risk assessments for each polymorphism were carried out separately.

The general statistical approach for such studies is the calculation of the odds ratio by comparing risk group with no-risk group with respect to the risk factor, in our case single nucleotide polymorphism. The effect of genotype on risk elevation was calculated by accepting heterozygous and homozygous mutated genotypes as risk group, which analyzed against the risk free group that composed of homozygous wild type genotype. Odds ratio is determined by proportioning the risky allele carrying individuals to those who are not carrier for this in both patient and control groups and proportioning the results respectively. The formula is given below:

$$OR=(\#\ Cases_{Risk}/\#\ Cases_{Riskless})\ \text{in Patients}\ /(\#\ Cases_{Risk}/\#\ Cases_{Riskless})\ \text{in Controls}$$

3.3.1 Genotypes and Allele Frequencies of Tyr113His Polymorphism of *EPHX1* Gene

Genotypes and allele frequencies of Tyr113His polymorphism of *EPHX1* gene in stroke patients and controls are listed in Table 3.3. The genotype distributions and frequencies show that 126 patients out of 237 (53.1%) and 61 controls out of 120 (50.8%) are homozygous wild type with a very similar frequency. On the other hand, 92 patients (38.8%) and 44 controls (36.6%) are heterozygous for Tyr113His polymorphism and 19 patients out of 237 (8%) and 15 controls (12.5%) have homozygous mutant genotype. Risk assessment was done by comparing the group that includes homozygous mutated genotypes and heterozygous ones with the group involving homozygous wild type individuals for patient and control groups. It was found that there is not any significant difference between these two groups in terms of genotype distribution (OR=0.911, CI=0.587-1.414; P=0.677).

Frequencies of wild type allele in patients and controls were found as 0.726 and 0.692, while those of mutant alleles are 0.274 and 0.308 respectively. Risk assessment shows that there is not any significant difference between wild type allele and mutant allele (OR=0.848, CI=0.603-1.911; P=0.341).

Table 3.3 Distribution of genotypes and allele frequencies of patient and control groups in terms of Tyr113His polymorphism.

	Patients (n=237)	Controls (n=120)	OR (95% CI)	*P*
Tyr113His polymorphism				
Genotypes, n (%)				
TT	126 (53.1)	61 (50.8)		
TC	92 (38.8)	44 (36.6)	0.911[a](0.587-1.414)	0.677
CC	19 (8.0)	15 (12.5)		
Allele frequency				
T	0.726	0.692	0.848[b](0.603-1.191)	0.341
C	0.274	0.308		

a. CC+TC vs. TT
b. C vs. T

3.3.2 Genotypes and Allele Frequencies of His139Arg Polymorphism of *EPHX1* Gene

Genotypes and allele frequencies of His139Arg polymorphism of *EPHX1* gene in stroke patients and controls are listed in Table 3.4. The genotype distributions and frequencies show that 163 patients out of 237 (68.8%) and 79 out of 120 (65.8%) are homozygous wild type. On the other hand, 65 patients (27.4%) and 36 controls (30%) are heterozygous for His139Arg polymorphism and 9 patients out of 237 (3.8%) and 5 controls (4.2%) have homozygous mutant genotype. Risk assessment was done by comparing the group that includes homozygous mutated genotypes and heterozygous ones with the group involving homozygous wild type individuals for patient and control groups. It was found that there is not any

significant difference between these groups in terms of genotype distribution (OR=0.875, CI=0.549-1.395; *P*=0.575).

Wild type allele frequencies in patients and controls were found as 0.825 and 0.808, while mutant allele frequencies are 0.175 and 0.192 respectively. Risk assessment shows that there is not any significant difference between allele frequencies (OR=0.895, CI=0.601-1.335; *P*=0.587).

Table 3.4 Distribution of genotypes and allele frequencies of patient and control groups in terms of His139Arg polymorphism.

	Patients (n=237)	**Controls (n=120)**	**OR (95% CI)**	***P***
His139Arg polymorphism				
Genotypes, n (%)				
AA	163 (68.8)	79 (65.8)		
AG	65 (27.4)	36 (30)	0.875[a](0.549-1.395)	0.575
GG	9 (3.8)	5 (4.2)		
Allele frequency				
A	0.825	0.808		
G	0.175	0.192	0.895[b](0.601-1.335)	0.587

a. GG+GA vs. AA
b. G vs. A

3.3.3 Genotypes and Allele Frequencies of Arg287Gln Polymorphism of *EPHX2* Gene

Genotypes and allele frequencies of Arg287Gln polymorphism of *EPHX2* gene in stroke patients and controls are listed in Table 3.5. The genotype distributions and frequencies show that 202 patients out of 237 (85.3%) and 99 out of 120 (82.5%) are homozygous wild type. On the other hand, 30 patients (12.7%) and 20 controls (16.6%) are heterozygous for His139Arg polymorphism and 5 patients (2.1%) and 1 controls (0.8%) have homozygous mutant genotype. Risk assessment was done by comparing the group that includes homozygous mutated genotypes and heterozygous ones with riskless group involving homozygous wild type individuals for patient and control groups. It was found that there is not any significant difference between two groups in terms of genotype distribution (OR=0.817, CI=0.452-1.477; P=0.503).

Wild type allele frequencies in patients and controls were found as 0.916 and 0.908, while mutant allele frequencies are 0.084 and 0.092 respectively. Risk assessment shows that there is not any significant difference between allele frequencies (OR=0.913, CI=0.530-1.575; P=0.744).

Table 3.5 Distribution of genotypes and allele frequencies of patient and control groups in terms of Arg287Gln polymorphism.

	Patients (n=237)	Controls (n=120)	OR (95% CI)	*P*
Arg287Gln polymorphism				
Genotypes, n (%)				
GG	202 (85.3)	99 (82.5)		
GA	30 (12.7)	20 (16.6)	0.817[a](0.452-1.477)	0.503
AA	5 (2.1)	1 (0.8)		
Allele frequency				
G	0.916	0.908	0.913[b](0.530-1.575)	0.744
A	0.084	0.092		

a. AA+GA vs. GG
b. A vs. G

3.4 Tyr113His and His139Arg Polymorphisms of *EPHX1* Gene and Arg287Gln Polymorphism of *EPHX2* Gene in Different Subgroups of Patients and Controls

Four different subgroup analyses were conducted to investigate distribution of the stroke cases according to the well established ischemic stroke risk factors. The analyses were carried out in hypertensive vs. normotensive, smoker vs. non-smoker, obese vs. non-obese and diabetic vs. non-diabetic subgroups. Each genotype was evaluated in terms of these vascular risk factors as well as an overall analysis which presented in Table 3.1.

The general statistical approach for such studies is the calculation of the odds ratio by comparing risk group with no-risk group with respect to the risk factor; in this case hypertension, diabetes, obesity and smoking status were determined as risk

factors for ischemic stroke development. The effect of genotype on risk elevation was calculated by accepting heterozygous and homozygous mutated genotypes as one group, which analyzed against the group that composed of homozygous wild type genotype. Odds ratio is determined by proportioning the mutant allele carrying individuals to those who are not carrier for this in both patient and control groups and then, proportioning the results respectively.

3.4.1 Subgroup Analysis for Hypertensive vs. Normotensive Cases with Respect to Tyr113His and His139Arg Polymorphisms of *EPHX1* Gene and Arg287Gln Polymorphism of *EPHX2* Gene in Ischemic Stroke Patients and Controls

The relation between hypertension and ischemic stroke was investigated by comparing hypertensive patient group with hypertensive control group and same comparison was done in normotensive group as well. This analysis was held for each of Tyr113His and His139Arg polymorphisms of *EPHX1* gene and Arg287Gln polymorphism of *EPHX2* gene separately. Genotypes and allele frequencies of 213 hypertensive and 144 normotensive subjects including both patients and controls are listed in Table 3.6.

The genotype distribution of Tyr113His polymorphism shows that there is not any significant difference between stroke patients and controls in terms of homozygous wild type (54.0% vs. 48.1%), heterozygous (40.4% vs. 34.6%) and homozygous mutant (5.6% vs. 17.3%) genotypes within the hypertensive subgroup (OR=0.788; CI=0.421-1.473; *P*=0.454). Distributions of the genotypes in normotensive subgroup show a similar pattern in patients (51.3% with homozygous wild type, 35.5% with heterozygous and 13.2% with homozygous mutant genotypes) and controls (52.9% with homozygous wild type, 38.3% with heterozygous and 8.8% with homozygous mutant genotypes) which not found to

be significant as well (OR=1.067; CI=0.554-2.055; P=0.845). Risk assessment was done by comparing the group that includes homozygous mutated genotypes (CC) and heterozygous (TC) ones with the group involving homozygous wild type (TT) individuals for patient and control groups.

Table 3.6 Distribution of genotypes and allele frequencies of hypertensive vs. normotensive groups in terms of Tyr113His, His139Arg and Arg287Gln polymorphisms.

	Hypertensive (n=213)				Normotensive (n=144)			
	Stroke (n=161)	**Control (n=52)**	**OR (95% CI)**	***P***	**Stroke (n=76)**	**Control (n=68)**	**OR (95% CI)**	***P***
Tyr113His polymorphism								
TT	87 (54.0%)	25 (48.1%)			39 (51.3%)	36 (52.9%)		
TC	65 (40.4%)	18 (34.6%)	0.788[a] (0.421-1.473)	0.454	27 (35.5%)	26 (38.3%)	1.067[a] (0.554-2.055)	0.845
CC	9(5.6%)	9 (17.3%)			10 (13.2%)	6 (8.8%)		
His139Arg polymorphism								
AA	113 (70.2%)	33 (63.5%)			50 (65.8%)	46 (67.6%)		
AG	41 (25.5%)	18 (34.6%)	0.738[b] (0.382-1.424)	0.364	24 (31.6%)	18 (26.5%)	1.087[b] (0.543-2.178)	0.813
GG	7 (4.3%)	1 (1.9%)			2 (2.6%)	4 (5.9%)		
Arg287Gln polymorphism								
GG	135 (83.8%)	44 (84.6%)			67 (88.2%)	55 (80.9%)		
GA	22 (13.7%)	8 (15.4%)	1.059[c] (0.447-2.509)	0.896	8 (10.5%)	12 (17.6%)	0.568[c] (0.226-1.428)	0.226
AA	4 (2.5%)	0 (0.0%)			1 (1.3%)	1 (1.5%)		

a. TC+CC vs. TT, **b**. AG+GG vs. AA, **c.** GA+AA vs. GG

The genotype distributions and frequencies of hypertensive subgroup in terms of His139Arg polymorphism show that 113 patients out of 161 (70.2%) and 33 controls out of 52 (63.5%) are homozygous wild type. On the other hand, 41 patients (25.5%) and 18 controls (34.6%) are heterozygous for His139Arg polymorphism and 7 patients (4.3%) and 1 control (1.9%) have homozygous mutant genotype. Comparison of stroke patients and controls within the subgroup reveals that there is not any significant relationship between genotypes and stroke risk in terms of His139Arg polymorphism (OR=0.738; CI=0.382-1.424; *P*=0.364). Additionally, the genotype distributions and frequencies of normotensive subgroup in terms of His139Arg polymorphism show that 50 patients out of 76 (65.8%) and 46 controls out of 68 (67.6%) are homozygous wild type. In the same group, 24 patients (31.6%) and 18 controls (26.5%) are heterozygous for His139Arg polymorphism and 2 patients (2.6%) and 4 controls (5.9%) have homozygous mutant genotype (Table 3.6). Comparison of stroke patients and controls within the normotensive subgroup reveals that there is not any significant relationship between genotypes and stroke risk in terms of His139Arg polymorphism (OR=1.087; CI=0.543-2.178; *P*=0.226). Risk assessment was done by comparing the group that includes homozygous mutated genotypes (GG) and heterozygous ones (AG) with the group involving homozygous wild type (AA) individuals for patient and control groups.

Finally, the genotype distributions and frequencies in terms of Arg287Gln polymorphism within hypertensive group show that 135 patients out of 161 (83.8%) and 44 controls out of 52 subjects (84.6%) are homozygous wild type (Table 3.6). On the other hand, 22 patients (13.7%) and 8 controls (15.4%) are heterozygous for Arg287Gln polymorphism and 4 patients (2.5%) have homozygous mutant genotype in the hypertensive subgroup. Risk assessment was done by comparing the group that includes homozygous mutated genotypes (AA) and heterozygous ones (GA) with the group involving homozygous wild type (GG) individuals for patient and control groups. It was found that there is not any

significant difference between two groups in terms of genotype distribution (OR=1.059, CI=0.447-2.509; *P*=0.896). The genotype distributions and frequencies in terms of Arg287Gln polymorphism within normotensive subgroup given in Table 3.6 show that 67 patients out of 76 (88.2%) and 55 out of 68 control subjects (80.9%) are homozygous wild type. In the same group, 8 patients (10.5%) and 12 controls (17.6%) are heterozygous for Arg287Gln polymorphism and 1 patient (1.3%) and 1 control (1.5%) have homozygous mutant genotype. It was found that there is not any significant difference between the groups in terms of genotype distribution (OR=0.568, CI=0.226-1.428); *P*=0.226).

3.4.2 Subgroup Analysis for Smoker vs. Non-smoker Cases with Respect to Tyr113His and His139Arg Polymorphisms of *EPHX1* Gene and Arg287Gln Polymorphism of *EPHX2* Gene in Ischemic Stroke Patients and Controls

The relation between smoking status and ischemic stroke was investigated by comparing smoker patient group with smoker control group and same comparison was done in non-smoker group as well. This analysis was held for each of Tyr113His and His139Arg polymorphisms of *EPHX1* gene and Arg287Gln polymorphism of *EPHX2* gene separately. Genotypes and allele frequencies of 77 smoker and 280 non-smoker subjects including both patients and controls are listed in Table 3.7.

Table 3.7 Distribution of genotypes and allele frequencies of smoker vs. non-smoker groups in terms of Tyr113His, His139Arg and Arg287Gln polymorphisms.

	Smoker (n=77)				Non-smoker (n=280)			
	Stroke (n=65)	**Control (n=12)**	**OR (95% CI)**	***P***	**Stroke (n=172)**	**Control (n=108)**	**OR (95% CI)**	***P***
Tyr113His polymorphism								
TT	33 (50.8%)	6 (50.0%)			93 (54.1%)	55 (50.9%)		
TC	26 (40.0%)	6 (50.0%)	0.970[a] (0.283-3.323)	0.961	66 (38.4%)	38 (35.2%)	0.882[a] (0.544-1.427)	0.608
CC	6 (9.2%)	0 (0.0%)			13 (7.6%)	15 (13.9%)		
His139Arg polymorphism								
AA	46 (70.8%)	6 (50.0%)			117 (68.0%)	73 (67.6%)		
AG	15 (23.0%)	5 (41.7%)	0.413[b] (0.118-1.444)	0.158	50 (29.1%)	31 (28.7%)	0.980[b] (0.586-1.641)	0.940
GG	4 (6.2%)	1 (8.3%)			5 (2.9%)	4 (3.7%)		
Arg287Gln polymorphism								
GG	52 (80.0%)	11 (91.7%)			150 (87.2%)	88 (81.5%)		
GA	12 (18.5%)	1 (8.3%)	2.750[c] (0.325-23.267)	0.336	18 (10.4%)	19 (17.6%)	0.645[c] (0.333-1.249)	0.191
AA	1 (1.5%)	0 (0.0%)			4 (2.3%)	1 (0.9%)		

a. TC+CC vs. TT, **b.** AG+GG vs. AA, **c**. GA+AA vs. GG

The genotype distribution given in Table 3.7 shows that there is not any significant difference between stroke patients and controls in terms of homozygous wild type (50.8% vs. 50.0), heterozygous (9.2% vs. 0.0%) and homozygous mutant genotypes (40.0% vs. 50.0%) within the smoker subgroup (OR=0.970; CI=0.283-3.323; *P*=0.961). Distributions of the genotypes in non-smoker subgroup show a similar pattern in patients (54.1% with homozygous wild type, 38.4% with heterozygous and 7.6% with homozygous mutant genotypes) and controls (50.9% with homozygous wild type, 35.2% with heterozygous and 13.9% with homozygous mutant genotypes) which not found to be significant as well (OR=0.882; CI=0.544-1.427; P=0.608). Risk assessment was done by comparing the group that includes homozygous mutated genotypes (CC) and heterozygous ones (TC) with the group involving homozygous wild type (TT) individuals for patient and control group.

The genotype distributions and frequencies of smoker subgroup in terms of His139Arg polymorphism given in Table 3.7 show that 46 patients out of 65 (70.8%) and 6 controls out of 12 (50.0%) are homozygous wild type. On the other hand, 15 patients (23.0%) and 5 controls (41.7%) are heterozygous for His139Arg polymorphism and 4 patients (6.2%) and 1 control (8.3%) have homozygous mutant genotype within the smoker subgroup. Comparison of stroke patients and controls within the subgroup of smokers reveals that there is not any significant relationship between genotypes and stroke risk in terms of His139Arg polymorphism (OR=0.413; CI=0.118-1.444; *P*=0.158). Additionally, the genotype distributions and frequencies of non-smoker subgroup in terms of His139Arg polymorphism show that 117 patients out of 172 (68.0%) and 73 controls out of 108 (67.6%) are homozygous wild type. In the same group, 50 patients (29.1%) and 31 controls (28.7%) are heterozygous for His139Arg polymorphism and 5 patients (2.9%) and 4 controls (3.7%) have homozygous mutant genotype (Table 3.7). Comparison of stroke patients and controls within the non-smoker subgroup reveals that although the genotype frequencies are similar for in both, there is not

any significant relationship between genotypes and stroke risk in terms of His139Arg polymorphism (OR=0.980; CI=0.586-1.641; *P*=0.940). Risk assessment was done by comparing the group that includes homozygous mutated genotypes (GG) and heterozygous ones (GA) with the group involving homozygous wild type (AA) individuals for patient and control groups.

Finally, the genotype distributions and frequencies in terms of Arg287Gln polymorphism within smoker subgroup show that 52 patients out of 65 (80.0%) and 11 controls out of 12 subjects (91.7%) are homozygous wild type (Table 3.7). On the other hand, 12 patients (18.5%) and 1 control (8.3%) are heterozygous for Arg287Gln polymorphism and 1 patient (1.5%) has homozygous mutant genotype in the smoker subgroup. Risk assessment was done by comparing the group that includes homozygous mutated genotypes (AA) and heterozygous ones (GA) with the group involving homozygous wild type (GG) individuals for patient and control groups. It was found that there is not any significant difference between two groups in terms of genotype distribution (OR=2.750, CI=0.325-23.267; *P*=0.336). While, the genotype distributions and frequencies in terms of Arg287Gln polymorphism within non-smoker group show that 150 patients out of 172 (87.2%) and 88 out of 108 control subjects (81.5%) are homozygous wild type. In the same subgroup, 18 patients (10.4%) and 19 controls (17.6%) are heterozygous for Arg287Gln polymorphism and 4 patients (2.3%) and 1 control (0.9%) have homozygous mutant genotype It was found that there is not any significant difference between the groups in terms of genotype distribution (OR=0.645, CI=0.333-1.249; *P*=0.191).

3.4.3 Subgroup Analysis for Obese vs. Non-obese Cases with Respect to Tyr113His and His139Arg Polymorphisms of *EPHX1* Gene and Arg287Gln Polymorphism of *EPHX2* Gene in Ischemic Stroke Patients and Controls

The relation between obesity status and ischemic stroke was determined in Tyr113His and His139Arg polymorphisms of *EPHX1* gene and Arg287Gln polymorphism of *EPHX2* gene. Genotypes and allele frequencies of 62 obese and 295 non-obese subjects including both patients and controls are listed in Table 3.8.

The genotype distribution given in Table 3.8 shows that there is not any significant difference between stroke patients and controls in terms of homozygous wild type (64.1% vs. 55.6%), heterozygous (30.2% vs. 22.2%) and homozygous mutant genotypes (5.7% vs. 22.2%) within the obese subgroup (OR=0.699; CI=0.167-2.918; *P*=0.622). Distributions of the genotypes in non-obese subgroup show a similar pattern in patients (50.0% with homozygous wild type, 41.3% with heterozygous and 8.7% with homozygous mutant genotypes) and controls (50.5% with homozygous wild type, 37.8% with heterozygous and 11.7% with homozygous mutant genotypes). Risk assessment was done by comparing the group that includes homozygous mutated genotypes (CC) and heterozygous ones (TC) with the group involving homozygous wild type (TT) individuals for patient and control group.

Table 3.8 Distribution of genotypes and allele frequencies of obese vs. non-obese groups in terms of Tyr113His, His139Arg and Arg287Gln polymorphisms.

	Obese (n= 62)				Non-obese (n=295)			
	Stroke (n=53)	**Control (n=9)**	**OR (95% CI)**	***P***	**Stroke (n=184)**	**Control (n=111)**	**OR (95% CI)**	***P***
Tyr113His polymorphism								
TT	34 (64.1%)	5 (55.6%)			92 (50%)	56 (50.5%)		
TC	16 (30.2%)	2 (22.2%)	0.699[a] (0.167-2.918)	0.622	76 (41.3%)	42 (37.8%)	-	-
CC	3 (5.7%)	2 (22.2%)			16 (8.7%)	13 (11.7%)		
His139Arg polymorphism								
AA	43 (81.1%)	7 (77.8%)			120 (65.2%)	72 (64.8%)		
AG	7 (13.2%)	1 (11.1%)	0.814[b] (0.146-4.525)	0.814	58 (31.5%)	35 (31.6%)	0.985[b] (0.601-1.613)	0.951
GG	3 (5.7%)	1 (11.1%)			6 (3.3%)	4 (3.6%)		
Arg287Gln polymorphism								
GG	44 (83.0%)	9 (100.0%)			158 (85.9%)	90 (81.1%)		
GA	8 (15.1%)	0 (0.0%)	NA	NA	22 (11.9%)	20 (18.0%)	0.705[c] (0.375-1.325)	0.276
AA	1 (1.9%)	0 (0.0%)			4 (2.2%)	1 (0.9%)		

a. TC+CC vs. TT, **b.** AG+GG vs. AA, **c.** GA+AA vs. GG

The genotype distributions and frequencies of obese subgroup in terms of His139Arg polymorphism given in Table 3.8 show that 43 patients out of 53 (81.1%) and 7 controls out of 9 (77.8%) are homozygous wild type. On the other hand, 7 patients (13.2%) and 1 control (11.1%) are heterozygous for His139Arg polymorphism and 3 patients (5.7%) and 1 control (11.1%) have homozygous mutant genotype within the obese subgroup. Comparison of stroke patients and controls within the subgroup of obese reveals that there is not any significant relationship between genotypes and stroke risk in terms of His139Arg polymorphism (OR=0.814; CI=0.146-4.525; *P*=0.814). Additionally, the genotype distributions and frequencies of non-obese subgroup in terms of His139Arg polymorphism show that 120 patients out of 184 (65.2%) and 72 controls out of 111 (64.8%) are homozygous wild type. In the same group, 58 patients (31.5%) and 35 controls (31.6%) are heterozygous for His139Arg polymorphism and 6 patients (3.3%) and 4 controls (3.6%) have homozygous mutant genotype (Table 3.8). Comparison of stroke patients and controls within the non-obese subgroup reveals that although the genotype frequencies are similar for in both, there is not any significant relationship between genotypes and stroke risk in terms of His139Arg polymorphism (OR=0.985; CI=0.601-1.613; *P*=0.951). Risk assessment was done by comparing the group that includes homozygous mutated genotypes (GG) and heterozygous ones (AG) with the group involving homozygous wild type (AA) individuals for patient and control groups.

Finally, the genotype distributions and frequencies in terms of Arg287Gln polymorphism within obese subgroup show that 44 patients out of 53 (83.0%) and 9 controls out of 9 subjects (100.0%) are homozygous wild type (Table 3.8). On the other hand, 8 patients (15.1%) are heterozygous when, 1 patient (1.9%) has homozygous mutant genotype in the obese subgroup. Risk assessment was done by comparing the group that includes homozygous mutated genotypes (AA) and heterozygous ones (GA) with the group involving homozygous wild type (GG) individuals for patient and control groups. It was found that there is not any

significant difference between two groups in terms of genotype distribution (P=0.181).

The genotype distributions and frequencies in terms of Arg287Gln polymorphism within non-obese group given in Table 3.8 show that 158 patients out of 184 (85.9%) and 90 out of 111 control subjects (81.1%) are homozygous wild type. In the same subgroup, 22 patients (11.9%) and 20 controls (18.0%) are heterozygous for Arg287Gln polymorphism and 4 patients (2.2%) and 1 control (0.9%) have homozygous mutant genotype. It was found that there is not any significant difference between the groups in terms of genotype distribution (OR=0.705; CI=0.375-1.325; P=0.276)

3.4.4 Subgroup Analysis for Diabetic vs. Non-diabetic Cases with Respect to Tyr113His and His139Arg Polymorphisms of *EPHX1* Gene and Arg287Gln Polymorphism of *EPHX2* Gene in Ischemic Stroke Patients and Controls

The relation between diabetes status and ischemic stroke was determined in Tyr113His and His139Arg polymorphisms of *EPHX1* gene and Arg287Gln polymorphism of *EPHX2* gene (Table 3.9).

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Table 3.9 Distribution of genotypes and allele frequencies of diabetic vs. non-diabetic groups in terms of Tyr113His, His139Arg and Arg287Gln polymorphisms.

	Diabetic (n=103)				Non-Diabetic (n=254)			
	Stroke (n=80)	**Control (n=23)**	**OR (95% CI)**	***P***	**Stroke (n=157)**	**Control (n=97)**	**OR (95% CI)**	***P***
Tyr113His polymorphism								
TT	44 (55.0%)	12 (52.2%)			82 (52.2%)	49 (50.5%)		
TC	32 (40.0%)	10 (43.5%)	0.893[a] (0.352-2.260)	0.810	60 (38.2%)	34 (35.1%)	0.934[a] (0.563-1.549)	0.791
CC	4 (5.0%)	1 (4.3%)			15 (9.6%)	14 (14.4%)		
His139Arg polymorphism								
AA	56 (70.0%)	15 (65.2%)			107 (68.2%)	64 (66.0%)		
AG	23 (28.8%)	8 (34.8%)	0.804[b] (0.301-2.146)	0.662	42 (26.7%)	28 (28.9%)	0.906[b] (0.529-1.552)	0.720
GG	1 (1.2%)	0 (0.0%)			8 (5.1%)	5 (5.1%)		
Arg287Gln polymorphism								
GG	70 (87.5%)	21 (91.3%)			132 (87.1%)	78 (80.4%)		
GA	8 (10.0%)	2 (8.7%)	1.500[c] (0.304-7.389)	0.616	22 (14.0%)	18 (18.6%)	0.778[c] (0.402-1.503)	0.453
AA	2 (2.5%)	0 (0.0%)			3 (1.9%)	1 (1.0%)		

a. TC+CC vs. TT, **b.** AG+GG vs. AA, **c.** GA+AA vs. GG

The genotype distributions and frequencies of diabetic subgroup in terms of His139Arg polymorphism given in Table 3.9 show that 56 patients out of 80 (70.0%) and 15 controls out of 23 (65.2%) are homozygous wild type. On the other hand, 23 patients (28.8%) and 8 controls (34.8%) are heterozygous for His139Arg polymorphism and 1 patient (1.2%) has homozygous mutant genotype within the diabetic subgroup. Comparison of stroke patients and controls within the subgroup of diabetics reveals that although the genotype frequencies are similar for in both, there is not any significant relationship between genotypes and stroke risk in terms of His139Arg polymorphism (OR=0.804; CI=0.301-2.146; *P*=0.662). Additionally, the genotype distributions and frequencies of non-diabetic subgroup in terms of His139Arg polymorphism show that 107 patients out of 157 (68.2%) and 64 controls out of 97 (66.0%) are homozygous wild type. In the same subgroup, 42 patients (26.7%) and 28 controls (28.9%) are heterozygous for His139Arg polymorphism and 8 patients (5.1%) and 5 controls (5.1%) have homozygous mutant genotype (Table 3.9). Comparison of stroke patients and controls within the non-diabetic subgroup reveals that although the genotype frequencies are similar for both, there is not any significant relationship between genotypes and stroke risk in terms of His139Arg polymorphism (OR=0.906; CI=0.529-1.552; *P*=0.720). Risk assessment was done by comparing the group that includes homozygous mutated genotypes (GG) and heterozygous ones (AG) with the group involving homozygous wild type (AA) individuals for patient and control groups.

Finally, the genotype distributions and frequencies in terms of Arg287Gln polymorphism within diabetic subgroup show that 70 patients out of 80 (87.5%) and 21 controls out of 23 control subjects (91.3%) are homozygous wild type (Table 3.9). On the other hand, 8 patients (10.0%) and 2 controls (8.7%) are heterozygous when 2 patients (2.5%) have homozygous mutant genotype in the diabetic subgroup. Risk assessment was done by comparing the group that includes homozygous mutated genotypes (AA) and heterozygous ones (GA) with

the group involving homozygous wild type (GG) individuals for patient and control groups. It was found that there is not any significant difference between two groups in terms of genotype distribution (OR=1.500; CI=0.304-7.389; *P*=0.616). The genotype distributions and frequencies in terms of Arg287Gln polymorphism within non-diabetic subgroup given in Table 3.9 show that 132 patients out of 157 (87.1%) and 78 out of 97 control subjects (80.4%) are homozygous wild type. In the same subgroup, 22 patients (14.0%) and 18 controls (18.6%) are heterozygous for Arg287Gln polymorphism and 3 patients (1.9%) and 1 control (1.0%) have homozygous mutant genotype. It was found that there is not any significant difference between two groups in terms of genotype distribution (OR=0.778; CI=0.402-1.503; *P*=0.453)

3.5 Effects of Conventional Risk Factors with Respect to Tyr113His and His139Arg Polymorphisms of *EPHX1* Gene and Arg287Gln Polymorphism of *EPHX2* Gene in Ischemic Stroke Patients and Controls

The conventional vascular risk factors namely hypertension, diabetes, obesity and smoking were analyzed in terms of proportion of ischemic stroke patients to the controls according to the genotype groups. First group were composed of homozygous and mutant heterozygous genotypes because number of subjects with mutant homozygous population was not satisfactory to carry out statistical analysis for all three polymorphisms. Second group was ascribed as wild type homozygous individuals with respect to their genotype (Table 3.10-3.11).

Table 3.10 Stratifications of hypertensive vs. normotensive groups and diabetic vs. non-diabetic groups in terms of Tyr113His, His139Arg and Arg287Gln polymorphisms and stroke-control status.

		Hypertensive Patient/Control	Normotensive Patient/Control	OR (95% CI)	*P*	Diabetic Patient/Control	Non-diabetic Patient/Control	OR (95% CI)	*P*
Tyr113His polymorphism	TT	87/25	39/36	3.21[a] (1.703-6.06)	0.00	44/12	82/49	2.19[b] (1.06-4.55)	0.03
	TC+CC	74/27	37/32	2.37[a] (1.24-4.52)	0.00	36/11	75/48	2.09[b] (0.97-4.51)	0.06
His139Arg polymorphism	AA	113/33	50/46	3.15[a] (1.80-5.50)	0.03	56/15	107/64	2.23[b] (1.17-4.27)	0.01
	AG+GG	48/19	26/22	2.14[a] (0.98-4.65)	0.05	24/8	50/33	1.98[b] (0.79-4.93)	0.12
Arg287Gln polymorphism	GG	135/44	67/55	2.52[a] (1.54-4.12)	0.00	70/21	132/78	1.97[b] (1.12-3.46)	0.02
	GA+AA	26/8	9/13	4.69[a] (1.47-15.00)	0.01	10/2	25/19	3.8[b] (0.74-19.42)	0.09

[a]OR calculated against normotensive, [b]OR calculated against non-diabetic

Table 3.11 Stratifications of smoker vs. non-smoker groups and obese vs. non-obese groups in terms of Tyr113His, His139Arg and Arg287Gln polymorphisms and stroke-control status.

		Smoker Patient/Control	Non-smoker Patient/Control	OR (95% CI)	*P*	Obese Patient/Control	Non-obese Patient/Control	OR (95% CI)	*P*
Tyr113His polymorphism	TT	33/6	93/55	3.25[a] (1.28-8.26)	0.01	34/5	92/56	4.14[b] (1.53-11.20)	0.00
	TC+CC	32/6	79/53	3.58[a] (1.40-9.15)	0.01	19/4	92/55	2.84[b] (0.92-8.78)	0.06
His139Arg polymorphism	AA	46/6	117/73	4.78[a] (1.95-11.76)	0.00	43/7	120/72	3.69[b] (1.57-8.63)	0.00
	AG+GG	19/6	55/35	2.02[a] (0.73-5.54)	0.17	10/2	64/39	3.05[b] (0.63-14.64)	0.15
Arg287Gln polymorphism	GG	52/11	150/88	2.77[a] (1.37-5.59)	0.00	44/9	158/90	2.78[b] (1.30- 5.97)	0.01
	GA+AA	13/1	22/20	11.81[a] (1.42-98.67)	0.01	9/0	26/21	-	-

[a]OR calculated against non-smoker, [b]OR calculated against non-obese

Risk analysis showed that having hypertension increases the incidence of ischemic stroke at least two times, regardless of genetic background (Table 3.10). Comparison of hypertensive patients/controls with normotensive patients/controls having wild type homozygous genotype (TT) for Tyr113His polymorphism results in 3.21 times higher risk for having stroke (*P*=0.00). Similarly, individuals with heterozygous and mutant homozygous genotype (TC+CC) in terms of Tyr113His polymorphism and hypertension were found to at risk of having stroke 2.37 times more than their counterpart (*P*=0.00). Analyses for His139Arg polymorphism for heterozygous and mutant homozygous genotype (AG+GG) showed that risk of having stroke is 2.14 times higher in hypertensive subjects than normotensive ones (*P*=0.05). In addition, risk analysis for wild type homozygous group revealed that hypertensive individuals with this genotype (AA) are prone to develop stroke 3.15 times more than normotensive individuals with the same genotype (*P*=0.03). Finally, effect of hypertension on having stroke was evaluated for different genotypes of Arg287Gln polymorphism. It was found that risk of having stroke is 2.52 times more for wild type homozygous genotype group (GG) and 4.69 times more for heterozygous and mutant homozygous genotype group (GA+AA) when hypertensive and normotensive population are compared in terms of stroke. (*P*=0.00, *P*=0.01).

Effect of diabetes on stroke was analyzed separately for heterozygous and mutant homozygous and wild type homozygous genotype groups of each polymorphism (Table 3.10). Risk analysis of Tyr113His polymorphism revealed that having diabetes insignificantly increases the risk of having stroke in the TC+CC genotypes group (OR=2.09; 95%CI=0.97-4.51; *P*= 0.06). On the other hand, analysis of wild type homozygous group (TT) in terms of Tyr113His polymorphism resulted that risk of having stroke is almost 2-fold higher for individuals with diabetes (*P*=0.03). The situation observed in Tyr113His polymorphism also holds for His139Arg polymorphism. It was observed that diabetes increases risk of having stroke in group of heterozygous and mutant

homozygous (AG+GG) individuals insignificantly (OR=1.98; 95% CI=0.79-4.93; *P*=0.12) while, risk is also increased more than two-fold in group of wild type homozygous (AA) ones but this time in significant manner (OR=2.23; 95% CI=1.17-4.27; *P*=0.01). The results of the Arg287Gln polymorphism were observed in same fashion with other polymorphisms in terms of diabetes. Once again diabetes is increasing the risk of stroke in both heterozygous (GA) and wild type homozygous (GG) genotypes but the former one is found to be statistically insignificant. Although the odds ratio is 3.8 for heterozygous genotype, result is not reliable (*P*=0.09). On the other hand, wild type homozygous genotype with lower odds ratio (1.97) than former one increases risk of stroke incidence two times (*P*=0.02).

Table 3.11 summerizes the stratification analysis held on smoker vs. non-smoker and obese vs. non-obese groups in terms of all three polymorphisms. Evaluation of smoking on stroke risk assessment in terms of Tyr113His polymorphism revealed that smoking significantly increases the risk of stroke development for more than three folds for both groups (TC+CC and TT) (*P*=0.01, *P*=0.01). Risk analysis on His139Arg polymorphism and smoking suggested that smoking increases the risk of having stroke almost five times for wild type homozygous genotype (AA) group (*P*=0.00) while, it is insignificantly increased for heterozygous and mutant homozygous genotype (AG+GG) group (OR=2.02; 95% CI=0.73-5.54; *P*=0.17). Effect of smoking on stroke incidence was determined for Arg287Gln polymorphism as well. Analysis on wild type homozygous genotype (GG) group showed that smoking increases the risk of having stroke about three times (*P*=0.00). Most dramatic effect of the tobacco usage was observed in heterozygous and mutant homozygous genotype (GA+AA) group of this polymorphism; smoking suggested to increase stroke risk for almost twelve times (*P*=0.01).

Risk analysis showed that having obesity increases the incidence of ischemic stroke, regardless of genetic background (Table 3.11). Comparison of obese group with non-obese group in terms of wild type homozygous (TT) genotype for Tyr113His polymorphism results in 4.14 times higher risk for having stroke (*P*=0.00). Similarly, individuals with risk of having stroke 2.84 times more in non-obese group (*P*=0.06). Analyses for His139Arg polymorphism for wild type homozygous genotype (AA) showed that risk of having stroke is 3.05 times higher for obese ones than non-obese ones but this result was found to be insignificant (*P*=0.15). In addition, risk analysis for wild type homozygous genotype (AA) group revealed that obese individuals with wild type homozygous genotype are prone to develop stroke 3.69 times more than their counterpart (*P*=0.00). Finally, effect of obesity on having stroke was evaluated for Arg287Gln polymorphism. It was found that risk of having stroke is 2.78 times more for wild type homozygous genotype (GG) while, risk analysis on heterozygous and mutant homozygous genotype (GA+AA) group cannot be calculated due to lack of obese control (*P*=0.01).

3.6 Logistic Regression Analysis

Logistic regression analyses with backward selection method (Backward likelihood ratio) were used to ascertain the effects of gender, age, diabetes, hypertension, obesity, smoking status, lipid parameters and genotypes in the stroke susceptibility.

Model 1

The first model was established with gender, age, diabetes, hypertension, obesity, smoking status, total cholesterol, triglyceride, LDL-cholesterol, HDL-cholesterol,

Tyr113His genotype, His139Arg genotype and Arg287Gln genotype covariates. Hypertension (OR=3.19; 95%CI, 1.92-5.30, P=0.00), smoking (OR=3.46; 95%CI, 1.66-7.21, P=0.00) and LDL (OR=1.46; 95%CI, 1.12-1.89, P=0.00) were found to be significant risk factors for ischemic stroke. In contrast, HDL (OR=0.27; 95%CI, 0.11-0.65, P=0.00) was found to be a protective factor for development of the disorder (Table 3.12).

Overall percentage of the correct cases was reported as 73.1% and Hosmer-Lemeshow goodness of fit test reveals that chi-square was 6.721 with 8 degrees of freedom while, significance was 0.567.

Table 3.12 Logistic regression analysis of gender, age, hypertension, obesity, smoking status, total cholesterol, triglyceride, LDL-cholesterol, HDL-cholesterol, Tyr113His genotype, His139Arg genotype and Arg287Gln genotype in all subjects.

Parameters	**OR**	**95% CI**	***P***
Hypertension	3.19	1.92-5.30	0.00
Smoking	3.46	1.66-7.21	0.00
LDL-cholesterol	1.46	1.12-1.89	0.00
HDL-cholesterol	0.27	0.12-0.65	0.02

Model 2

The second model was established with male subjects and age, diabetes, hypertension, obesity, smoking status, total cholesterol, triglyceride, LDL-cholesterol, HDL-cholesterol, Tyr113His genotype, His139Arg genotype and Arg287Gln genotype were selected as covariates. Age (OR=0.97; 95%CI, 0.94-0.99, P=0.02), hypertension (OR=3.43; 95%CI, 1.65-7.12, P=0.00), obesity

(OR=5.21; 95%CI, 1.13-23.99, P=0.03) and smoking (OR=3.19; 95%CI, 1.36-7.47, P=0.01) were found to be significant risk factors for ischemic stroke (Table 3.13).

Overall percentage of the correct cases was reported as 74.2% and Hosmer-Lemeshow goodness of fit test reveals that chi-square was 5.499 with 8 degrees of freedom while, significance was 0.703.

Table 3.13 Logistic regression analysis of age, diabetes, hypertension, obesity, smoking status, total cholesterol, triglyceride, LDL-cholesterol, HDL-cholesterol, Tyr113His genotype, His139Arg genotype and Arg287Gln genotype in male subjects.

Parameters	**OR**	**95% CI**	***P***
Age	0.97	0.94-0.99	0.02
Hypertension	3.43	1.65-7.12	0.00
Obesity	5.21	1.13-23.99	0.03
Smoking	3.19	1.36-7.47	0.01

Model 3

The third model was established with female subjects and age, diabetes, hypertension, obesity, smoking status, total cholesterol, triglyceride, LDL-cholesterol, HDL-cholesterol, Tyr113His genotype, His139Arg genotype and Arg287Gln genotype were selected as covariates. Hypertension (OR=4.27; 95%CI, 1.92-9.48, *P*=0.00), diabetes (OR=2.78 95%CI 1.15-6.75, *P*=0.02), smoking (OR=8.16; 95%CI, 1.71-38.99, *P*=0.01), LDL-cholesterol (OR=2.02; 95%CI, 1.31-3.11, *P*=0.00) were found to be significant risk factors for ischemic

stroke whereas, HDL-cholesterol (OR=0.16; 95%CI, 0.04-0.60, *P*=0.01) was found to be significant protective risk factor (Table 3.12).

Overall percentage of the correct cases was reported as 74.2% and Hosmer-Lemeshow goodness of fit test reveals that chi-square was 4.170 with 8 degrees of freedom while, significance was 0.841.

Table 3.14 Logistic regression analysis of age, diabetes, hypertension, obesity, smoking status, total cholesterol, triglyceride, LDL-cholesterol, HDL-cholesterol, Tyr113His genotype, His139Arg genotype and Arg287Gln genotype in female subjects.

Parameters	**OR**	**95% CI**	***P***
Hypertension	4.27	1.92-9.48	0.00
Diabetes	2.78	1.15-6.75	0.02
Smoking	8.16	1.71-38.99	0.01
LDL-cholesterol	2.02	1.31-3.11	0.00
HDL-cholesterol	0.16	0.04-0.60	0.01

CHAPTER 4

DISCUSSION

Ischemic stroke is caused by blockage of arteries which supplies blood in other words oxygen and nutrients to the brain. Obstruction within an artery is generally originated from disruption of atherosclerotic lesions which are asymmetrical focal thickenings of the intima composed of lipids, cells, debris and connective tissue elements. Several risk factors, which are players of a complex network, have been associated with development of stroke. These factors are grouped into three; alterable risk factors, unalterable risk factors and the others. Hypertension, diabetes, smoking and obesity are examples of modifiable ones whereas; age, gender and ethnic origin are listed in the non-modifiable ones. Socioeconomic factors, geographic location, alcohol or drug abuse were reported as other risk factors. Identification and investigation of these factors and their relations with genetic factors not only improves our understanding of underlying mechanism of this complex disorder, but also gives rise to development of new treatment strategies.

Soluble epoxide hydrolase is responsible for conversion of epoxyeicosatrienoic acids (EETs) to their corresponding diols. EETs are potent vasodilators that participate in the regulation of vascular tone and cerebral blood flow. Consequently, they were suspected to play a role in predisposition to and/or recovery from cerebrovascular injury (Zhang L. et al. 2008a). One of the six single nucleotide polymorphisms that results in amino acid substitution have been shown to cause a significant reduce in enzyme activity. Arg287Gln polymorphism

results in 25-75% decrease in enzyme activity which is expected to be a preventive effect on disease susceptibility (Przybyla-Zawislak et al. 2003).

Microsomal epoxide hydrolase (mEH) is a crucial phase I enzyme that catalyzes the conversion of various xenobiotic epoxides and polycyclic aromatic hydrocarbons (PAHs) to more polar diol metabolites. Hydrolysis of epoxides through the action of mEH might be result in either detoxification or activation of active mutagen compounds. If the conversion of epoxides to less active diol intermediates is followed by further reactions by CYPs, highly mutagenic and toxic compounds are produced. In contrast, if the diol metabolites is conjugated with phase II enzymes such as GSTs, detoxification takes place. The activity levels of mEH in elimination or production of carcinogens is not very clear. Two of the SNPs with opposite influences on enzyme activity namely Tyr113His and His139Arg were studied to understand the nature of the enzyme in different conditions.

The aim of this study is to investigate the possible associations between interested single nucleotide polymorphisms of soluble and microsomal epoxide hydrolases and ischemic stroke risk in Turkish population. The relation of ischemic stroke with lipid parameters, conventional risk factors and three polymorphisms were evaluated as well.

The study population with 237 ischemic stroke patients and 120 healthy controls were considered to satisfy the requirements that mentioned in previous chapters. Stroke is a late onset disorder and its already known that age is one of the determinants in development of the disorder. Thus, there should not be any significant difference between ages of patients and controls to make meaningful conclusions. Representation of two genders in significant numbers is another important aspect of association studies. Our study population was consisted of patients and controls with similar proportions of female subjects to male subjects (58.2% vs. 46.6%).

It is known that males prone to develop stroke more than females (Stegmayr et al. 1997). Our investigation on male individulas confirms this statement. It was found that number of males in patient group is 1.5 times more than the number of males in control group (P=0.038).

The effect of the conventional risk factors were found to be significant on development of the ischemic stroke according to the comparison of the patient and control groups in terms of hypertension, diabetes, smoking status and obesity. It is accepted that these factors increase the stroke susceptibility. Our findings about all of these factors were found in a parallel fashion with previous statements in a significant way as expected.

Previous studies on the relation of high blood pressure and stroke revealed that stroke incidence is proportional to the level of hypertension (Hebert et al. 1988). Hypertension was found as a significant risk factor for stroke susceptibility and current study estimated the relative risk as 2.770 based on comparison of patient group with the controls (P=0.000). The general statement on association between these two health problems was confirmed in our study.

Diabetes is another conventional risk factor of the ischemic stroke. It was proposed that negative effect of diabetes on ischemic stroke is mediated by high blood pressure, high blood cholesterol and obesity which increase likelihood of having stroke. Analysis of the results in our study group showed that 33.7% of the stroke patients and 19.1% of the controls suffer from this disorder. Comparison reveals that the frequency of diabetic people was significantly higher in patient group then controls (P=0.003). Estimated relative risk of ischemic stroke according to diabetes mellitus was found to be more then 2-fold based on comparison of patient group with the controls.

Tobacco usage was also found to be a significant conventional risk factor in terms of stroke development. In this study, it was found that smoking increases stroke

susceptibility almost three times according to comparison of smoker patients (27.4%) with controls (10%) and the frequency of smoking was significantly higher in patient group (P=0.000).

It is known that obesity generally brings the burden of elevated blood cholesterol, hypertension and diabetes. These side effects of the obesity contribute the elevation of ischemic stroke risk in obese people. The association between body mass index (kg/m^2) and ischemic stroke was found to be significantly increasing the ischemic stroke risk. The percentage of obesity in patients was reported as 22.3%, while it is 7.5% in controls. Obesity exhibited more then 3-fold relative risk in patients. The frequency of the obesity in patients was found to be significantly higher than obesity in controls (P=0.000).

Possible associations between blood cholesterol level and stroke were investigated in several studies. Clinical characteristics of patient group were also compared with the controls (Table 3.2). Elevated total cholesterol level was reported to be weakly associated with nonfatal ischemic stroke risk by other groups and result of our study on effect of total cholesterol level as a risk factor on ischemic stroke susceptibility supports this hypothesis. Total cholesterol (4.8 ± 1.3 vs. 4.6 ± 1.3) and triglycerides (1.4 ± 0.2 vs. 1.3 ± 0.2) levels were not found to significantly differ between patient and control groups. On the other hand, results reveal that HDL-cholesterol level of the patient group (1.1 ± 0.3 mmol/L) is significantly lower than that of controls (1.2 ± 0.3 mmol/L, P=0.003), while LDL-cholesterol level of the patient group (2.9 ± 1.1 mmol/L) is significantly higher than that of controls (2.7 ± 1.0 mmol/L, P=0.009) as expected. Cholesterol which is one of the components in atherosclerotic lesions is a sticky substance and travelled by carrier lipoproteins in blood stream. LDL is responsible for distribution of cholesterol to the different parts of the body while HDL is responsible for collection of excess amounts. It is not wrong to hypostatize that increased levels of LDL-cholesterol and/or decreased levels of HDL-cholesterol promotes the possible accumulation

of debris within the atheroma. Our findings on LDL and HDL seem to be reasonable in this perspective.

Genotype distribution and allele frequencies differ among the populations with different ethnic origin. Collected data is used to map distribution of genetic variation over the world so; determination of these aspects in each population for interested polymorphisms has another scientific merit. The allele frequencies of all three polymorphisms in healthy members of Turkish population were determined in this study and compared with other populations.

The comparison of Tyr113His polymorphism of *EPHX1* in Turkish population with previous studies and different ethnic population is represented in Table 4.1. The mutant allele frequency was found to be similar to that were found in other Turkish studies except from the results of the very recent one (Ada et al. 2007, Pınarbaşı et al. 2007, Erkişi et al. 2010). It is natural to see neglectable variations within the same population due to different sizes of study populations and ethnic composition. However, study of Erkişi et al. on Tyr113His polymorphism in Turkish population indicates a distant difference. Small population size (41 individuals) should result in representation of a minor group in population or possible non-homogenous sampling of individuals in their study might explain this distinction. The allele frequencies in Turkish population are generally found to be consistent with Caucasians. In this study, mutant allele frequency of our population was found to be highly similar with those of German, British and French people (Smith and Harrison 1997,Harms et al. 2004, Clavel et al. 2005). Lastly, mutant allele frequencies in Asian populations were found to be slightly higher than that of determined in our study (Cheng et al. 2004, Xiao et al. 2004, Yoshikawa et al. 2000).

Table 4.1 Comparison of allele frequencies of *EPHX1* Tyr113His single nucleotide polymorphism in different populations.

Populations	Tyr113His Polymorphism		N	Reference
	Wild Type	Mutant Allele		
Turkish	**0.69**	**0.31**	**120**	**This study**
Turkish	0.65	0.35	310	Pınarbaşı et al., 2007
Turkish	0.72	0.28	266	Ada et al., 2007
Turkish	0.33	0.67	41	Erkişi et al.,2010
German	0.74	0.26	238	Harms et al., 2004
France	0.65	0.35	210	Clavel et al.,2005
British	0.69	0.31	406	Smith, Harrison, 1997
Taiwan	0.52	0.48	424	Cheng et al., 2004
Chinese	0.55	0.45	200	Xiao et al. 2004
Japanese	0.56	0.44	716	Yoshikawa et al.,2000

The comparison of His139Arg polymorphism of *EPHX1* in Turkish population with previous studies and different ethnic population is represented in Table 4.2. The mutant allele frequency in terms of His139Arg single nucleotide polymorphism was found to less than one of Tyr113His polymorphism. Our findings on allele frequencies of His139Arg are similar to that were found in other Turkish studies and Caucasian nations (Smith and Harrison 1997, Harms et al. 2004, Clavel et al. 2005, Ada et al. 2007, Pınarbaşı et al. 2007). Only exception was seen in results of Erkişi et al. in Turkish population as in Tyr113His polymorphism that discussed before (Erkişi et al. 2010). In addition to this, mutant allele frequencies in Asian populations other than Chinese population were

found to be similar with that of determined in our study (Cheng et al. 2004, Xiao et al. 2004, Yoshikawa et al. 2000).

Table 4.2 Comparison of allele frequencies of *EPHX1* His139Arg single nucleotide poymorphism in different populations.

Populations	His139Arg Polymorphism		N	Reference
	Wild Type	Mutant Allele		
Turkish	**0.81**	**0.19**	**120**	**This study**
Turkish	0.87	0.13	310	Pınarbaşı et al., 2007
Turkish	0.84	0.16	266	Ada et al., 2007
Turkish	0.75	0.25	41	Erkişi et al., 2010
German	0.82	0.18	238	Harms et al., 2004
France	0.79	0.21	210	Clavel et al.,2005
British	0.86	0.14	406	Smith, Harrison, 1997
Taiwan	0.81	0.19	424	Cheng et al., 2004
Chinese	0.91	0.09	200	Xiao et al. 2004
Japanese	0.86	0.14	716	Yoshikawa et al.,2000

The comparison of Arg287His polymorphism of *EPHX2* in Turkish population with different ethnic groups is represented in Table 4.3. Arg287Gln polymorphism has been associated with protective effect on stroke development and low mutant allele frequency in the population might be one of the contributors of high susceptibility of a population to ischemic stroke. Although there are

several studies on the association between Arg287Gln polymorphism and ischemic stroke risk in literature, just a few publications on population studies are available.

Table 4.3 Comparison of allele frequencies of *EPHX2* Arg287Gln single nucleotide poymorphism in different populations

Populations	Arg287Gln Polymorphism		N	Reference
	Wild Type	Mutant Allele		
Turkish	**0.91**	**0.09**	**120**	**This study**
African-American	0.92	0.08	412	Fornage et al, 2004
Caucasian	0.81	0.19	310	Fornage et al., 2004
African-American	0.91	0.09	1337	Wei et al, 2006
Caucasian	0.84	0.16	1645	Wei et al., 2006
White Europeans	0.84	0.16	736	Gschwendtner et al., 2008
Chinese	0.76	0.24	458	Sun et al., 2008

The frequency of mutant allele of Turkish population was found to be very similar to African-American population which stated to be more prone to suffer from ischemic stroke than other populations (Fornage et al. 2004, Wei et al., 2005). On the other hand, mutant allele frequency of Turkish population was found to be distant from other Caucasian populations (Fornage et al. 2004, Wei et al., 2005,

Gschwendtner et al. 2008). Meta analysis of Fornage et al. on Arg287Gln was carried on in United States of America where people with different ethnicity lives. The distinction of subjects as Caucasians was possibly made by phenotypic characteristics and this group might represent a genetic mosaic. Similar problem might be seen in study on allele frequencies of white Europeans. Mutant allele frequency in Chinese population was found to be higher than the one determined in Turks as well (Sun et al. 2007). The new data on Arg287Gln polymorphism in Turkish population would clarify our understanding about reasons of the allele frequency differences.

Comparison of Tyr113His genotype and allele distributions of ischemic stroke patients and control revealed that there is not any significant difference between two groups in neither the genotype nor the allele distributions. Genotype distribution in patients and controls was found as following; 53.1% vs. 50.8% homozygous wild type, 38.8% vs. 36.6% heterozygous and 8.0% vs. 12.5% mutant homozygous, respectively. Minor allele frequency of Tyr113His was 0.278 for patients and 0.308 for controls, no significant difference between two groups is observed. His139Arg genotypes and alleles were compared and contrasted as well. The genotype distributions of patients and controls were found highly similar for all three genotypes (AA, AG and GG) and statistically there was not any significant difference between genotype profiles of these groups. Thus, minor allele frequencies of patients and controls are almost same (0.175 and 0.192, respectively). These two polymorphisms were investigated to evaluate the possible associations between variations of mEH and stroke risk for the first time hence; there is not any available study in literature to compare with our findings. Microsomal epoxide hydrolase enzyme plays a dual role in the metabolism of hazardous compounds. Conjugation of mEH with phase II enzymes such as GSTs results in detoxification of the carcinogens alternatively, conjugation might take place with CYPs and more mutagenic compounds should be produced. This complicated mechanism prevents us to make an strict conclusion on effects of the

variations in enzyme activity. Both slow (Tyr113His) and fast (His139Arg) enzyme variants were not found to be significantly associated with ischemic stroke risk. Previous studies on association of these SNPs with various types of disorders reveals that risk assessments should be done carefully. Not only polymorphisms on mEH but also polymorphisms on its conjugated enzymes in other words, more complex network should be taken in account and investigation of combined effects of polymorphisms might be more meaningful.

Investigation on Arg287Gln polymorphism and effect of the genotypes in ischemic stroke risk shows that genotype (OR=0.817; 95%CI=0.452-1.477; P=0.503) and allele frequency (OR=0.913; 95%CI=0.530-1.575; P=0.744) distributions are in a similar fashion. Comparison of the patients and controls in terms of mentioned characteristics observed as statistically insignificant. Arg287Gln variant of sEH is characterized with decreased enzyme activity and increased EET levels *in vivo.* Anti-inflammatory and vasodilatory effects of EETs in body are proposed to have a protective effect against ischemic injury which supported by studies in animals. However, our findings did not show this and effect of Arg287Gln allele on ischemic stroke risk is almost same with wild type allele. A study on white Europeans with 10,352 subjects revealed an increased risk of ischemic stroke was found to be associated with Arg287Gln polymorphism (Gschwendtner et al. 2008). In addition, the coronary artery risk development in young adults (CARDIA) study that carried out by Fornage et al., 2004 to identify possible associations between artery calcification and Arg287Gln polymorphism was in line with findings on white Europeans (Fornage et al. 2004). Further research on effect of this SNP on enzyme activity and possible gene interactions should improve our understanding about the mechanism.

Effects of well established ischemic stroke risk factors were investigated to determine possible associations between interested risk factor and genotypes in our study population. Four different subgroup analyses were held in hypertensive

vs. normotensive (Table 3.6), smoker vs. non-smoker (Table 3.7), obese vs. non-obese (Table3.8) and diabetic vs. non-diabetic (Table 3.9) subgroups. Even though effects of hypertension, smoking, obesity and diabetes on ischemic stroke risk were determined significantly in comparison of patient group with control population (Table 3.1), subgroup analysis which compares cases in terms of same vascular factors suggest that there is not any significant difference based on genotypes and allele frequencies.

Population stratification was done to analyze the effects of the conventional vascular risk factors in detail. Risk analyses of population who have the same genotype were done by comparing stroke vs. control cases with respect to hypertension, diabetes, smoking status and obesity (Tables 3.10-3.11). The conventional vascular risk factors were analyzed in terms of proportion of ischemic stroke patients to the controls for mutant and heterozygous genotype group and wild type homozygous genotype groups. First genotype group was composed of homozygous and mutant heterozygous genotypes because number of subjects with mutant homozygous population was not statistically significant for all three polymorphisms.

Hypertension was found to have a significant impact on development of the ischemic stroke regardless of the genetic background. The risk of having stroke in hypertensive individuals is 3.21 fold higher than normotensive ones within the wild type homozygous genotype group of Tyr113His polymorphism (P=0.00). The same risk is 2.37 for the other genotype group of Tyr113His polymorphism (P=0.00). Similar situation was observed for His139Arg polymorphism as well; risk analysis revealed that hypertensive individuals with His139Arg wild type homozygous genotype are 3.15 times more prone to develop stroke while, it is 2.14 for the second genotype group (P=0.00, P=0.05). These two polymorphisms have contrasting influences on enzyme activity but, interestingly wild type enzyme increases the stroke susceptibility more than fast or slow variants when

hypertension is the case. This situation may become clear with a combined analysis because existence of both polymorphisms on the same gene might result in wild type like phenotype. Finally, the relation between Arg287Gln genotypes and ischemic stroke risk was investigated. It was found that hypertensive individuals with wild type Arg287Gln genotype are 2.52 times more prone to develop stroke when, risk is doubled for the ones with heterozygous and mutated genotype (P=0.00, P=0.01). In this case, reduced sEH activity was found to cause a slight decrease in stroke risk in hypertensives. Investigation on roles and interactions of sEH with lipid metabolism by Newman et al.,2005 demonstrates the possible influence of sEH activity as a modifier of blood pressure (Newman et al., 2005). We can conclude that reduced sEH activity may alter the levels of blood pressure and decrease the negative effect of hypertension on stroke development.

Risk assessment on diabetes was also stratified as well. Analysis on Tyr113His wild type homozygous genotype group showed that stroke risk is two times elevated when people suffer from diabetes (P=0.03). Although the same situation is in account for the other genotypes, result is not found to be statistically significant (P=0.06). The risk of having stroke of diabetic individuals in the wild type homozygous group of His139Arg polymorphism is twice as much as ones without diabetes (P=0.03). Even though the risk assessment for latter group was found in a similar way with former group, result was insignificant (P=0.12). These results imply that neither decrease nor increase in mEH activity modifies the ischemic stroke risk of diabetic individuals. Analyses on Arg287Gln polymorphism showed that diabetes doubles the stroke incidence for ones with wild type genotype while risk is 3.8 higher for the ones in other genotypes group in an insignificant way (P=0.02, P=0.09). It was reported that Arg287Gln polymorphism and ischemic stroke risk was associated in African American subjects as well but the mechanism remains unclear (Wei et al., 2007).

Tobacco usage was expected to increase stroke incidence more other vascular risk factors with respect to interested genotypes because, subjected microsomal epoxide hydrolase enzyme is directly related with metabolism of polyaromatic hydrocarbons that involved in cigarette smoke with more than 90 hazardous compounds. Changes in enzyme activity of microsomal epoxide hydrolase are expected to have an impact on harmful effects of smoking. Analysis on the first polymorphism on mEH, Tyr113His revealed that risk of having stroke is 3.25 when ones with wild type genotype are smoking and it is 3.58 for the ones with other genotypes (P=0.01, P=0.01). The risk of stroke was found to be 4.78 times increased in the case of smoking within the wild type genotype group of His139Arg polymorphism in a significant manner while, risk is insignificantly increased for members of risky genotype group (P=0.00, P=0.17). Finally, relation between stroke risk and smoking with respect to Arg287Gln polymorphism of sEH was investigated. It is known that exposure to cigarette smoke promotes the expression of soluble epoxide hydrolase significantly. This up regulation suggests a possible role of sEH in prevention of hazardous effects of smoking. It was found that smoking increases the risk 2.77 times for wild type genotype group (P=0.00). Most dramatic stroke risk was observed in heterozygous and mutated genotypes of Arg287Gln polymorphism which is almost 12 fold increased with smoking (P=0.01). None the less reduced enzyme activity is expected to have a protective effect against ischemic stroke development, decreased detoxification of hazardous components in cigarette smoke increases the risk. This result indicates the importance of interactions among genetic and environmental factors in development of ischemic stroke.

Body mass index (kg/m^2) was found to be associated with ischemic stroke risk by elevated blood cholesterol, hypertension and diabetes. Risk analysis showed that having obesity increases the incidence of ischemic stroke, regardless of genetic background (Table 3.11). Comparison of obese group with non-obese group in terms of wild type homozygous genotype for Tyr113His polymorphism results in

4.14 times higher risk for having stroke (*P*=0.00). Similarly, individuals with other genotypes in terms of Tyr113His polymorphism were found to be 2.84 times more at risk of having stroke than non-obese group (*P*=0.06). Analyses for His139Arg polymorphism for heterozygous and mutant genotypes showed that risk of having stroke is 3.05 times higher for obese individuals than non-obese ones but this result was found to be insignificant as well (*P*=0.15). In addition, risk analysis for the other group revealed that obese individuals with wild type homozygous genotype are prone to develop stroke 3.69 times more than their counterpart (*P*=0.00). Finally, effect of obesity on having stroke was evaluated for Arg287Gln polymorphism. It was found that risk of having stroke is 2.78 times more for wild type genotype while, risk analysis on other genotypes cannot be calculated due to lack of obese control (*P*=0.01). Results show that obesity has a negative impact on vascular health which might be resulted from elevation of fat deposits in body and promotion of effects of diabetes and hypertension on ischemic stroke risk.

Logistic regression analyses with backward selection method were used to address the effects of gender, age, diabetes, hypertension, obesity, smoking status, lipid parameters and genotypes in the stroke susceptibility. Three models were established to predict effect of the factors in overall population and to investigate role of the parameters in gender dependent manner. Result of the first analysis in all subjects denoted that hypertension, obesity, smoking and LDL cholesterol were significant risk factors for ischemic stroke. In contrast, HDL cholesterol was found to be a protective factor for development of the disorder (Table 3.10). Strong effects of hypertension, smoking, obesity, diabetes, LDL cholesterol and HDL cholesterol on ischemic stroke were stated previously. Conclusion of binary logistic regression test regardless of the gender exposed well established risk factors; hypertension, obesity, smoking, LDL cholesterol and a protective factor against the development of the disorder which is HDL cholesterol. These findings overlap with the expectations because these factors are strongest determinants in

stroke risk and protection. The second model was established with male subjects who are expected to be more prone to develop stroke. Age, hypertension, obesity and smoking were found to be significant risk factors for ischemic stroke (Table 3.11). Males are more susceptible to suffer from stroke and regression analysis denoted that hypertension, smoking, obesity and age are the determinants of the stroke development in this group. It is known that every decade of life doubles the risk of ischemic stroke after the age of 55 and stroke incidence rates of males are greater than women's at younger ages while, it becomes closer with aging. The third model was established with female subjects and hypertension, diabetes, smoking, LDL cholesterol were found to be significant risk factors for ischemic stroke whereas, HDL cholesterol and Arg287Gln mutant genotype were found to be protective risk factors (Table 3.12). Women are expected to be less effected from obesity than males because estrogen hormone which is abundant in woman promotes vascular flexibility. In this group hypertension, diabetes, smoking, LDL cholesterol and HDL cholesterol were found to be significantly associated with stroke.

CHAPTER 5

CONCLUSION

Microsomal epoxide hydrolase (*EPHX1*) is a critical phase I enzyme that catalyzes the conversion of various xenobiotic epoxide substrates and polycyclic aromatic hydrocarbons (PAHs) to more polar diol metabolites. Hydrolysis of epoxides through the action of *EPHX1* enzyme gives rise to either detoxification after conjugation by phase II enzymes or production of highly carcinogenic compounds. Metabolisms of carcinogenic and mutagenic compounds in tobacco smoke and variations in its activity have been associated with development atherosclerotic lesions caused by such compounds. Studies suggest that mutagens found in tobacco smoke stimulate the formation of DNA adducts, which lead to genetic alterations in blood vessels. Animal studies show that tobacco smoke mutagens such as PAHs and heterocyclic amines directly increase the development of atherosclerotic lesions. Two of the single nucleotide polymorphisms in *EPHX1* gene result in critical amino acid substitutions; Tyr113His causes reduction in enzyme activity, while His139Arg results in increase in enzyme activity

Soluble epoxide hydrolase (*EPHX2*) enzyme catalyzes the conversion of active epoxides to less active polar diols. One of these substrates of sEH is epoxyeicosatrienoic acids (EETs) which are lipid metabolites of arachidonic acid, synthesized in vascular endothelial cells by the cytochrome P450 system. EETs are potent vasodilators that participate in the regulation of vascular tone and they have been shown to regulate cerebral blood flow. Consequently, they are suspected to play a role in predisposition to and/or recovery from cerebrovascular injury. Hydrolysis of the EETs to their corresponding diols by sEH regulates

EETs levels and represents a major mechanism by which the biological effects of EETs are attenuated. Arg287Gln polymorphism within *EPHX2* gene results in alteration in its corresponding enzyme activity.

Blood samples of 237 ischemic stroke patients and 120 controls were collected and all polymorphisms were determined by PCR-RFLP method. There was not any significant difference between two groups in terms of mean value of the age (P=0.444). Analysis revealed that number of individuals with hypertension, diabetes mellitus, smoking status and obesity in patient group was significantly higher than ones in the control group. In addition, mean LDL-cholesterol level of patient group was found to be significantly higher when compared to the control group. On the contrary, HDL-cholesterol level of the patients was fond to be significantly lower than their counterpart.

Risky allele frequencies of Tyr113His polymorphism which is C allele were found as 0.274 for patient group and 0.308 for controls. Analysis revealed that there was not any significant difference between two groups. Risky allele frequencies in terms of His139Arg polymorphism (G) were found as 0.175 in patients and 0.192 in controls which was not found to be statistically significant. Finally, risky allele frequencies regarding Arg287Gln polymorphism (A) were found as 0.084 in patients and 0.092 in controls and it was found to be statistically insignificant as well. Therefore, we can conclude that having these risky alleles are not risk factors for the development of ischemic stroke.

Subgroup analyses with respect to hypertension, smoking, diabetes and obesity were carried out to determine the effect of these vascular risk factors. Analysis on hypertension revealed that having hypertension increases the risk of stroke for 2 times and hypertensive individuals within the wild type homozygous genotype groups of Tyr113His (TT) and His139Arg (AA) and heterozygous and mutant homozygous genotype group of Arg287Gln (GA+AA) are more prone to suffer from stroke. Tyr113His and His139Arg polymorphisms affect the enzyme activity

in opposite manners so, either increase or decrease in mEH activity was found to be related with increased risk of ischemic stroke. Heterozygus and mutant homozygous genotype of the Arg287Gln polymorphism was expected to have a protective effect on having stroke but this finding suggested that effect of hypertension on these variants of sEH is more remarkable than one on wild type enzyme. Investigation on diabetes showed that having risky or riskless genotypes do not affect the risk of having ischemic stroke, it doubles the risk regardless of the genotype. Smoking was found to be dramatically increasing the risk of stroke in patients with risky genotype pf Agr287Gln polymorphism which is about 12 times more than non-smoker patients.

Logistic regression analyses were used to ascertain the effects of vascular factors, lipid parameters and Tyr113His, His139Arg and Arg287Gln genotypes in the stroke susceptibility. LDL (OR=1.46; 95%CI, 1.12-1.89, *P*=0.00), smoking (OR=3.46; 95%CI, 1.66-7.21, *P*=0.00) and hypertension (OR=3.19; 95%CI, 1.92-5.30, *P*=0.00) were found to be significant risk factors for ischemic stroke, whereas HDL (OR=0.27; 95%CI, 0.12-0.65, *P*=0.02) was found to be a protective factor.

In this study, possible associations of Tyr113His and His139Arg polymorphisms of *EPHX1* gene and Arg287Gln polymorphism of *EPHX2* gene with ischemic stroke risk in Turkish population were investigated. Variability of allele frequencies in different populations and lack of studies about relation of these polymorphisms and ischemic stroke risk made determination of Tyr113His and His139Arg polymorphisms of *EPHX1* gene and Arg287Gln polymorphism of *EPHX2* gene and possible associations of them with ischemic stroke come into prominence. In this study, the relation of Tyr113His and His139Arg polymorphisms of *EPHX1* gene and risk of ischemic stroke is investigated for the first time in literature while, Arg287Gln polymorphism and ischemic stroke risk in Turkish population was studied for the first time.

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APPENDIX A

ETHICAL COMMITTEE APPROVAL FORM

HİZMETE ÖZEL

T.C.
GENELKURMAY BAŞKANLIĞI
GÜLHANE ASKERİ TIP AKADEMİSİ KOMUTANLIĞI
A N K A R A

22 Şubat 2008

Y. ETİK KRL. : 1491 - 567 - 08
KONU : GATA Etik Kurulu

Doç. Dr. Şeref DEMİRKAYA

20 Eylül 2005 tarihli 43. Oturumda GATA Etik Kurulu'ndan onay almış olan **"Paraoksonaz 1'in Aktivite ve Gen Polimorfizmlerinin İskemik Strok Üzerindeki Etkisinin Araştırılması"** başlıklı çalışmanın adının **"HMG-Co Redüktaz, Lesitin Kolesterol Asetil Transferaz, GST Transferazlar, Lipoproteinler ve Sitokrom P450 Enzimlerinin Genetik Polimorfizmlerinin İskemik Strok Üzerindeki Etkisinin Araştırılması"** olarak değiştirilmesi ile ilgili protokol değişikliği başvurunuz ile ilgili, GATA Etik Kurulu'nun kararı EK'tedir.

Rica ederim.

Ali Uğur URAL
Prof. Tbp. Kd. Alb.
GATA Etik Kurulu Başkanı

EK :
1 Adet Etik Kurul Raporu

HİZMETE ÖZEL

T.C.
GENELKURMAY BAŞKANLIĞI
GÜLHANE ASKERİ TIP AKADEMİSİ KOMUTANLIĞI
ETİK KURUL TOPLANTI RAPORU

OTURUM NO : 103
OTURUM TARİHİ : 15 Şubat 2008
OTURUM BAŞKANI : Prof. Tbp. Kd. Alb. Ali Uğur URAL
OTURUM SEKRETERİ : Doç. Dr. Ecz. Kd. Alb. Adnan ATAÇ

GATA Etik Kurulu'nun 15 Şubat 2008 günü yapılan 103. oturumunda; GATA Nöroloji AD'dan Doç.Dr. Şeref Deirkaya'nın sorumlu araştırmacılığını yaptığı 20 Eylül 2005 tarihli 43. Oturumda GATA Etik Kurulu'ndan onay almış olan **"Paraoksonaz 1'in Aktivite ve Gen Polimorfizmlerinin İskemik Strok Üzerindeki Etkisinin Araştırılması"** başlıklı çalışmanın adının **"HMG-Co Redüktaz, Lesitin Kolesterol Asetil Transferaz, GST Transferazlar, Lipoproteinler ve Sitokrom P450 Enzimlerinin Genetik Polimorfizmlerinin İskemik Strok Üzerindeki Etkisinin Araştırılması"** olarak değiştirilmesi ile ilgili protokol değişikliği değerlendirildi.

Protokol değişikliğinin amaç, yöntem ve yaklaşım bakımından etik ilkelere UYGUN olduğuna karar verildi.

BAŞKAN	**ÜYE**	**ÜYE**	**ÜYE**
Ali Uğur URAL Prof.Tbp.Kd.Alb.	Ali İhsan UZAR Prof.Hv.Tbp.Kd.Alb.	Ayhan KUBAR Prof.Tbp.Alb.	Adnan ATAÇ Doç.Dr.Ecz.Kd.Alb.
ÜYE	**ÜYE**	**ÜYE**	
Mükerrem SAFALI Doç.Tbp.Kd.Alb.	K. Melih AKAY Doç.Tbp.Kd. Alb.	Mustafa ÖZER Doç.Tbp.Alb.	
ÜYE	**ÜYE**	**ÜYE**	
Ergün TOZKOPARAN Doç.Tbp. Alb.	Nalan AKBAYRAK Prof. Dr. Sağ. Yb.	Muharrem UÇAR Yrd.Doç.J.Tbp.Yb.	

APPENDIX B

INFORMED CONSENT FOR PATIENTS

BİLGİLENDİRİLMİŞ ONAM (RIZA) FORMU

İnme-felç hastalığı için risk oluşturan faktörleri bulmak üzere yeni bir araştırma yapmaktayız. Araştırmanın ismi "Paroxsanaz 1'in Aktivite Ve Gen Polimorfizmlerinin İskemik Strok Üzerindeki Etkilerinin Araştırılması"dır.

Sizin de bu araştırmaya katılmanızı öneriyoruz. Bu araştırmaya katılıp katılmamakta serbestsiniz. Çalışmaya katılım gönüllülük esasına dayalıdır. Kararınızdan once araştırma hakkında sizi bilgilendirmek istiyoruz. Bu bilgileri okuyup anladıktan sonra araştırmaya katılmak isterseniz formu imzalayınız.

Araştırmaya davet edimenizin nedeni sizde bu hastalığın bulunmasıdır. Size gerekli tetkikleri yaptiktan sonra bu hastalık için kabul görmüş klasik bir tedavi başlayacağız.

Eğer araştırmaya katılmayı kabul ederseniz Prof. Dr. Okay Vural, Doç. Dr. Şeref Demirkaya ve Uz. Öğ. V. Semai Bek veya onların görevlendirdiği bir hekim tarafindan muayene edilecek ve bulgularınız kaydedilecektir. Bu çalışmayı yapabilmek için kolunuzdan 10 ml (2 tüp) kadar kan alamamız gerekmektedir. Bu kandan çalışmada kullanılacak olan tetkikler çalışılacaktır. Bu çalışmaya katılmaniz için sizden herhangi bir ücret istenilmeyecektir. Çalışmaya katıldığınız için size ek bir ödeme de yapılmayacakıir. Kan alımı sizin hastalığınız klinik

takibi sırasında alınacak kanlar alınır iken 2 tüp fazladan alınacaktır. Dolayısı ile size ek bir işlem yapılmayacaktır.

Yapılacak arastırmanın getireceği olası yararlar: böyle bir analiz hastalığınıza sebep olan beyin damarlarınızın tıkanmasina yol açan veya damarınızın tıkanması için risk oluşturan faktörlerin tespit edilmesinin öğrenilmesinde yaralı olacaktır. Şu anda bu çalışmanin hemen size bir fayda olarak dönüp dönmeyeceğini bilmiyoruz. Ancak ilgili hastalığın temelinde yatan nedenlerin öğrenilmesinde ve gelecekte yeni tedavi yaklaşımlarının geliştirilmesi, bu hastalik geçirme riski olan hastaların önceden tespit edilmesi ve belki de hastalik geçirmeden once önlem alınmasında fayda sağlayacaktır.

Bu çalışmaya katılmayı reddedebilirsiniz. Bu araştırmaya katılmak tamamen isteğe bağlıdır ve reddettiğiniz takdirde size uygulanan tedavide ya da bundan sonra kliniğimizde size karşı davranışlarımızda herhangi bir değişiklik olmayacaktır. Yine çalışmanın herhangi bir aşamasında onayınızı çekme hakkına sahipsiniz.

HASTANIN BEYANI

Sayın Prof. Dr. Okay Vural, Doç. Dr. Şeref Demirkaya ve Uz. Öğ. V. Semai Bek tarafından Gülhane Askeri Tıp Akademisi Nöroloji Anabilim Dalı'nda tıbbi bir araştırma yapılacağı belirtilerek bu araştırma ile ilgili yukarıdaki bilgiler bana aktarıldı. Bu bilgilerden sonra böyle bir araştırmaya "katılımcı" olarak davet edildim.

Eğer bu araştırmaya katılırsam hekim ile aramda kalması gereken bana ait bilgilerin gizliliğine bu araştırma sırasında da büyük özen ve saygı ile yaklaşılacağına inanıyorum. Araştırma sonuçlarının eğitim ve bilimsel amaçlarla

kullanımı sırasında kişisel bilgilerimin ihtimamla korunacağı konusunda bana yeterli güven verildi.

Araştırma için yapılacak harcamalarla ilgili herhangi bir parasal sorumluluk altına girmiyorum. Bana da bir ödeme yapılmayacaktır.

İster doğrudan, ister dolaylı olsun araştırma uygulamasından kaynaklanan nedenlerle meydana gelebilecek herhangi bir sağlık sorunumun ortaya çıkması halinde, her türlü tıbbi müdahalenin sağlanacağı konusunda gerekli güvence verildi. (Bu tıbbi müdahalelerle ilgili olarak da parasal bir yük altına girmeyeceğim).

Bu araştırmaya kat lmak zorunda değilim ve katılmayabilirim. Araştırmaya katılmam konusunda zorlayıcı bir davranışla karşılaşmış değilim. Eğer katılmayı reddedersem, bu durumun tıbbi bakımıma ve hekim ile olan ilişkime herhangi bir zarar getrmeyeceğini de biliyorum.

Bana yapılan tüm açıklamaları ayrıntılarıyla anlamış bulunmaktayım. Kendi başıma belli bir düşünme süresi sonunda adı geçen bu araştırma projesinde "katılımcı" olarak yer alma kararı aldım. Bu konuda yapılan daveti büyük bir memnuniyet ve gönüllük içerisinde kabul ediyorum.

Katılımcı

Adı, Soyadı:

Adres:

Tel:

İmza:

Görüşme Tanığı

Adı, Soyadı:

Adres:

Tel:

İmza:

Katılımcı ile Görüşen Hekim

Adı, Soyadı:

Adres:

Tel:

İmza:

APPENDIX C

INFORMED CONSENT FOR CONTROLS

GÖNÜLLÜ BİLGİLENDİRİLMESİ

Araştırma beyin damar tıkanması sonucu oluşan felç-inme hastalığına sebep olan veya katkıda bulunan durumların ortaya konmasına yönelik bir çalışmadır. İnme-felç için risk oluşturan birçok hastalık ve durumu şu an için biliyoruz. Bizim yapacağımız çalışma bunların dışında da bu hastalık için risk oluşturabilecek faktörlerin olup olmadığının araştırılmasıdır. Bu amaçla kanda yüksek yoğunluktaki yağ proteinine (HDL) bağlı olarak bulunan ve eksikliğinde damar sertliği ve sonuçta damar tıkanmasına sebep olabilen paraoksonaz 1 ve benzeri enzimlerin aktivitesi ve genetik durumu incelenecektir. Yapacağımız çalışma daha önce temelde aynı mekanizmaya dayanan kalp krizi için yapılmış ve anlamlı sonuçlar bulunmuştur. Bu işlem için sizden 2 tüp 10 ml kan alınacak ve çalışmalar buradan yapılacaktır. Kan alımı sizin hastalığınızın klinik takibi sırasında alınacak kanlar ile birlikte alınacak ve size ek bir işlem yapılmayacaktır. Sizden 2 tüp kan alımı dışında her hangi bir işlem veya bu çalışmayla ilişkili ek bir tedavi yapılmayacaktır. Araştırma sırasında oluşabilecek herhangi bir zararlı durumu yoktur. Sizden sadece kan alınacaktır. Araştırmaya gönüllü olarak katılmaktasınız ve araştırmaya katılmakta tamamen serbestsiniz. Çalışmada yer alacak gönüllü sayısı yaklaşık 150 hasta ve 150 sağlıklı kişi olacaktır.

Çalışmada yer aldığınız ve bilimsel gelişmelere katkılarınızdan dolayı teşekkür ediyoruz.

Açıklamaları Yapan Araştırmacının

Adı, Soyadı:

Görevi:

İmzası:

Açıklamayı başından sonuna kadar tanıklık eden kişinin

Adı, Soyadı:

Adresi:

İmzası:

Çalışmaya katılan gönüllünün

Adı, soyadı:

Adres:

İmzası:

APPENDIX D

REAGENTS

Reagents used in human genomic DNA isolation from human whole blood samples were described below.

1. **TKM Buffer (pH 7.6, 200 mL)**

   10 mM Trizma base (pH 7.6)

   10 mM KCl

   2 mM EDTA.H_2O

   4 mM $MgCl_2$

   242.20 mg Trizma base, 148.87 mg EDTA, 149.10 mg KCl and 162.64 mg $MgCl_2$ are separately dissolved in appropriate amount of distilled water. All solutions were collected in one beaker and pH was adjusted to 7.6 with HCl. Total volume was completed to 200 mL and buffer was autoclaved before use.

2. **10% SDS Solution**

   0.1g of SDS detergent in molecular grade was dissolved in each mL of distilled water. Solution is not sterilized.

3. Saturated NaCl Solution

35.06 g NaCl in molecular grade was dissolved in 100 mL of distilled water.

4. TE Buffer, pH 8.

100 mM Tris-HCl

500 mM EDTA

10 mL of 100 mM Tris-HCl, pH 8.0 solution is mixed with 0.2 mL of 500 mM EDTA solution and total volume was completed to 100 mL.

5. Tris-HCl (pH 8.0, 100 mM, 100 mL)

1.21 g trizma base was dissolved in appropriate amount of distilled water, pH was adjusted to 8.0 with HCl and total volume is completed to 100 mL. Buffer was autoclaved before use.

6. EDTA (pH 8.0, 500 mM, 100 mL)

18.61 g EDTA was dissolved in appropriate amount of distilled water, pH was adjusted to 8.0 with NaOH and total volume is completed to 100 mL. Buffer was autoclaved before use.

Reagents used in qualification of human genomic DNA samples by agarose gel electrophoresis were described below.

1. **Tris-Borate-EDTA Buffer (5X TBE Buffer, pH 8.3, 1 L)**

   450 mM Trizma base

   450 mM Boric acid

   10 mM EDTA.H_2O

   54 g Trizma base and 27.5 g boric acid were separately dissolved in appropriate amount of distilled water. All solutions were collected in one beaker and 20 μL of 500 mM EDTA solution was added. pH was adjusted to 8.3 and total volume was completed to 1 L, autoclaved before use.

APPENDIX E

LIST OF STUDY POPULATION

Table E.1 List of study population consisted of 237 stroke patients and 120 controls. P: patient, C: control, M: male, F: female, Y:yes, N:no, LDL-C: low density lipoprotein cholesterol, HDL-C: high density lipoprotein cholesterol, TT: wild type homozygous for Tyr113His polymorphism, TC: heterozygous for Tyr113His polymorphism, CC: mutant homozygous for Tyr113His polymorphism, AA: wild type homozygous for His139Arg polymorphism, AG: heterozygous for His139Arg polymorphism, CC: mutant homozygous for His139Arg polymorphism, GG: wild type homozygous for Arg287Gln polymorphism, GA: heterozygous for Arg287Gln polymorphism, AA: mutant homozygous for Arg287Gln polymorphism.

No	Patient Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total Cholesterol	Trigl1cerides	LDL-C	HDL-C	Tyr113His Polymorphism	His139Arg Polymorphism	Arg287Gln Polymorphism
1	P	75	M	Y	Y	Y	N	119	114	72	24	TC	AA	GG
2	P	57	F	Y	Y	N	N	210	136	132	51	TT	AG	GG
3	P	41	M	N	N	Y	N	166	140	104	34	CC	AG	GG
4	P	73	M	Y	N	N	N	143	64	74	57	TT	AA	GG
5	P	53	M	Y	Y	N	N	401	231	296	59	TT	AA	GG
6	P	66	M	Y	Y	Y	N	130	126	85	20	TC	AG	GG
7	P	56	F	Y	Y	N	N	138	212	70	38	TT	AG	GA
8	P	54	M	Y	N	Y	N	200	217	115	42	TT	AA	GG
9	P	67	F	Y	N	N	N	209	106	139	49	TC	AA	GG
10	P	76	M	Y	N	Y	N	155	68	91	50	TC	AA	GG
11	P	78	F	Y	Y	N	N	142	137	79	36	TC	AA	GG
12	P	75	F	Y	N	N	N	182	104	127	34	TC	AA	GG
13	P	74	F	Y	Y	N	N	167	62	107	48	TC	AA	GG

Table E.1 (continued)

No	Patient Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total Cholesterol	Trigl1cerides	LDL-C	HDL-C	Tyr113His Polymorphism	His139Arg Polymorphism	Arg287Gln Polymorphism
14	P	73	F	Y	Y	N	N	207	202	125	42	TT	AA	GG
15	P	73	F	Y	N	N	Y	180	88	107	55	WM	AA	GG
16	P	67	F	Y	N	N	N	208	124	143	40	TT	AA	GG
17	C	71	M	Y	N	N	N	148	119	82	42	CC	AA	GG
18	P	61	M	Y	Y	N	N	169	122	108	37	TT	AA	GG
19	P	40	M	N	N	Y	N	145	104	78	47	TT	AA	GG
20	C	61	F	Y	N	N	N	130	83	46	68	CC	AG	GG
21	P	60	F	Y	Y	N	Y	192	150	119	43	TT	AA	GG
22	P	75	M	N	N	N	N	175	96	71	85	TT	AA	GG
23	P	76	F	Y	N	N	N	124	135	50	46	TT	AA	GG
24	P	76	M	Y	Y	N	N	132	93	74	39	TC	AG	GG
25	C	51	M	N	N	Y	N	142	115	83	35	TT	AA	GG
26	P	50	F	N	N	N	N	139	180	73	30	TC	AA	GA
27	C	42	F	N	N	N	N	104	113	57	24	TT	AA	GA
28	C	45	F	N	N	Y	N	141	53	73	57	TT	AG	GA
29	P	70	M	Y	N	N	N	116	73	59	42	TC	AG	GG
30	P	76	M	N	N	Y	N	128	124	73	30	TC	AA	GA
31	C	63	M	Y	Y	Y	N	117	100	60	37	TC	AA	GG
32	C	63	F	N	N	N	N	200	221	122	34	TC	AG	GG
33	C	75	M	Y	Y	N	N	182	153	100	51	TT	AG	GG
34	C	58	F	Y	N	N	N	104	97	43	42	TT	AG	GG
35	C	78	F	Y	N	N	N	139	95	71	49	TT	AG	GA
36	C	74	M	N	N	N	N	167	82	100	51	TC	AA	GG
37	P	71	M	Y	N	N	N	207	232	126	35	TC	GG	GA
38	C	61	M	N	N	N	N	228	166	155	40	TT	AA	GG
39	C	85	M	Y	N	N	N	119	160	24	63	TT	AG	GG
40	C	65	F	N	Y	N	N	235	126	126	84	TC	AG	GG
41	C	65	M	N	N	N	N	191	57	128	52	TC	AG	GA
42	C	58	M	N	Y	Y	N	229	107	169	39	TT	AG	GG
43	C	61	F	N	N	N	Y	262	163	170	59	TT	AA	GG

Table E.1 (continued)

No	Patient Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total Cholesterol	Trigl1cerides	LDL-C	HDL-C	Tyr113His Polymorphism	His139Arg Polymorphism	Arg287Gln Polymorphism
44	C	67	F	Y	Y	N	N	151	127	85	41	TT	AA	GG
45	C	76	M	N	N	N	N	231	206	143	47	TC	AA	GG
46	C	66	M	Y	Y	N	N	158	127	98	35	TC	AG	GG
47	P	61	M	N	Y	N	N	148	144	76	43	TT	AG	GG
48	C	66	M	N	Y	Y	N	268	349	160	38	TC	AA	GG
49	C	60	F	Y	N	N	N	191	123	121	45	TC	AA	GG
50	P	64	F	N	N	Y	Y	166	94	99	48	TT	AA	GG
51	P	58	F	Y	Y	N	N	350	360	228	50	TC	AA	GG
52	P	74	F	Y	N	N	Y	241	154	162	48	TC	AA	GG
53	C	71	M	N	N	N	N	165	140	80	55	TT	AG	GG
54	P	80	F	Y	N	N	Y	145	86	90	38	TC	AA	GG
55	P	62	M	Y	Y	N	N	188	107	128	39	TC	AA	GG
56	C	68	M	N	N	N	N	210	115	123	64	TT	AG	GG
57	C	65	F	N	N	N	N	200	157	123	46	CC	AA	GG
58	C	72	F	Y	N	N	N	234	149	158	46	TT	AG	GA
59	C	65	M	N	N	N	N	117	80	64	37	TC	GG	GG
60	C	63	F	Y	Y	N	Y	193	328	66	43	CC	AA	GG
61	C	70	F	N	N	N	N	187	132	118	43	TT	AA	GG
62	C	70	F	Y	N	N	N	202	105	130	51	TT	AA	GG
63	P	63	M	Y	N	N	N	131	89	72	41	TT	AA	GG
64	C	65	F	Y	N	N	N	174	113	85	66	TC	AA	GG
65	C	78	F	Y	N	N	N	169	111	93	54	TT	AA	GG
66	P	68	M	Y	N	N	N	171	80	95	60	TC	AA	GG
67	C	47	M	N	N	N	N	241	272	130	57	TC	AA	GG
68	P	77	F	Y	N	N	N	195	95	137	39	TT	AA	GG
69	C	77	F	Y	Y	N	N	156	87	103	36	TT	AA	GG
70	P	80	F	Y	N	N	N	201	94	120	62	CC	AA	GG
71	C	71	M	N	N	N	N	168	131	90	52	TT	AA	GG
72	P	55	M	Y	N	N	N	157	124	95	37	TT	AA	GA
73	C	73	M	Y	N	N	N	140	220	76	20	CC	AG	GG
74	C	61	M	N	N	Y	N	180	35	114	59	TC	AG	GG
75	P	62	M	N	N	N	N	142	90	54	43	TC	AA	GG

Table E.1 (continued)

No	Patient Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total Cholesterol	Trigl1cerides	LDL-C	HDL-C	Tyr113His Polymorphism	His139Arg Polymorphism	Arg287Gln Polymorphism
76	P	77	M	N	N	N	N	147	54	68	68	TC	AA	GG
77	P	24	M	N	N	N	N	235	255	143	41	TT	AG	GG
78	P	53	F	N	N	Y	N	96	192	23	35	TT	AA	GG
79	P	61	M	N	N	N	N	186	142	121	37	TT	AG	GG
80	C	52	M	N	N	N	N	267	103	183	63	TT	AA	GG
81	P	78	M	Y	N	Y	N	201	183	120	44	TC	AA	GG
82	C	65	M	N	N	N	Y	160	76	113	32	TC	AA	GG
83	P	81	M	Y	N	N	N	133	270	37	42	CC	AG	GG
84	P	80	F	Y	N	N	Y	105	113	57	25	TT	AA	GG
85	C	50	F	Y	N	N	N	123	76	84	24	TC	AA	GG
86	C	87	F	Y	N	N	Y	106	91	54	34	TC	AA	GG
87	C	67	M	N	N	N	N	168	102	82	6	TT	GG	GG
88	C	38	M	N	N	Y	N	184	50	111	63	TC	AA	GG
89	C	50	M	N	N	N	N	147	55	79	57	CC	AA	GG
90	P	75	F	Y	N	N	N	110	58	52	46	TC	AA	GG
91	P	26	M	N	N	N	N	162	95	80	63	TT	AA	GG
92	P	55	M	Y	N	N	N	194	83	123	54	TC	AA	GG
93	P	26	M	N	N	N	N	156	64	114	29	CC	AG	GG
94	P	73	F	Y	N	N	N	154	101	99	35	TT	AG	GG
95	C	80	F	N	Y	N	N	153	123	77	51	TT	AG	GG
96	P	36	M	N	N	N	N	187	140	116	43	TC	AA	GG
97	P	56	M	N	N	N	N	167	109	100	45	TT	AA	GG
98	P	47	F	N	N	N	N	184	123	123	36	TT	AA	GA
99	P	81	F	Y	Y	N	N	139	193	69	31	TC	AA	GG
100	P	73	M	Y	Y	N	N	198	224	118	35	TT	AA	GG
101	P	73	F	Y	N	N	N	154	101	99	35	TT	AG	GG
102	P	66	F	N	N	N	N	129	248	58	21	TT	AG	GG
103	P	74	F	Y	N	N	N	230	127	154	51	TT	AA	GG
104	P	44	M	N	N	Y	N	200	212	96	62	TC	AA	GG
105	C	51	F	N	N	N	N	168	51	110	48	TT	AA	GG
106	P	67	M	Y	N	N	N	172	148	101	41	TT	AA	GG

Table E.1 (continued)

No	Patient Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total Cholesterol	Trigl1cerides	LDL-C	HDL-C	Tyr113His Polymorphism	His139Arg Polymorphism	Arg287Gln Polymorphism
107	P	73	F	Y	Y	N	N	227	206	143	43	TT	AG	GG
108	C	88	F	Y	N	N	N	154	71	100	40	TT	AG	GG
109	P	66	M	Y	Y	N	N	129	254	49	29	TC	AA	GG
110	P	61	F	Y	Y	N	N	124	178	63	25	TT	AG	GG
111	P	78	M	N	N	Y	N	130	110	70	40	TC	AG	GG
112	C	59	M	N	N	N	N	157	110	90	45	TT	AG	GG
113	C	69	F	N	Y	N	N	241	119	157	60	TT	AG	GA
114	P	80	M	Y	N	N	N	304	74	239	50	TT	AG	GG
115	P	76	F	Y	Y	N	N	158	110	98	38	CC	AG	GG
116	P	79	F	Y	N	N	N	202	136	129	46	TT	AA	GG
117	C	79	M	Y	N	N	N	145	147	80	42	TT	AG	AA
118	C	51	F	N	N	N	N	110	90	54	38	CC	AA	GA
119	P	21	M	N	N	N	N	149	83	100	32	CC	AA	GG
120	P	76	M	N	N	N	Y	193	106	132	40	TT	GG	GG
121	P	20	M	N	N	Y	N	166	80	100	50	TC	AG	GG
122	P	80	F	Y	N	N	N	193	251	86	57	TC	AG	GG
123	P	64	F	Y	N	N	N	145	144	77	39	TC	AA	GG
124	P	71	M	N	N	Y	N	190	81	132	42	TT	AA	GG
125	P	67	F	Y	Y	N	Y	391	226	297	49	TT	AA	GG
126	P	58	F	Y	Y	N	N	274	256	169	54	TT	AG	GG
127	C	77	M	N	N	N	N	179	167	80	46	TT	AA	GG
128	P	49	M	N	N	Y	N	226	204	139	46	TC	AA	GG
129	C	70	M	Y	N	N	N	168	147	98	41	TT	AG	GG
130	P	78	F	N	N	N	N	178	88	103	57	TT	AA	GG
131	P	65	M	N	N	N	N	154	164	79	42	TC	AA	GG
132	P	75	M	Y	N	N	N	195	142	130	37	TC	AG	GG
133	P	79	M	N	N	Y	N	105	80	54	35	TC	AG	GG
134	P	73	F	Y	Y	N	N	291	315	205	23	TT	AA	GG
135	P	25	M	N	N	N	N	177	140	107	43	TC	AG	GG
135	P	73	M	Y	N	N	N	195	79	119	60	TC	AA	GG
136	P	74	M	Y	N	N	N	128	118	70	34	TC	AA	GG
137	P	78	F	Y	Y	N	Y	181	138	109	44	TC	AG	GG

Table E.1 (continued)

No	Patient Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total Cholesterol	Trigl1cerides	LDL-C	HDL-C	Tyr113His Polymorphism	His139Arg Polymorphism	Arg287Gln Polymorphism
138	C	80	F	Y	Y	N	N	324	317	196	65	TT	GG	GG
139	P	56	M	Y	Y	N	N	177	157	105	41	TC	AA	GG
140	P	67	M	Y	N	N	N	165	154	89	43	TC	AG	GA
141	P	74	M	Y	N	N	N	169	201	79	50	TC	GG	GG
142	P	64	M	Y	Y	N	N	168	170	93	41	TC	AA	GG
143	P	73	M	Y	Y	N	N	178	173	100	43	TC	AA	GG
144	C	52	F	N	N	N	N	147	100	73	54	TT	AG	GG
145	C	74	M	Y	N	N	N	119	95	67	33	TC	AA	AA
146	P	57	M	N	N	Y	N	187	149	116	41	TT	AA	GG
147	P	76	M	N	N	N	N	161	171	94	33	TC	AG	GA
148	P	61	M	N	N	Y	N	187	124	116	46	TC	AA	GG
149	C	77	F	Y	N	N	N	162	178	79	47	TC	AA	GG
150	P	62	F	N	Y	N	N	157	145	91	37	TC	AA	GG
151	P	73	M	N	N	Y	N	304	265	204	47	TC	AG	GA
152	P	63	M	N	N	Y	N	133	103	66	46	TT	AA	GA
153	P	52	M	N	N	Y	N	158	98	108	31	TT	AG	GG
154	C	79	M	N	N	N	N	202	77	150	37	TC	AG	GG
155	P	61	F	Y	N	N	N	121	124	62	34	TC	AA	GG
156	C	46	M	Y	N	Y	N	304	252	207	47	TT	AG	GG
157	P	45	M	N	N	Y	N	151	333	51	33	TT	AA	GG
158	P	64	M	N	N	Y	N	166	97	103	36	TT	AG	GA
159	P	56	F	N	Y	N	N	270	284	169	44	TT	AA	GG
160	P	67	M	Y	Y	N	N	266	253	171	44	TC	AA	GA
161	P	53	M	Y	N	N	N	284	302	176	48	TC	GG	GG
162	P	80	M	N	N	N	N	220	119	146	50	TC	AA	GG
163	P	62	M	Y	Y	N	N	214	110	143	46	TC	AG	GG
164	C	41	F	N	N	N	N	150	130	84	40	TT	AG	GG
165	P	80	M	N	N	N	N	203	150	123	50	TT	AG	GG
166	P	61	M	N	N	Y	N	131	120	75	34	CC	AG	AA
167	P	67	F	N	Y	N	N	190	135	125	38	TT	AA	GG
168	C	48	F	N	N	N	Y	100	86	55	28	TT	AG	GG
169	P	65	F	N	N	Y	N	222	105	149	52	TC	AA	GG
170	P	79	F	Y	Y	N	N	164	150	103	31	TC	AA	GG

Table E.1 (continued)

No	Patient Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total Cholesterol	Trigl1cerides	LDL-C	HDL-C	Tyr113His Polymorphism	His139Arg Polymorphism	Arg287Gln Polymorphism
171	P	61	M	Y	N	Y	N	181	107	119	41	TC	AG	GG
172	P	80	M	Y	Y	N	N	289	377	173	41	TT	AA	GG
173	P	61	M	N	Y	Y	N	143	102	84	39	TT	AG	GG
174	P	69	M	Y	N	N	N	156	77	105	36	CC	AG	GG
175	P	76	F	Y	Y	N	N	158	68	104	40	TT	AG	GA
176	P	69	F	N	N	N	N	123	99	70	33	TT	AA	GG
177	C	43	F	N	N	Y	N	222	164	137	52	TC	AG	GG
178	P	77	M	Y	Y	Y	N	148	73	102	31	TC	AG	GA
179	P	78	F	Y	N	N	N	188	62	124	52	TC	GG	GG
180	C	64	F	Y	N	N	Y	269	133	186	56	TT	AA	GG
181	P	58	F	N	N	N	N	166	98	94	52	TT	AA	GG
182	C	66	M	N	N	N	N	204	62	135	57	TT	AA	GG
183	P	59	F	Y	Y	N	N	200	167	133	33	CC	AA	GG
184	C	69	F	Y	N	N	N	187	104	78	47	TC	AA	GG
185	P	53	M	N	N	Y	N	139	33	86	46	TC	AA	GG
186	C	42	M	N	N	Y	N	178	244	96	33	TT	AA	GG
187	C	65	M	Y	N	N	N	148	115	76	49	CC	AG	GG
188	C	87	M	Y	Y	N	N	104	110	38	44	TT	AA	GG
189	C	75	F	N	N	Y	Y	184	81	113	55	TT	GG	GG
190	P	54	M	N	N	N	N	170	59	121	37	TT	AG	GG
191	C	77	M	Y	N	N	N	139	60	95	32	TC	AA	GG
192	C	69	F	N	N	N	N	178	115	106	49	TC	AA	GG
193	P	75	F	N	Y	N	N	189	87	118	54	TC	AA	GG
194	P	78	M	Y	Y	N	N	163	135	100	36	TT	AA	GG
195	P	40	F	N	N	Y	Y	192	94	127	46	TT	AA	GG
196	P	48	M	Y	N	N	N	211	72	147	50	TT	AG	GG
197	P	80	F	Y	Y	N	N	184	77	112	54	TT	AG	GA
198	P	41	M	N	N	N	N	145	120	88	33	CC	AA	GG
199	P	77	F	Y	Y	N	N	192	129	123	43	TC	AA	GG
200	P	63	F	Y	Y	N	Y	151	69	100	37	TT	AA	GG
201	P	55	M	Y	N	Y	N	277	163	207	37	TT	AA	GA
202	P	79	M	Y	Y	N	N	120	68	61	45	TT	AA	GG
203	P	67	F	Y	Y	N	N	197	127	114	58	TT	AG	GG

Table E.1 (continued)

No	Patient Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total Cholesterol	Trigl1cerides	LDL-C	HDL-C	Tyr113His Polymorphism	His139Arg Polymorphism	Arg287Gln Polymorphism
204	P	63	M	Y	Y	N	N	145	157	83	31	TT	AG	GG
205	P	75	F	Y	N	N	N	228	169	152	42	TT	AA	GG
206	C	65	M	N	N	N	N	159	62	103	44	TT	AA	GG
207	C	78	M	N	N	N	N	158	82	87	55	TT	AA	GG
208	C	81	M	Y	N	N	N	108	54	48	49	TT	AA	GG
209	C	56	M	N	Y	N	N	177	122	110	43	TT	AA	GG
210	C	64	F	Y	Y	N	N	166	246	86	31	TC	AA	GG
211	C	79	F	Y	N	N	N	181	110	110	49	CC	AG	GG
212	C	67	F	N	N	N	N	298	102	228	50	TT	AA	GA
213	C	76	F	Y	N	N	N	166	110	85	59	TT	AG	GA
214	C	64	M	N	N	Y	N	130	91	86	26	TC	AA	GG
215	C	77	M	N	Y	N	N	338	158	243	63	TC	AA	GG
216	C	75	M	N	N	N	N	191	149	109	52	TT	AA	GG
217	P	54	F	N	N	N	N	201	301	114	27	TC	AA	GG
218	P	61	F	Y	N	N	N	180	110	123	35	TT	AA	GG
219	P	71	M	Y	N	N	N	125	71	73	38	TT	AA	AA
220	C	68	F	Y	N	N	Y	186	170	97	55	CC	AA	GG
221	P	74	F	Y	N	N	N	211	165	123	55	TT	AG	GG
222	P	59	M	N	N	N	N	128	127	60	23	TT	AA	GG
223	P	62	M	N	N	Y	N	154	70	103	37	TT	GG	GG
224	C	71	F	Y	N	N	N	149	97	90	40	TT	AA	GA
225	C	52	F	N	N	N	N	154	71	100	40	CC	AA	GG
226	C	78	M	Y	N	N	N	159	124	97	37	TC	AA	GG
227	P	70	M	Y	N	N	N	198	76	134	49	TT	AG	GG
228	P	69	M	Y	N	N	N	170	83	111	42	TT	AA	GG
229	C	80	M	Y	N	N	N	197	86	126	54	TT	AG	GG
230	P	57	F	N	Y	N	N	210	154	135	44	TT	AG	GG
231	P	58	F	Y	N	N	N	123	112	60	41	TC	AA	GG
232	P	80	M	Y	N	N	N	136	62	78	46	TT	AA	GA
233	C	78	F	N	N	N	N	157	84	98	41	TT	AA	GG
234	C	57	F	N	N	N	N	94	93	19	56	TT	AA	GG
235	C	60	F	Y	N	N	N	206	198	112	54	CC	AA	GA
236	C	63	F	Y	N	N	N	257	246	160	48	TC	AA	GG

Table E.1 (continued)

No	Patient Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total Cholesterol	Trigl1cerides	LDL-C	HDL-C	Tyr113His Polymorphism	His139Arg Polymorphism	Arg287Gln Polymorphism
237	P	74	M	N	Y	N	N	130	65	65	52	TC	AG	GG
238	P	50	M	Y	N	N	N	77	75	24	38	TT	AG	GG
239	C	58	M	Y	N	N	N	150	200	80	30	CC	AA	GG
240	C	54	M	Y	N	N	N	166	103	94	51	TT	GG	GA
241	C	78	M	N	N	N	N	142	60	80	50	CC	AA	GG
242	P	57	M	N	Y	N	N	193	127	139	29	TT	AA	GG
243	C	75	M	Y	Y	N	N	185	71	115	56	TT	AA	GG
244	C	77	M	Y	N	N	N	118	88	64	36	TT	AG	GA
245	P	62	M	Y	N	N	N	195	233	119	29	TT	AA	AA
246	P	81	F	Y	Y	N	N	161	85	96	48	TC	AG	GG
247	P	77	F	Y	Y	N	N	116	187	61	18	TT	AA	GG
248	P	54	M	Y	Y	N	N	159	119	111	24	CC	AA	GG
249	P	82	F	Y	Y	N	N	167	90	102	47	TC	AA	GG
250	P	71	F	Y	Y	N	N	163	172	91	38	TT	AA	GG
251	P	80	F	Y	N	N	N	165	139	90	47	TT	AA	GG
252	C	59	F	N	N	N	N	213	126	126	62	TT	AG	GG
253	C	57	F	N	N	N	N	168	166	100	35	TC	AA	GG
254	C	79	F	Y	Y	N	N	163	147	86	48	TC	AA	GG
255	C	52	F	N	N	N	N	233	114	163	47	TC	AA	GA
256	C	79	F	Y	N	N	N	59	99	23	16	TC	AA	GG
257	P	69	M	Y	Y	N	N	207	223	109	53	TT	AG	GG
258	C	54	M	N	Y	N	N	282	220	191	47	TT	AA	GG
259	P	70	F	Y	N	N	N	181	145	95	57	TT	AA	GG
260	C	67	F	N	N	N	N	160	70	92	52	TC	AA	GA
261	C	50	F	N	N	N	N	131	246	68	14	TC	AA	GG
262	P	31	F	N	N	N	N	246	268	141	51	TT	AA	GG
263	P	55	F	N	N	N	N	273	62	190	71	TT	AA	GG
264	C	90	F	N	Y	N	N	214	146	133	52	TC	AA	GG
265	P	71	M	Y	Y	N	N	216	178	146	34	TC	AG	GG
266	C	77	F	Y	N	N	N	314	173	202	77	TT	AA	GG
267	C	37	M	N	N	Y	N	99	71	14	71	TC	AA	GG
268	P	77	M	Y	Y	N	N	176	193	92	45	TC	AA	GG
269	C	57	M	Y	Y	N	N	174	49	103	61	TC	AA	GA

Table E.1 (continued)

No	Patient Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total Cholesterol	Trigl1cerides	LDL-C	HDL-C	Tyr113His Polymorphism	His139Arg Polymorphism	Arg287Gln Polymorphism
270	C	68	M	N	N	N	N	136	52	72	54	TC	GG	GG
271	P	74	F	Y	Y	N	N	285	277	173	57	TT	AG	GG
272	P	47	M	Y	Y	N	N	175	129	95	54	TC	AG	GG
273	P	43	M	N	N	Y	N	301	310	185	54	TT	AA	GG
274	P	67	M	Y	Y	N	N	212	87	151	44	TT	AA	GG
275	P	69	F	N	N	N	N	248	147	170	49	CC	AA	GG
276	C	60	M	Y	N	N	N	154	89	92	44	TC	AA	GG
277	P	74	M	N	N	Y	N	154	96	106	29	TT	AA	GG
278	P	71	M	Y	Y	Y	N	229	234	141	41	TC	AA	GG
279	P	74	M	Y	N	N	N	140	122	80	36	TC	AA	GA
280	P	76	F	Y	Y	N	N	170	174	86	49	TT	AA	GG
281	C	44	M	N	N	N	Y	171	109	110	39	TT	AA	GG
282	P	68	F	Y	Y	Y	N	117	109	55	40	TT	AA	GG
283	C	52	F	Y	N	N	N	200	173	111	54	TC	AG	GG
284	P	68	F	Y	N	N	N	205	92	141	46	TT	AA	GG
285	P	74	F	Y	N	N	Y	150	70	97	39	TC	AA	GA
286	C	58	F	N	N	N	N	157	149	90	37	TC	AA	GA
287	P	57	M	Y	Y	Y	N	237	253	152	34	TC	AA	GG
288	C	79	M	Y	Y	N	N	199	173	129	35	TT	AA	GG
289	C	73	F	N	N	N	N	203	111	141	40	TT	AG	GG
290	P	34	F	N	N	N	N	223	174	103	24	CC	AG	GA
291	P	61	M	Y	N	N	N	176	132	114	36	TT	AA	GG
292	P	62	M	N	N	N	N	181	188	105	38	TC	AG	GG
293	P	62	M	Y	N	N	Y	171	219	135	148	TT	AA	GG
294	P	63	F	Y	Y	N	N	117	246	164	176	TT	AA	GA
295	P	68	M	Y	N	Y	Y	137	187	94	140	TT	AA	GG
296	P	71	F	N	N	N	N	200	229	98	173	TT	AG	GG
297	P	70	M	Y	Y	Y	N	205	215	90	149	TC	AA	GG
298	P	69	F	Y	N	N	N	150	206	46	149	TC	AA	GG
299	P	71	M	Y	N	N	N	157	218	123	159	TT	AG	GG
300	P	79	M	Y	Y	Y	Y	237	168	127	119	CC	AA	GG
301	P	38	M	Y	N	Y	N	199	255	150	184	TT	AA	GA
302	P	71	F	Y	N	N	Y	203	202	126	125	TT	AG	GG

Table E.1 (continued)

No	Patient Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total Cholesterol	Trigl1cerides	LDL-C	HDL-C	Tyr113His Polymorphism	His139Arg Polymorphism	Arg287Gln Polymorphism
303	P	73	M	Y	N	Y	Y	230	245	114	177	TT	AA	GG
304	P	63	M	N	N	Y	Y	262	172	69	101	TC	AA	GG
305	P	30	M	N	N	N	Y	92	155	118	97	TC	AA	GG
306	P	68	M	Y	N	Y	Y	238	175	100	118	TC	AA	GG
307	P	61	F	Y	N	N	Y	181	190	115	100	TT	AG	GG
308	P	75	M	N	N	N	N	220	140	61	88	TT	AA	GG
309	P	42	M	N	N	N	N	197	230	78	167	TC	AA	GG
310	P	59	F	Y	N	N	Y	216	262	162	158	TC	AA	GG
311	P	73	M	Y	N	Y	Y	195	238	175	152	TT	GG	GG
312	P	78	M	N	N	N	Y	169	181	118	121	TT	AG	GA
313	P	71	M	Y	Y	Y	Y	229	220	336	144	TT	GG	GG
314	P	79	M	Y	Y	N	Y	159	195	96	147	TT	AA	GA
315	P	59	M	Y	N	N	Y	203	169	114	109	TT	AA	GG
316	P	70	F	Y	Y	N	Y	261	229	160	156	TT	AA	GG
317	P	21	M	N	N	N	N	128	159	91	98	TT	AA	GG
318	P	52	M	N	N	N	N	175	203	61	130	TT	AA	GG
319	P	77	F	Y	Y	Y	Y	175	261	146	185	CC	AA	GG
320	P	77	M	Y	N	Y	N	224	128	143	74	TC	AA	GG
321	P	79	F	Y	N	N	N	244	175	72	119	TC	AA	GG
322	P	68	M	Y	N	Y	Y	210	244	105	181	CC	AA	GG
323	P	79	N	Y	Y	N	Y	247	247	152	180	TC	AA	GA
324	P	46	M	Y	Y	N	Y	271	193	103	123	TC	AA	GG
325	P	73	M	Y	N	Y	Y	193	168	143	101	TT	AA	GG
326	P	70	M	Y	Y	N	Y	168	254	194	176	TT	AA	GG
327	P	73	N	Y	Y	N	N	254	129	96	85	TT	AA	GA
328	P	79	N	N	N	Y	Y	129	255	92	155	TC	AA	AA
329	P	81	N	N	N	N	N	255	217	209	138	CC	AA	GG
33N	P	79	M	Y	Y	N	Y	199	303	59	238	TC	AA	GG
331	P	66	M	Y	Y	Y	N	217	152	396	51	TC	AA	GG
332	P	75	M	Y	N	Y	Y	303	249	247	156	TT	AA	GG
333	P	64	M	Y	N	Y	Y	152	108	77	69	TT	AA	GA
334	P	72	N	Y	N	Y	N	249	195	96	124	TT	AG	GG
335	P	64	N	Y	Y	N	Y	197	175	82	121	TC	AG	GG

Table E.1 (continued)

No	Patient Control	Age	Gender	Hypertension	Diabetes	Smoking	Obesity	Total Cholesterol	Trigl1cerides	LDL-C	HDL-C	Tyr113His Polymorphism	His139Arg Polymorphism	Arg287Gln Polymorphism
336	P	64	N	Y	N	N	Y	229	229	112	163	TT	AA	GG
337	P	51	M	Y	N	Y	Y	174	174	171	114	TC	AA	GG
338	P	65	M	Y	Y	Y	Y	207	207	334	97	TT	AA	GG
339	P	53	M	Y	N	Y	Y	226	205	162	129	TT	AG	GG
340	P	77	N	Y	N	N	N	205	195	111	128	TC	AA	GG
341	P	71	N	Y	Y	N	Y	239	135	96	79	TT	AA	GG
342	P	75	M	Y	N	Y	Y	195	191	99	114	CC	AA	GG
343	P	65	M	Y	Y	Y	Y	289	208	187	130	TT	AA	GG
344	P	57	M	N	N	N	Y	135	256	104	180	TC	AA	GG
345	P	67	N	Y	Y	N	N	191	187	140	131	TT	AA	GA
346	P	32	M	N	N	Y	N	208	146	110	89	TT	AA	GG
347	P	80	M	Y	N	N	N	164	123	47	66	TT	AA	GG
348	C	55	N	N	N	N	N	213	173	103	99	TT	GG	GG
349	C	52	N	N	N	N	N	256	170	122	105	TT	AG	GG
350	C	51	M	N	N	N	N	145	152	105	92	TT	AA	GA
351	P	61	N	Y	N	Y	N	187	172	106	98	TT	AG	GG
352	P	68	F	Y	N	Y	N	140	202	75	25	TC	AG	GA
353	P	72	F	N	N	N	N	256	253	165	40	TC	AA	GG
354	P	81	F	Y	N	N	N	188	156	115	42	TC	AA	GG
355	C	80	F	Y	N	N	N	233	202	83	24	TT	AA	GG
356	C	80	F	N	N	N	N	129	63	46	71	TT	AA	GA
357	P	75	N	Y	Y	N	Y	239	135	96	79	TC	AA	GA