Genetic polymorphisms of vitamin D3 metabolising CYP24A1 and CYP2R1 enzymes in Turkish patients with ischemic stroke

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Objective: Vitamin D deficiency is known as an important risk factor in pathogenesis of atherosclerosis, which contributes to stroke development. Genetic variations including single nucleotide polymorphisms (SNPs) in enzymes involved in vitamin D metabolism can affect susceptibility to the development of stroke. Therefore, the objective of this study was to investigate the association between polymorphisms of vitamin D metabolising enzymes (rs927650 SNP in *CYP24A1* and rs10741657 SNP in *CYP2R1* genes,) and ischemic stroke risk in Turkish population.
Materials and Methods: To test this hypothesis, we designed a case-control study which consisted of 256 ischemic stroke patients and 132 controls. Genotypes were determined by PCR-RFLP technique.
Results: No significant differences were found between patients and controls in terms of CYP24A1 rs927650 and CYP2R1 rs10741657 genotype frequencies. Polymorphic allele frequencies of CYP24A1 rs927650 and CYP2R1 rs10741657 were 0.414 and 0.660 in stroke patients, respectively.
Conclusion: This is the first study conducted regarding the association of CYP24A1 rs927650 and CYP2R1 rs10741657 genetic polymorphisms and ischemic stroke risk. The polymorphic genotypes of these polymorphisms, together with hypertension, diabetes, smoking and obesity, were found as significant risk factors for ischemic stroke.

Keywords: CYP24A1, CYP2R1, single nucleotide polymorphism, vitamin D, ischemic stroke

Introduction

Stroke, a serious and life-threatening neurological medical condition, also known as

brain attack, occurs when the blood flow to an area of the brain is interrupted or reduced which is generally caused by a clot or blood vessel that bursts or leaks. There are lots of risk factors for the development of stroke such as, age, gender, obesity, cigarette smoking, hypertension, diabetes mellitus, atherosclerosis and dyslipidemias.

Vitamin D is one of the oldest steroid hormones including the principal circulatory form 25 hydroxyvitamin D (25-OH-D_3) and the hormonal form, 1,25-dihydroxyvitamin D (1α -25-$(OH)_2D_3$) [1]. The best known function of vitamin D is in bone and calcium metabolism. Apart from this well-documented role, vitamin D has been identified as an important factor in cardiovascular health [2]. Kendrick et al. (2009) and Durup et al. (2015) have reported that vitamin D deficiency is associated with cardiovascular disease events [3,4]. Vitamin D has a direct role in systemic inflammatory process [5,6]. Endothelial cells (EC) have vitamin D receptors and express 1α-hydroxylase to produce 1α,25$(OH)_2D_3$ [7]. Besides, vitamin D takes part in an inflammatory process by applying protective effects on endothelial activation and dysfunction. Decrease in nitric oxide (NO) and increase in oxidative stress are the main contributors to endothelial disfunctioning [8]. It has been revealed that vitamin D stimulates NO synthesis [9] and also reduces oxidative stress in endothelium by suppressing the superoxide synthesis [10]. Vascular tone is also regulated by vitamin D by modulating discharge of endothelium derived contracting factors [6] and vitamin D prevents the proliferation of vascular smooth muscle cells by inhibiting release of epidermal growth factor and endothelin. Moreover, vitamin D has an effect on regulation of coagulation process through upregulating the expression of the anticoagulant and down regulating the expression of a critical coagulation factors in monocytes [11].

Some researchers reported that vitamin D deficiency triggers hypertension, heart attack, diabetes and atherosclerosis [2, 12, 13]. With all these evidence, vitamin D deficiency is appearing as a risk factor for stroke among the other risk factors like hypertension, smoking, hyperlipidemia, obesity, age, and gender. CYP24A1 and CYP2R1, important cytochrome P450 enzymes, expressed in the kidney and liver, respectively, are known to take role in vitamin D metabolism. CYP2R1 takes role in the biological activation process of vitamin D; on the other hand, CYP24A1 carries out the degradation of vitamin D [14]. Genetic variation in the genes which express CYP24A1 and CYP2R1 enzymes can change the enzyme activity in the organism. So, concentration of vitamin D can vary depending on the effect of these variations on the gene. The level of vitamin D has been reported as an important parameter for stroke risk and severity [12, 15].

Approximately 50 polymorphisms of *CYP24A1* gene were identified up to now. Jones et al. (2012) reported that four polymorphisms are associated with lower concentrations of vitamin D [16]. It was shown that *CYP24A1* promoter region SNP reduces the expression of *CYP24A1* in human lymphocytes cell culture [17]. The relationship between polymorphism and reduction of CYP24A1 enzyme activity was also shown by Jacobs et al. (2013) with the study of vitamin D metabolism and cancer cells [18]. Studies showed that CYP2R1 enzyme has been related with the vitamin D deficiency [19-22]. CYP2R1 rs10741657 polymorphism affects vitamin D level and it has been associated with lower 25(OH)D_3 synthesis [23]. Ramos-Lopez et al. (2007) reported that CYP2R1 rs10741657 polymorphism of GG or GA genotyped patients have lower levels of 25(OH)D_3 when compared to AA genotyped patients [24]. Similar results were obtained from study conducted by Hassanein *et al*. (2014) [25]. They were

also reported that low level of 25(OH)D_3 was found in GG or GA genotyped individuals [25].

In the present study, rs927650 polymorphism in *CYP24A1* gene and rs10741657 polymorphism in *CYP2R1* gene were investigated for the risk of ischemic stroke. To our current knowledge, this is the first study concerning the association of genetic polymorphisms of CYP24A1 and CYP2R1 enzymes and the risk of ischemic stroke susceptibility.

Materials and methods

Study Population

The study population was comprised of 256 consecutive unrelated adult Caucasian acute hemispheric ischemic stroke patients from central Anatolia (Turkey) and 132 symptom-free Caucasian controls from the same geographic region. Informed consent was obtained from all participants before study entry. Cases were selected among patients suffering stroke admitted to the neurology services of Gülhane Military Medical Faculty, Ankara, within 24 h after onset. These patients had an anterior circulation stroke resulted from carotid artery atherosclerotic disease. The cerebral infarction was initially diagnosed on the basis of neurological examination and brain computer tomography (CT) scan and then transthoracic echocardiographic examination, Holter study and Transcranial Doppler emboli detection procedure to rule out emboli source. In order to be considered eligible, the patients should meet following criteria: having anterior circulation stroke, no other major illnesses, including autoimmune diseases, neoplasms, coagulopathies, hepatic or renal failure, no known embolic source (aortic arch, cardiac or carotid), no family history of hematological, autoimmune or chronic inflammatory diseases, no history of myocardial infarction within 3 weeks or of

transient ischemic attack or stroke at any time. The details of the inclusion and exclusion criteria were as described before [26]. The study was approved by the Ethical Committee of the Gülhane Medical Academy and was carried out according to the principles of the Declaration of Helsinki. Our classification system is similar to the Trial of ORG 10172 in Acute Stroke Treatment (TOAST). We included TOAST "Large-vessel disease" group and Oxfordshire Community Stroke Project (OCSP) "total anterior" and "partial anterior circulation infarcts" groups into the study. Control subjects were selected randomly from neurology outpatient clinics and did not have stroke or transient ischemic attack at any time. All exclusion criteria were applied to the controls exactly, plus not having carotid stenosis (lumen narrowing) > 70% or ulcerated carotid plaque.

Genotype Determination

Genomic DNA was extracted from peripheral blood leukocytes by salt extraction [27] and stored at -20°C till use. Standard PCR protocols, followed by restriction enzyme digestions, were used to genotype the *CYP24A1* gene (rs927650) and *CYP2R1* gene (rs10741657) polymorphisms. The primer pairs used for PCR amplification of the CYP24A1 rs927650 SNP were 5'-GGGAAGAGCAATGACATGGA-3' and 5'-GCCCTGGAAGACTCATTTTG-3' and those for the CYP2R1 rs10741657 SNP were 5′ TGGTTGCATAACACAAACCTA3′ and 5′ CTGAAAGCCAGTAACAATGGT3′. For the amplification of both CYP24A1 rs927650 SNP and CYP2R1 rs10741657 SNP regions PCR mixture in a total volume of 50 μL contained 200 ng genomic DNA, 200 μM dNTPs, 30 pmol of each primer, 1.5 mM $MgCl_2$ and 2.5 U of Taq polymerase and the PCR conditions consisted of an initial melting temperature of 94°C for 2 min followed by 35 cycles of melting (94°C, 30 s), annealing (60 °C, 30 s) and extension

(72°C, 1 min). A final extension step (72°C, 3 min) terminates the reaction. Genotyping for CYP24A1 rs927650 polymorphism involved digestion of PCR products with 2 U *Bg1*II at 37ºC for 18 h. For the CYP2R1 rs10741657 polymorphism, PCR products were digested with 2U *Mn*II at 37ºC for 18 h. All of the digestion products were visualized by electrophoresis on a 3% agarose gel.

Statistical Analyses

Continuous variables were expressed as mean ± SD. Normality of the sample distribution of each continuous variable was tested with the Kolmogorov–Smirnov test. Differences of continuous variables were evaluated by the independent samples t or Mann-Whitney *U* test. Categorical variables were expressed as proportions and compared using $\chi 2$ test. Allele distributions were compared using $\chi 2$ test. The associations of the effects of vascular risk factors, genotypes in ischemic stroke cases and controls were assessed by odds ratios (ORs) and 95% confidence intervals (CIs). The OR and CI were calculated by using logistic regression analysis with backward selection method. *P* values <0.05 were considered statistically significant. These statistical analyses were performed with SPSS 20.0 software (SPSS Inc., Chicago, IL, USA).

Results

Table 1 presents the clinical laboratory test results and some risk factors of ischemic stroke patients and controls. The age and sex status of patients and controls were not significantly different. The prevalence of risk factors for stroke such as hypertension, diabetes, cigarette smoking, obesity and levels of total cholesterol, triglyceride and LDL-cholesterol were found to be higher in patient group than control group. On the other hand HDL-cholesterol level was significantly lower in ischemic stroke patients

(1.1±0.3 mmol/L) when compared to the control group (1.2±0.3 mmol/L, P< 0.01).

There was no statistically significant difference in distribution of CYP24A1 rs927650 and CYP2R1 rs10741657 genotypes and allele frequencies in ischemic stroke patients and controls (Table 2).

In Table 3, genotype data were stratified into hypertensive/normotensive, diabetic/non-diabetic, smoker/non-smoker and obese/non-obese sub-groups. While making comparison between the genotypes of CYP24A1 rs927650, the CT and TT were attributed as a single group. Thus, CT+TT were compared against the wild type genotype (CC). Similar practice was applied for CYP2R1 rs10741657 genotypes; AG+GG were taken as a single group and compared against the wild type (AA).

In CYP24A1 polymorphism CC wild-type genotype group, the proportion of stroke patients to controls was found to be higher in the hypertensive, diabetic, smoker and obese groups than the respective normotensive, non-diabetic, non-smoker and non-obese group. For CYP24A1 CC and CT+TT genotypes, the risk of having stroke is 3.6 times and 3.1 times higher (P<0.01, P<0.01) in hypertensives when compared to normotensives, respectively. As a result, the risk of having stroke increases with hypertension for all genotypes of CYP24A1 rs927650 polymorphism. In diabetics and smokers group, CT+TT risky genotype had 2.4-fold and 3.7-fold increased risk for stroke, respectively (P<0.01, P<0.01). The risk ratio increased up to 4.1 in obese group when compared to non-obese group (P<0.01).

When Table 3 was examined in terms of CYP2R1 rs10741657 AG+GG genotyped individuals, the risk of having stroke was 3.4 times higher in hypertensives when compared to normotensives (P<0.01). In diabetics, AG+GG genotype had 2.8-fold increased risk for ischemic stroke (P<0.01). As can be seen in Table 3, AG+GG genotype (OR = 2.226, P=0.01) was found to be a significant risk factor of stroke in

smokers. Among obese subjects, for AG+GG genotype, the risk of having stroke was 4.8 times higher than for non-obese subjects (P<0.01).

Logistic regression analysis with backward likelihood method was used to determine the effects of vascular risk factors, lipid parameters and CYP24A1 rs927650 and CYP2R1 rs10741657 genotypes in the prediction of ischemic stroke and the results are given in Table 4. Age, sex, hypertension, smoking status, diabetes, obesity, lipid parameters (total cholesterol, triglycerides, HDL-cholesterol, LDL-cholesterol) were added as covariates together with CYP24A1 rs927650 and CYP2R1 rs10741657 genotypes. The analysis showed that hypertension (OR=3.387, 95%CI=2.095-5.475, P<0.01), smoking (OR=3.074, 95%CI=1.598-5.915, P<0.01), obesity (OR=2.730, 95%CI=1.211-6.155, P=0.015) and LDL-cholesterol (OR=1.397, 95%CI=1.086-1.796 P<0.01) were the significant determinants of ischemic stroke. However, HDL-cholesterol (OR=0.276, 95%CI=0.119-0.640, P<0.01) served as preventive factor for stroke. The model predicted 71.1% of cases correctly and the Hosmer-Lemeshow goodness of fit test pointed out that the calibration of the model was satisfactory (chi-square=7.237; 8 degrees of freedom; P=0.511).

Discussion

In this study, the relationship between vitamin D metabolising enzymes CYP24A1 rs927650 and CYP2R1 rs10741657 genetic polymorphisms and risk of ischemic stroke was investigated. Stroke is the term employed to describe the acute neurological and irreversible manifestations of cerebrovascular disease which results from interruptions to blood flow in the brain and is the second leading cause of death in the world. Atherosclerosis is a progressive disease characterized by the accumulation of cholesterol deposits in arteries and is an important risk factor for stroke [28]. Oxidative stress, which has a pivotal role in the pathogenesis of atherosclerotic process, is a

condition occuring when the physiological balance between oxidants and antioxidants is disrupted. So, oxidative stress and atherosclerosis are the most important risk factors for stroke development. Vitamin D deficiency promotes atheroma formation and vitamin D supplementation prevents the vascular risk and low levels of 25-hydroxyvitamin D ($25(OH)D_3$) are associated with peripheral artery disease [29, 30]. In addition, Carvalho and Sposito (2015) reported that vitamin D may prevent oxidative stress in endothelial cells and reduces the expression of apoptosis related genes [29]. Also vitamin D modulates the expression of oxidative stress and atherogenesis regulatory proteins [31]. Vitamin D deficiency has been found to trigger hypertension, heart attack [12] and also cardiovascular-related diseases [32], like diabetes [13], heart failure [33], atherosclerosis [2], peripheral vascular disease and pathological state of the endothelium [34] and many studies reported that low level vitamin D increases stroke severity and stroke risk [12, 15]. Therefore, understanding the effect of polymorphisms of vitamin D metabolising enzymes in ischemic stroke risk is of crucial importance.

The cytochrome P450s (CYP450) are a large superfamily of phase I enzymes. Actually, all organisms from bacteria to humans and plants can have them. The cytochrome P450 enzymes are monooxgenases and generally introduce oxygen, usually in the form of a hydroxyl group, to the substrate. In addition to hydroxylation, the CYP450 enzymes are able to perform other reactions such as dealkylation, carbon-carbon bond cleavage, dehydrogenation and epoxidation [35]. They are responsible for the oxidative metabolism of endogenous and exogenous compounds because of this role they act as a body's defense system against xenobiotic exposure [36]. CYP450 enzymes take role in generation of epoxyeicosatrienoic acids (EETs), important regulators of coronary circulation. In addition, they catalyse the oxidation of low density lipoprotein in liver and arterial walls that is important in atherogenesis [37]. Because of these

properties there are some association studies related with the CYP450 enzyme polymorphisms and ischemic stroke risk in the literature [26, 38-40]. We have previously shown that in Turkish population variants of CYP1A1 gene were significantly related with the risk of ischemic stroke, especially in subgroups [40]. According to results of our other study, the minor allele of CYP2E1*5B polymorphism increases the risk of having stroke 8-times compared to wild type allele [26]. Catabolism and hydroxylation of vitamin D is carried out by CYP24A1 and CYP2R1 enzymes, respectively [14]. Change in normal activity of the enzymes taking part in activation or inactivation of vitamin D can affect the risk of having stroke. It was shown that diabetic individuals having G allele for the rs10741657 polymorphism of CYP2R1 had lower level of 25-OH-D_3 concentration than AA genotyped individuals [24]. It was also shown that individuals with AA genotype had higher concentration of 25-OH-D_3 than AG and GG genotyped individuals [25].

In the present study, there was no significant difference between patients and controls with respect to genotype distribution of CYP24A1 rs927650 and CYP2R1 rs10741657 polymorphisms (Table 2). Therefore, these the SNPs of CYP24A1 and CYP2R1 cannot be considered as independent risk factors for ischemic stroke in Turkish population. There is no study to evaluate effect of CYP24A1 and CYP2R1 polymorphism on ischemic stroke risk in the literature. However, the association between vitamin D status and cardiovascular risk have been investigated and found that vitamin D deficiency is associated with the cardiovascular disease in some small cross-sectional studies ([12, 13, 32]. The connection between vitamin D deficiency and cardiovascular disease was explained by several mechanisms in a number of studies conducted. The study of Li *et al*. showed that direct involvement of 1-25$(OH)_2D_3$ in suppression of renin gene provides the regulation of renin-angiotensin system directly suppressing renin gene

expression [41]. Disruption in renin-angiotensin system affects development of heart diseases [42]. Modulation of calcium and phosphate homeostasis, smooth muscle cell proliferation, inflammation, and thrombosis can be included to the functions and physiological actions of vitamin D. The study of Kasuga *et al*. (2002) indicated that overexpression of 24-hydroxlyase (CYP24A1), taking part in catabolism of 1-25$(OH)_2$-D_3, increases the risk of atherosclerosis [43]. Transgenic rats generated for the study were constitutively expressing CYP24A1. Atherosclerotic lesions were examined in aorta of rats which were either fed normal diet or high fat diet [43]. Besides, Hassanein *et al*. (2014) observed that patients with coronary heart disease had significantly lower level of 25-OH-D_3 when compared to control group without coronary artery disease [25]. The study also revealed an association between rs10741657 SNP of *CYP2R1* gene and coronary artery disease incidence [25]. The association of the CYP2R1 rs10741657 SNP with coronary artery disease incidence was found in individuals having the G variant in their genotypes. Also, individuals with AA genotype had higher concentration of 25-OH-D_3 than AG and GG genotyped individuals [25]. So, it can be concluded that the polymorphisms in CYP24A1 and CYP2R1 also increase the incidence of coronary artery disease due to decreased level of vitamin D.

The study population was grouped under hypertensive/normotensive, diabetic/non-diabetic, smoker/non-smoker and obese/non-obese subgroups to analyze the effect of hypertension, diabetes, smoking status and obesity together with CYP24A1 rs927650 and CYP2R1 rs10741657 genotypes on the risk of ischemic stroke (Table 3). The risk of having stroke in hypertensive individuals was three-fold higher than normotensives within the CT+TT genotype of CYP24A1 rs927650 polymorphism group. The individuals carrying wild type CC genotype have 3.6-fold increased risk of having hypertension-related ischemic stroke when compared to normotensives.

CYP24A1 has been clearly established as the main enzyme responsible for the degradation of active vitamin D. Tarcin *et al*. (2009) reported that vitamin D deficiency is associated with increased prevalence of hypertension and endothelial dysfunction in healthy subjects [44]. Polymorphic enzyme may enhance the degradation of active vitamin D and decreased level of vitamin D may increase the risk of stroke development together with hypertension.

In the study of Mauf *et al*. (2015) association between the lower level of vitamin D and incidence of type 1 diabetes (T1D) was examined [45]. T1D is a chronic autoimmune disease which is mainly carried out by immunogenic dendritic cells [46]. It was suggested increased plasma levels of 25-OH-D_3 may inhibit formation of dendritic cell (DC) and increase immunomodulatory cells so decrease incidence of T1D. So, 25-OH-D_3 shows immunomodulatory effects at the cellular level in patients with T1D. For CYP24A1 rs927650 polymorphism, when compared to TT genotyped individuals, individuals with CC genotype showed a significantly higher increase of immunomodulatory cells [45]. Individuals with TT genotype in CYP24A1 rs927650 SNP had an increased frequency of mature dendritic cells, which increase the risk of T1D. Besides, the maturation of DCs is disrupted in the presence of 1-25$(OH)_2D_3$ [47] and also 1 -25$(OH)_2D_3$ treatment has been shown to induce apoptosis of mature DCs [48]. According to our results; in diabetics, CT+TT genotypes significantly increase the risk of stroke 2.4-fold when compared to non-diabetics. Smokers with CT+TT risky genotype had 3.7-fold higher risk than non-smokers with CT+TT risky genotype. Similarly, we observed that CT+TT genotyped obese group had 4-fold higher risk for ischemic stroke.

When Table 3 was examined for CYP2R1 rs10741657 polymorphism AG+GG genotype group was found to be significant risk factor for ischemic stroke in all

subgroups like hypertensives, smokers, diabetics and obese. For CYP2R1, two different studies showed the association of the G variant of the rs10741657 SNP with type 1 diabetes [20, 24]. Moreover, the study of Ramos-Lopez et al., (2007) demonstrated that individuals having G allele had lower level of 25-OH-D_3 concentration than AA genotyped individuals [24]. Also, the other studies have revealed the association of the CYP2R1 rs10741657 SNP with incidence of pancreatic cancer [49] as well as hepatitis C virus- related hepatocellular carcinoma [50].

Logistic regression analysis confirmed hypertension, smoking, obesity and LDL-cholesterol as significant risk factors for stroke, while HDL-cholesterol as significant protective factor against ischemic stroke. Similar results were obtained from our previous studies [26, 40].

Limitations of the present study include number of control subjects. However, it should be noted that the sample was clinically well defined and strictly selected so as to keep mean age of controls and stroke patients similar, as age is the strongest determinant of stroke. It was highly difficult to find elderly subjects who meet the required criteria as controls, i.e. no history of ischemic stroke, transient ischemic attack and ischemic heart disease at any time or myocardial infarction within 3 weeks and no more than 70 % carotid stenosis. Confirmation of these results requires further studies involving larger populations. Nevertheless, statistical tests showed that our sample size was enough to obtain reliable statistical results.

Conclusion

In conclusion, to the best of our knowledge, this is the first study to evaluate the possible association between the polymorphisms of vitamin D metabolising enzymes, CYP24A1 rs927650 and CYP2R1 rs10741657, and ischemic stroke risk. These

polymorphisms had no role in the development of ischemic stroke in the studied Turkish population. But, significant results were obtained in hypertensive, smoker, diabetic and obese individuals carrying the variant allele.

Disclosure statement

The authors have no conflict of interest.

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