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Research Article

THROMBOPHYLIC GENE MUTATION ANALYSIS OF CAROTID ARTERY DISEASES; WESTERN TURKEY

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ABSTRACT

We evaluated the effects of 12 genes polymorphisms on CAD(Carotid Artery Diseases) formation by using a ViennaLab CVD Strip Assay. Group A includes 41 patients (70.2 ± 8.6 years, 30 men) with CAD and Group B includes 39 healthy controls (67.3 ± 9.2 years, 28 men). Twenty patients had transient ischemic attack or stroke, 21 had carotid artery stenosis, more than 50 % in Group A. Hyperlipidemia is more frequent in Group A compared Group B (71%, 49%; $p<0.05$). Heterozygote form of Factor V H1299R, Factor XIII V34L, B-Fibrinogen -455G>A, MTHFR C677T and MTHFR A1298C were more frequent in Group A compared with Group B significantly [(2.6%, 7.3% $p<0.05$), (12.8%, 19.5% $p<0.05$), (12.8%, 19.5% $p<0.05$), (20.5%, 34.1% $p<0.05$), (25.6%, 46.3% $p<0.05$)]. The results of study showed that the formation of CAD, Factor V H1299R, Factor XIII V34L, B-Fibrinogen -455 G>A, MTHFR C677T and MTHFR A1298C heterozygous mutation seems to be determinant ($p<0.05$).

INTRODUCTION

Cerebrovascular disease (CVD) is a group of diseases with high disability rates (Folsom *et al*, 1994), which is the third most common cause of all deaths after heart and cancer diseases. In the United States, more than 700,000 cases of stroke are reported each year and the annual cost of the economy is approximately $ 51 billion (Abou-Chebl *et al,* 2005). The mortality and morbidity caused by stroke cause significant labor force and economic damage.

Ischemic strokes (88%) constitute the majority of strokes, followed by primary intracerebral hemorrhage (9%) and subarachnoid hemorrhage (3%) (Thom, 2006). Approximately 90% of cerebral thromboembolic events are caused by atherosclerosis. Craniocervical atherosclerosis does not commonly hold the carotid artery, the involvement is focal to the segment, usually individual or multiple segments. The lesion is often located in the carotid bifurcation and fold area. Craniocervical atherosclerosis is responsible for 20-30% of all stroke cases. Atherosclerotic plaques in the carotid artery bifurcation cause more ulceration and plaque rupture than plaques in other segments. Carotid artery stenosis is frequently embolic and rarely hemodynamically induced ischemia in the brain (Walker *et al*, 1995; De Weerd *et al,* 2014; Debakey, Lawrie, and Glaser 1985).

The course of patients with atherosclerotic carotid artery stenosis varies with plaque character and degree of stenosis. Cases with a stenosis rating of more than 75% have a 2-5% risk of stroke each year. If ulcerated plaque is present, this rate rises to 7-8%. High fat-containing, soft, hypoechoic plaques were associated with stroke and transient ischemic attack (TIA). Heterogeneous plaques are suggested to be more in symptomatic cases. It has been described that patients with calcified plaques with less than 75% stenosis and soft plaques with less stenosis with a lower risk of stroke have lower risk (De Weerd *et al*, 2014). Patients with untreated persistent neurological deficits with stroke and TIA have a 20-fold higher

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risk of developing a new cerebrovascular event (CVE) than the asymptomatic population.

The risk factors that cause carotid artery atherosclerosis are generally similar to the risk factors in the development of coronary artery disease. The most important ones are; advanced age, male gender, genetic factors, smoking, hypertension (HT), diabetes mellitus (DM), hyperlipidemia (HL), obesity, ischemic heart disease, Peripheral Artey Disease (PAD), past ischemic stroke and the presence of transient ischemic attacks. These structural and acquired risk factors accelerate the development of atherosclerosis and increase their severity.

Atherosclerosis and CAD are known to be multifactorial diseases. The presence of acquired atherosclerotic risk factors besides genetic factors is also important in the development of CAD. In our study, Carotid Color Doppler USG (CCDUS) and conventional carotid angiography in the control group without CAD and in the patient group determined by conventional carotid angiography with CAD gene blood samples were taken to determine 12 gene polymorphisms. In our study, the effect of genetic polymorphism on the development of CAD was studied.

MATERIALS AND METHODS

Design and subjects

This study was performed in the Medical Genetics Department, Afyon Kocatepe University Hospital, Afyonkarahisar, Turkey. The study was approved by the Ethics Committee of the Afyon Kocatepe University Hospital, and all individuals signed an informed consent form. The study was designed as a retrospective case-control study and was conducted between March 2013-December 2016 in Afyon Kocatepe University Education and Research Hospital. The patients included in the study were selected from the patients who presented to the Cardiology and Neurology Department of Afyon Kocatepe University with the clinical findings suggesting CVE, TIA and cerebral ischemia. Patients knews could be withdrawn from the study without giving any reason when they wanted.

Study Criteria

The patients included in the study were selected from patients who applied to Cardiology and Neurology outpatient clinics. Patients who had carotid artery stenosis more than 50% in carotid angiography and those diagnosed with carotid artery stenosis with less than 50% carotid artery stenosis were included in the study (Figure 1a,1b,1c). Risk factors for atherosclerosis were determined by performing biochemical routine examinations. Classic risk factors were considered as age, gender, HT, HL, DM and smoking. The diagnosis of HT was made when the patient was taking antihypertensive medication or blood pressure was >140/90 mmHg. Diagnosis of fasting blood glucose 2 times more than 126 mg / dl, oral glucose tolerance test 2 hours or in patients with blood glucose levels above 200 mg / dl in the measurement of random glucose, HbA1c level above 6.5% and insulin or when antidiabetic medication is used. Patients who were diagnosed with DM such as prediabetes or metformin due to obesity or impaired glucose tolerance test were not included in the study. The diagnosis of HL was based on the use of statin and / or fibrate derivatives of the patient and TC(Total Cholesterol)> 200 mg / dl, LDL-C(Low-Density Lipoprotein-Cholesterol) > 130 mg / dl, TG> 150 mg / dl, or HDL-C(High-density lipoprotein cholesterol) <40 mg / dl in men, <50 mg/dl in women. The study group consisted of 41 patients and 39 control groups. In the patient and control groups, the number of subjects were more likely.

Exclusion Criteria

- Patients without carotid angiography and carotid USG,
- CVE, TIA and cerebral ischemic symptoms do not occur due to CAD,
- under 30
- Breastfeeding mothers were not accepted.

DNA isolation, PCR, and reverse hybridisation

DNA samples which that originated from Afyonkarahisar was collected by Cardiology Department, was sent to Medical Genetics Department. Genomic DNA of the 160 samples was originally extracted from fresh blood anticoagulated with EDTA using either the CVD strip assay lysis solution and GENTRACT resin (ViennaLab, Vienna, Austria) or the QIAamp DNAblood Midi (Qiagen, Hilden, Germany) extraction kit, using a silica membrane-based DNA purification method that yields up to 60 mg of DNA from 2 ml initial blood volume following the manufacturer's instructions. The CVD strip assay (ViennaLab) screens for gene mutations, which have already been mentioned based on a reverse hybridization principle. The different target gene sequences were concurrently amplified and biotin labeled in a single amplification reaction. The reaction consisted of 0.1 mg of DNA added to 15 mL already prepared PCR amplification mix, including primers that flank the target sequences and dNTPs in the presence of 1 U Taq polymerase. The PCR cycles were optimized as follows: 2 min at 94 ^{0}C of initial denaturation followed by 35 cycles of amplification (15 s denaturation at 94 ^{0}C, 30 s annealing at 58 ^{0}C, and 30 s extension at 72 ^{0}C), and a final extension of 3 min at 72 ^{0}C. The amplification products were denatured and selectively hybridized to a test strip that contains allele- specific oligonucleotide probes (wild type and mutant) immobilized as an array of parallel lines. Bound biotinylated sequences were detected using streptavidin alkaline phosphatase and color substrates.

Statistical analysis

Statistical analysis was performed using SPSS 20.0 computer software tests listed below. The mean age of the individual, the sex ratio in the group risk factors, identifying blood biochemical parameters and HT, DM and HL smoking rates were evaluated by statistical methods. It viewed whether significant differences in the first quantitative data revealed that there are differences in many variables. Because of the data were not normally distributed to the non-parametric test which Mann-Whitney test was used. Categorical variables were determined as polymorphisms of HT, DM, HL, smoking and all genes. For these variables, Pearson Chi-Square test was performed among the groups. p value <0.05 was considered statistically significant. Data were shown as mean ± standard deviation. Hardy-Weinberg equilibrium was tested for each genotype within groups by means of test X^2. Statistical significance was defined by two tailed P<0.05.

RESULTS

Comparison of Demographic Variables of the Participated Groups

General clinical characteristics of the population and demographic data of all participants are shown in Tables 1 and 2. There was no significant difference in the Mann-Whitney test for age groups. Table 3 shows according to the Hardy-Weinberg equilibrium was tested for each genotype within groups by means of X^2 test results; statistically significant differences were found between the results in CAD.

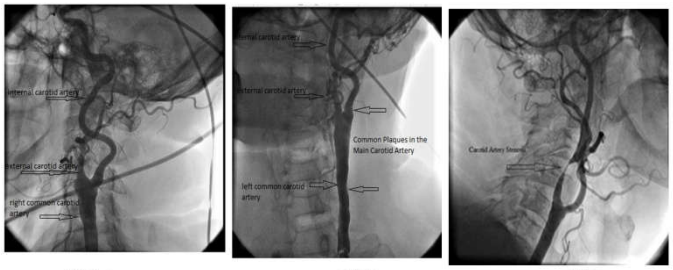

Fig 1 a Fig b Fig c

Figure 1 Carotid angiography is performed using X-rays, especially in severe and / or symptomatic carotid artery stenosis or carotid ultrasonography where no healthy information can be obtained. This examination method is still considered the gold standard for the diagnosis of carotid artery disease. During the procedure, a catheter is advanced from the groin or arm artery and the carotid arteries are injected into the carotid arteries. All images belong to Afyon Kocatepe University Faculty of Medicine Cardiology Department Angiography Laboratory. **Fig1a.** Normal carotid artery angiography image. Right carotid artery and its branches. **Fig1b.** Left carotid artery and its branches; Common Plates in the Main Carotid Artery. **Fig1c:** Carotid Artery Stenosis.

Table 1 Age and gender distribution of groups

Working group	Gender	Number	Age average	Standard deviation	%
Control	Woman	11	65,00	10,23	28,2
	Male	28	68,14	8,86	71,8
	Total	39	67,26	9,24	100
Patient	Woman	11	67,00	8,33	26,8
	Male	30	71,33	8,53	73,2
	Total	41	70,17	8,59	100
Total	Woman	22	66,00	9,16	27,5
	Male	58	69,79	8,76	72,5
	Total	80	68,75	8,98	100

Table 2 Demographic characteristics of all groups included in the study

Demographic variables	Patient	Control	Patient	Control	P
HT	8 (%72,7)	7 (%63,6)	16 (%53,3)	18 (%64,3)	0,610
HL	8 (%72,7)	4 (%36,4)	21 (%70,0)	15 (%53,6)	0,045
DM	3 (%27,3)	5 (%45,5)	11 (%36,7)	11 (%39,3)	0,525
Smoke	-	1 (%9,1)	17 (%56,7)	14 (%50)	0,784

Comparison of Genetic Results of the Groups

There was no statistically significant difference between these 2 groups in terms of 12 gene polymorphism showed a statistically significant difference. The number of patients in the prothrombin G20210A and PAI-1 4G/5G group were higher than the patients. In our study, the association of HL with CAD was second after HT, there was a significant difference between the patient and control groups (p=0,045<0.05). This showed us that there was a significant relationship between hyperglycemia and CAD. There was no significant difference between groups in terms of smoking (p=0.784>0.05) and between the with DM groups in the two groups (p = 0.525>0.05).

When the results were compared Factor V H1299R mutation was found to be significant related to CAD (p=0.024<0.05). Factor FXIII V34L mutation was found to be a risk factor for CAD when compared with each other (p=0.019<0.05). Factor FXIII V34L mutation was found to be a risk factor for CAD when compared with the groups with hyperlipidemia (p=0.005<0.05).

When the groups with hyperlipidemia were compared, β-fibrinogen -455 GA mutation was found to be a risk for CAD (p= 0.001) and β-Fibrinogen-455 GA mutation was found to be associated with CAD when the patients with hyperlipidemia (p= 0.000). MTHFR C677T mutation was associated with CAD and we was found to be significant (p= 0.008<0.05).

Table 3 Genotype and allel frequency candidate genes for the risk of developing in the CAD Patients

Genes	Patients	Control	Total	p-value
Factor V G1691A(Leiden)				
Normal	37	32	69	0,483
Heterozygote	1	3	4	
Homozygote	3	4	7	
Factor V H1299R(R2)				
Normal	38	32	70	
Heterozygote	3	1	4	0,024
Homozygote	-	6	6	
Protrombin G20210A				
Normal	37	38	75	
Heterozygote	4	-	4	0,083
Homozygote	1	1		
Factor XIII V34L				
Normal	32	25	57	
Heterozygote	8	5	13	0,019
Homozygote	1	9	10	
β-fibrinojen -455G>A				
Normal	26	18	44	
Heterozygote	14	8	22	0,001
Homozygote	1	13	14	
PAI-1 4G-5G				
Normal	14	10	24	
Heterozygote	26	22	48	0,065
Homozygote	1	7	8	
HPA1 a/b				
Normal	27	31	58	
Heterozygote	13	8	21	0,299
Homozygote	1	-	1	
MTHFR C677T				
Normal	18	14	32	
Heterozygote	19	10	29	0,008
Homozygote	4	15	19	
MTHFR A1298C				
Normal	17	21	38	
Heterozygote	20	8	28	0,018
Homozygote	4	10	14	
ACE I/D				
Normal	10	10	20	0,892
Heterozygote	21	18	39	
Homozygote	10	11	21	
APOB R3500Q				
Normal	41	39	80	

APOE genotip				
2/3	4	8	12	
2/4	1	1	2	
3/3	30	26	56	0,723
3/4	5	3	8	
4/4	1	1	2	

DISCUSSION

Ischemic stroke is currently the major cause of death and long-term disability. The incidence of CVE development is very high in the world due to CAD that develops on the basis of atherosclerosis. Preventive treatment methods against classical vascular risk factors are insufficient to prevent ischemic stroke. The development of individual protective strategies can be achieved by defining the human genome and disease biology. More effective preventive and therapeutic methods can be developed which will enable us to identify genetic pathologies that may cause disease and to identify individuals at risk of disease. Thus, side effects associated with multiple treatment strategies can be reduced. In our study, we evaluated the effects of 12 gene polymorphisms which may be effective in the occurrence of CAD and compared them with other risk factors which are effective in CAD.

There was a significant difference in gender between the groups included in the study. In the study of Shinkowa *et al*., the incidence of stroke in men between the ages of 55-64 was 2-3 times higher than in women, while it was found that this difference decreased in older ages (Shinkawa *et al*, 1990). This may be due to the high average age of the control group patients and the increased frequency of hypertension with age.
In the study of Mark A. Espeland *et al*., gender difference was significant in terms of atherosclerosis in the Internal Carotid Artery (ICA) segment. Atherosclerosis in the ICA segment was more common in men. There was no significant difference in atherosclerosis in Arteria serebralis anteriyor (ASA) and bifurcation segments (Espeland *et al*, 1999). These studies show that women are less prone to atherosclerosis before menopause. This can be explained by the fact that blood pressure and TC or LDL-K levels are lower in women than men before menopausal high blood estrogen levels (Onat *et al*, 1997). In our study, we found that male gender was higher in the group with CAD, and all of the women with CAD were in the postmenopausal period.

HT is an important risk factor for CVE development. G.Parrinello *et al* reported early onset of carotid atherosclerosis in individuals with HT (Parrinello *et al*, 2004). In Su-Ta-Chen *et al*. studies, HT has been shown to be a major risk factor for carotid atherosclerosis (Su *et al*, 2001). In a meta-analysis of Mac Mahon and his collaborates which included fourteen randomized trials, a 5-6 mmHg reduction in blood pressure was shown to reduce the chances of stroke by 42% in a period of 4-6 years (MacMahon *et al*, 1990). Weber suggested that systolic blood pressure is an important factor in the development of early carotid atherosclerosis (Weber, 2002). We found that a significant relationship between the diagnosis of HL and CAD.
In the presence of hyperlipidemia (HL), which is another important risk factor for atherosclerosis, the incidence of CAD increases. In the study of "Atherosclerosis Risk in Communities", it was found that HDL-C levels were lower, plasma TC, LDL-C and TG levels were higher in cases with carotid artery atherosclerosis (Tell, 1991). In the Tromso study by Mathiesen *et al*., the relation between low HDL-C level and the frequency of lipid-rich echolucent atherosclerotic plaque formation in the carotid artery was demonstrated (Mathiesen, Bonaa, and Joakimsen, 2001). Smilde *et al*. reduced LDL-C levels in patients with familial hypercholesterolemia reported that intima-media thickening associated with carotid artery atherosclerosis was significantly reduced (Smilde *et al*, 2000).

Smoking is one of the factors that increase the risk of developing atherosclerosis by increasing inflammation in the serum (Ross 1999; Zevin *et al*, 2001). In large-scale studies such as Framingham, The Cardiovascular Health Study, The Honolulu Heart Study, smoking has been shown to be a strong risk factor for ischemic stroke, and it has been shown to increase the risk by about 2-fold after correction for other risk factor (Wood *et al*, 1970). Case-control and prospective epidemiological studies have shown that DM has an independent effect in ischemic stroke and the relative risk increase varies between 1.8 and 6 times (Bogousslavsky, 1985). Dempsey *et al*, however, did not find a significant relationship between carotid plate thickness and DM (Dempsey and Moore,1992).

Factor V H1299R mutation decreases the level of FV and develops APC resistance. Factor V H1299R polymorphism shows broad ethnic variation. In Europe, Asia, Australia and Africa, the incidence in the healthy population is reported to be 5-17% (Zaatari *et al*, 2006). Factor H1299R did not have a strong association with cerebrovascular diseases and its association with increased VTE (Aleksova *et al*., 2015). Recently, FXIII V34L mutation, mild venous and arterial thrombosis protective effects are mentione (F. Rosendaal, 2002). FXIII V34L mutation increased the risk of hemorrhagic stroke, but it was reported to be protecting cerebral infarction (González-Conejero *et al*, 2006). Recent studies support our findings that the Val34Leu polymorphism may be protective against MI and venous thrombosis, except to patients has got to family history and hyperlipidemic (Onrat *et al*, 2012).

In studies, increased fibrinogen levels were associated with arterial thrombolytic disorders. In prospective studies in patients without disease and in patients with vascular disease, increased fibrinogen level was determined in relation to CVE, myocardial infarction and peripheral vascular diseases (Heinrich *et al*, 1994). Martiskainen *et al*. they found that β-Fibrinogen-455 GA mutation was reported to be a risk factor for the development of lacunar infarct (Martiskainen *et al*, 2003). Nishiuma *et al*. was concluded that there was a significant relationship between stroke patients and β-Fibrinogen-455 GA mutation independent of other risk factor (Nishiuma S, 1998). These findings were supported our results and in our study was very similar to the reported data.

The most common mutation that reduces MTHFR enzyme activity is the C677T genotype, while the second most common mutation is found in the A1298C genotype. There is evidence that increased levels of homocysteine with decreased MTHFR enzyme activity is a risk factor for vascular diseases such as coronary artery disease and peripheral artery disease (Boushey *et al,* 1995). In some studies, MTHFR C677T and A1298C gene polymorphisms are controversial both on plasma homocysteine levels and as a single risk factor. These polymorphisms have been reported in healthy individuals (Li *et al,* 2003). These results concluded that homozygosity for this

frequent mutation in the MTHFR gene is associated with a threefold increase in risk for premature CVD, this results similar to our study. Uçar *et al*., reported that the MTHFR C677T gene mutation has not been associated with ischemic stroke in the Turkish population in the Black Sea Region (Uçar *et al*, 2004). Sazcı *et al*., in their study on 1004 women and 680 men, the incidence of MTHFR A1298C was 43.7%, MTHFR A1298A incidence was 46.3%, MTHFR C1298C incidence was found to be 10.0%. In the same study, Turkey, "the MTHFR C677T/A1298C together incidence of heterozygous mutations were determined as 21.6% (Sazci *et al*, 2005). In our study was very similar to the these reported datas.

Increased PAI-1 activity is associated with vascular inflammation and atherosclerosis, especially in obesity and DM patients, and serum levels increase in conditions such as metabolic syndrome (Aso 2007; Mehta and Shapiro 2008). In other studies, when compared with stroke and control groups, there was no increase in the risk of arterial thrombosis with MTHFR (C677T, A1298C) and PAI-1 polymorphisms (Cao *et al*, 2014). Our data support the concept that the PAI-1 gene is a susceptibility locus, i.e., it is neither necessary nor sufficient for the disease to occur, but makes it more likely that one will become ill. The extent to which this polymorphism confers an additional coronary risk has to be addressed in prospective studies.

The prothrombin G20210A mutation increases plasma prothrombin level by 30% compared to normal controls and is prone to venous thrombosis. In a study, it was reported that the risk of myocardial infarction was 4 times higher in smokers and 40-fold in smokers in the prothrombin G20210A allele (F. R. Rosendaal *et al*, 1997). In other study of young patients with ischemic stroke, Madonna *et al*. found that the prothrombin G20210A mutation was 9.4% in the patient group and 12.4% in the control group (Madonna *et al*, 2002). In another study, prothrombin G20210A mutation was detected in young ischemic stroke women (Longstreth Jr. *et al*, 1998). De Stefano *et al.* were been found that prothrombin G20210A mutation may be a factor increasing the risk of cerebral ischemia (De Stefano *et al*, 1998).

CONCLUSION

Our data support the idea that, while individual genetic susceptibility variants are of limited clinical use, the combined information from a number of these variants can permit the identification of groups of people at high and low risk of developing a complex trait such as CAD. The polygenic model used in this study, considering the cumulative effect of hemostatic gene variants, was significantly associated to some in vitro measurements of thrombin generation. In the specific context of advanced CAD, similar approaches may be useful as surrogate markers of the propensity to form blood clots leading to MI. Further studies on larger samples are needed to confirm this intriguing working-hypothesis, as well as to improve predictive modelling.

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