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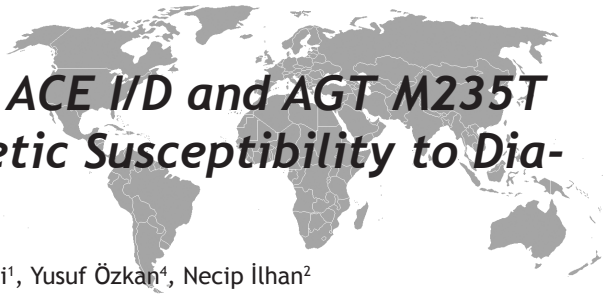

Role of ADDUCIN Gly460Trp, ACE I/D and AGT M235T Gene Polymorphisms in Genetic Susceptibility to Diabetic Nephropathy

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ABSTRACT

Diabetes mellitus is a metabolic disease with a high incidence of morbidity and mortality lowering the quality of life with acute and chronic complications and it needs to be followed up and treated throughout the lifespan. Concordant with the frequency of DM and the increase in the length of life frequency of DNP is also increased. The I allele of the ACE I/D polymorphism, AGT M235T's T allele and ADD G460W's W allele presence are purported to present a predisposition to DNP. In our study we aimed to investigate the effect of the AGT M235T, ACE I/D and ADD 460W polymorphisms in the development of DNP in patients with diabetes mellitus. The study group consisted of 194 patients with Diabetic nephropathy and the control group contained 100 DM patients. In the diabetic group the DD genotype of the ACE I/D polymorphism was 54 (54.0%) and the D allele was 69.0% and in the nephropathy group II genotype of the ID were 78 (40.2%) and 51 (26.3%) and the I allele was 46.4 respectively and the presence of the I allele was associated with the presence of nephropathy. There was no significance between the genotypes in the presence of a coexistence of AGT M235T and ACE I/D polymorphisms between the groups the MD alleles (42%) demonstrated significance in the diabetic and the T/I alleles (28.1) demonstrated significance in the nephropathy group. In the ADD G640W polymorphism on its own, subjects having GG (77.3%) and WW (7.7%) genotypes (p=0.025) might have been predisposed to nephropathy however when in combination with the ACE I/D, the presence of the DD/GG (45%) in the diabetic group and the presence of the ID/GG (29.9%) and II/GG (19.6%) combination in the DNP group were statistically significant. The D/G (64%) alleles were significant in the diabetic and the I/G (36.6) alleles were significant in the DNP groups respectively. As a conclusion we think that the II-ID/GG genotype and the I/G alleles in the presence of the ACE-ADD combination and TT/ID genotype and T/I alleles in the ACE-AGT combination may be effective in the predisposition of the diabetic patients to nephropathy.

Key words: *DM, diabetic nephropathy, ACE I/D polymorphism, AGT M235T polymorphism, ADD G460W polymorphism*

Diyabetik Nefropatiye Eğilimde Adducin Gly4460trp, Ace I/D And Agt M235t Gen Polimorfizmlerinin Rolü

ÖZET

Diabetes mellitus (DM), yasam boyu sürekli izlem ve tedavi gerektiren, akut ve kronik komplikasyonları ile yasam kalitesini azaltan morbiditesi ve mortalitesi yüksek kronik metabolik bir hastalıktır. DM sıklığındaki ve yasam süresindeki artısa bağlı DNP sıklığı da artmaktadır. ACE I/D polimorfizminden I-alleli, AGT M235T'den T-alleli ve ADD G460W'den W-allel varlığının DNP'ye yatkınlık olusturduğu ileri sürülmektedir. Çalısmamızda diyabetli kiŞilerin, DNP'ye gidislerinde, AGT M235T, ACE I/D ve ADD G460W polimorfizmlerinin etkisini incelemek amaçlanmıstır. Kontrol grubu olarak 100 DM'li hasta, çalısma grubu olarak 194 nefropatili hasta incelendi. ACE I/D polimorfizminin DD genotipi %54.0 ve D alleli %69.0 diyabetik grupta, nefropatili grupta ise ID, II genotipi sırası ile 78 (%40.2) ve 51(%26.3) ve I alleli %46.4 ile I allelinin varlığı nefropati ile iliskiliydi. Gruplar arasında AGT M235T ve ACE I/D polimorfizminin birlikteliğinde; genotipler arasında anlamlılık yokken, M/D allelleri (%42) diyabetik; T/I allelleri (%28.1) nefropatik grupta anlamlıydı. Tek basına ADD G460W polimorfizminde GG (%77.3) ve WW (%7.7) genotiplerinin nefropatiye yatkın olabileceği, ACE I/D birlikteliğinde; diyabetik grupta DD/GG (%45); DNP'li grupta ID/GG (%29.9) ve II/GG (%19.6) birlikteliği anlamlıydı. D/G (%64) allelleri diyabetik; I/G allelleri ise (%36.6) DNP'li grupta anlamlıydı. Sonuç olarak; diyabetik hastalarda nefropatiye olan yatkınlıkta; ACE-ADD birlikteliğinde II-ID/GG genotip ile I/G allellerinin ve ACE-AGT birlikteliğinde ise TT/ID genotipi ile T/I allellerinin etkili olabileceğini düsünmekteyiz.

Anahtar kelimeler: *DM, Diyabetik Nefropati, ACE I/D polimorfizmi, AGT M235T polimorfizmi, ADD G460W polimorfizmi*

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INTRODUCTION

Diabetic nephropathy (DN) is one of the most serious complications of diabetes, and the number of patients with diabetic nephropathy is increasing in parallel with the increase in the prevalence of diabetes mellitus (DM) (1). The pathogenesis of DN appears to be multifactorial, and several environmental, and/or genetic factors might be responsible for the development and progression of the disease (2); however, precise mechanisms have not been elucidated yet. Cumulative evidences suggest that genetic susceptibility plays an important role in the pathogenesis of diabetic nephropathy (3, 4), and worldwide efforts have been made to identify the genes conferring susceptibility to diabetic nephropathy.

As demonstrated in several studies with type 1 and type 2 DM, some genetic predispositions increase the risk of diabetic nephropathy (4-6). Among these genes, the genes encoding the renin-angiotensin system (RAS), especially the angiotensin-converting enzyme (ACE) gene, have been extensively evaluated (7). RAS acts a key regulator of sodium homeostasis and may play an important role in blood pressure (BP) regulation. There is an association between the D allele and higher plasma levels of ACE that also seems to be associated with diabetic complications (8, 9). ACE not only plays an effective role in nephropathy, but also participates in cardiac complications and hypertension (10, 11).

Angiotensinogen (AGT) is another RAS gene. Its well known M235T polymorphism, characterized by a methionine (M) to threonine (T) substitution at position 235, has been associated with the presence of essential hypertension, higher plasma AGT concentrations, and blunted renal vascular response to angiotensin II infusion (12, 13). A number of studies have investigated a possible role of M235T polymorphism in genetic predisposition to DN (14, 15). A higher 24-hour urinary albumin excretion (UAE) was found in homozygous TT patients with type 2 DM (16) and a correlation between the T allele and DN was also found in type I DM (14, 17). By contrast, in other studies AGT polymorphism does not appear to be a risk factor for DN both in type 1 (14) and type 2 DM (15, 16). Marre` et al. (9) found a significant interaction between AGT M235T polymorphism and the Insertion/Deletion (I/D) polymorphism in the ACE gene: they found indeed that type I DM patients with D and T allele had a major risk to develop DN.

In humans, the α-adducin (ADD1) gene is located on chromosome 4 and a G→T polymorphism at position 217 in exon 10 of the gene results in a glycine to tryptophan substitution at amino-acid position 460 (Gly460Trp) in the protein. This polymorphism has been linked to hypertension in some (18, 19) but not all studies (20, 21). The majority of studies addressing blood pressure in general, predominantly normotensive populations failed to show associations with adducing polymorphisms, reported positive associations for subgroups only (e.g. postmenopausal women), or depended on epistatic interactions with other polymorphisms, especially the ACE I/D polymorphism (22). Two studies (23, 24) demonstrated a reduction of GFR and effective renal plasma flow in hypertensive carriers of the ADD1 W/W genotype on low-salt diet and a reduction of GFR and an increase of urinary proteins in carriers of the ADD1 W allele and ACE/DD genotype in a general predominantly normotensive population.

This report describes the investigation of genetic variants in the ACE I/D, AGT T235M and ADD Gly460Trp gene for association with diabetic nephropathy in a case–control collection in Turkish population.

MATERIALS AND METHODS

NIDDM patients (294) having nephropathy or not were selected among the diabetic patients followed by Firat University Medical Faculty Endocrine Polyclinic while

Table 1. *Demographic, clinical and biochemical values of diabetic and diabetic nephropathy patients.*

General Characteristics	*DM*	*DN*	*p value*
Age (year)	*55.8±10.8*	*60.3±11.0*	*<0.001*
Weight (kg)	*76.9±16.0*	*74.8±15.7*	*>0.05*
Height (cm)	*162.7±12.5*	*152.3±44.5*	*>0.05*
Gender (M/F)	*45/55*	*84/110*	*>0.05*
Disease duration (years)	*8.2±6.29*	*15.7±9.5*	*<0.0001*
Fasting blood glucose (mg/dL)	*193.8±114.7*	*185.4±116.8*	*>0.05*
HbA1C (%)	*9.5±7.5*	*8.4±2.0*	*>0.05*
Urine protein/creatinine ratio	*0.4±0.1*	*3.9±0.1*	*<0.001*

Table 2. *Regression analysis between genetic polymorphism and disease durations in DN group*

	B		*SE*		*t*		*p value*	
	NND	*DN*	*NND*	*DN*	*NND*	*DN*	*NND*	*DN*
ACE I/D	*0.41*	*-0.78*	*0.72*	*0.61*	*0.57*	*1.27*	*0.569*	*0.204*
ADD G460W	*-0.75*	*0.27*	*1.18*	*0.78*	*0.64*	*0.35*	*0.524*	*0.730*
AGT M235T	*0.41*	*-0.15*	*0.80*	*0.67*	*0.50*	*0.23*	*0.613*	*0.816*

those having diabetic nephropathy were selected among the diabetic nephropathy patients followed by the nephrology polyclinic. All subjects were divided into two groups as follows: (1) diabetes with nephropathy (DN) (n=194); (2) diabetes without nephropathy (NND) (n=100). After consenting to participate in the study, each subject underwent an examination by a physician. And provided a diabetes history regarding its diagnosis, treatment and the occurrence of complications recorded by the same physician. Spot urine protein and creatinine levels were determined and urine protein/creatinine ratio calculated for DN diagnosis (25). Each subject provided a blood sample for biochemical measurements and DNA extraction. Written informed consent was obtained from all subjects and study protocol was approved by the Firat University Hospital Human Ethics Committee.

Table 3. *Genotype and allele frequency distribution of ADD, AGT and ACE gene polymorphisms in DM and DN groups.*

	DM	*DN*	*p value*
ACE I/D			
DD	*54 (54.0%)*	*65 (33.5%)*	*0.003*
ID	*30 (30.0%)*	*78 (40.2%)*	
II	*16 (16.0%)*	*51 (26.3%)*	
Allele frequency			
D	*69.0%*	*53.6%*	*<0.0001*
I	*31.0%*	*46.4%*	
AGT M235T			
TT	*25 (25.0%)*	*51 (26.3%)*	*0.68*
TM	*55 (55.0%)*	*97 (50.0%)*	
MM	*20 (20.0%)*	*46 (23.7%)*	
Allele frequency			
M	*52.5%*	*51.3%*	*0.68*
T	*47.5%*	*48.7%*	
ADD G460W			
GG	*77 (77.0%)*	*150 (77.3%)*	*0.025*
GW	*22 (22.0%)*	*29 (14.9%)*	
WW	*1(1%)*	*15 (7.7%)*	
Allele frequency			
G	*88.0%*	*4.8%*	*0.290*
W	*12.0%*	*15.2%*	

ACE; Angiotensin converting enzyme, ADD; Adducin, AGT; Angiotensinogen, DM; Diabetes Mellitus, DN; Diabetic Nephropathy

Genetic and biochemical analysis

Genomic DNA was purified from 200 µl of human whole blood, using a Wizard Genomic DNA purification Kit (Lot: 253596, Cat: A1125). Blood was obtained from a superficial vein of forearm after a 12-hour fast and serum was separated. Urine protein and urine creatinine were studied by Olympus AU600 in serum glucose, HbA1C and spot urine.

Detection of ACE I/D polymorphism

A genomic DNA fragment on intron 16 of the ACE gene was amplified by polymerase chain reaction (PCR) using a flanking primer pair (26) and a primer pair that recognizes insertion-specific sequence (27). The flanking primer pair consisted of 5′-CTGGAGACCA CTCCCATCCTTTCT-3′ and 5′-GATGTGGCCATCACATTCGTCAGAT-3′. PCR amplification products were obtained using 50 µl of the reaction mixture. initial melting step of 5 min at 94 °C; followed by 30 cycles of 1-minute at 94 °C, 1-minute 45 s at 60 °C, and 1-minute 30 s at 72 °C; and a final elongation step of 5 min at 72 °C. Fragments without insertion (D allele) and with insertion (I allele) of 190 and 490 bp, respectively, were detected on a 1.5% agarose gel containing ethidium bromide.

Detection of AGT M235T polymorphism

For detection of AGT M235T polymorphism AGT amplification of DNA was performed by PCR methodology (28). This was followed by digestion of 248 bp PCR product with restriction enzyme Tth 111I at 370C for 2 h. Digested products were separated on a 3.5% agarose gel. The heterozygous form was indicated by the appearance of two fragments of 165 and 141 bp, while the homozygous form revealed only one band of 141 bp and individuals lacking this mutation showed only one band of 165 pb.

Detection of ADD G460W polymorphism

ADD gene polymorphism was genotyped by method of Morrison et al. (29). Briefly, two allele-specific primers were mixed and used for the PCR amplification in a single

Table 4. Frequency distribution of coexistence of ACE-ADD, AGT-ADD and AGT-ACE in DM and DN groups.

	DM	DN	p
coexistence of ACE-ADD genotypes			
DD/GG	45.0%	27.8%	0.006
DD/GW	8.0%	3.1%	
DD/WW	1.0%	2.6%	
ID/GG	20.0%	29.9%	
ID/GW	10.0%	7.7%	
ID/WW	4.0%	2.6%	
II/GG	8.0%	19.6%	
II/GW	4.0%	4.1%	
II/WW	0%	2.6%	
coexistence of ACE-ADD alleles			
D/G	64.0%	47.9%	0.003
D/W	5.0%	5.7%	
I/G	24.0%	36.6%	
I/W	7.0%	9.8%	
coexistence of AGT-ADD genotypes			
MM/GG	18.0%	20.1%	0.27
MM/GW	7.0%	4.1%	
MM/WW	0%	2.1%	
MT/GG	46.0%	40.2%	
MT/GW	9.0%	5.7%	
MT/WW	0%	4.1%	
TT/GG	13.0%	17.5%	
TT/GW	6.0%	5.2%	
TT/WW	1.0%	1.0%	
coexistence of AGT-ADD alleles			
M/G	49.0%	44.6%	0.74
M/W	5.0%	6.4%	
T/G	37.5%	40.2%	
T/W	8.5%	8.8%	
coexistence of AGT-ACE genotypes			
MM/DD	15.0%	9.3%	0.055
MM/ID	7.0%	8.2%	
MM/II	1.0%	8.2%	
MT/ DD	28.0%	17.5%	
MT/ID	18.0%	20.6%	
MT/II	11.0%	11.9%	
TT/DD	10.0%	6.7%	
TT/ID	6.0%	11.3%	
TT/II	4.0%	6.2%	
coexistence of AGT-ACE alleles			
M/D	42.0%	32.0%	0.004
M/I	10.5%	18.3%	
T/D	27.0%	21.6%	
T/I	20.5%	28.1%	

reaction. The following primer sets were used; Forward primer 5'-CTCCTTTGCTA GTGACGGTGATTC-3' and the Reverse primer 5'-GACTTGGGACTGCTTCCATTCGGC-3'. PCR conditions: Amplification was carried out on a PCR System (Bio-Rad Laboratories, ABD; Moleculer Imager Gel Doc XR) in a 25 µl reaction mixture. The size of PCR products was 147 bp, 122 bp and 25 bp, respectively, which were clearly resolved on a 3% agarose gel (Bioron GmbH, Ludwigshafen, Germany).

Statistical methods

Continuous variables were expressed as mean ± Standard deviation (SD). Independent sample t tests were used to analyse differences in continuous variables between diabetics without nephropathy and with nephropathy. Chi-square test was used for comparison of nominal variables between groups. P<0.05 were accepted as statistically significant. Statistical significance of the observed genotype frequencies was evaluated according to Hardy-Weinberg rule compared to the expected genotype frequencies. Hardy–Weinberg equilibrium was evaluated by the chi-square test. The effect of the difference between the duration of disease, regression analysis was used to determine. Regression analysis was used, between NND and DN groups, for disease duration. All analysis was carried out using SPSS 12.0 software (Statistical Package for Social Sciences, SPSS Inc., IL, USA).

RESULTS

Characteristics of the subjects

The 294 participants included 194 DN and 100 NND patients shown in Table 1. Duration of diabetes and ratio of urine protein/creatinine were statistically significant and higher in the group having DN when compared to the diabetes mellitus group. There were no significant differences in the levels of serum lipids, glucose, HbA1C, chloride and blood pressure. Compared to NND group, disease duration was significantly higher in DN group. But with regression analysis it has detected that ACE, ADD and AGT polymorphisms have not an impact on disease durations (Table 2).

Distribution of ACE, Angiotensinogen and adducin genotypes and alleles within the study population

Genotypic and allelic frequencies of ACE I/D, AGT M235T and ADD G460W for the study groups are shown in Table 3. There was no deviation from Hardy–Weinberg equilibrium for the polymorphisms considered. The ID and II genotype frequency were higher in DN while DD genotype was higher in patients with NND and the difference was statistically significant (p=0.003). The D allele was more frequent in NND compared to DN and this was also significant (p <0.0001). The TT genotype and T allele of the Angiotensinogen M235T tended to be slightly more frequent in the diabetic patients with nephropathy but this was not significant (p= 0.68). In adducin G460W gene

polymorphism, WW genotype was significantly higher in patients with nephropathy compared with diabetics without nephropathy (p=0.025) but there was no significant difference in G and W alleles among groups.

Results of combined genetic variation in the ACE, ADD and AGT genes were shown in table 4. Combination of II homozygosity of the ACE gene and GG homozygosity of the ADD gene was more frequent in diabetics with nephropathy while combination of DD homozygosity of the ACE gene and GG homozygosity of the ADD gene was more frequent in NND (p= 0.006). The frequency of D/G allele combination was higher in diabetics without nephropathy (p=0.003). Among the diabetic groups, there were no significant relation between AGT and ADD combination. While there was no statistically significant difference between the groups in terms of simultaneous presence of ACE and AGT (p=0.055), M/D alleles were significantly higher in NND group and simultaneous presence of T/I alleles was significantly higher in DN group (p=0.004).

DISCUSSION

There are great ethnic differences in ACE gene polymorphism while there is also a heterogeneity among ethnic groups (30, 31). When compared to Europe, D allele has been found to be higher in the African American population living in the United States of America and lower in the Japanese population (32). In a meta-analysis of Fujisawa et al. (33) and a study of Ahluwalia et al. (34) on type 2 patients having DN, a strong association has been shown between presence of D allele and DN. Marre et al. (9) found no significant difference in terms of ID and DD genotypes in ACE gene polymorphism studies on DM patients grouped according to nephropathy but showed that the frequency of II genotype decreased with the stage of the disease. Eroglu et al. (15) studied ACE gen polymorphism in DM patients with or without DN and found no significant difference between the groups. Else Schmidt et al. (35) and Buraczynska et al. (36) finds similar results. Contrary to the findings of Fugisawa et al. (33) and Ahluwalia et al. (34), we found that the frequencies of DD genotype in NND group and ID and II genotype in DN group were statistically significantly different. Different results obtained could be explained by different genetic structures due to race and ethnical backgrounds or not being able to fully eliminate the factors (hypertension, glomerular diseases etc.) effective in the development of nephropathy.

Eroglu et al. (15) and Tarnow et al. (37) found no significant difference between the groups in terms of the distribution of genotypes and alleles of AGT M235T polymorphism. However, Ahluwalia et al. (34) found in their study that TT genotype was significantly associated with MT/TT genotype while Freire at el. (38) found that TT genotype was significantly associated with DN group. In our study, similar to the findings of Eroglu et al. (15) and Tarnow et al. (37), we could not find any statistically significant association between NND and DN groups in terms of the distribution of genotypes and alleles of AGT M235T polymorphism.

Gutierrez et al. (39) studied the association between the development of nephropathy and ACE I/D and AGT M235T polymorphisms and showed that neither of the polymorphisms caused a significant difference in terms of development of nephropathy. Marre et al. (9) claimed in their studies that ID or DD genotype, especially in ACE I/D polymorphism, interact with TT genotype of AGT M235T polymorphism and could contribute to the development of nephropathy. Nicod et al. (40) found that simultaneous presence of ACE I/D polymorphism and AGT-MM genotype has an increased contribution to the development of renal failure. In our study, we too studied the effects of genotypes and alleles of ACE I/D and AGT M235T polymorphisms on the development of nephropathy. Although the combination of MM/DD and MT/DD genotypes in DM group and the combination of TT/ID genotypes in DN group were found to be high, but this was not statistically significant compared with NND. With respect to alleles, coexistence of M/D alleles in NND group and T/I alleles in DN group were statistically significantly.

In a study on hypertensive patients, Beeks et al. (24) showed that ADD-WW genotype increased GFR and renal plasma flow significantly when compared to ADD-GG genotype. Conway et al. (41) showed that there was no significant difference between type 1 NND group and DN group in terms of ADD G460W gene polymorphism in progression to nephropathy. In another study, it was suggested that ADD G460W polymorphism did not directly cause renal disease but increased the rate of progression to renal failure together with other polymorphisms (40). In our study, we found the higher frequency of WW genotype of ADD G460W gene polymorphism to be statistically significantly in DN group when compared to the NND group. This suggests us that WW genotype could be effective in development of DN in DM patients.

In a study, it was suggested that ACE-II genotypes and ADD-460WW genotypes, through increasing hypertensive effect, could act jointly in progression of renal disease in patients having IgA nephropathy (42). In other studies, combination of ADD G460W and ACE I/D polymorphisms were found to have joint role on blood pressure and progression of renal disease (43, 44) and coexistence of DD/GG genotypes were shown to be more effective in progression to renal diseases (40). In studies conducted on different ethnic groups, no significant association was found between progression to renal disease and simultaneous presence of ADD and ACE gene polymorphisms (20, 45). In our study, with respect to combination of both ADD G460W and ACE I/D polymorphisms, we found that combination of DD/GG genotype was significantly high in NND while combination of ID/GG and II/GG were significantly high in DN group. With respect to simultaneous presence of alleles, D/G alleles were significantly high in without nephropathic diabetic group and I/G alleles were significantly high in DN group. In a study conducted on Caucasian women, AGT M235T, ADD G460W and AT-II type 1 receptor polymorphisms were reported to contribute to renal function loss (46). Nicod et al. (40) showed that coexistence of the genotypes of ADD-AGT polymorphism had no effect in progression to nephropathy. In our study, simultaneous presence of genotype and alleles of ADD G460W and AGT M235T polymorphisms were not found to be statistically significantly different in NND and DN groups.

As a result, we believe, in simultaneous presence of AGT M235T, ACE I/D and ADD G460W gene polymorphisms, II-ID/GG genotypes and I/G alleles in simultaneous presence of ACE-ADD gene polymorphism along with TT/ID genotype and T/I alleles in simultaneous presence of ACE-AGT could be effective in susceptibility to diabetic nephropathy in diabetic patients. The reason of having similarities and differences with other studies could be explained by the genetic differences of the study groups. Thus, our findings should be supported by further studies having larger number of cases.

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