Renal Failure

ISSN: 0886-022X (Print) 1525-6049 (Online) Journal homepage: https://www.tandfonline.com/loi/irnf20

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To cite this article: Kadriye Altok Reis, Fatma Ayerden Ebinç, Eyüp Koç, Hüseyin Demirci, Yasemin Erten, Galip Güz, Ülver Boztepe Derici, Musa Bali, Oğuz Söylemezoğlu, Turgay Arınsoy & Şükrü Sindel (2011) Association of the Angiotensinogen M235T and APO E Gene Polymorphisms in Turkish Type 2 Diabetic Patients with and without Nephropathy, Renal Failure, 33:5, 469-474, DOI: 10.3109/0886022X.2011.568133

To link to this article: https://doi.org/10.3109/0886022X.2011.568133

Published online: 18 Apr 2011.

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Renal Failure, 33(5): 469–474, (2011)
Copyright © Informa Healthcare USA, Inc.
ISSN 0886-022X print/1525-6049 online
DOI: 10.3109/0886022X.2011.568133

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CLINICAL STUDY

Association of the Angiotensinogen M235T and APO E Gene Polymorphisms in Turkish Type 2 Diabetic Patients with and without Nephropathy

Kadriye Altok Reis[1], Fatma Ayerden Ebinç[1], Eyüp Koç[1], Hüseyin Demirci[2], Yasemin Erten[1], Galip Güz[1], Ülver Boztepe Derici[1], Musa Bali[1], Oğuz Söylemezoğlu[3], Turgay Arınsoy[1] and Şükrü Sindel[1]

[1]Department of Nephrology, Faculty of Medicine, Gazi University, Ankara, Turkey; [2]Department of Endocrinology, Faculty of Medicine, Gazi University, Ankara, Turkey; [3]Department of Pediatric Nephrology, Faculty of Medicine, Gazi University, Ankara, Turkey

Abstract

Background: Diabetic nephropathy (DN) is a leading cause of diabetes-related morbidity and mortality. The aim of this study was to evaluate the relationship of AGT M235T and apoprotein E (APO E) gene polymorphism with DN in Turkish patients of Type 2 diabetes, and to compare genotype and allele distributions among DN patients, non-DN patients, and healthy controls. *Methods*: AGT M235T and APO E genotype and allele analysis were performed in 111 DN patients, 108 non-DN patients, 106 healthy control subjects for APO E genotype, and 100 for AGT M235T genotype polymorphism. APO E and AGT M235T genotype were determined by RFLP-PCR. *Results*: The frequencies of APO E ε2/3, ε 3/3, ε 3/4 genotypes were 22.7%, 60%, 60%, respectively, among DN patients and 6.6%, 80%, 10.4%, respectively ($p < 0.001$), in the non-DN patients. The frequencies of AGT M235T MM, MT, TT genotypes among the same groups were 17%, 46%, 37% and 21%, 63%, 16%, respectively ($p < 0.02$). Having the ε2/3 genotype and TT genotype increased the risk for DN nephropathy [4.8-fold (95% CI: 1.94–11.67), 2.9-fold (95% CI: 1.27–6.69), respectively]. *Conclusion*: Our study has shown that AGT M235T TT genotype and APO E ε 2/3 genotype may be linked to a risk for DN among Turkish population.

Keywords: Diabetic nephropathy, gene polymorphism, APO E, angiotensinogen

INTRODUCTION

Diabetic nephropathy (DN) is a leading cause of diabetes-related morbidity and mortality.[1] The two most significant risk factors known today in its development are hyperglycemia and high blood pressure.[2] Genetic predisposition has also been recently suggested as another cause of DN.[3–5] A major reason behind this suggestion is the presence of DN only among 1/3 of long-term diabetic patients.[6] In addition, the higher prevalence of DN among patients with hypertensive family members and among different ethnic groups (Indian immigrants living in the United Kingdom, African Americans, and Mexican Americans) also suggests the role of genetic predisposition in the development of DN. This proposition is also corroborated by epidemiological studies.[7–12] However, despite this belief that genetic factors may be influential in the development of DN, no gene has yet been clearly defined.[11] Studies are being conducted into the renin–angiotensin system (RAS) and lipid metabolism genes.[11–15]

RAS is a system that influences blood pressure regulation, sodium metabolism, and renal hemodynamics. Angiotensin II, which emerges via RAS, increases mesangial cell proliferation and matrix production, as well as being a strong vasoconstrictor.[16,17] Therefore, the main genes whose DN associations are most commonly studied are angiotensinogen-converting enzyme, angiotensinogen (AGT), and angiotensin II Type 1 receptor genes, which code parts of the RAS system.[18]

AGT M235T gene polymorphism in RAS is related to elevated AGT levels and hypertension.[19–21] Due to the association between this gene polymorphism and

Address correspondence to Fatma Ayerden Ebinç, Department of Nephrology, Faculty of Medicine, Gazi University, 06510 Besevler, Ankara, Turkey. Fax: +90312 2129006; E-mail: fayerdenebin@yahoo.com

Received 15 August 2010; Revised 24 January 2011; Accepted 27 February 2011

hypertension it has also been linked to DN. However, research results have remained inconclusive[22–25] and no consensus exists as of yet.

Large-scale studies have shown that the coexistence of lipotoxicity and glucotoxicity plays a role in the development of micro and macrovascular complications of diabetes.[26,27] Apoprotein E (APO E) is a glucoprotein with a significant role in lipid metabolism control. APO E gene polymorphism is closely related to a disruption in lipid metabolism[28] and is thus claimed to play a role in the development of DN despite similarly inconclusive research results.[13,29–31]

Our aim in this study was to study the relationship of AGT M235T and APO E gene polymorphism with DN in Turkish patients of Type 2 diabetes, and to compare genotype and allele distributions among DN patients, normoalbuminuria Type 2 diabetes patients, and healthy controls.

SUBJECTS AND METHOD

Participants were patients who had been suffering from diabetes for a minimum of 10 years and were being followed up in Gazi University Medical School's Nephrology and Endocrinology Clinic, and healthy individuals in the control group. The study protocol was approved by Gazi University Medical School's Ethics Committee. Participants' serum blood urea nitrogen, creatinine, glucose, total cholesterol, LDL cholesterol, HDL cholesterol, triglyceride, hemoglobin, and hemoglobin A_1C levels (twice with a 3-month gap) were measured from blood samples drawn after a 12 h starvation by using the standard method. Creatinine and protein measurements were made from 24 h urine specimens (twice with a 1-month gap and 2 days after antihypertensive drugs were stopped). Glomerular filtration rate was calculated by using the creatinine clearance method. The following formula was used for creatinine clearance (CCr) measurement: CCr = [urine creatinine (mgI/dL) × urine volume (mL)]/serum creatinine (mg/dL) × 1440. DN was defined by macroalbuminuria with a urinary albumin excretion of more than 300 mg in a 24 h collection of urine.

Patients' age, gender, and cigarette use were recorded. Height, weight, waist circumference measurements were also made. Their body mass index (BMI) was calculated. All participants were informed verbally about the study and their signed consent was obtained along with the consent of the ethics commission of the university. Participants included 111 DN patients, 108 non-DN patients, 106 healthy control subjects for APO E gene polymorphism, and 100 for AGT M235T polymorphism. All patients in the study were assessed with respect to hypertension, hyperlipidemia, and diabetic retinopathy.

AGT and APO E gene polymorphism genotype and allele analysis were performed by using the RFLP-PCR method. Genomic DNA was isolated by a denaturation–precipitation process from peripheral blood cells by using ammonium bromide salts. Polymerase chain reaction (PCR) amplification of relevant parts was performed with the mixture prepared with 75 mM Tris–HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% Tween-20, 2.0 mM NH_4MgCl_2, 50 mM of each dNTP, 50 pmol from each specific primer, 1.25 units taq DNA polymerase, and 0.2–0.5 μg DNA specimen. Thirty cycles were completed in the Perkin Elmer 9600 thermal cycler at 94°C for 30 s, 60°C for 45 s, and 68°C for 1 min. For angiotensinogen genotype (M235T) identification, 5′-TGACAGGATGGAA GACTGGCT-GCTCCCTGC-3′ and 5′-AGCAGA-GAGGTTTGC-CTTACCTTG-3′ primer sequences were used and 104 bp PCR products were cut with Msp 1 restriction enzyme (RFLP). These products were analyzed by using 73 and 31 bp 4% High Melt-Small Fragment AgarGel (CLP, Inc., USA) electrophoresis. The nonsense point mutation in exon 2 at position 235 (M235T) causes methionine (M) to threonine (T) substitution. Although heterozygotes (MT) display three bands, T-type variants did not break down and only displayed 104 bp band (Figure 1).

APO E was determined by using the standard procedure for genomic DNA. APO E genotypes were studied with the PCR method by using previously defined primers in the 227 bp fragment of the APO E gene.[24] During statistical analyses, the chi-square test was used to compare AGT and PAI-1 genotype allele distribution between groups, and the Hardy–Weinberg chi-square test was used to determine whether there was normal genetic distribution.

RESULTS

DN was detected in 111 of the 219 diabetic patients in the study and was not present in 108. When the DN and non-DN groups were compared regarding

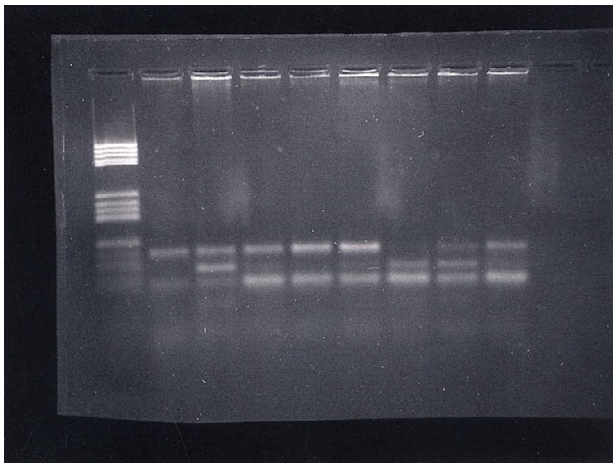


Figure 1. Angiotensinogen M235T-Msp 1 DNA bands – 3rd and 8th bands: MT (104 bp, 736 bp, 31 bp); 4th, 5th, 6th, and 9th bands: MM; 7th band: TT (73 bp, 31 bp).

demographics, anthropometric measurements, and biochemical parameters, no meaningful difference was observed in age, BMI, waist circumference, cigarette smoking, and Hb A_1C and Hb levels. On the other hand, the two groups differed meaningfully with respect to gender distribution, duration of diabetes, hypertension, hyperlipidemia and diabetic retinopathy, serum creatinine level, and creatinine clearance measurement. At the same time, fasting glucose levels between the two groups were marginally meaningfully different (Table 1). Of the DN group, 51.4% were females and 48.6% males; of the non-DN group, 67.6% were females and 32.4% males. Of the diabetic patients, the nephropathy group displayed 90% hypertension, 55.9% hyperlipidemia, 46.8% background diabetic retinopathy, and 33.3% proliferative retinopathy, whereas the non-nephropathy group displayed 78.7% hypertension, 37.4% hyperlipidemia, 27.8% background diabetic retinopathy, and 1.9% proliferative retinopathy.

When our cases were compared against the control subjects, no statistical difference was found between their APO E genotype (ε2/2, ε3/3, ε4/4, ε2/3, ε2/4, ε3/4) distributions, whereas a meaningful difference was observed regarding AGT genotypes (MM, MT, TT). MM, MT, and TT genotypes were 61%, 37%, 2%, respectively, in the control group, whereas those among diabetic patients were 19.2%, 54.3%, 26.5%, respectively ($p < 0.0001$). A comparison of DN and non-DN patients yielded the following results: ε2/3 genotype (22.7% vs. 6.6%), ε3/3 genotype (60% vs. 80%), ε3/4 genotype (17.3% vs. 10.4%) ($p < 0.001$) (Table 2). According to this, having the ε2/3 genotype increased the risk for DN by 4.8-fold (95% CI: 1.94–11.67). When DN and non-DN patients were compared with respect to AGT polymorphism, the results were MM genotype (17% vs. 21%), MT genotype (46% vs. 63%), TT genotype (37% vs. 16%) ($p < 0.002$) (Table 2). Having the TT genotype increased the risk for DN by 2.9-fold (95% CI: 1.27–6.69). Carrying T allele was associated with a risk for DN (odds ratio: 1.67; 95% CI: 1.14–2.44) (Table 3).

Table 2. Distribution of M235T and APO E genotype in diabetic nephropathy (DN) and non-diabetic nephropathy (non-DN) patients.

	DN (n = 108)	Non-DN (n = 111)
APO E genotype		
ε2/3	7 (6.6%)	25 (22.7%)*
ε3/3	88 (83%)	66 (60%)
ε3/4	11 (10.4%)	19 (17.3%)
M235T genotype		
MM	23 (21.3%)	19 (17%)
MT	68 (63%)	51 (46%)
TT	17 (15.7%)	41 (37%)**

Notes: *$p = 0.001$; odds ratio (95% CI) = 4.76 (1.9–11.6).
**$p = 0.002$; odds ratio (95% CI) = 2.9 (1.27–6.69).

Table 3. Distribution of M235T and APO E allele frequencies in diabetic nephropathy (DN) and non-diabetic nephropathy (non-DN) patients.

	DN (n = 222)	Non-DN (n = 216)
APO E		
E4	20 (9.0%)	13 (6.0%)
E3	176 (79.3%)	194 (89.8%)
E2	26 (11.7%)	9 (4.2%)
M235T		
M	89 (40.1%)	114 (52.8%)
T	133 (59.9%)	102 (47.2%)*

Note: *$p = 0.008$; odds ratio (95% CI) = 1.67 (1.14–2.44).

DISCUSSION

We found in our study that the AGT TT genotype increased the risk for DN 2.9 times in diabetic patients. Also, the comparison of healthy individuals and diabetic patients showed that the MM genotype was less frequent and the TT genotype more frequent in diabetic patients.

In the development and progression of DN, systemic high blood pressure and elevated glomerular capillary pressure both play a significant role.[32] RAS is important for the control of both systemic and intrarenal blood pressure. Therefore, RAS is thought to be closely

Table 1. Comparison of selected characteristics between diabetic nephropathy (DN) and non-diabetic nephropathy (non-DN) groups.

	DN (n = 111)	Non-DN (n = 108)	p
Gender (F/M)	57/54	73/35	0.01
Age (years)	58.39 ± 9.63	59.06 ± 8.80	0.92
Duration of diabetes (years)	16.16 ± 5.46	14.20 ± 4.25	<0.01
Hypertension (Y/N)	101/10	85/23	0.01
Diabetic retinopathy (no/background/proliferative)	22/52/37	74/30/2	<0.01
Cigarette smoking (Y/N)	35/76	22/86	0.10
Hyperlipidemia (Y/N)	62/49	41/67	<0.01
Waist circumference (cm)	99.450 ± 10.43	96.95 ± 10.43	0.09
BMI (kg/m^2)	29.086 ± 8.20	28.464 ± 5.16	0.93
Hemoglobin (g/dL)	11.61 ± 1.74	12.67 ± 1.75	<0.01
Fasting blood glucose (mg/dL)	170.31 ± 59.90	186.36 ± 63.15	0.05
Hemoglobin A_1C	7.92 ± 1.65	7.86 ± 1.49	0.66
Serum creatinine (mg/dL)	2.33 ± 1.53	1.05 ± 0.66	0.000
Creatinine clearance (mL/min)	47.09 ± 31.65	79.46 ± 26.62	<0.01

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related to DN.[33] In addition, inhibition of this system prevents the formation of DN.[34]

AGT M235T gene polymorphism is one of the gene polymorphisms seen in RAS. This polymorphism is closely related with essential hypertension. In the presence of AGT M235T gene polymorphism, elevated plasma AGT concentration is seen.[18,35] TT homozygote genotype results in the highest AGT elevation, which in turn increases angiotensin II level.[36] Such high angiotensin II levels may cause glomerular injury due to both systemic and glomerular pressure increase.[36] According to this, the increased risk for DN observed in this study when TT genotype was present may have occurred due to RAS activation. Lower MM genotype among diabetic patients also leads to the conclusion that RAS is less active in non-diabetics.

Despite these hypotheses, inconclusive results have been obtained in many studies of AGT M235T gene polymorphism and DN. Similar to our study, Wang et al., Young et al., and Wu et al., claimed that TT genotype might increase the risk for DN in Type 2 diabetics, whereas Fogarty et al. claimed the same for Type 1 diabetics.[22–24,37] On the other hand, certain other studies found no such relationship.[14,22,25] In another study conducted with a Turkish population, Eroğlu et al.[25] found no relationship between AGT M235T gene polymorphism and DN. Despite these conflicting results, the effects of RAS still suggest that the gene polymorphisms which affect this system may be closely related to DN. Prospective studies focusing particularly on this aspect of the issue would undoubtedly bring meaningful results.

Another result of our study has been that although APO E genotype distribution does not differ meaningfully between the control group and diabetic patients, APO E2 gene polymorphism (ε2/3 genotype) increases the risk for DN 4.8 times among diabetics. In addition, e2 and e4 allele frequency was *higher* among DN patients, albeit not statistically meaningful.

APO E is a glycoprotein that controls the lipid metabolism consisting of 299 amino acids. It is a part of VLDL, IDL, chylomicrons, chylomicron traces, and HDL.[1,28] It carries lipids to the intracellular environment via LDL and APO E receptors. The APO E gene is localized in the 19th chromosome and consists of three isoforms (E2, E3, and E4) coded by three codominant alleles (ε2, ε3, and ε4).[38,39] The lipid profile is determined by APO E's genetic variations. Although APO e3 allele functions normally, e2 and e4 alleles have different features. Accordingly, e4 allele is associated with an increase in the affinity of APO E to receptors, whereas e2 allele is associated with a decrease in its affinity to receptors. When APO E2 polymorphism is present, triglycerides and their traces are increased due to the decrease in the catabolism of lipoproteins containing triglycerides; and when APO E4 polymorphism is present, triglyceride levels fall due to the increase in the metabolism of triglyceride-rich lipoproteins and, thus, total and LDL cholesterol levels increase.[28,38–41]

APO E also plays a role in central and peripheral nerve regeneration, immunoregulation, cellular growth and differentiation, and recovery of tissue damage.[42] APO E is further claimed to decrease vascular smooth muscle cell proliferation capacity and increase vascular heparan sulfate proteoglycan level.[43] Elevated heparan sulfate proteoglycan levels inhibit mesangial cell proliferation and assist the formation of glomerular basal membrane and main filtration barrier in the interstitium.[43] Therefore, it is believed that a possible defect with the existence of APO E gene polymorphism may lead to an increase in mesangial cell proliferation.[43] In experimental studies, APO E gene polymorphism has been observed to be associated with mesangial cell proliferation and mesangial matrix increase.[43]

Recently, APO E gene polymorphism has been claimed to play a role in DN development. However, the large number of studies into this issue has failed to reach a definitive conclusion.[1,6,13,15,42] As was the case in our study, several others also claim that carrying e2 allele may increase the risk for DN. For instance, Araki et al. showed in their prospective study that the presence of e2 allele increased the risk for DN. They claimed that e2 allele was a factor both in the development and in the progression of DN.[29] Case–control studies with similar results also exist.[1,15,30,44] Additionally, the single histopathological study conducted by Guan et al.[13] also found an association between diabetes-related glomerulopathy and APO E2. On the other hand, some other studies found no relationship between APO E2 and DN.[6,42,45,46] Conversely, Boizel et al.[47] claimed that the risk for DN was lower with APO E2 than it was with E3 and E4. In another study conducted with a Turkish population, İlhan et al.[31] claimed that there might be a relationship between APO E4 isoform and DN.

The factors leading to such contradicting results in studies about DN and gene polymorphism are not yet clear. It is considered that the presence of different ethnic groups in these studies is one noteworthy factor. However, there are also studies with contradicting results which were conducted on individuals from similar ethnic groups. Other factors may be the use of different criteria in the definition of DN (microalbuminuria vs. end-stage renal failure), as well as the use of different techniques in the identification of albuminuria and serum creatinine measurement. In addition to these, effects of other genetic factors may also be influential.[13,30,48]

The possible effect mechanism of APO E2 gene polymorphism on DN is not yet clear. Despite the lack of evidence, an increase in APO E2 and lipoprotein-rich triglycerides and their traces may be related to DN development.[38–40] Also, mesangial cells and the triglyceride-rich lipoproteins accumulated in the macrophages may cause the release of various cytokines. These cytokines may result in an increase in the production of extracellular matrix proteins and enlarged mesangium.

The macrophages accumulating due to cytokines may produce more oxidized lipoproteins and increase renal injury.[49–54] It is also believed that, with APO E2 polymorphism, there will be less protection against growth factors that induce mesangial cell proliferation. In short, APO E2 might mean fewer autocrine protective effects in the case of diabetes.[55]

When the risk factors were assessed, we found that the risk for DN was increased by being male, a longer duration of DM, hypertension, and low hemoglobin levels, whereas BMI, waist circumference, and smoking did not seem to have any effect on DN. Even though Hb A_1C levels were higher in the DN group, this difference was not statistically meaningful. This result may have been due to the small number of patients and the relatively short-term assessment of Hb A_1C levels.

The main limitation of our study is that this is a cross-sectional study, and this type of study may find associations with the disease but not identify specific causes. Therefore, the possible roles of gene polymorphisms in the development or progression of DN should be confirmed by an observational follow-up study.

In sum, our study has shown that AGT M235T TT genotype and APO E ε2/3 genotype may be linked to a risk for DN among Turkish population. Studying AGT and APO E genotypes in diabetic patients may help in deciding whether patients fall in a DN-risk group. Furthermore, synergistic or combined effects in individuals with AGT and APO E genotypes may also contribute the present risk factors for the development of DN. But our study was not designed for investigating this relationship. Findings verified by population studies with a higher number of cases may contribute to the emergence of new treatment models, which will affect pathogenesis among at-risk patients.

ACKNOWLEDGMENT

This study has been supported by the Turkish Hypertension and Renal Diseases Association and Gazi University.

***Declaration of interest*:** The authors declare that there is no interest that could be perceived as prejudicing the impartiality of the research reported.

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