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A Relation Between the Apolipoprotein E Genotypes and Microalbuminuria in Type 2 Diabetes Mellitus

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Abstract : Various relations between apolipoprotein E (apo E) and diabetic nephropathy have been reported. In this study, the question of whether there is any relation between apo E genotypes and microalbuminuria in patients with type-2 diabetes mellitus was examined. Twenty-one male and 25 female patients, with ages the range of 37-62 years (mean 51.7±7.5) with type-2 diabetes mellitus were studied. The apo E genotypes were determined by PCR amplification of the 227 bp region followed by CfoI digestion to release specific band patterns. While 24 patients had microalbuminuria (microalbuminuria group), 22 patients had normoalbuminuria (normoalbuminuria group). The patients with microalbuminuria had suffered from diabetes for a longer period of time (median 13 vs 11 years $p<0.005$) and had higher levels of serum total cholesterol (median 199 vs 161 mg/dl, $p<0.01$) and LDL cholesterol (median 123.5 vs 95 mg/dl, $p<0.01$) than the other group. In the microalbuminuria group, the distribution of apo E genotypes was revealed as ε2/ε2 2 (8.3%), ε3/ε2 7 (29.2%), ε4/ε2 0 (0%), ε3/ε3 12 (50.0%), ε4/ε3 2 (8.3%) and ε4/ε4 1 (4.2%). In the normoalbuminuria group, the distribution of apo E genotypes was revealed as ε2/ε2 0 (0%), ε3/ε2 3 (13.6%), ε4/ε2 0 (0%), ε3/ε3 14 (63.6%), ε4/ε3 4 (18.1%) and ε4/ε4 1 (4.5%). In these two groups of patients, in terms of the distribution of apo E genotypes, no significant difference could be found ($p>0.05$). However, the apo ε2 allele frequency in the microalbuminuria group in comparison to the normoalbuminuria group was found to be quite high (22.9% vs 6.8%, odd ratio 4.89, $p<0.05$).

As a result, we concluded that the ε2 allele of apo E may play a role in the mechanism of nephropathy in type-2 diabetes mellitus

Key Words: Diabetes mellitus, microalbuminuria, apolipoprotein E genotypes

Introduction

Apolipoprotein E (apo E) is found on the surface of all of the major classes of circulating lipoproteins. It is necessary for the normal clearance of cylomicron remnants, very-low-density lipoprotein (VLDL), LDL, and intermediate-density lipoprotein (IDL) and may play a role in reverse cholesterol transport (1). There are three common alleles for the apo E gene: ε2, ε3, and ε4. The most common phenotypes are ε3/ε3, ε4/ε3, and ε2/ε3. The frequency of apo E alleles in Turkey is 86% for ε3, 7.9% for ε4 and 6.1% ε2 according to Turkish heart study (2).

Although the preventive effect of apo E on plasma lipids has been shown (3), the effects of apo E genotypes and alleles on the lipids are clearly different. The ε2 isoform is associated with lower cholesterol but higher triglyceride levels than the ε3 isoform, and the ε4 isoform is associated with higher cholesterol but lower triglyceride levels (4). On the other hand, there are relations between apo E and several diseases (5). It has been suggested that there is an association between apo E polymorphism and macroalbuminuria in patients with non-insulin dependent diabetes mellitus (6). This polymorphism is associated with the progression of diabetic nephropathy. The presence of the ε4 allele is a protective factor, and other alleles are risk factors (7).

The aim of this study was to investigate the association between the apo E genotypes and microalbuminuria in patients with type-2 diabetes mellitus.

Materials and Methods

Forty-six patients with type-2 diabetes mellitus (21 M and 25 F, aged 37 to 62 years) were investigated. The

duration of diabetes in the patients was determined. All of the patients were taking oral hypoglycemic agents. In order to compare the serum lipid values, 30 healthy people (15 M and 15 F, aged 35 to 65 years) were included in the study.

Hypertension was diagnosed when systolic blood pressure>140 mmHg and/or diastolic blood pressure>90 mmHg was found (8). Patients who had a history of coronary artery disease or ischemic findings on electrocardiography were not included in the study. Retinopathy was evaluated according to the following classification (9): 1 - no signs of diabetic retinopathy; 2 - nonproliferative diabetic retinopathy (NPDR); and 3 - proliferative diabetic nephropathy (PDR). Patients exhibiting diabetic neuropathy signs and symptoms were not included in the study.

Serum concentrations of total cholesterol (total-C), high-density lipoprotein cholesterol (HDL-C) and triglyceride (TG) were measured from fresh serum samples drawn after a 12-hour overnight fast, using commercially available kits from the Boehringer Mannheim company. The levels of glycated hemoglobin (HbA1c) were measured with the same autoanalyzer. Serum levels of LDL-C were calculated from the measurements of total-C, HDL-C and TG by the Fridewald formula (10). Microalbuminuria was measured by the nephelometric method (Beckman Array System) in 24-hour urine samples (11). Cases with ketoacidosis, bacteriuria, hematuria, excessive trained and those using angiotensin-converting enzyme inhibitors were not included in the study. Urine samples were centrifuged at 3000xg for 10 minutes before microalbuminuria was measured in order to remove any cells or debris.

Microalbuminuria was defined as AER 20-200 mg/min (or approximately 30-300 mg/day) and overt proteinuria as AER>200 mg/min in at least two out of three measurements (12).

Genomic DNA was isolated from blood (13). The apo E genotypes were determined by PCR amplification of the 227 bp region followed by CfoI digestion to release specific band patterns (14). Amplification of the apo E target sequence was performed by using the 5-primer TCCAAGGAGCTGCAGGCGGCGCA and 3 primer ACAGAATTCGCCCCGGCCTGGTACACTGCCA. These primer pairs amplify a 227 bp region of DNA that spans both apo E polymorphic sites. About 15 ml of PCR products were run on 3% agarose gel. The major band was seen to correspond to a molecular weight of approximately 227 bp. Then, 15 µl of amplification products was subjected to restriction by CfoI. The digested products were electrophoresed, stained with ethidium bromide and viewed.

Lipid parameters were compared between the healthy control group and the patients, and also between the microalbuminuria and normoalbuminuria groups. The distribution of apo E genotypes and the allele frequency were investigated in these two groups of diabetic patients. Statistical analyses were performed with the SPSS 6.1 computer program by using the unpaired Student's t test, Mann Whitney-U test, Fisher's exact test, Kruskal Wallis test and odd ratio where necessary. The level $p<0.05$ was considered to be statistically significant.

Results

The numbers of males and females were similar to each other in both the patient and control groups. (M/F ratio, 21/25 vs 15/15; Fisher's exact test, $p>0.05$). There was no significant difference between the patients and control groups in terms of mean age (51.7±7.5 vs 50.5±7.9 years), or in the serum levels of total-C (172.9±30.4 vs 171.4±31.1 mg/dl), HDL-C (34.6±5.9 vs 33.7±5.6 mg/dl), LDL-C (104.7±24.9 vs 106.5±27.4 mg/dl) and TG (169.4±45.1 vs 156.0±48.4 mg/dl) (unpaired Student's t test, $p>0.05$).

The clinical and laboratory characteristics of the patients are shown in Table 1.

The patients with microalbuminuria showed a longer duration of diabetes ($p<0.05$) and higher levels of serum total-C and LDL-C ($p<0.01$) than the other group, but there were no significant differences between the other parameters seen in Table 1.

Fundoscopic findings were normal in 7 (31.8%) of the normoalbuminuric patients, and NPDR was determined in 15 (68.2 %) of them. NPDR and PDR were detected in 19 (79.2%) and 5 (20.8%) of the microalbuminuric patients, respectively.

Figure I shows PCR amplification of apo E. Figure II presents CfoI restriction endonuclease cleavage maps for each apo E isoform and the size of fragments from polymorphic sites. Each apo E allele has specific combinations of CfoI fragment sizes. Different sizes of DNA fragments are generated by CfoI restriction of the amplified 227 bp sequence.

Table 1. Some characteristics of the patients.

	Microalbuminuria	Normoalbuminuria	P
N	24	22	-
Sex (M/F)	11/13	10/12	NS
Age (years)	46.5 (37-62)	48 (41-60)	NS
BMI (kg/m^2)	28 (25-30)	27.5 (25-32)	NS
Diabetes duration (years)	13 (8-19)	11 (7-15)	<0.05
Hypertensive/normotensive	10/14	9/13	NS
HbA1c (%)	8 (6-16)	8.5 (5-15)	NS
Total-C (mg/dl)	199 (118-224)	161 (100-207)	<0.001
HDL-C (mg/dl)	35 (24-46)	33.5 (23-42)	NS
TG (mg/dl)	180 (85-260)	152.5 (105-220)	NS
LDL-C (mg/dl)	123.5 (62-145)	95 (53-122)	<0.01

NS: non-significant

Fisher's exact test was applied to determine whether there was any difference in terms of sex distribution and hypertension frequency in the patient groups in Table 1. Other parameters in this table are shown as median (minimum-maximum) values and were evaluated by Mann Whitney U test.

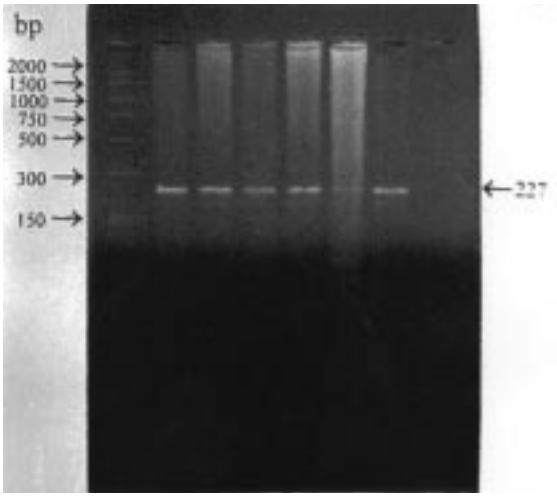

Figure 1. Agarose gel electrophoresis analysis of PCR-amplified genomic DNA by using Apo E primers (see methods section). The size of the amplified fragment is 227 bp. Lanes show the amplified fragment using 1 µg genomic DNA from blood.

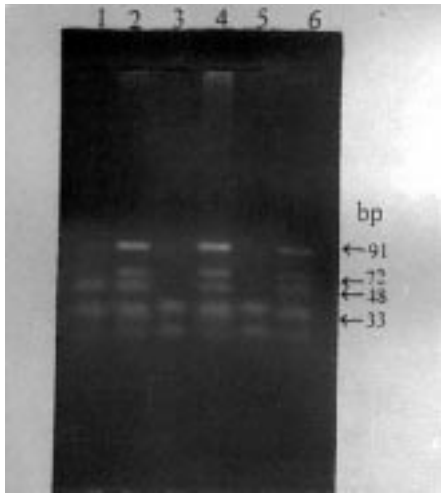

Figure 2. Gel electrophoresis analysis of CfoI digestion of PCR product. The 227 bp PCR products were cut by CfoI (see methods section). Lanes 2,4,6: Apo E genotype is ε4/ε3. Lanes 3,5: Apo E genotype is ε4/ε4. Lane 1: Apo E genotype is ε3/ε3

The distribution of apo E genotypes and frequency of alleles in microalbuminuric and normoalbuminuric diabetic patients are shown in Table 2.

There was no significant difference in terms of apo E genotype distribution between the microalbuminuria and normoalbuminuria groups (Kruskal Wallis one-way ANOVA test, p>0.05). However, the frequencies of apo E alleles were found to be different between these two groups (Kruskal Wallis one-way ANOVA test, χ^2 = 4.09 and p<0.05). The frequency of the ε2 allele in patients with microalbuminuria was significantly higher than in the normoalbuminuric patients (odd ratio= 4.89 and p<0.05). No significant difference in terms of the frequencies of ε3 and ε4 existed between these two groups of patients (p>0.05).

Table 2. Apolipoprotein E genotypes and allele frequencies in patients.

Genotype	Microalbuminuria n (%)	Normoalbuminuria n (%)	
ε4/ε4	1 (4.2)	1 (4.5)	
ε4/ε3	2 (8.3)	4 (18.1)	
ε4/ε2	0 (0)	0 (0)	
ε3/ε3	12 (50.0)	14 (63.6)	
ε3/ε2	7 (29.2)	3 (13.6)	
ε2/ε2	2 (8.3)	0 (0)	
Allele			Odd ratio
ε4	4 (8.3)	6 (13.6)	0.67
ε3	33 (68.8)	35 (79.5)	1.40
ε2	11 (22.9)	3 (6.8)	4.89

Discussion

Diabetes is a chronic condition which creates the risk of three major complications. These are diabetic retinopathy, nephropathy and neuropathy. Almost one third of diabetic patients (IDDM or NIDDM) develop diabetic nephropathy in their lifetime (15). Chronic hyperglycaemia stands with diabetes duration as the main predicting factor for the development of nephropathy in IDDM. In contrast, nephropathy in NIDDM presents with a different natural history and, as well as atherosclerosis, can precede diabetes diagnosis and even the onset of patent hyperglycemia. It has been suggested that low-density lipoprotein cholesterol levels in ε2 allele carriers (whether they are diabetic or not) are lower than in ε2 non-carriers. The 2-fold increase in nephropathy in ε2 non-carriers with NIDDM argues for a role for LDL in the development of human nephropathy in NIDDM patients. However, the role of lipid abnormalities in this remains a matter for debate (16). The levels of total-C and LDL-C in our patients with microalbuminuria were found to be high ($p<0.01$). Since the number of patients was insufficient, the relation with the apolipoprotein E genotypes and apolipoprotein E alleles with serum lipids (total-C, LDL-C, HDL-C and TG) could not be determined. However, it would be useful to take into consideration the results of the Turkish heart study. In the study in question, the ε2 allele is related to the low level of total-C and LDL-C. But the effect of the ε4 allele in increasing plasma cholesterol levels in men has been found to be limited. Nevertheless, it has been found out that homozygosity for apo ε4 has a significant effect on the increase in cholesterol levels in women. It has been demonstrated that homozygosity for apo e2 in men is related to the considerable increase in TG level. One interesting finding is that apo ε4/ε3 is related to TG increases both in men and women when compared to apo ε3/ε3, whereas homozygosity for ε4/ε4 has not been found to have a considerable effect on TG levels. Moreover, it has been observed that ε2 and ε4 alleles have no significant relation with HDL-C levels in the Turkish population (2).

The increased frequency of the ε2 allele in type-2 diabetes mellitus with nephropathy has been suggested (17). Likewise, Chowdhury et al. (18) reported a relation between ε2 and diabetic nephropathy, but this allele was positive in 23.4% of their type-1 diabetic patients with nephropathy. They asserted that it was not a single genetic locus that had caused the nephropathy. Our results are comparable to those of these studies.

Sheet et al. (15) concluded that poor glycemic control along with elevated systolic blood pressure are powerful predictors for the development of overt proteinuria in microalbuminuric patients with NIDDM. In the present study, microalbuminuric and normoalbuminuric patients, both in terms of blood pressure and glycemic control, had similar peculiarities. Of course, here it should be remembered that glycemic control could decrease the albuminuria, and in some of the patients with microalbuminuria it could supply normoalbuminuria. Elible et al. (19) suggest that when metabolic control is improved, incipient albuminuria remains constant, but advanced albuminuria shows progression. Tanaka et al.

(20) stated that glycemic control is a more potent factor than blood pressure level in the development of microalbuminuria. However, as far as the progression of microalbuminuria to overt proteinuria is concerned, hypertension is the most crucial factor in elderly NIDDM patients. De Pablos et al. (21) in a study they did on type-2 diabetic patients, reported that the albumin excretion rate has no relation with age, BMI, diabetes duration, or the level of HbA1c, but that it is related to blood pressure.

Albuminuria and its relation to the male sex in type-2 diabetes has been discussed (22). In our study, the male sex frequencies in microalbuminuric and normoalbuminuric patients were similar to each other (Table 1, $p>0.05$).

The frequencies of nephropathy and retinopathy being very close to each other in our diabetic patients is in accordance with the findings in the literature (23). But there are some who suggest that diabetic nephropathy and diabetic retinopathy cannot develop with the same mechanism (24).

In summary, our results suggest that the ε2 allele of the apo E gene could be related to the occurrence of microalbuminuria in patients with type 2-diabetes mellitus. More detailed studies are necessary to help clarify this relation.

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