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DNA polymorphism of Pvu II site in the lipoprotein lipase gene in patients with non-insulin dependent diabetes mellitus

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We studied the effect of variation at the lipoprotein lipase (LPL) gene locus on the susceptibility of individuals with non-insulin dependent diabetes mellitus (NIDDM) in a population of 110 NIDDM patients and 91 controls. Our objective was to study the relationship between the LPL–Pvu II polymorphism and NIDDM and lipid metabolism. PCR-RFLP was used to determine the DNA polymorphism of the sixth intron of the LPL gene. The frequencies of the genotypes in case and control groups were 29.1 and 30.8% for P+/P+; 45.5 and 36.3% for P+/P−; 25.5 and 33% for P−/P− respectively. There was no significant difference in frequencies of genotypes between the two groups. Logistic regression analysis revealed that triacylglycerol (TAG) and apolipoprotein E levels were associated with NIDDM, whereas Pvu II genotypes were not found as independent risk factors for the disease. Overall this study demonstrates the role of the Pvu II polymorphism in the LPL gene in modulating plasma lipid/lipoprotein levels in patients with NIDDM. Copyright © 2004 John Wiley & Sons, Ltd.

KEY WORDS — NIDDM; lipoprotein lipase; polymorphism; Pvu II; lipoprotein

INTRODUCTION

One of the important risk factors in the development of NIDDM is the high level of lipids (cholesterol and TAG) in the plasma. Although environmental factors, for example dietary fat intake, physical activity and smoking, influence the plasma lipids, genetic factors also contribute to the modulation of plasma lipid levels. Increased understanding of pathophysiology and molecular biology has led to identification of a number of candidate genes involved in glucose and lipid homeostasis. Lipoprotein lipase (LPL) is a major determinant of plasma lipoprotein profiles because it affects all classes of lipoprotein particles. The action of LPL is essential not only for the hydrolysis of TAGs in chylomicrons and very low density lipoproteins (VLDL) but also for the maturation of high density lipoproteins (HDL) and low density lipoproteins (LDL).[1,2] Human LPL is a glycoprotein of 448 amino acids in its mature form,[3] and the corresponding gene has a span of 30 kilobases (kb) comprising 10 exons.[4–6] Several DNA polymorphisms that generate restriction fragment length polymorphisms (RFLP) have been identified in the human LPL gene. These include polymorphisms identified with Bam HI,[7] Pvu II,[7,8] Hind III,[9] Bst NI,[10] Bst I,[11] Bgl II,[12] and Xba I.[13] Pvu II polymorphism is the result of a C → T transition in the restriction site of the LPL gene sixth intron, 1.57 kb from the splice acceptor (SA) site.[14] The region containing the Pvu II site resembles a splicing site in its homology to the consensus sequence required for 3′-splicing and the formation of the lariat structure, suggesting that the $C^{497} \rightarrow T$ (CAG CTG ⇒ TAG CTG) change may interfere with correct splicing of mRNA.

Trials have been carried out to explore associations between LPL gene polymorphisms and lipoprotein phenotypes. The results provided evidence of an association of the genotypes identified by the Pvu II RFLP

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with plasma TAG levels.[15,16] Polymorphisms of LPL–Pvu II have been reported to be associated with circulating TAG levels in some studies[15–22] but not in others.[23–26] These polymorphisms are common in White populations.[17,23,24]

We evaluated the impact of Pvu II, a common polymorphism in the LPL gene in a representative group of type 2 diabetic Turkish subjects, and investigated their influence on plasma lipids.

MATERIAL AND METHODS

Subjects

We studied 110 NIDDM patients (49 men and 63 women) who were referred to the Turkish Diabetes Hospital, Istanbul for their routine clinical examination (every 1–2 months). The diagnosis of NIDDM was based on the criteria of The Expert Committee on the diagnosis of diabetes mellitus.[27] Written consent was obtained from every patient after a full explanation of the study, which was approved by the Ethics Committee of the University of Istanbul, Cerrahpasa Faculty of Medicine. Control subjects consisted of 91 apparently healthy people (30 men and 61 women) not taking medication, who either attended a routine health check at a general practice or were staff of the Cerrahpasa Medical Faculty (Istanbul University, Turkey). No patients in the study were related. All had normal hepatic and endocrine functions and were relatively well controlled with glycosylated haemoglobin (HbA_{1c}) $\leq$6–7% (normal range $\leq$8%). Tobacco and alcohol consumers were not included in the study. The patients with macro- and microangiopathic complications were excluded from this study. Total cholesterol, TAG, HDL-cholesterol, LDL-cholesterol, and plasma glucose levels were measured after overnight fasting.

Blood collection

Blood samples were taken between 08.00 and 10.00 hours by venipuncture after overnight fasting. Serum was obtained after allowing samples to clot for 30 min at room temperature followed by a 10-min centrifugation.

Analytical methods

Lipid and apolipoprotein assays. All the analytical measurements were performed after overnight fasting. Serum TAG and total cholesterol levels were measured using standard enzymic methods (Merck, Darmstadt, Germany) of automated analysis on an AU5021 (Olympus, Merck). Serum apolipoprotein E was determined by turbidimetry (automated Cobas-Mira analyser, Roche, Meylan, France); serum apolipoprotein AI, apolipoprotein B and lipoprotein (a) were determined by immunonephelometry on a Behring Nephelometer analyser with Behring reagents (Behringwerke, Marburg, Germany). Sera were analysed without pretreatment and diluted in double-distilled water when lipid or apolipoprotein levels exceeded reference values. Pooled sera were included in each series of measurements for apolipoprotein E. Between assays coefficients of variation of these methods were 2.14, 4.66, 0.95, 1.52, 2.92, 4.34 and 1.53% respectively for total cholesterol, TAG, glucose, apolipoprotein E, apolipoprotein AI, apolipoprotein B and lipoprotein (a).

DNA analysis. Blood was drawn into tubes containing EDTA as an anticoagulant. Human DNA was isolated from white blood cells of the subjects by a standard salting out method.[28] Polymerase chain reaction (PCR) analysis of the sixth intron was performed in a DNA thermocycler using 25 µl reaction mixtures with commercially available buffer composed of $MgCl_2$, 300 µmol l^{-1} of deoxynucleotide triphosphates (dNTPs), 5.6 µmol l^{-1} of forward and reverse primers, and 1.25 units of thermostable DNA polymerase from *Thermus aquaticus*. The selected sequences for 5′ and 3′ oligomers were SB-75: 5′-ATG GCA CCC ATG TGT AAG GTG-3′, and SB-76: 5′-GTG AAC TTC TGA TAA CAA TCT C-3′.[20] The quality of the PCR products was checked by 1.5% agarose gel electrophoresis (90 V h^{-1}) with a 50 bp marker. Samples of 430 bp-long PCR products (8 µl) were then incubated with Pvu II restriction endonuclease overnight at 37°C. The digested DNA was run on a 2% agarose gel (90 V h^{-1}). The 430-bp product was digested to 320- and 110-bp products if a Pvu II restriction site was present.

Statistical analysis

Statistical analyses were conducted using Unistat 5.1 software. Serum TAG and lipoprotein (a) were logarithmically transformed before the analysis to obtain a normal distribution of data. A comparison of variables between two groups or among three groups was carried out using the unpaired *t*-test or one-way ANOVA, respectively. The Hardy–Weinberg equilibrium was tested by a chi-square test. Genotype frequencies were estimated by a chi-square test. The variables across the various genotypes and groups were estimated by two-way ANOVA with an

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Table 1. Demographic information of the study groups

	Patient ($n=110$)	Control ($n=91$)
Age (years)	57.99 ± 0.87	55.46 ± 1.18
BMI ($kg\,m^{-2}$)	27.39 ± 0.41	26.84 ± 0.44
Glucose ($mmol\,l^{-1}$)	8.25 ± 0.35*	3.49 ± 0.06
Total cholesterol ($mmol\,l^{-1}$)	5.46 ± 0.24	5.42 ± 0.14
Triacylglycerol ($mmol\,l^{-1}$)	1.82 ± 0.13	1.75 ± 0.09
Apolipoprotein E ($mg\,l^{-1}$)	42.31 ± 1.40	49.24 ± 3.36†
Apolipoprotein AI ($g\,l^{-1}$)	1.43 ± 0.03	1.42 ± 0.03
Apolipoprotein B ($g\,l^{-1}$)	1.15 ± 0.03	1.13 ± 0.03
Lp(a) ($g\,l^{-1}$)	0.15 ± 0.01	0.18 ± 0.02

Values are represented as mean ± SE. *$p < 0.001$; †$p < 0.05$.

interaction term to test the influence of Pvu II genotype on the lipid profile. *P* values less than 0.05 were considered significant.

RESULTS

Genotype distribution of Pvu II polymorphism and its relation with serum lipid parameters (total cholesterol, TAG, apolipoprotein E, apolipoprotein AI, apolipoprotein B and Lp(a)) were investigated in a total of 110 NIDDM and 91 control unrelated subjects. The demographic information of both patient and control groups is given in Table 1. The frequencies of major NIDDM risk factors are summarized in Table 2. The frequency distributions of the risk factors examined did not show any significant difference in the patient and control groups. After PCR analysis, 430-bp fragments were obtained by agarose gel electrophoresis. PCR products are spliced to 320- and 110-bp fragments in the presence of the respective restriction sites. Determination of Pvu II polymorphisms by PCR and RFLP showed the respective frequencies for −/−, +/− and +/+ genotypes to be 25.5, 45.5, and 29.1 in subjects with NIDDM, and 33, 36.3, and 30.8 in the control group (Table 3). The chi-square test showed no significant difference between genotype frequencies, the Hardy–Weinberg equilibrium was tested on a whole study population ($n = 201$). According to the Hardy–Weinberg proportion, the various genotypes are in equilibrium (chi-square = 2.04, $p = 0.361$) and the proportions may remain constant over generations.

Table 2. Risk factors for non-insulin dependent diabetes mellitus in patients and control subjects

	Diabetes *n* (%)	Control *n* (%)	*p*
Gender			
Male	48 (43.2)	30 (33)	n.s.
Female	63 (56.8)	61 (67)	n.s.
Dyslipidaemia	25 (22.5)	17 (18.7)	n.s.
Obesity	75 (67.6)	57 (62.6)	n.s.
Smokers	15 (18.8)	—	—

The variables were compared with the χ^2-test among groups. n.s., not statistically significant.

Table 3. LPL–Pvu II genotype frequency distributions in non-insulin dependent diabetes mellitus patients and control subjects

	LPL gene Pvu II genotype frequencies		
	+/− *n* (%)	−/− *n* (%)	+/+ *n* (%)
Patient	50 (45.5)	28 (25.5)	32 (29.1)
Control	33 (36.3)	30 (33)	28 (30.8)

The Pvu II genotype frequency distributions were compared with χ^2 test.

The relationship between LPL gene Pvu II genotypes and lipid parameters of NIDDM and control subjects are presented in Table 4. Serum TAGs for −/−, +/−, and +/+ genotypes in subjects with NIDDM, expressed as mean ± SE in $mmol\,l^{-1}$ were 1.60 ± 0.14, 2.55 ± 0.48, and 1.66 ± 0.13, respectively. There was also a significant difference in serum apolipoprotein E concentrations between the Pvu II genotypes where the lowest level was observed in −/−. Other parameters did not show any significant differences. Gender, BMI, plasma glucose, total cholesterol, TAG, apolipoprotein E, apolipoprotein AI, apolipoprotein B, smoking habit and LPL genotypes were selected as conventional risk factors for NIDDM to be analysed in multiple logistic regression analysis (Table 5). Plasma glucose, apolipoprotein E and apolipoprotein B were found as risk factors for NIDDM whereas no such association was observed for LPL–Pvu II genotypes.

DISCUSSION

Our present study explored the association between Pvu II polymorphisms of the LPL gene and lipid/lipoprotein levels in NIDDM. As far as we are aware, the present study represents the first investigation of the common polymorphism (Pvu II) of the lipoprotein lipase gene in type 2 diabetic Turkish patients and their influence on lipid parameters. To date, a number of studies have been reported which explored possible associations between LPL polymorphism and lipid parameters in NIDDM.[18,19,29,30] The results are not consistent and suggest that the effect of this variant is context-dependent (ethnicity and sex).

Hypertriacylglycerolemia and decreased adipose tissue LPL activity occur commonly in diabetic

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Table 4. Effects of LPL gene Pvu II polymorphism on clinical parameters in non-insulin dependent diabetes mellitus patients and control subjects

	LPL gene Pvu II polymorphism			
Patients	+/− (50)	−/− (28)	+/+ (40)	*p*
Glucose ($mmol\,l^{-1}$)	9.98 ± 0.58	9.18 ± 0.62	9.67 ± 0.68	n.s.
Total cholesterol ($mmol\,l^{-1}$)	5.61 ± 0.17	5.26 ± 0.24	5.37 ± 0.18	n.s.
Triacylglycerol ($mmol\,l^{-1}$)	2.55 ± 0.48	1.60 ± 0.14	1.66 ± 0.13	<0.05
Apolipoprotein E ($mg\,l^{-1}$)	48.56 ± 3.49	38.35 ± 2.32	42.07 ± 2.31	<0.05
Apolipoprotein AI ($g\,l^{-1}$)	1.46 ± 0.038	1.37 ± 0.055	1.43 ± 0.047	n.s.
Apolipoprotein B ($g\,l^{-1}$)	1.19 ± 0.072	1.14 ± 0.044	1.12 ± 0.045	n.s.
Lp(a) ($g\,l^{-1}$)	0.15 ± 0.026	0.13 ± 0.03	0.13 ± 0.021	n.s.
Controls	+/− (33)	−/− (30)	+/+ (28)	
Glucose ($mmol\,l^{-1}$)	3.54 ± 0.20	3.52 ± 0.14	3.69 ± 0.14	n.s.
Total cholesterol ($mmol\,l^{-1}$)	5.37 ± 0.25	5.40 ± 0.22	5.42 ± 0.23	n.s.
Triacylglycerol ($mmol\,l^{-1}$)	1.75 ± 0.13	1.68 ± 0.14	1.74 ± 0.19	n.s.
Apolipoprotein E ($mg\,l^{-1}$)	47.83 ± 3.18	42.98 ± 2.13	45.76 ± 3.36	n.s.
Apolipoprotein AI ($g\,l^{-1}$)	1.41 ± 0.04	1.42 ± 0.056	1.40 ± 0.04	n.s.
Apolipoprotein B ($g\,l^{-1}$)	1.15 ± 0.05	1.10 ± 0.054	1.06 ± 0.052	n.s.
Lp(a) ($g\,l^{-1}$)	0.15 ± 0.025	0.17 ± 0.041	0.18 ± 0.028	n.s.

Values are represented as mean ± SE. n.s., not statistically significant.

subjects.[31] Normal levels of apo AI found in our diabetic group may be because TAG-containing HDL are better substrates for hepatic lipase, so the lipid-poor apo AI is more rapidly cleared from the circulation.[32]

Some previously published results suggest that the LPL +/+ genotype is associated with an unfavourable plasma lipid profile.[15,20] In contrast to previous reports, the Pvu II +/+ genotype was not found to correlate with unfavourable lipid levels in the present study, whereas NIDDM patients carrying the +/− genotype were associated with higher apolipoprotein E, apolipoprotein B, TAG, total cholesterol and Lp(a) levels. An explanation for this inconsistency could be based on the different genetic background of this cohort. It is also likely that the higher animal fat intake of the Turkish subjects could mask the effect of LPL polymorphism.

Table 5. Association of risk factors with non-insulin dependent diabetes mellitus by multiple logistic regression analysis

	All			
	β	SE	OR	*p*
Sex	−0.50	1.157	0.606	0.665
BMI	0.057	0.090	1.059	0.521
Plasma glucose	4.908	1.226	135.406	0.000*
Cholesterol	−0.255	0.176	0.775	0.142
Triacylglycerol	0.730	0.573	2.074	0.203
Apolipoprotein E	−0.192	0.060	0.825	0.001*
Apolipoprotein AI	−0.223	1.686	0.800	0.895
Apolipoprotein B	4.173	2.097	64.910	0.047*
Smoking habit	11.72	29.851	1.229	0.695
LPL				
(−/−)	−1.691	1.020	0.184	0.097
(+/−)	−0.353	1.093	0.703	0.747
(+/+)	−20.647	6.719	0.262	0.240

The multivariate logistic regression model included sex, BMI, plasma glucose, cholesterol, triacylglycerol, apolipoprotein E, apolipoprotein AI, apolipoprotein B, smoking habit, and LPL–Pvu II genotype variables. β indicates estimated coefficient; SE, standard error; OR, adjusted odds ratio. *Statistically significant.

Differential gene analysis showed the importance of investigating clinical characteristics for mutations of coding and non-coding nucleotide sequences. Intron gene polymorphisms do not affect phenotype characteristic of the mature protein, but they could affect the maturation and turnover of mRNA, its size, translatability, and the nature and number of the protein products formed.[33] Such polymorphisms in nucleic acids can be readily detected if they lead to an alteration at the restriction sites. The relation of NIDDM and Pvu II RFLP polymorphism of the LPL gene has been investigated in different populations. An association between the extent of NIDDM and the LPL–Pvu II polymorphism was reported in European,[34] and Spanish[18] subjects, whereas Pvu II polymorphism did not exhibit any significant association with NIDDM in a Chinese population.[19] Our results are in agreement with the findings of Shen

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et al.[19] in that no association between Pvu II and NIDDM was observed.

A study carried out in Spanish subjects had comparable gene frequencies for the Pvu II genotype distribution in diabetic patients and controls[18] in which a higher frequency of the +/− genotype in patients with NIDDM was observed, whereas, Shen and coworkers[19] failed to show a significant difference in Pvu II genotype frequencies between the NIDDM and control individuals they studied. In our study we did not find any significant difference among the patient and control groups when Pvu II genotype frequencies were taken into account.

In conclusion, LPL–Pvu II polymorphism was not found to be a genetic risk factor for non-insulin dependent diabetes mellitus, whereas the +/− genotype was found to be more associated with higher TAG and apo E levels than the +/+ and −/− genotypes in this selected study group.

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