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ASSOCIATION OF *LPL-HIND*III POLYMORPHISM WITH CORONARY ARTERY DISEASE IN MACEDONIAN POPULATION

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Abstract: **Objective:** Coronary artery disease (CAD) is a leading cause of high mortality and morbidity in worldwide. The *Hind*III polymorphism of the *LPL* gene (*LPL-Hind*III) is a common variant and has been associated with plasma lipid and lipoprotein variability in population studies.

Aim: Evaluation of the *LPL-Hind*III polymorphism as an independent risk factor for coronary artery disease in Macedonian population.

Material and Methods: A polymerase chain reaction amplification and consecutive restriction enzyme digestion was used to reveal lipoprotein lipase, the intron 8 *LPL-Hind*III polymorphism. Study group included 114 randomized subjects with angiographically documented coronary artery stenosis (CAD group: 87 males, 27 females). Control group consisted of 35 patients (21 males and 14 females) without significant stenosis in coronary arteries.

Results: Independent multiple regression analysis of LDL plasma level and their correlation with *LPL-Hind*III polymorphism and analyzed risk factors: hypertension, diabetes, family history of CAD, physical activity, antilipidemic drugs and alcohol consumption, LDL, show statistically significant correlation with BMI, and also between *LPL-Hind*III and LDL plasma level. In the examined group, only triglycerides reached a statistically significant association with the *LPL-Hind*III polymorphism.

Conclusion: In our study, the *LPL-Hind*III polymorphism was not identified as independent risk factor for CAD, but showed association with high triglycerides and LDL levels.

Key words: coronary artery disease, DNA analysis, *LPL-Hind*III polymorphism.

INTRODUCTION

Many factors are involved in the etiology of coronary artery disease (CAD) as a result of complex reaction between genetic predisposition and environmental influences. Individual evaluation for CAD includes not only risk factors but also relevant phenotype classification for particular mutations and polymorphism. In the majority of cases polymorphism and gene mutations are present in CAD (1, 2).

Lipoprotein lipase hydrolyses triglycerides from chylomicrons and very low density lipoproteins (VLDL) to the free fatty acids and glycerol. Frequency of individual *LPL* gene mutations significantly varied between various populations. *LPL* polymorphism has minor expression, environmental factors mainly determine intensity and outcome of clinically manifested atherosclerosis and CAD (3, 4, 5, 6).

The replacement of a thymine (T) with a guanine (G) base in intron 8 of the *LPL* gene abolishes the *LPL-Hind*III restriction site. *LPL-Hind*III polymorphism in the *LPL* gene demonstrates strong correlation with CAD in population from various nationalities: Saudi Arabia (7), North Europe (8), Russia (9), Italia (10), and China (11).

The aim of the study was to assess *LPL- Hind*III polymorphism as an independent risk factor and predictor of CAD in Macedonian population.

Hypothesis: It is not possible to LPL-HindIII polimorhism has influence on hyperlipidemia and appearance of coronary artery disease?

MATERIAL AND METHODS

Two institutions participated in our prospective, randomized study: Cardiology Clinic and the Laboratory for molecular biology. On University Cardiology Clinic the patients were chosen consecutively following coronary angiogram if they satisfied the inclusion and exclusion criteria from the study. The study group included 114 patients with angiographically documen-

ted significant (≥ 70% stenosis) coronary artery stenosis. Demographics characteristics (age, sex, place of living, and nationality), risk factors for CAD (family history of CAD, the presence of hyperlipidemia, hypertension or diabetes mellitus, smoking), and Body-Mass Index (BMI) have been questioned by uniform questionnaire. Physical activity, education, use of antilipemic drugs, and alcohol consumption were also considered. The patients with hypertensive heart disease, heart defect or hypertrophic cardiomiopathy were excluded from the study. The control group consisted of 35 patients with normal coronary structure and with similar ethnical structure as in CAD group.

The following biochemical blood analyses were performed at the Institute of Clinical Biochemistry: total cholesterol, LDL-cholesterol, HDL-cholesterol, total lipids and triglycerides.

This study was conducted as a part of the scientific and research project "Molecular-genetic Examinations of the Coronary Artery Disease in Macedonian Population". The study was approved (No. 14–151/2) by the Ethical Committee of the Medical Doctor's Chamber of Republic of Macedonia. All patients signed written informed consent before the study.

*DNA analysis and detection of LPL-Hind*III *polymorphism*

Molecular-genetic analyses were performed at the Laboratory for Molecular Biology, Institute of Biology using the established molecular techniques for detection of mutations and polymorphism (12).

A 5 ml of venous blood sample was drawn in vacuum sterilized test tube (Vaccutainer®) with anticoagulant (EDTA, disodium salt). The standard isolation of genomic DNA was performed from nucleated cells, using DNA extraction with sodium chloride and chloroform, following by ethanol precipitation. The *LPL-Hind*III polymorphism in intron 8 was identified by restriction enzyme digestion of the PCR-amplified segment of the *LPL* gene (13, 14). The primers were derived from sequences between exons 8 and 9 in *LPL* to amplify the sequence around a *LPL-Hind*III restriction site in intron 8 (the forward primer was 5'- TAG AGG TTG AGG CAC CTG TGC -3' and the reverse primer was 5'- GTG GGT GAA TCA CCT GAG GTC -3'). Each PCR-amplification reaction was performed using 100-250 ng genomic DNA; 10 pmol of each primer; 200 mmol/L each of a dATP, dCTP, dGTP, and dTTP; and 0.05 U of Taq polymerase in a total reaction volume of 20 L. Amplification was performed in a GeneAmp PCR System 9400 (Perkin Elmer). In the case of amplification of exon 9, initial denaturation at 94°C for 5 minutes was followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 57°C for 1 minute, and extension at 72°C for 1 minute, with final extension at 72°C for 10 minutes. For amplification of intron 8, initial denaturation at 94°C for 5 minutes was followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 57°C for 1 minute, and extension at 72°C for 1 minute, with final extension at 72°C for 10 minutes. The *LPL-Hind*III polymorphism was identified by restriction analysis using *Hind*III endonuclease digestion (Figure 1). The restriction products were resolved by agarose electrophoresis and the gels were recorded by digital camera (Canon A70). Images were analyzed by GelPro software.

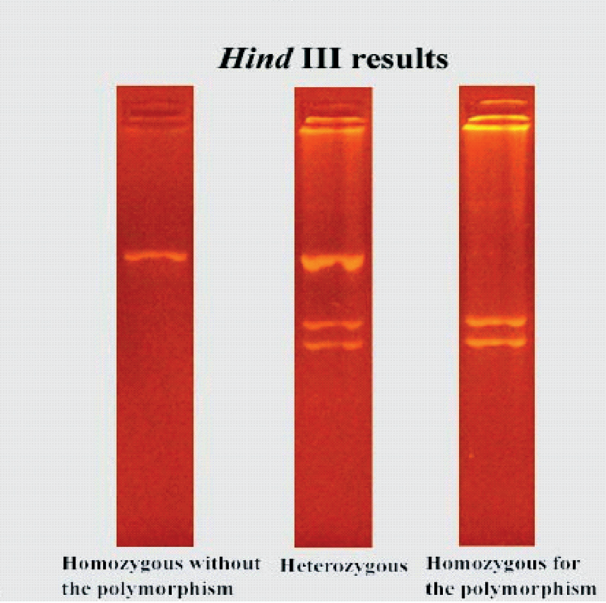

Figure 1. *Electrophoresed pattern of LPL-HindIII polymorphism*

Statistical data processing. T-test was used to analyze differences in continuous variables, and the chi-square and Fisher's exact tests were used to analyze distribution of categorical variables. Odds ratio (OR) and 95% CI were used to estimate risks of HTG associated with environmental factors or serum blood chemistry. Those variables with significant OR in the univariate analysis were further examined by multivariate regression model to obtain adjusted OR and adjusted 95% CI. Differences were considered statistically significant when $p < 0.05$ or when the 95% CI range was not included in the unity. SPSS 8.0 for MS Windows was used to complete the statistical analyses.

RESULTS

Study group included 114 patients (87 males, 27 females). The control group consisted of 35 patients (21 males, 14 females) with normal coronary arteries. The results of the genetic detection of *LPL-Hind*III polymorphism, as well as of the demographics, biochemical and

other parameters between groups are summarized and compared in table 1. There was no significant difference in the sex distribution, ethnical structure and mean age between groups. Hyperlipidemia ($p < 0.001$), diabetes ($p < 0.05$) and the use of antilipidemic drugs ($p < 0.05$) were more common in study group. For both CAD and control groups,the genotype distributions were in accordance with the Hardy-Weinberg expectation (equilibrium). Genetic equilibrium is an ideal state that provides a baseline against which to measure change.

It seems that the *LPL-Hind*III polymorphism is not a risk factor for the presence of CAD in Macedonian population ($\chi2 = 0,0355$; df = 1; p=0,851), (Fisher's exact test p = 0,587) (Table 1).

Chi-square and Fisher's Exact test did not reveal statistical significant relationship between the *LPL--Hind*III polymorphism and the analyzed risk factors (hyperlipidemia, hypertension, diabetes, family history of CAD, physical activity, use of antilipidemic drugs and alcohol consumption) (Table 2).

Among the plasma levels of triglycerides, total lipids, HDL and LDL in the examined group, only triglycerides reached a statistically significant association with the *LPL-Hind*III polymorphism ($p < 0.05$) (Table 3).

Table 1. *Comparison of the demographics and other parameters between groups*

Parameter		The study group (n = 114)	The control group (n = 35)	P value
Male/female		87 (76.3%)/27 (23.7%)	21 (60%)/14 (40%)	NS
Age (year)		59.4 ± 9.2	57.9 ± 6.6	NS
BMI (kg/m^2)		27.26 ± 3.45	26.22 ± 3.11	NS
Family history of CAD		42	7	$\chi2 = 3.44$ $p < 0.064$
Hiperlipidemia		64	6	$\chi2 = 16.3$ $p < 0.001$
Diabetes		31	4	$\chi2 = 3.70$ $p < 0.05$
Hypertension		71	23	$\chi2 = 0.136$ $p < 0.713$
Use of antilipidemic drug		47	8	$\chi2 = 3.88$ $p < 0.049$
Use of Alcohol		29	6	$\chi2 = 1.03$ $p < 0.311$
Smoking				
Never		38	14	$\chi2 = 0.524$ $p < 0.770$
Ex-smoker		29	8	
Current smoker		47	13	
Physical activity				
Low		38	11	$\chi2 = 0.601$ $p < 0.740$
Moderate		65	19	
High		11	5	
Educational level				
Low (n)		91	26	$\chi2 = 0.487$ $p < 0.485$
High (n)		23	9	
***Hind* III polymorphism**	$H^{+/+}$ or $H^{+/-}$ (n)	95	27	NS
	$H^{-/-}$ (n)	12	3	NS
Unamplificable by PCR		7	5	NS

NS: Not Significant, PCR: Polymerase Chain Reaction

(Homozygous, $H^{+/+}$, heterozygous, $H^{+/-}$). The presence of the *LPL-Hind*III polymorphism homozygous, $H^{-/-}$ — the absence of the *LPL-Hind*III polymorphism

Table 2. *Coronary risk factors and comparison to the presence and the absence ($H^{+/+}$ or $H^{+/-}$; $H^{-/-}$) of the Hind III polymorphism*

Risk factors	Study group	$H^{+/+}$ or $H^{+/-}$ (n = 95)	$H^{-/-}$ (n = 12)	P value
Hyperlipidemia	No	41	4	< 0.372
	Yes	54	8	
Hypertension	No	35	3	< 0.321
	Yes	60	9	
Diabetes	No	68	10	< 0.315
	Yes	27	2	
Family anamnesis	No	62	7	< 0.430
	Yes	33	5	
Physical activity	Low	29	6	< 0.394
	Moderate	57	5	
	High	9	1	
Smoker	Never	31	5	< 0.694
	In the past	26	2	
	Present	38	5	
Antilipidemics	No	56	7	< 0.601
	Yes	39	5	
Alcohol	No	69	10	< 0.342
	Yes	26	2	

Table 3. *The frequency of the lipid fractions (Triglycerides, Total lipids, HDL, LDL) and their relation to Hind III polymorphism in the examined group*

Lipids/HindIII	$H^{+/+}$ [n=54]	$H^{+/-}$ or $H^{-/-}$ [n=53]	p-level
Triglycerides	2.796 ± 1.887	2.219 ± 0.909	P < 0.0469
Total lipids	9.244 ± 3.085	8.931 ± 2.102	P < 0.5471
HDL	1.013 ± 0.299	1.090 ± 0.313	P < 0.1968
LDL	3.485 ± 0.967	3.608 ± 0.982	P < 0.5221

t = t - test; df = degrees of freedom; p = p level of the model

Table 4. *Multiple regression analysis of LDL and their relation to risk factors and Hind III polymorphism in the examined group*

LDL	R = 0,47 R^2 = 0,22 F = 2,220 p = 0,02		
	Beta	T-test	p-level
Hypertension	0.014	0.141	0.887
Diabetes	0.076	0.704	0.482
Family anamnesis	0.147	1.472	0.144
Smoker	0.147	1.402	0.164
Physical activity	0.029	0.271	0.786
Alcohol	0.051	0.465	0.642
BMI	0.210	2.018	0.046*
Hind III	0.274	2.606	0.010*

* statistically significant correlation of LDL plasma level with BMI and LPL-HindIII polymorphism
R: Multiple R; R2 Square multiple R; F — Ratio; P — p level of the model

Independent multiple regression analysis of LDL plasma level and their correlation with *LPL-Hind*III polymorphism and analyzed risk factors: hypertension, diabetes, family history of CAD, physical activity, antilipidemic drugs and alcohol consumption, LDL show statistically significant correlation with BMI ($p < 0.05$), and also between *LPL-Hind*III and LDL plasma level ($p < 0.05$) (Table 4).

DISCUSSION

As it's well known, numerous studies and recent research has shown that LPL plays a key role in removes triglycerides from the circulation, and generates fatty acids for storage in the adipose tissue or for oxidation in the skeletal muscle. In our study, the accent was placed on the examination of *LPL* as a gene-candidate for coronary artery disease. Some genetic variants have been described in the sequence of *LPL* gene. *LPL-Hind*III polymorphism as one of these genetic variants, has important connection to CAD. So, the goal of this study was to assess the influence of *LPL-Hind*III polymorphism over the appearance of the coronary artery disease in Macedonian population. This original study represents the first investigation in Republic of Macedonia for this purpose.

Our study investigates *LPL-Hind*III polymorphism as independent risk factor for CAD. *LPL-Hind*III polymorphisms of the *LPL* gene in numerous studies have demonstrated strong correlation with CAD in population from various nationalities like Saudi Arabia (7), North Europe (8), Russia (9), Italia (10), and China (11). In our study, *LPL-Hind*III polymorphism was not found to be a risk factor for significant CAD in Macedonian population. Our findings support that *LPL-Hind*III polymorphism varies across different populations as detected by other studies (2, 8). Our study analysis shows little difference in distribution of *LPL-Hind*III polymorphism between study and control group. But, the presence of *LPL-Hind*III polymorphism does not expose the patient into a significant risk for appearance of CAD in Macedonian population (table 1).

In the etiology of CAD, many risk factors are involved and result in complex interaction between genetic predisposition and environmental influences (3, 4, 7, 8, 15, 16). The environmental factors determine the intensity and result of the clinically manifested CAD. Comparison of the analyzed risk factors between groups are summarized in table 1, where there is significant influence of hyperlipidemia ($p < 0.001$), diabetes ($p < 0.05$) and use of antilipidemic drugs ($p < 0.049$). In study we also analyzed coronary risk factors and compared with the presence (homozygous, $H^{+/+}$ or heterozygous, $H^{+/-}$) and the absence (homozygous, $H^{-/-}$) of the *LPL-Hind*III polymorphism in study group, and we don't found statistical significant influence in patients with CAD (table 2). It is supposed that the presence of polymorphisms in several genes at the same time (as those for the enzymes involved in the lipid metabolism, coagulation factors, inflammatory mediators and other proteins), is the cause for the multi-gene nature of arteriosclerosis and CAD (3, 6, 17).

Lipoprotein lipase plays an important role in the lipid metabolism, by hydrolyzing core triglycerides of circulating chylomicrons and VLDL. There are more authors and studies which analyze the connection of serum lipids with CAD. Also, it is known that LPL polymorphism correlated with LPL activity is associated with plasma lipid and lipoprotein levels (3, 7, 12, 18). Many studies analyzed correlation of *LPL-Hind*III polymorphism and level of serum lipids in patients with CAD (2, 3, 7, 12, 18, 19). In our study, on table 3, the analysis showed triglycerides level, as a significant factor linked with process of atherosclerosis and indirectly with appearance of CAD in Macedonian population ($p < 0.0469$). With independent multiple regression statistical analysis of LDL plasma level and their correlation with *LPL-Hind*III polymorphism and response variables of the independent factors: hypertension, diabetes, family history of CAD, physical activity, antilipidemic drugs and alcohol consumption, we found that LDL show statistically significant correlation with BMI ($p < 0.046$), and also between LPL-HindIII and LDL plasma level (table 4; $p < 0.010$). In our study, with our population, i.e. Macedonian population, we found that *LPL-Hind*III polymorphism was associated with high triglycerides and LDL levels, as a significant factor associated with process of appearance of CAD.

CONCLUSION

In our study *LPL-Hind*III polymorphism was not identified as an independent risk factor for CAD in Macedonian population.

Our study also confirmed that LPL-HindIII polymorphism was associated with high triglycerides and LDL levels, what is significant factor linked with process of atherosclerosis and appearance of CAD.

Modern molecular, biologic and genetic techniques gradually contribute in discovering etiologic and pathogenetic mechanisms of cardiovascular diseases. Defined genetics polymorphisms and mutations are expected to play a role in accurate early risk stratification and diagnostic of cardiovascular diseases giving more opportunities for prediction and may lead to new treatments for this life-threatening disease.

Abbreviations

Coronary artery disease (CAD)
Deoxyribonucleic acid (DNA)
Polymerase chain reaction (PCR)
Lipoprotein lipase (LPL)
riglycerides (TG)
High density lipoproteins (HDL)
Low density lipoproteins (VLDL)
Very low density lipoproteins (VLDL)
Body-Mass Index (BMI)
Odds ratio (OR)
Confidence interval (CI)
Chi-square (Chi)
t-test (t)
Degrees of freedom (df)
p level of the model (p)
Not Significant (NS)

Sažetak

POVEZANOST LPL-HIND III POLIMORFIZMA SA ARTERIJSKOM BOLEŠĆU U MAKEDONSKOJ POPULACIJI

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Uvod: Koronarna arterijska bolest (CAD) je vodeći uzrok visokog mortaliteta i morbiditeta širom sveta. Hind III polimorfizam LPL gena (LPL-Hind III) je čest i povezan je sa varijantama gena za lipide i lipoproteine u ispitivanoj populaciji.

Cilj rada: Evaluacija Hind III polimorfizma kao nezavisnog faktora rizika za koronarnu arterijsku bolest u makedonskoj populaciji.

Materijal i Metode: Polimerazna lančana reakcija (PCR) i enzimska digestija DNK su korišćene za otkrivanje lipoprotein lipaze, intron 8 LPL-Hind III polimorfizma. Ispitivanu grupu je činilo 114 randomiziranih pacijenata kod kojih je angiografski potvrđena stenoza koronarne arterije (CAD grupa: 87 muškog pola, 27 ženskog pola). Kontrolna grupa se sastojala od od 35 pacijenata (21 muškog pola, 14 ženskog pola) bez signifikantne stenoze koronarne arterije.

Rezultati: Multiplom linearnom regresijom utvrđena je statistički značajna korelacija između LDL i BMI, kao i između LPL-Hind III i LDL nivoa u plazmi. U ispitivanoj grupi je dobijena statistički značajna povezanost samo između triglicerida i LPL-Hind III polimorfizma.

Zaključak: U našoj studiji, Hind III polimorfizam nije identifikovan kao nezavisan faktor rizika za koronarnu arterijsku bolest, ali je dobijena povezanost sa visokim nivoom triglicerida i nivoom LDLholesterola.

Klučne reči: koronarna arterijska bolest, DNK analiza, LPL-Hind III polimorfizam.

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