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IS INTRON 3 POLYMORPHISM OF *CD36* GENE ASSOCIATED WITH HYPERCHOLESTEROLEMIA RISK IN OVERWEIGHT CHILDREN? A PRELIMINARY STUDY

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Abstract

Introduction. The functions of CD36 membrane receptor include removal of oxidized low-density lipoproteins from plasma. The aim of our study was to search association between the IVS3-6 C allele and hypercholesterolemia in overweight children.

Material and Methods. The study groups comprised 55 Caucasian children with (33) and without hypercholesterolemia (22). Amplicons of exon 4 including fragments of introns 3 and 4 were studied using denaturing high-performance liquid chromatography (DHPLC).

Results. Polymorphism detected by DHPLC was single nucleotide substitution in intron 3 (IVS3-6 T/C - rs3173798). The IVS3-6 T/C polymorphism is located in the region encoding the oxidized LDL binding domain, at a conserved splice site. Total serum cholesterol concentrations were significantly lower in the IVS3-6 TC heterozygotes than in the TT patients. Furthermore we found tendency (p=0.06) to lower LDL-cholesterol level in IVS3-6 TC heterozygotes than in wild-type homozygotes.

Conclusion. The results of our preliminary study suggest that the IVS3-6 C allele of *CD36* rs3173798 polymorphism may be associated with lower serum total and LDL-cholesterol in overweight children diagnosed with hypercholesterolemia.

Keywords. *CD36* gene, children, hypercholesterolemia, genetic risk factor, DHPLC.

INTRODUCTION

The functions of CD36 membrane receptor include removal of oxidized low-density lipoproteins (oxLDL) from plasma (1). Recently, CD36 has been reported to play an important role in atherogenicity (2), but there is no clear indication whether mutations of the CD36 receptor gene protect against or increase the risk of hypercholesterolemia

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and atherosclerosis (3).There have been suggestions that polymorphisms of the *CD36* gene modulate lipid metabolism in Caucasians (4). Some reports suggest that **CD36** deficiency elevates serum LDL-cholesterol (5).

The IVS3-6 T/C (rs3173798) polymorphism is located in the region encoding the oxidized LDL binding domain, at a conserved splice site (6). The aim of our study was to search association between the IVS3-6 C allele and hypercholesterolemia in overweight children.

PATIENTS AND METHODS

The study groups comprised 55 Caucasian children with overweight or obesity treated at the Independent Laboratory of Propedeutics in Pediatrics of Pomeranian Medical University in Szczecin (northwestern Poland) in 2008-2010. The patients were Polish residents, aged no more than 15 years. Clinically stable 33 patients (21 girls and 12 boys) with hypercholesterolemia were included in the study group and 22 (11 girls and 11 boys) without hypercholesterolemia were included in the control group. The criteria for hypercholesterolemia diagnosis was total cholesterol (CH) >170 mg/dL and low density lipoprotein cholesterol (LDL) >110 mg/dL (7). None of the study subjects presented clinical criteria for familial hypercholesterolemia, according to the Dutch Lipid Clinic Network criteria (8). Both of the study groups comprised only children on the hypolipemic diet given for at least half a year before the study, due to their overweight. This inclusion criterion was used to minimize the impact of differences in diet on serum lipids and glucose. Patients with diabetes mellitus, renal or liver failure, thyroid dysfunction (current hypo- or hyperthyroidism) or malignancy were excluded from the study. The fasting blood sample was taken for DNA extraction, complete blood count, erythrocyte sedimentation rate, glycated hemoglobin and measurements of serum lipid profile (total, high density lipoprotein (HDL)-, low density lipoprotein (LDL-cholesterol), and triglycerides, apolipoproteins: ApoA1, ApoB, lipoprotein a {$Lp_{(a)}$}, glucose, insulin, high-sensitivity C-reactive protein (hsCRP), creatinine, homocysteine, uric acid, alanine and aspartate transaminases (ALT, AST), plasma fibrinogen and von Willebrand factor. Each patient's weight, height, waist and hip circumference, systolic and diastolic blood pressure were measured. The body mass index (BMI), waist-to-hip ratio (WHR), mean arterial pressure (MAP), the homeostatic model assessment parameters (HOMA IR and HOMA β to quantify insulin resistance and beta-cell function, respectively) were calculated. Genomic DNA was isolated as previously described (9). Amplicons of exon 4 including fragments of introns 3 and 4 were studied using denaturing high-performance liquid chromatography DHPLC technique as previously described (10). The PCR products with alterations detected by DHPLC were bidirectionally sequenced using the Applied Biosystems Dye-terminator Cycle Sequencing Ready Reaction kit,

Table 1. Clinical and morphometric parameters of patients with hypercholesterolemia stratified by IVS3-6 T/C *CD36* genotype

CD36 genotype	IVS3-6 T/C		
	TT (n=24)	TC (n=9)	p-value
Gender (% males)	33%	67%	0.16
Age (years)	12.5 ± 3.0	12.0 ± 3.0	0.59
Systolic BP (mmHg)	119.4 ± 12.8	119.0 ± 12.9	0.77
Diastolic BP (mmHg)	75.2 ± 11.4	75.0 ± 11.7	0.74
MAP (mmHg)	89.9 ± 11.0	89.7 ± 11.6	0.66
HR (1/min)	80.4 ± 11.4	74.8 ± 8.5	0.22
Weight (kg)	63.9 ± 20.4	64.6 ± 30.8	1.0
BMI (kg/m^2)	25.6 ± 4.9	25.9 ± 8.0	0.83
BMI.≥ 25 kg/m^2	44%	58%	0.67
Waist (cm)	90.5 ± 14.0	89.2 ± 22.4	0.57
Hip (cm)	98.4 ± 8.9	94.0 ± 18.3	0.28
WHR	0.92 ± 0.09	0.94 ± 0.06	0.64

Data are given as mean ± SD or percentage of patients with the indicated genotype. BP = arterial blood pressure, MAP = mean arterial pressure, HR = heart rate, BMI = body mass index, WHR = waist-to-hip ratio.

according to the manufacturer's protocol. Semi-automated sequence analysis was performed using a 373A DNA fragment analyzer (Applied Biosystem, Foster City, CA).

The study complies with the principles outlined in the Declaration of Helsinki and was approved by our institutional Ethics Committee. Informed consent was obtained from each patient and their parents.

Differences between subgroups of patients classified according to the intron 3 polymorphism (IVS3-6 T/C) were tested with the Mann-Whitney test for quantitative variables and the Fisher's exact test for qualitative variables. We did not use any correction for multiple comparisons. $P<0.05$ was considered statistically significant. The minimal detectable difference (MDD) of means between IVS3-6 T/C genotype groups is 45 mg/dL for total serum cholesterol concentration, assuming 80% statistical power and 40 mg/dL standard deviation.

RESULTS

There were no significant differences between the study and control groups as regards age (12.3±3.0 and 11.5±3.0 years, respectively, p=0.29) or BMI (25.7±5.3 and 24.8±6.5 kg/m^2, respectively, p=0.59) or gender (50% and 36%, respectively, p=0.21).

Polymorphism detected by DHPLC was single nucleotide substitution in intron 3 (IVS3-6 T/C - rs3173798). Genotype frequencies were 81.8% TT and 18.2% TC in the control group and 72.7% TT and 27.3% TC in the group with hypercholesterolemia. Genotype distributions in both groups were consistent with the Hardy-

Table 2. Biochemical data of patients with hypercholesterolemia stratified by IVS3-6 T/C *CD36* genotype

CD36 genotype	IVS3-6 T/C		
	TT (n=24)	TC (n=9)	p-value
WBC (G/L)	6.51 ± 1.5	6.38 ± 1.8	0.87
RBC (T/L)	4.97 ± 0.3	5.02 ± 0.4	0.84
Hemoglobin (g/dL)	13.60 ± 2.8	13.9 ± 0.9	0.73
Hematocrit (%)	41.0 ± 1.0	41.8 ±2.8	0.57
Platelets (G/L)	288.4 ± 64.3	290.7 ± 53.6	0.98
Total cholesterol (mg/dL)	214.9 ± 40.4	187.3 ± 28.2	0.024
HDL-cholesterol (mg/dL)	52.8 ± 10.2	46.5 ± 10.3	0.17
LDL-cholesterol (mg/dL)	139.4 ± 36.3	118.0 ± 22.5	0.064
Triacylglycerols (mg/dL)	113.6 ± 72.8	119.2 ± 24.8	0.098
Lp(a) (mg/dL)	41.9 ± 42.9	60.2 ± 54.3	0.42
ApoA1 (mg/dL)	147.8 ± 22.6	139.5 ± 26.6	0.61
ApoB (mg/dL)	93.6 ± 21.0	82.2 ± 16.3	0.34
ApoA1/ApoB ratio	0.64±0.15	0.61 ± 0.16	0.75
hsCRP (mg/L)	1.93 ± 2.3	1.80 ± 2.4	0.83
ESR (mm/h)	11.8±7.71	4.33±2.50	0.0091
UA (mg/dL)	5.71±3.38	4.48±0.93	0.27
Creatinine (mg/dL)	0.59±0.08	0.59±0.17	0.84
Fibrinogen (mg/dL)	304.5±57.7	290.5±77.7	0.41
ALT (IU/L)	19.2±9.30	15.0±4.86	0.45
AST (IU/L)	23.6±6.75	20.17±5.53	0.24
vWF	97.9±25.5	100.4±18.6	0.68
Homocysteine (μmol/L)	8.86±2.23	8.59±1.55	0.69
Fasting glucose (mg/dL)	84.8 ± 7.2	87.3 ± 5.55	0.45
OGTT glucose at 60 min	115.6±33.8	116.8±42.4	1.0
OGTT glucose at 120 min	107.0±33.0	101.7±1.91	0.89
Fasting insulin μIU/mL	14.4±9.53	15.2±11.9	0.84
HOMA IR	3.12±2.35	3.38±2.78	0.88
HOMA β	233.4±116.9	207.7±134.5	0.51
HbA1c (%)	5.32±1.16	5.44±0.19	0.56

Data are given as mean ± SD. WBC = white blood cells, RBC = red blood cells, ESR = erythrocyte sedimentation rate, Lp(a) = lipoprotein a, UA = uric acid, ALT = alanine transaminase, AST = aspartate transaminase, vWF = von Willebrand factor, OGTT = oral glucose tolerance test, HOMA (homeostatic model assessment), IR = insulin resistance, β = beta-cell function, HbA1c = glycated hemoglobin.

Weinberg equilibrium (p = 1). There was no significant difference in genotype frequencies between groups (p=0.51). No sequence alterations were found in exon 4.

The clinical data and morphometric parameters of patients with hypercholesterolemia stratified by the intron 3 polymorphism are presented in Table 1. There were no significant differences between the genotype subgroups in terms of any of the analyzed parameters.

The biochemical data of patients with hypercholesterolemia stratified by the *CD36* genotype are presented in Table 2. Total cholesterol concentrations were significantly lower in the IVS3-6 TC heterozygotes than in the TT patients. The IVS3-6 TC genotype was also associated with lower erythrocyte sedimentation rate. Furthermore, we found tendency (p=0.064) to lower LDL-cholesterol level in IVS3-6 TC heterozygotes than in wild-type homozygotes. No other differences were found between genotype subgroups.

The presented data suggest that the IVS3-6C allele of *CD36* is not associated with the risk of hypercholesterolemia, but may contribute to cholesterol level modulation in children with hypercholesterolemia.

DISCUSSION

The IVS3-6C allele frequency in control group (13.6%) was slightly higher, but in the group with hypercholesterolemia (9.1%) it was similar to that described earlier in the Caucasian populations (6.2% to 11.2%), according to the NCBI dbSNP database. The frequency of CC variant homozygotes in the Caucasian population is 0.0% - 1.8%. It is much more frequent in the Asian and African populations (7.0% - 22.1%). In our previous study (10) on three hundred six Caucasian infants we observed 0.7% frequency of CC genotype. In the current study CC homozygotes were not found. No data have been published so far, which would suggest an association between IVS3-6 T/C polymorphism in the *CD36* gene and hyperlipidemia in children. Some authors (11) report that the minor allele C is associated with protection against neovascular age-related macular degeneration. Other authors (12) report that *CD36* intron polymorphisms may contribute to individual and population variability in blood lipids. It was reported that some promoter (rs10499859, rs109654) and intron (rs1358337) *CD36* polymorphisms in African-American adults may contribute to increase the HDL-cholesterol plasma concentrations. Other authors (13) have shown an association between *CD36* promoter single nucleotide polymorphism (rs2151916) and LDL-cholesterol levels in the UK cohort of adult twins. Mean levels of LDL-cholesterol were significantly lower in minor allele homozygotes. IVS3-6 T/C polymorphism was not analyzed in that study.

It seems that *CD36* protein plays a significant role in the pathogenesis of atherosclerosis by serving as a highly specific receptor for oxidized phospholipids prevalent in oxLDL. The interaction of oxLDL with *CD36* triggers a signaling cascade that is

necessary for oxLDL uptake and foam cell formation within the atherosclerotic plaque (14, 15).

We have demonstrated recently (16) that the IVS3-6C allele of *CD36* is associated with cardiovascular risk factors such as high CRP, body mass index, and type 2 diabetes in adults. On the other hand, this variant is associated with low Lp(a), suggesting its protective effect in terms of development of atherosclerosis. The IVS3-6C allele is associated also with younger age at myocardial infarction. In the present study the IVS3-6C allele of *CD36* is not associated with the risk of hypercholesterolemia, but may contribute to cholesterol level modulation in children with hypercholesterolemia. The IVS3-6C allele of *CD36* is not associated also with the measurements of glucose, insulin or HOMA IR and HOMA β, but similar values of these parameters in obesity children are reported by other researchers (17).

In conclusion, the results of our preliminary study suggest that the IVS3-6 C allele of *CD36* rs3173798 polymorphism may be associated with lower serum total and LDL-cholesterol in overweight children diagnosed with hypercholesterolemia. Because the functional effects of the polymorphism have not been elucidated so far, further research is necessary to assess its functional implications for the risk and clinical course of hypercholesterolemia.

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Conflict of interest

The authors declare that there is no conflict of interest.

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