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Comparison of Lipid Profiles in Relation to *APOB* EcoRI Polymorphism in Obese Children with Hyperlipidemia

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Abstract. *Background: We aimed to evaluate apolipoprotein B-100 (APOB) EcoRI polymorphism and plasma lipid parameters together in children and adolescents. This is the first such study in Turkey to determine possible relationships of these parameters. Materials and Methods: Three separate groups were studied: a group of obese children with hyperlipidemia, a group of obese children without hyperlipidemia, and a group of healthy children neither with hyperlipidemia nor obesity. Polymerase chain reaction (PCR), denaturating gradient gel electrophoresis (DGGE) and automatic sequence analysis techniques were used. Sequencing results were examined by Proseq and BioEdit computer programmes. Results: Mutant A allele was not observed in the healthy control group, whereas it was more frequent in the hyperlipidemic obese children; the GA genotype was correlated with total and low density lipoprotein-cholesterol levels. Conclusion: In this study, we suggest that obese child patients having the A allele could have a higher risk for developing hyperlipidemia.*

Hyperlipidemia is the elevation of lipids including fats, fatty acids, cholesterol, cholesterol esters, phospholipids and triglycerides in the bloodstream (1). There are various complications of hyperlipidemia such as coronary artery diseases (CAD) related to obesity and atherosclerosis (2).

Obesity is increasing at an alarming rate, and its associated disorders are placing a considerable strain on our healthcare systems (3). Obesity is often accompanied by hyperlipidemia. Both obesity and hyperlipidemia are independently associated with atherosclerosis. In parallel to adults, the prevalence of obesity in children and adolescents has also increased during recent decades (4). From cross-sectional and longitudinal studies of many countries and different ethnic groups, there is enough evidence that in children and adolescents, obesity is associated with lipid and lipoprotein changes and with other well-known risk factors of cardiac disease in adulthood, *e.g.* insulin resistance and high blood pressure (5). As the duration of hyperlipidemia is higher, obesity in early life is associated with early stages of atherosclerosis (6).

In childhood obesity, genetic factors should be considered much more than in adult cases; enviromental factors are not assumed to be influential as genetic factors (4). In particular, the genes involved in lipid metabolism, such as those encoding the major apolipoproteins, are thought to be candidate genes for CAD with obesity (7).

Apolipoprotein B (Apo B) is a large, amphipathic glycoprotein playing a central role in human lipoprotein metabolism (7) and is coded by *APOB* gene located on chromosome 2. One of the two Apo B forms is Apo B-100, which is required for very low-density lipoprotein (VLDL) production in the liver. In addition to being an essential structural component of VLDL, Apo B-100 is also the ligand for LDL-receptor-mediated endocytosis of LDL particles (8). It was proven that even one amino acid change in the carboxyl end of the Apo B protein can destroy its binding capacity to LDL receptors; it was also shown that Apo B was defective in some hyperlipidemic and/or hypercholesterolemic patients in binding to the receptor (9). There are also several known *APOB* polymorphisms that were proven to cause hyperlipidemia and cardiovascular disease (CVD) (10-14). One such polymorphism is the EcoRI polymorphism that results in Glu4154Lys amino acid substitution in the 26th exon (7). In previous studies, a significant direct relationship between EcoRI polymorphism and the serum levels of cholesterol and triglyceride was found. It was thought that these polymorphisms reduce the binding capacity of Apo B to LDL receptors and so cause a decrement in LDL clearance (15, 16).

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Key Words: Apo B-100, hyperlipidemia, obesity, DGGE, gene polymorphism, *APOB*.

Table I. *Primers and DGGE screening conditions.*

Primers*	Exon	Fragment size (bp)	Gradient %**	Running Temp (˚C)
Forward: 5'-GC*-ATCGACGTGAGGTTCCAGAAAGCA3' Reverse: 5'-GAAAGGTGTAATCACTAGGTCTT3'	29	390	30-50	60

*GC=5'GCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGC-3'; **100% denaturant=7 M urea and 40% (v/v) formamide in TAE buffer.

Therefore, we aimed to evaluate both the *APOB* EcoRI polymorphism and plasma lipid parameters together in children and adolescent cases. This is the first study in a Turkish population evaluating these aspects together.

Materials and Methods

Participants. Informed consent was obtained from the families of the participants, and the study was approved by the Ethical Committee of Istanbul University, The Istanbul Faculty of Medicine (No: 1688).

Three study groups who were followed up in Pediatric Endocrinology clinics of participating centers were enrolled in the study. The ages of the 90 children included were from 8 to 15 years for females and 9 to 16 years for males. For our study, we divided the children into three separate groups: one of them was the group of obese children with hyperlipidemia (patient group), the second was that of obese children without hyperlipidemia (control I group) and the last group comprised healthy children neither with hyperlipidemia nor obesity (control II group). Standard deviation score (SDS) was preferred instead of BMI values (17). Cholesterol and triglyceride values change depending on the age and gender, thus we preferred to use 95th percentiles as the limit value (18). Genomic DNA was extracted from venous whole blood samples (from leukocytes) using a Roche Blood Kit (Roche MagNa Pure Compact Nucleic Acid Izolation Kit-I (Manheim, Germany).

Method of genotyping. The coding regions of exon 29 of the Apo B-100 gene were amplified using specific primers (Table I). In each pair, a 40-base GC-rich segment ('GC-clamp') was attached to the 5' end of the forward primer to facilitate the detection of mutations by denaturating gradient gel electrophoresis (DGGE). The PCR reaction was performed under standard conditions in a volume of 50 μl containing 30 μl sterile distiled water, 3 μl 10xPCR Buffer (contains no $MgCl_2$), 5 μl (25 mM) $MgCl_2$, 5 μl (1 millimoles) of each deoxyribonucleoside triphosphate, 2 μl (10 picomoles) of each primer (Table I), 2 μl (1.5 units) of DNA Taq polymerase and 3 μl (25-175 ng) of human genomic DNA. The reaction consisted of denaturation at 94˚C for 4 minutes, followed by 35 cycles of 95˚C for 30 seconds, 62˚C for 30 seconds and 72˚C for 60 seconds, with a final extension at 72˚C for 10 minutes. Thermal cycling was performed by an Applied Biosystems 9700 (CA, USA). Electrophoresis of 10 μl of final PCR reaction products was performed on 2% agarose gel.

Polymorphism detection. In the first stage, mutation analysis of the coding region of exon 29 of the *APOB* was carried out by DGGE. DGGE is a tool that was developed to analyze DNA. It is very sensitive at detecting changes (mutations) in the genetic sequence within a sample, and can detect even a one base pair difference between strands of DNA. Table I shows the electrophoretic conditions, including the running temperature and the denaturing gradient of the formamide/ urea concentration range for each different PCR product. DGGE analysis reveals migration differences of homoduplexes and heteroduplexes of PCR-generated amplicons. For DGGE, 15 μl PCR mixes were analyzed using an 8% polyacrylamide gel (acrylamide-to-bisacrylamide ratio 37.5:1; W×H×L: 0.08×19×25 cm) with a 30-50% denaturing gradient of urea formamide (UF) A solution of 100% UF was 7 M urea and 40% deionized formamide in 1×TAE buffer (1×TAE: 40 mM Tris–acetate, 1 mM EDTA, pH 8.0). Electrophoresis was performed at 70 V for 16 hours at a constant temperature of 60˚C. After electrophoresis, the gels were stained with ethidium bromide and viewed under a UV source.

Sequencing. After the mutation analysis, PCR products with different band patterns were purified using the Roche High Pure PCR Product Purification Kit for DNA sequencing. A total of 50 ng of the purified PCR product was used for cycle sequencing with the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham, London). The amplified products were purified with Template Suppression Reagent solution and sequenced on an ABI PRISM 310 Genetic Analyzer. Analyses were done by using the Proseq and BioEdit programmes (McMaster University, Hamilton, Canada).

Lipid profiles. Blood samples were obtained in the morning after a 12-hour overnight fast. Total cholesterol, triglyceride, LDL-cholesterol, HDL-cholesterol and VLDL-cholesterol were analyzed by a biochemical autoanalyser at the Clinical Laboratory of Pediatric Endocrinology, Faculty of Medicine, Istanbul University within 4 hours.

Statistical analysis. Statistical analyses were performed using the SPSS software package (SPSS Inc., Chicago, IL, U.S.A.), revision 8.5. Clinical laboratory data were expressed as means±standard deviation (SD). Mean values were compared between patients and controls subjects by the unpaired Students *t*-test. The *APOB* genotypes and alleles between groups were tested using Chi-square test. Allele frequencies were estimated by gene counting methods. A value of $p<0.05$ was considered statistically significant.

Results

Demographic characteristics are summarized in Table II. There were significant differences in total cholesterol, triglyceride, LDL-cholesterol, and VLDL-cholesterol levels between patients and the controls, as was expected.

Table II. *Demographic characteristics of the study population.*

	Patients n=38	Control I n=39	Control II n=13	Overall n=90
Gender (female/male)	26/12	24/15	5/8	55/35
Age (years)	11.50±3.61	11.43±3.23	8.15±2.15	10.98±3.44
BMI (kg/m^2)-SDS	6.51±3.10^{b1}	6.26±3.12^{c1}	0.14±1.06	5.48±3.63
Triglyceride (mg/dl)	149.92±64.53$^{a1, b1}$	80.33±24.80	86.69±38.74	110.63±57.80
Total cholesterol (mg/dl)	181.78±35.64^{a1}	145.87±18^{c2}	161.53±31.29	163.30±32.83
HDL-cholesterol (mg/dl)	49.57±13.23	48.84±10.34	54.53±9.98	49.97±11.64
LDL-cholesterol (mg/dl)	104.10±26.25^{a1}	82.01±14.97	88.84±26.19	92.32±24.13
VLDL-cholesterol (mg/dl)	30.50±13.35$^{a2, b1}$	20.48±26.79	18.30±6.19	24.40±20.34
Total cholesterol/HDL-cholesterol (mg/dl)	3.96±1.14	3.09±0.63	2.99±0.48	3.44±0.97

n: Number of individuals; BMI, body mass index; SDS, standard deviation score; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL; very high density lipoprotein; Patient group: obese children with hyperlipidemia, Control I group: obese children without hyperlipidemia, Control II Group: healthy children neither with hyperlipidemia nor obesity. The results are shown as means±SD. Between patient and control I, $^{a1}p<0.001$, $^{a2}p<0.05$; Between patient and control II, $^{b1}p<0.001$; Between control I and control II, $^{c1}p<0.001$, $^{c2}p<0.05$.

We determined G4154A polymorphism in exon 29 of *APOB* in the patient and control I groups. The genotype and allele frequencies for G4154A in exon 29 of *APOB* in the patients and controls are given in Table III. There were no significant differences in genotype or allele frequencies between patients and controls ($p>0.05$). Moreover, there was no individual with the AA genotype among the study group participants. Nor did we find mutant A allele in the healthy control II group (Table III).

We found that total cholesterol ($p=0.013$, 95% Cl: 15.67-86.82) and LDL-cholesterol ($p<0.05$, 95% Cl: 8.57-68.91) levels were higher in patients with the GA genotype compared to those of control I with the same genotype (Table IV). However, we could not find any relationship between any genotype and the lipid profile in the control groups.

We found total cholesterol and LDL-cholesterol levels to be higher in patients with the GA genotype than in those with the GG genotype ($p<0.05$). Mutant GA genotype was found in the patient group and there was a correlation between total cholesterol and LDL-cholesterol levels (Table IV).

Table III. *Prevalence of the APOB (G4154A) genotypes and alleles in the study groups.*

APOB (G4154A)	Patients (n=38)	Control I (n=39)	Control II (n=13)
Genotypes			
GG	34 (89.5%)	35 (89.7%)	13 (100%)
GA	4 (10.5%)	4 (10.3%)	0
AA	0	0	0
Alleles			
G	72 (92.3%)	74 (94.9%)	26 (100%)
A	4 (7.7%)	4 (5.1%)	0

n: Number of individuals. Patient group: obese children with hyperlipidemia, Control I Group: obese children without hyperlipidemia, Control II Group: healthy children neither with hyperlipidemia nor obesity. Chi-square test, $p>0.05$.

Discussion

The prevalence of obesity in children and adolescents has increased dramatically in the past three decades (19). Childhood obesity is important for public health for many reasons (6). First of all, obesity during childhood and adolescence is associated with a number of CVD risk factors. Some of these risk factors include type 2 diabetes mellitus, hypertension, and hyperlipidemia. Additionally, obesity and many of the associated CVD risk factors have a strong predisposition to persist through adulthood. Thus, obesity during childhood and adolescence increases adult risk of CVD (20, 21). For this reason, a considerable amount of research resources have been devoted to this topic in recent years.

CVD is the leading cause of death and morbidity worldwide (22). It is believed that atherosclerosis, predisposing CVD, is initiated early in childhood (23). Furthermore, obesity has been associated with hyperlipidemia, a high LDL cholesterol and a low level of HDL cholesterol. Overweight and obesity were associated with hyperlipidemia in children (24). Previously it was shown that cholesterol levels and obesity could track well from childhood to adulthood (20, 21, 25, 26). In the Muscatine Study, 75% of school-aged children who had total cholesterol concentrations higher than the 90th percentile at baseline had total cholesterol concentrations of >200 mg/dl in their early twenties (22, 27, 28). In the Bogalusa Heart Study, approxiametly 70% of the children with elevated cholesterol levels continued to have increased cholesterol levels in young adulthood (22, 29).

Table IV. *Comparison of G4154A genotypes frequencies and biochemical parameters.*

	Patients (n=38)			Control I (n=39)			Control II (n=13)		
G4154A genotypes	GG (n=34)	GA (n=4)	AA (n=0)	GG (n=35)	GA (n=4)	AA (n=0)	GG (n=13)	GA (n=0)	AA (n=0)
Total cholesterol (mg/dl)	180.05±36.69	196.50±23.17	0	145.94±18.41	145.22±16.11	0	161.53±31.29	0	0
LDL-cholesterol (mg/dl)	102.82±26.63	115±22.80	0	82.18±15.26	80.50±13.91	0	88.84±26.19	0	0

n: Number of individuals. LDL, low density lipoprotein; Patient group: obese children with hyperlipidemia, Control I Group: obese children without hyperlipidemia, Control II Group: healthy children neither with hyperlipidemia nor obesity. Chi-square test, $p<0.05$.

We considered the demographic characteristics of our study groups. There were significant differences in total cholesterol, triglyceride, LDL-cholesterol, and VLDL-cholesterol between the groups, as expected.

In our study, we had chosen *APOB* as a candidate gene of lipid metabolism. In the literature there were several *APOB* polymorphisms associated with hyperlipidemia (10-14) We have studied the EcoRI polymorphism at the 4154 position, causing a glutamine to lysine substutition in the 26th exon (7). Only a few studies have investigated the possible mechanisms whereby the EcoRI polymorphism of the *APOB* gene affects serum cholesterol and Apo B levels. Studying LDL kinetics in relation to *APOB* polymorphism in five different populations, Houlston *et al*. observe that in four out of these five populations, the LDL fractional catabolic rate was lower in those carrying the minor EcoRI allele, the difference reaching statistical significance in one population. These data show that variation in *APOB* may influence LDL metabolism and that the EcoRI polymorphism may influence the LDL catabolic rate (15). However, Gallagher and Myant did not encourage this hypothesis. Indeed, whereas the major pathway for removal of LDL from the plasma is through binding of Apo B-100 on LDL particles to the LDL receptor, Gallagher and Myant found no difference between binding affinities to human skin fibroblasts of LDL particles from individuals homozygous for the major EcoRI allele and those from individuals homozygous for the minor EcoRI allele (16).

In our study, when we compared genotype and allele prevalence, there were no significant differences between patients and controls. Additionally, we compared allele frequencies between patient and control groups although there was no individual with the AA genotype in the study groups. Interestingly, we found no A allele (mutant) carriers in the healthy control group. Furthermore, we also compared G4154A genotype frequencies and biochemical parameters and observed that total cholesterol ($p=0.013$, 95% Cl: 15.67-86.82) and LDL-cholesterol ($p<0.05$, 95% Cl: 8.57-68.91) levels were higher in patients with GA genotype compared to those of control I group with the same genotype. Unfortunately, we could not find any relationship between G4154A genotype and lipid profiles in the control groups.

Pouliot *et al*. investigated whether the *APOB* EcoRI polymorphism influenced the associations described among obesity, regional adipose tissue distribution, and plasma lipoprotein levels in 56 healthy men. After adjusting for age and BMI rate, they observed that total cholesterol levels were significantly higher in heterozygous individuals compared to homozygous individuals (30). Similar to this study, we found increased total cholesterol and LDL-cholesterol levels in patients with GA genotype compared to GG genotype ($p<0.05$).

In conclusion, we suggest that obese pediatric patients having mutant A allele could have a higher risk of having hyperlipidemia in the future. Accordingly, this is the first study to determine the relationships between hyperlipidemia, *APOB* EcoRI polymorphism and childhood obesity. For future research, the study groups are planned to be enlarged to obtain more precise results.

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