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Stepwise Positive Association Between *APOA5* Minor Allele Frequencies and Increasing Plasma Triglyceride Quartiles in Random Patients with Hypertriglyceridemia of Unclarified Origin

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Abstract Apolipoprotein A5 (ApoA5) gene and its protein product play a central role in the complex regulation of circulating triglyceride levels in humans. Naturally occurring variants of the apolipoprotein A5 gene have been associated with increased triglyceride levels and have been found to confer risk for cardiovascular diseases. In our study, four polymorphisms, the T-1131C, IVS3+G476A, T1259C, and C56G alleles of *APOA5* were analyzed in a total of 436 patients by polymerase chain reaction—restriction fragment length polymorphism methods. The randomly selected patients were classified into four quartile (q) groups based on triglyceride levels (q1: TG<1.31 mmol/l; q2: 1.31–2.90 mmol/l; q3: 2.91–4.85 mmol/l; q4: TG>4.85 mmol/l). We observed significant stepwise increasing association between the four *APOA5* minor allele carrier frequencies and plasma triglyceride quartiles: -1131C (q1: 4.44%; q2: 8.95%; q3: 12.9%; q4: 20.6%), IVS3+476A (q1: 4.44%; q2: 5.79%; q3: 11.1%; q4: 19.7%), 1259C (q1: 4.44%; q2: 6.84%; q3: 11.1%; q4: 20.6%) and 56G (q1: 5.64%; q2: 6.31%; q3: 11.16%; q4: 11.9%). The serum total cholesterol and high density lipoprotein-cholesterol levels also showed allele-dependent differences in the quartiles. The findings presented here revealed a special arrangement of APOA5 minor alleles in patients with different serum triglyceride ranges in Hungarians.

Keywords ApoA5 · Hypertriglyceridemia · T-1131C · IVS3+G476A · T1259C · C56G · PCR-RFLP

Abbreviations

APOA5	Apolipoprotein A5
HTG	Hypertrigyceridaemia
LPL	Lipoprotein lipase
SNP	Single nucleotide polymorphism
VLDL	Very-low-density lipoprotein

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Introduction

Hypertriglyceridemia (HTG) is a common metabolic problem in industrialized countries. Serum triglyceride

levels over 1.7 mmol/L are also associated with dyslipidemia (e.g. cholesterolemia and lipoproteinemia) and metabolic syndrome [1, 2]. HTG exacerbates the risk for pancreatitis, coronary artery disease and other vascular diseases [3–5].

Apolipoprotein A-V protein is encoded by the *APOA5* gene. It is a key apolipoprotein whose physiological role has been demonstrated in studies in knockout mice showing elevated triglyceride levels. The *APOA5* gene is located on chromosome 11q23 within the *APOAI-CIII-AIV-AV* gene cluster, and comprises 3 exons, encoding 366 amino acids [6, 7]. Numerous studies confirmed that naturally occurring variants of the *APOA5* gene (like *-1131C, IVS3+476A, 1259C*, *56G* alleles) associate with elevated triglyceride levels [8–11]. Besides, some of them were found to confer risk for the development of coronary heart disease and stroke [10, 12–17].

This apolipoprotein appears to play a key role in the hydrolysis of triglyceride-rich lipoproteins by increasing the activity of lipoprotein lipase (LPL). Rare mutations of apoA-V can cause familial chylomicronemia [18, 19]. In most populations the SNPs (Single nucleotide polymorphism) S19W, IVS3+G476A, T1259C and -1131T>C in *APOA5* are relatively common (approximately 5–10% allele frequency). They are associated with *in vivo* dysfunction of apoA-V and as a consequence, with elevated triglyceride levels [20, 21].

In a recent study patients with classic hyperlipoproteinemia phenotypes were characterized for *APOA5* S19W, –T1131C, IVS3+G476A and T1259C minor alleles. Our preliminary observations of metabolic syndrome patients prompted us to do these series of experiments.

Materials and Methods

Study Population

In the present study 436 unrelated random adult Hungarian patients (235 males and 201 females, mean age: 60.5± 10.08 years, range: 23–74 years) were selected for the study. The patients were categorized into four quartile (q) groups based on serum triglyceride levels (q1: TG<1.31 mmol/l; q2: 1.31–2.90 mmol/l; q3: 2.91–4.85 mmol/l; q4: TG>4.85 mmol/l).

The DNA with the clinical dataset from the patients was deposited into the local biobank. The patients gave their informed consent for the future genetic tests of the samples and for data analysis. The local biobank was established with the authorization of the National Ethics Committee.

Genetic Analysis

DNA was isolated from peripheral blood leukocytes by a standard salting method. *T-1131C* alleles were determined as previously described [16]. For all SNPs we considered the principle to design primers creating an obligatory cleavage site within the PCR product, which enabled monitoring of digestion efficacy.

To test the *IVS3+G476A* variant the following oligonucleotides were used for amplification: 5′-CTC AAG GCT GTC TTC AG-3′ (sense) and 5′-CCT TTG ATT CTG GGG ACTG G-3′ (antisense). The PCR product (15 μl) was digested with 1U of *MnlI* restriction endonuclease at 37°C overnight. The restriction fragments were analyzed using 3% agarose gel stained with ethidium bromide, and visualized by an UV transilluminator. With GG genotype, the digestion resulted in 25 bp, 114 bp, 141 bp fragments, and in homozygous samples in 25 bp, 41 bp, 73 bp and 141 bp long products were detected. The *T1259C* polymorphism was detected using the primers 5′-TCA GTC CTT GAA AGT GGC CT-3′ (sense) and 5′-ATG TAG TGG CAC AGG CTT CC-3′ (antisense). The PCR product was digested with 1U of *BseGI* restriction endonuclease at 55°C overnight. After the digestion, the normal (TT) genotype gave fragments of 122 bp and 165 bp, whereas the homozygous form (CC) resulted in 35 bp, 87 bp, 165 bp fragments. The region containing the *C56G* polymorphism was amplified with 5′-AGA GCT AGC ACC GCT CCT TT and 5′-TAG TCC CTC TCC ACA GCG TT primers. The 256 bp amplicon was digested with Cfr13I enzyme; after digestion 79, 177 bp fragments were detected in the

Table 1 Major clinical parameters of the four quartile groups

	Quartiles of plasma triglycerides			
	TG<1.31 *n*=124	TG=1.31–2.90 *n*=95	TG=2.91–4.85 *n*=108	TG>4.85 *n*=109
Males/females	45/79	45/50	64/44	81/28
Age (yrs)	55.3±1.69	57.2±1.68	53.7±1.53	52.6±1.30
HDL cholesterol (mmol/l)	1.40±0.05	1.12±0.03[a;#]	1.02±0.02[a;#]	1.01±0.08[a;#]
Triglycerides (mmol/l)	1.01±0.02	2.10±0.05[a;#]	3.70±0.05[a;#]	7.06±0.25[a;#]
Total serum cholesterol (mmol/l)	4.76±0.10	5.20±0.10[a;#]	5.62±0.11[a;#]	6.10±0.14[a;#]

[a] adjusted for gender; [#] $p<0.05$ vs. TG<1.31

Table 2 APOA5 genotypes and allele frequencies in the quartile groups

		TG<1.31 *n= 124*	TG=1.31–2.90 *n= 95*	TG=2.91–4.85 *n= 108*	TG>4.85 *n= 109*
T-1131C	TT	113 (91.9%)	81 (85.3%)	80 (74.1%)	69 (63.3%)
	TC+CC	11+0 (8.9%)	11+3 (14.7%)	28+0 (25.9%)	35+5 (33.7%)
	C allele frequency	4.44%	8.95%	12.9%[#]	20.6%*
T1259C	CC	115 (91.1%)	82 (86.3%)	86 (79.6%)	70 (64.2%)
	CG+GG	11+0 (8.9%)	13+0 (13.7%)	20+2 (20.4%)	33+6 (35.8%)
	G allele frequency	4.44%	6.84%	11.1%[##]	20.6%*
C56G	TT	111 (89.5%)	84 (88.4%)	83 (76.9%)	85 (78.0%)
	TC+CC	12+1 (10.5%)	10+1 (11.6%)	15+0 (23.1%)	22+2 (22.0%)
	C allele frequency	5.64%	6.31%	11.55%	11.9%**
IVS3+G476A	GG	113 (91.1%)	85 (89.5%)	84 (77.8%)	71 (65.1%)
	GA+AA	11+0 (8.9%)	9+1 (10.5%)	24+0 (22.2%)	33+5 (34.9%)
	A allele frequency	4.44%	5.79%	11.1%[§]	19.7%*

[#] p=0.001; *p<0.001; [##] p=0.003; **p=0.019; [§] p=0.006

samples with CC genotype, while in homozygous GG samples 26, 79, 151 bp products were detected.

PTC 200 PCR (Bio-Rad, Hercules, CA, USA) equipments were used for amplification. The conditions were similar for all polymorphisms: a 2-minute initial denaturation at 96°C was followed by 35 cycles of 20 seconds at 96°C; 20 s at 60°C; and 20 s at 72°C; the final extension at 72°C was 5 min long. The amplification was carried out in a final volume of 50 µl containing 5 µl reaction buffer (500 mM KCl, 14 mM $MgCl_2$, 10 mM Tris-HCl, pH 9.0), 1 µl 50 mM $MgCl_2$, 0.2 mM of each dNTP, 1 U of Taq polymerase, 0.2 mM of each reaction specific primers and 1 µg DNA [22].

Statistical Analysis

Results are expressed as mean ± SEM. Statistical significance was assessed by the Mann-Whitney U test to compare the differences between groups. χ^2 tests were used to compare discrete data. A value of $p<0.05$ was considered to indicate statistical significance. All statistical analyses were performed using SPSS 13.0 software (SPSS Inc, Chicago, IL, USA).

Results

Clinical characteristics of the four quartile groups are shown in Table 1. Serum triglycerides and total cholesterol were significantly elevated while serum HDL-cholesterol levels were significantly lower in q2, q3 and q4 compared to the q1. The age ranges of the four groups show no significant differences.

Table 2 demonstrates the APOA5 genotypes and allele frequencies in the four quartile groups.

Table 3 Triglycerides levels of different genotypes in each quartile groups

	T-1131C		IVS3+G473A		T1259C		C56G	
mmol/l	TT	TC+CC	GG	GA+AA	TT	TC+CC	CC	CG+GG
TG<1.31	1.01±0.02	0.98±0.02	1.01±0.02	1.03±0.02	1.01±0.02	1.04±0.02	1.01±0.02	0.99±0.02
TG=1.31–2.90	2.05±0.03	2.39±0.04	2.07±0.42	2.31±0.05	2.07±0.03	2.31±0.05	2.11±0.03	2.05±0.04
TG=2.91–4.85	3.63±0.05	3.89±0.07	3.62±0.04	3.96±0.05	3.59±0.04	4.06±0.06	3.69±0.04	3.72±0.06
TG>4.85	6.97±0.11	7.20±0.14	6.92±0.13	7.40±0.18	6.29±0.11	7.26±0.14	6.91±0.09	7.33±0.10

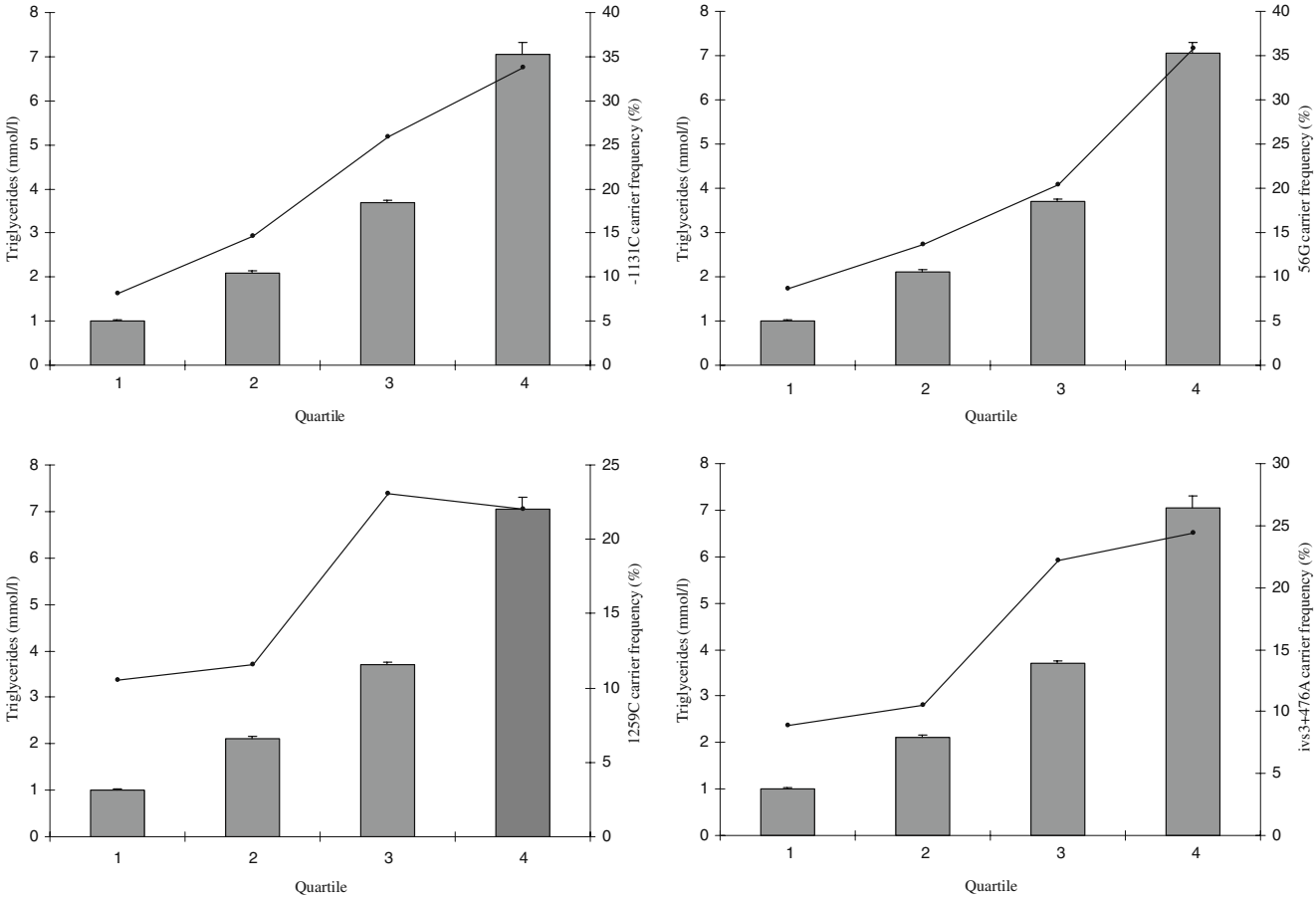

Fig. 1 Frequency of carriers of APOA5 variants according to the serum triglycerides

Table 3 shows triglyceride levels of different genotypes in each quartile. Figure 1 shows the frequency of carriers of APOA5 variants according to the serum triglycerides of each quartile. The frequencies of the minor variants of three APOA5 alleles (-1131C, IVS3+476A, 1259C) are higher in q3 and q4, while in the case of variant 56G higher frequency can be found only in q4 group. The allele frequencies of all APOA5 variants studied were consistent with Hardy-Weinberg equilibrium expectation in every group.

Table 4 indicates the frequencies of APOA5 haplotypes in the quartiles. The frequency of APOA5*2 haplotype is higher in q3 and q4 and APOA5*3 haplotype is rifer in q4.

Discussion

Most HTGs are present in the context of obesity, diabetes, and metabolic syndrome [23, 24]. Under these circum-

Table 4 Frequencies of APOA5 haplotypes in the quartiles

	<1.38 mmol/l *n*= 124	1.38–1.93 mmol/l *n* =95	1.94–2.83 mmol/l *n*= 108	>2.83 mmol/l *n*= 109
*APOA5*1/1*	100 (80.65%)	70 (73.68%)	63 (58.33%)	44 (40.37%)
*APOA5*1/2-2/2*	11 (8.87%)	10 (10.53%)	22* (20.37%)	38* (34.86%)
*APOA5*1/3-3/3*	13 (10.48%)	11 (11.58%)	15 (13.89%)	24* (22.02%)
Other haplotype variants	–	4 (4.21%)	8 (7.41%)	3 (2.75%)

* $p \leq 0.05$ vs. TG<1.38

stances, the overflow of free fatty acids from the visceral adipose tissue to the liver leads to an increased production and accumulation of triglycerides, and finally, to an increased VLDL (very-low-density lipoprotein) production [19]. Altered triglyceride metabolism can be involved in the abnormal accumulation of lipids in vascular endothelial cells under pathologic conditions, and can also be implicated in the formation of atherogenic plaques that are associated with pathologic processes leading to the development of ischemic vascular diseases [25–27].

APOA5 was identified as part of the *APOAI-CIII-AIV-AV* gene cluster on 11q23 locus. Several SNPs in this gene cluster have been reported to have a significant influence on serum triglyceride levels [28, 29]. As APOA5 affects triglyceride metabolism, the naturally occurring variants of the APOA5 gene have been widely studied over the past few years.

Several SNPs within the APOA5 locus have been identified in humans. Four of them, *T-1131C, IVS3 +G476A, T1259C*, and *C56G* represent the most common variants. As these natural genetic variants have effect on the activity of their protein transcripts, some of these alleles have been reported to associate with elevated fasting or postprandial circulating triglyceride levels [8–11, 17]. It is generally accepted, that while some genetic polymorphisms can act independently from their genetic surroundings, the majority of them exert their effects in accord with other polymorphisms as haplogroups.

The APOAV protein has special coexisting roles in the complex regulation of circulating triglyceride levels in humans. First, APOA5 interacts with LPL, the central enzyme involved in the regulation of circulating triglyceride levels, and thereby it is an activator of the intravascular triglyceride hydrolysis. This interaction represents the major mechanism by which APOA5 exerts its modifier activity. Secondly, as a part of the triglyceride level-lowering effect of APOA5, it also inhibits hepatic VLDL production [19].

Wang et al. found a higher frequency of carriers of APOA5 variants in lipid clinic patients than in controls, a significant stepwise relation-ship between APOA5 minor allele carrier frequencies and plasma triglyceride quartiles, and higher APOA5 S19W and APOA5 –T1131C allele and carrier frequencies in lipid clinic patients than in controls for hyperlipoproteinemia types 2B, 3, 4 and 5. These findings indicate that APOA5 variants S19W and -1131T>C are strongly and specifically associated with HTG in lipid clinic patients and with several hyperlipoproteinemia phenotypes defined by elevated plasma triglyceride concentration. Hyperlipoproteinemia type 2A, which is not characterized by elevated triglyceride levels, was not associated with APOA5 minor alleles [30].

In our study, we found a significant stepwise relationship between APOA5 minor allele carrier frequencies and serum triglyceride quartiles. The frequency of the minor variants of three APOA5 alleles (-1131C, IVS3+476A, 1259C) are higher in q3 and q4, while in case of the fourth (56G variant) higher frequency could be only found in the q4 group. The frequency of APOA5*2 haplotype is higher in q3 and q4 and APOA5*3 haplotype is rifer in q4. Thus, alleles of APOA5 are risk factors for HTG in the general Hungarian population, and the results show, that the triglyceride level driven accumulation of rare alleles can not be restricted to specific type of hyperlipoproteinemia. It should be noted, that the lack of multivariable analysis to show the possible independent association of APOA5 locus and triglyceride levels is a real limitation of the study; and since the exact explanation of the findings is not known, further studies are required to clarify the background.

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