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APOA5 Ala315>Val, identified in patients with severe hypertriglyceridemia, is a common mutation with no major effects on plasma lipid levels

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Abstract

Background: The importance of the apolipoprotein A5 (*APOA5*) gene in determining plasma triglyceride (TG) levels has been demonstrated in transgenic and knockout mice and confirmed by human association studies in different ethnic groups.
Methods: We screened for nonsynonymous *APOA5* mutations in patients with plasma TG levels >10 mmol/L. The coding sequence and promoter region of the *APOA5* gene were sequenced in 95 individuals with severe hypertriglyceridemia (HTG). A large population sample of 3202 individuals was screened by PCR and restriction analysis for presence of detected mutation.
Results: In total, three heterozygous carriers of 944C>T (Ala315>Val) were identified in the severe HTG patients, while 22 carriers were identified in the population sample. The rare allele frequency of the Val315 was significantly higher in the HTG sample than in controls (0.016 vs. 0.003, $p<0.01$, respectively). Most of the control Ala315Val carriers, however, had plasma lipid levels (TGs, total cholesterol and high-density lipoprotein cholesterol) within the usual range detected in the population.
Conclusions: *APOA5* Ala315>Val does not play any dominant/important role in the genetic determination of plasma TG levels, but the increased frequency in HTG patients compared to controls suggests that it might interact with other gene variants to cause HTG. Clin Chem Lab Med 2008;46:773–7.

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Introduction

The apolipoprotein A5 gene (*APOA5*) was identified by comparative sequencing of human and mice DNA in 2001 (1). Generation of transgenic and knockout mice demonstrated the role of this gene in determining plasma triglyceride (TG) levels. The transgenic mice exhibited diminished, and the knockout mice elevated levels of plasma TG, while the plasma cholesterol levels were not significantly influenced.

ApoA5 is located on TG-rich (chylomicrons and very-low-density lipoprotein) and high-density lipoprotein (HDL) particles. In comparison to other apolipoproteins, plasma concentration of apoA5 in humans is low – ranging from ~25 to 400 μ/L (2). Nevertheless, *ApoA5-ApoC3* double knockout and double transgenic mice have normal TG levels (3). In the double transgenic mice, despite the fact that plasma levels of human apoA5 are approximately 500 times lower than that of apoC3, apoA5 still has a significant effect on plasma TG levels (3).

Almost 20 *APOA5* single nucleotide polymorphisms (SNPs)/mutations have been identified [most of them reviewed in (4)]. Two TG-raising haplotypes have been identified, *APOA5*2* and *APOA5*3*, which can be tagged by T-1131>C and the Ser19>Trp variants, respectively, and which have been studied in a wide range of human populations, although some gender-specific and ethnicity-specific differences have been described (4–7). *APOA5*–1131C and 19Trp alleles have also been associated with myocardial infarction risk and coronary artery disease development (8, 9).

ApoA5 binds to, and enhances the activity of, lipoprotein lipase in mice (10, 11). A similar function of apoA5 in humans was confirmed by Priore Oliva et al. (12) and by Marcais et al. (13) in their studies of patients with premature truncation of *APOA5*. Two patients with rare *APOA5* mutations, resulting in premature stop codons at positions 145 [C433>T; Gln145>Stop; (12)] and 139 [C417>T; Gln139>Stop; (13)] were reported. Plasma of the patients activated lipoprotein lipase in vitro less efficiently than control plasma from healthy individuals. These results confirmed that APOA5 is an activator of lipoprotein lipase.

To explore whether rare *APOA5* mutations impacted on severe hypertriglyceridemia (HTG), we screened by resequencing the *APOA5* gene (coding sequence and exon/intron boundaries) in a group of 95 patients with severe HTG.

Materials and methods

Study groups

A total of 95 unrelated Czech patients (77 males and 18 females, mean age 51.6 years, TG levels >10 mmol/L) with severe HTG were selected from the database of the Lipid Clinic of the Third Department of Medicine, Charles University, Prague and were screened for the presence of rare *APOA5* mutations.

As a control population we genotyped 3202 unrelated Caucasians (1490 males and 1712 females, aged 25–67 years, plasma TG levels <10 mmol/L). These individuals were selected according to the World Health Organization (WHO) protocol for the MONICA study (Manual WHO/MNC 82.2, Nov-1983) as representative of 1% of the Czech Caucasian population sample (from nine selected districts). All participants gave their informed consent prior to the start of the study. The study was approved by the local Ethics Committee.

DNA analysis

DNA was isolated from frozen EDTA blood by the standard salt-out method (14).

Four fragments which covered the coding region of *APOA5* (exons 1–3) were amplified using the following four pairs of primers: AVF5 (5′-TAA CAG GAT TTC GGG CAG TT-3′) and AVR5 (5′-AGA GCT AGC ACC GCT CCT TT-3′) for AV5 (524 bp, cover exon 1 and exon 2); AVR3 (5′-CCT CGA CCC TGG GGC CAA CGC-3′) and AVF3 (5′-CAA GCC TCG TCC ACG CCC CC-3′) for AV3 (486 bp, cover 5′ half of exon 3); AVR2 (5′-GCA GCA ACT GAA GCC CTA CAC GA-3′) and AVF2 (5′-TCA GTC TCC TGG TCG ATG GCG-3′) for AV2 (546 bp, cover middle part of exon 3); AVR1 (5′-AGG AGG TGC GCC AGC GAC TT-3′) and AVF1 (5′-TGG GAG GCT GGG TTT GCA AAG-3′) for AV1 (493 bp, cover 3′ half of exon 3). Overlapping fragments of AV3, AV2 and AV1 span the whole sequence of exon 3. The amplification conditions were hot start at 95°C for 5 min followed by 30 cycles at 95°C/30 s, 55°C (except 65°C for AV3)/30 s, 72°C/45 s, ending by 72°C/3 min.

The PCR products were purified by GFX PCR DNA and a Gel Band Purification Kit (Amersham Biosciences, Little Chalfont, England, Cat. No. 27-9602-01) or AMPure PCR Purification System (Agencourt Bioscience, Bewerly, MA, USA, Cat. No. 000146).

Sequencing reaction was proceeded by a DYEnamic ET Dye Terminator Cycle Sequencing Kit for MegaBACE™ DNA Analysis Systems (Amersham Biosciences, Cat. No. US81090) under the condition of 95°C/20 s, 50°C/15 s and 60°C/60 s, 25 cycles. Purified by a CleanSEQ Dye-Terminator Removal (Agencourt Bioscience, Cat. No. 000145), the products of sequencing reaction were run on a MegaBACE™ 1000 DNA Analysis System (Amersham Biosciences).

To genotype the Ala315>Val (944C>T) variant within the *APOA5* gene, oppositely oriented oligonucleotides *APOA5* 315F 5′ TTC ACT CGC GCC ATC GAC CAG GAG ACT GAG G and *APOA5* 315R 5′ ATC CAG ACG GGC CTG CAG CTT GCT CAG were used. PCR product (141 bp) was cleaved by restriction enzyme *Hin*6I (Fermentas, Burlington, Canada) and separated on 10% MADGE gel (15) in 0.5× Tris/borate/EDTA (TBE) buffer. The rare allele Val315 is characterized by an uncut fragment of 141 bp, the common allele Ala315 by fragments 91 bp and 50 bp.

Biochemical analysis

The lipoprotein parameters were measured enzymatically by the WHO Regional Lipid Reference Center, IKEM, Prague on a Roche COBAS MIRA autoanalyzer (Haffmann-La Roche, Basel, Switzerland), using conventional enzymatic methods with reagents from Hoffmann-La Roche.

Statistical analysis

Statistical analysis was performed using the χ^2-test and analysis of variance. TGs were logarithmically transformed before analysis to obtain the normal distribution of data.

Results

A C to T change was detected in the heterozygous state in *APOA5* cDNA position 944 in three out of 95 HTG patients, representing a carrier frequency of 0.03 (allele frequency of 0.016). This mutation resulted in the substitution of alanine at position 315 for valine. All three carriers were middle-aged men; two of them were current smokers and had treated hypertension. One of them was obese, while the other two were overweight. None of them had diabetes or cardiovascular disease. Two of them had *APO* $\varepsilon3\varepsilon3$ and one *APO* $\varepsilon2\varepsilon3$ genotypes.

To clarify whether this variant was associated with plasma TG level, a large population sample was screened for the presence of this mutation. In 3202 individuals, we detected 22 Ala315/Val315 heterozygotes. The carrier frequency in this sample population was 0.007 (rare allele frequency of 0.003) and this frequency was lower than in the HTG group ($p<0.01$).

The carriers of Ala315>Val from the patient and control groups were comparable for age, body mass index, smoking and diet. Therefore, it is unlikely that these conditions could have been significant environmental and non-genetic factors in the development of HTG.

The Ala315>Val variant showed no significant linkage disequilibrium with other *APOA5* variants previously genotyped in the same population group [$D'=0.752$ and LOD score 0.1 for T-1131>C variant; $D'=1.0$ and LOD score 0.26 for Ser19>Trp; and $D'=1.0$ and LOD score 0.28 for Val153>Met).

In this sample population, Ala315Val was not associated with significant TG raising effects, if anything, Ala315Val carriers tended to have slightly elevated plasma TG levels than Ala315 homozygotes, although these differences did not reach significance ($p=0.3$). There were no effects on any other lipid parameters. Table 1 shows the basic characteristics and lipid levels of all Val315 allele carriers.

Discussion

APOA5 variants are amongst the strongest genetic determinants of plasma TGs identified so far, although some ethnic and sex differences have been described. It is also not completely clear if their effect is mediated through the linkage disequilibrium with neighboring *APOC3* variants. Interestingly, only three functional mutations in the *APOA5* gene (Gln139> Stop and Gln145>Stop; c.161+3g>c variant completely abolished the function of the donor splice site

Table 1 Basic characteristics and lipid levels of all *APOA5* Val315 allele carriers.

Sex	Age	*APOE*	ApoA5 T-1131>C	ApoA5 Ser19>Trp	Smoking	HL treatment	Menopausal status	BMI, kg/m^2	TG	Total cholesterol	HDL cholesterol
M	**46**	**ε3ε3**	**TT**	SerSer	**Prev**	**No**	–	**29.4**	**12.50**	**5.25**	**0.93**
M	**57**	**ε3ε2**	**TT**	SerSer	**Yes**	**No**	–	**27.5**	**22.80**	**12.85**	**0.84**
M	**49**	**ε3ε3**	**TT**	SerSer	**Yes**	**No**	–	**34.5**	**24.00**	**11.60**	**1.55**
M	47	ε3ε3	TT	SerSer	No	No	–	24.3	0.74	4.50	0.88
M	52	ε3ε3	TT	SerSer	No	No	–	32.3	5.00	7.65	1.08
M	39	ε4ε3	TT	SerSer	No	No	–	26.2	1.79	4.59	0.79
M	53	ε3ε3	TT	SerSer	No	No	–	26.2	2.01	5.73	0.84
M	61	ε3ε3	TT	SerSer	No	No	–	22.6	0.83	5.12	1.34
M	57	ε3ε3	TC	SerSer	Yes	No	–	28.0	2.32	6.89	0.72
M	57	ε3ε2	TT	SerSer	Prev	No	–	30.9	1.46	3.64	1.15
M	49	ε2ε2	TT	SerSer	No	No	–	26.0	0.86	5.27	2.45
M	50	ε3ε3	TT	SerSer	No	No	–	23.2	0.80	5.31	1.35
F	62	ε3ε3	TT	SerSer	No	No	Post	37.2	3.12	6.76	1.10
F	52	ε3ε3	TT	SerSer	Prev	No	Post	22.5	1.59	6.31	1.49
F	67	ε3ε2	TT	SerSer	Prev	No	Post	31.5	1.62	6.81	1.39
F	61	ε3ε3	TC	SerSer	No	No	Post	40.9	6.24	7.23	0.82
F	55	ε3ε2	TT	SerSer	No	No	Post	27.4	2.65	6.02	1.58
F	30	ε4ε3	TT	SerSer	No	No	Pre	20.4	0.48	4.30	1.81
F	48	ε3ε3	TT	SerSer	Yes	No	Pre	24.5	1.51	5.55	1.23
F	39	ε3ε2	TT	SerSer	No	No	Pre	21.9	1.50	6.01	2.27
F	37	ε3ε3	TC	SerSer	No	No	Pre	27.1	0.55	4.95	1.67
F	40	ε3ε3	TC	SerSer	Yes	Yes	Pre	28.9	7.79	4.82	1.00
F	27	ε3ε3	TT	SerTrp	Yes	No	Pre	21.2	1.59	5.35	1.03
F	54	ε4ε3	TT	SerSer	No	Yes	Pre	26.9	1.42	5.64	1.17
F	38	ε3ε3	TT	SerSer	No	No	Pre	38.9	1.78	5.41	1.36

A total of 25 individuals heterozygous for the *APOA5* Val315 allele were identified. No homozygotes were detected. The common *APOA5* Ala315>Val variant was not significantly associated with TG levels ($p=0.3$) and other lipid parameters (given in mmol/L). The first three individuals (in bold) were detected in 95 HL type 5 patients, other carriers of the Val315 allele have been detected in the MONICA population sample. BMI, body mass index; HL, hypolipidemic.

in intron 3, suggesting a splicing defect) associated with extreme TG levels have been described so far (12, 13, 16).

The existence of the Ala315>Val variant was first mentioned by Pennacchio et al. (5) but was not analyzed further in detail. We identified a non-synonymous Ala315>Val change in the *APOA5* gene in three individuals with TG levels >10 mmol/L. The Val315 allele was also detected in 22 individuals out of the total 3202 screened from a general population study, the majority of whom had lipid levels within the normal range.

We were unable to analyze the lipid profile of carriers of this mutation in detail. All patients with HTG were under treatment according to the guidelines and their current lipid and lipoprotein levels were significantly lower than the pretreatment levels, which were used for inclusion into the study. The controls from the general population sample were screened only for levels of total, HDL and low-density lipoprotein cholesterol (calculated) and TGs. Interestingly, most of the carriers of the Ala315Val from the control group had, if anything, lower TG levels than the non-carriers. We also considered whether carriage of a rare allele of either −1131T>C or Ser19Trp SNPs could account for this difference. However, none of the HTG Ala315Val carriers were heterozygous for either of these SNPs. In the population sample, five out of 22 Ala315Val carriers carried a rare allele of either T-1131>C or Ser19>Trp; however, while some of these individuals had amongst the highest TG levels for the group, several had very low TG levels, thus eliminating this as an explanation.

Despite this lack of association with plasma TG levels, the frequency of the mutation was significantly higher in the patients with HTG than in controls ($p<0.01$). It is possible that in order to have effects of plasma TG levels, Ala315>Val needs to be associated with some other, as yet unidentified mutation, either situated in the *APOA5* gene cluster or in another gene region resulting in an interaction which leads to HTG.

We examined whether the apolipoprotein E (*APOE*) gene would influence this, as an association between the *APOA5* and *APOE* variants has been reported in patients with different forms of dyslipidemias (17–19).

We determined *APOE* genotypes in all individuals. In the HTG group, two Val315 carriers had *APOE* $\varepsilon3\varepsilon3$ genotypes and one was *APOE* $\varepsilon3\varepsilon2$ heterozygote. In the control group, there were 14 $\varepsilon3\varepsilon3$ homozygotes, three $\varepsilon4\varepsilon3$ heterozygotes, four $\varepsilon3\varepsilon2$ heterozygotes and one $\varepsilon2\varepsilon2$ homozygote who were Val315 carriers. Thus, it is unlikely that the *APOE* gene acts synergistically with this *APOA5* mutation in the development of HTG and this suggests that some other genes are implicated.

In our Caucasian control sample population, this mutation did not influence plasma TG levels significantly. Molecular modeling of apoA5 suggests that the residues around amino acid 315 do not play a part in either lipid binding or lipoprotein lipase activation, and the Ala>Val change is rather conservative (20). Out of 25 carriers of this mutation, three were from the sample of HTG patients and consequently had very high TG levels, while the other carriers had levels of TG that did not correspond to this being a variant, which had generally strong effect on plasma TG levels. The higher frequency of the Ala315>Val in the HTG patients could be a chance event as the number of tested individuals was small, but further research is needed to elucidate if the presence of another genetic variant(s) or environmental factors are necessary to cause manifest HTG in *APOA5* Ala315>Val carriers.

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References

1. Pennacchio LA, Olivier M, Hubacek JA, Cohen JC, Cox JC, Fruchart JC, et al. An apolipoprotein influencing triglycerides in humans and mice revealed by comparative sequencing. Science 2001;294:169–73.
2. O'Brien PJ, Alborn WE, Sloan JH, Ulmer M, Boodhoo A, Knoerman MD, et al. The novel apolipoprotein A5 is present in human serum, is associated with VLDL, HDL, and chylomicrons, and circulates at very low concentrations compared with other apolipoproteins. Clin Chem 2005;51:351–9.
3. Baroukh N, Bauge E, Akiyama J, Chang J, Afzal V, Fruchart JC, et al. Analysis of apolipoprotein A5, C3, and plasma triglyceride concentrations in genetically engineered mice. Arterioscler Thromb Vasc Biol 2004;24: 1297–302.
4. Hubacek JA. Apolipoprotein A5 and triglyceridemia. Focus on the effects of the common variants. Clin Chem Lab Med 2005;43:897–902.
5. Pennacchio LA, Olivier M, Hubacek JA, Krauss RM, Rubin EM, Cohen JC. Two independent apolipoprotein AV haplotypes influence human plasma triglyceride levels. Hum Mol Genet 2002;11:3031–8.
6. Talmud PJ, Hawe E, Martin S, Olivier M, Miller GJ, Rubin EM, et al. Relative contribution of variation within the APOC3/A4/A5 gene cluster in determining plasma triglycerides. Hum Mol Genet 2002;11:3039–46.
7. Lai CQ, Tai ES, Tan CE, Cutter J, Chew SK, Zhu YP, et al. The APOA5 locus is a strong determinant of plasma triglyceride concentrations across ethnic groups in Singapore. J Lipid Res 2003;44:2365–73.
8. Hubáček JA, Škodová Z, Adámková V, Lánská V, Poledne R. The influence of APOAV polymorphisms (T-1131>C and S19>W) on plasma triglyceride levels and risk of myocardial infarction. Clin Genet 2004;65:126–30.
9. Szalai C, Keszei M, Duba J, Proházska Z, Kozma GT, Császár A, et al. Polymorphism in the promoter region of the apolipoprotein A5 gene is associated with an increased susceptibility for coronary artery disease. Atherosclerosis 2004;173:109–14.
10. Schaap FG, Rensen PC, Voshol PJ, Vrins C, van der Vliet HN, Chamuleau RA, et al. ApoAV reduces plasma triglycerides by inhibiting very low density lipoprotein-triglyceride (VLDL-TG) production and stimulating lipoprotein lipase-mediated VLDL-TG hydrolysis. J Biol Chem 2004;279:27941–7.
11. Fruchart-Najib J, Bauge E, Niculescu LS, Pham T, Tho-

mas B, Rommens C, et al. Mechanism of triglyceride lowering in mice expressing human apolipoprotein A5. Biochem Biophys Res Commun 2004;319:397–404.
12. Priore Oliva CP, Pisciotta L, Li Volti G, Sambataro MP, Cantafora A, Bellocchio A, et al. Inherited apolipoprotein A-V deficiency in severe hypertriglyceridemia. Arterioscler Thromb Vasc Biol 2005;25:411–7.
13. Marcais C, Verges B, Charriere S, Pruneta V, Merlin M, Billon S, et al. Apoa5 Q139X truncation predisposes to late-onset hyperchylomicronemia due to lipoprotein lipase impairment. J Clin Invest 2005;115:2862–9.
14. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for DNA extraction from human nucleated cells. Nucleic Acid Res 1988;16:1215.
15. Day IN, Humphries SE. Electrophoresis for genotyping: microtiter array diagonal gel electrophoresis on horizontal polyacrylamide gels, hydrolink, or agarose. Anal Biochem 1994;222:389–95.
16. Priore Oliva CP, Tarugi P, Calandra S, Pisciotta L, Bellocchio A, Bertolini S, et al. A novel sequence variant in APOA5 gene found in patients with severe hypertriglyceridemia. Atherosclerosis 2006;188:215–7.
17. Schaefer JR, Sattler AM, Hackler B, Kurt B, Hackler R, Maisch B, et al. Hyperlipidemia in patients with apolipoprotein E 2/2 phenotype: apolipoprotein A5 S19W mutation as a cofactor. Clin Chem 2004;50:2214.
18. Hubacek JA, Horinek A, Skodova Z, Adamkova V, Ceska R, Zlatohlavek L, et al. Hypertriglyceridemia: interaction between APOE and APOAV variants. Clin Chem 2005; 51:1311–3.
19. Evans D, Seedorf U, Beil FU. Polymorphisms in the apolipoprotein A5 (APOA5) gene and type III hyperlipidemia. Clin Genet 2005;68:369–72.
20. Talmud PJ. Rare APOA5 mutations – clinical consequences, metabolic and functional effects. Atherosclerosis 2007;194:287–92.