Strong Linkage Disequilibrium and Association of –1131T>C and c.553G>T Polymorphisms of the Apolipoprotein A5 Gene With Hypertriglyceridemia in a Japanese Population

Akira Matsunaga, MD; Hiroaki Arishima, MD; Hideya Niimura, MD;
Bo Zhang, PhD; Yoshinari Uehara, MD; Kazuo Ohwaki, MD;
Mie Morita; Kazue Hayashida; Keijiro Saku, MD

Background The apolipoprotein A5 gene (*ApoA5*) plays an important role in modulating triglyceride metabolism. Polymorphisms of *ApoA5*, including –1131T>C and c.553G>T (G185C), have been reported to correlate with hypertriglyceridemia (HTG). In the present study the relationships of 5 single nucleotide polymorphisms, including the –1131T>C, c.56C>G, IVS3+476G>A, c.553G>T, and c.1259T>C polymorphisms of *ApoA5*, with HTG were investigated.

Methods and Results The study group comprised 95 Japanese patients with HTG and 119 unrelated normolipidemic subjects. Frequencies of the C allele of –1131T>C (0.511) and the T allele of c.553G>T (0.205) in the hypertriglyceridemic patients were significantly higher than in the normolipidemic subjects (0.315 and 0.105, respectively). The c.56C>G (S19W) polymorphism was not observed, and the other 4 polymorphic sites were in strong linkage disequilibrium. Five of the 8 detected haplotypes with the C allele of –1131T>C correlated with HTG. Promoter activities of *ApoA5*, including that with the –1131T>C polymorphism, were estimated using a luciferase assay. Analysis of *ApoA5* promoters showed that the –1131T>C polymorphism alone had no effect. Comparison of expression of mutant G185C and wild-type *ApoA5*-green fluorescent protein (GFP) in HepG2 cells showed that *ApoA5*-GFP was abundant in punctate endosome-like structures, and *ApoA5* (G185C)-GFP expression resembled that of the wild type.

Conclusions The –1131T>C and c.553G>T (G185C) polymorphisms correlated with HTG in this Japanese population, but neither polymorphism directly affected *ApoA5* expression. (Circ J 2007; 71: 746–752)

Key Words: Apolipoprotein A5; Hypertriglyceridemia; Linkage disequilibrium; Polymorphism

Hypertriglyceridemia (HTG) and low plasma levels of high-density lipoprotein (HDL) are common metabolic disorders. HTG is recognized as an independent risk factor for coronary artery disease (CAD);¹ in middle-aged Japanese the fasting triglyceride level is a significant risk factor for CAD.² A secondary prevention study of CAD treated with statins suggested that it might be prudent to target fasting triglycerides to less than 150 mg/dl.³ Apolipoprotein A5 (*ApoA5*) is a new member of the apolipoprotein gene family, found by a combined approach of comparative sequence analysis⁴ and differential gene expression, and it is highly upregulated in the early phase of liver regeneration.⁵ Triglyceride levels are 4-fold higher in *ApoA5* knockout mice and significantly lower in transgenic mice⁴ or adenovirus-treated mice expressing human *ApoA5* than in wild-type mice.⁶ Several groups have investigated the mechanisms underlying effects of *ApoA5* on triglyceride metabolism: it may lower plasma triglyceride levels by increasing lipoprotein lipase activity,^{7,8} or reduce the hepatic

level of very-low-density lipoprotein (VLDL) triglycerides.⁹

The size and molecular weight of mature human *ApoA5* protein are 343 amino acid residues and 39 kDa, respectively. Protein structure analysis predicts that several amphipathic helical domains and an N-terminal signal peptide are characteristic features of apolipoproteins. Human *ApoA5* is located approximately 30 kb downstream from the *ApoA1/C3/A4* gene cluster on chromosome 11q23. It contains 4 exons encoding a 1,107-bp open reading frame. Interestingly, 2 initiation codons have been reported, the first was identified by Pennachio et al;⁴ and is known to be followed by the second ATG codon, 9 nucleotides downstream, as reported by van der Vliet et al.⁵ The National Center for Biotechnology Information reference protein sequence (NP_443200) starts at this second initiation codon. The *ApoC3* SstI polymorphism has been associated with altered plasma triglyceride concentrations.¹⁰ Recently, definitive N-terminal protein sequences obtained from *ApoA5* purified from human serum were demonstrated.¹¹ The actual N-terminal sequence of *ApoA5* corresponds to that expected from cleavage of the predicted signal peptide, indicating that the mature protein is an intact molecule with a predicted relative molecular mass (*M_r*) of 39 kDa. Human *ApoA5* is fairly polymorphic and 3 common haplotypes have been identified in Caucasians: wild-type haplotype *ApoA5**1; *ApoA5**2, defined by rare alleles of –1131T>C, –3A>G, IVS3+476G>T,

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Department of Cardiology, Fukuoka University School of Medicine, Fukuoka, Japan

Mailing address: Akira Matsunaga, MD, Department of Cardiology, Fukuoka University School of Medicine, 7-45-1 Nanakuma, Johnan-ku, Fukuoka 814-0180, Japan. E-mail: matsunag@cis.fukuoka-u.ac.jp

and c.1259T>C; and ApoA5*3, defined by a rare allele of S19W. Therefore, genotyping for -1131T>C or S19W essentially serves to tag single nucleotide polymorphisms (SNPs).¹² The rare C allele of -1131T>C (called SNP3) is more frequent in Japanese (0.34) than in Caucasians (0.08).¹³ We previously identified a polymorphism of ApoA5, c.553G>T as ApoA5(G162C), using the amino acid number of mature ApoA5 in Japanese patients with hypertriglyceridemia (abstract; Circulation J 2003 67, Supplement 1: 270). Moreover, Kao et al reported the same polymorphism as ApoA5(G185C) in a Chinese population.¹⁴

This study aimed to evaluate the association of SNPs, including -1131T>C and c.553G>T, of ApoA5 with hypertriglyceridemia in a Japanese population.

Methods

Subjects

The first patient with the ApoA5 polymorphism c.553G>T in our Japanese population was a 62-year-old man who came to the ambulatory clinic of Fukuoka University Hospital because of high plasma levels of triglycerides and glucose. Investigations revealed poorly controlled diabetes mellitus (fasting blood glucose, 187 mg/dl; hemoglobin A1c, 9.4%) and hyperlipidemia (total plasma cholesterol, 529 mg/dl; triglycerides, 2,580 mg/dl; HDL-cholesterol, 18 mg/dl). He also had mild fatty liver on echography, and elevated serum levels of aspartate aminotransferase (39 IU/L) and alanine aminotransferase (36 IU/L). No blood samples from his family were available.

For analysis of the frequencies of polymorphisms in subjects with/without HTG, blood specimens of 119 normolipidemic Japanese individuals (80 men, 39 women; age, 58±14 years), and 95 Japanese patients with high levels of fasting plasma triglycerides (76 men, 18 women; age, 56±12 years) were recruited. Blood was drawn after a 10-h fast. All subjects admitted to the Fukuoka University Hospital were grouped as follows: (1) individuals with HTG: plasma triglyceride level >200 mg/dl; (2) normolipidemic individuals: plasma total cholesterol (TC) level <220 mg/dl and triglyceride <150 mg/dl. The 2 groups did not significantly differ with respect to age and gender. The HTG group had significantly higher TC ($p<0.001$) and significantly lower HDL-cholesterol ($p<0.0001$) than the normolipidemic group.

This study was approved by the Ethics Review Committee of Fukuoka University Hospital.

DNA Amplification by Polymerase Chain Reaction (PCR)

Genomic DNA was isolated from 120-μl samples of peripheral blood obtained from all subjects. Oligodeoxynucleotide primers used for amplification and sequencing were chosen according to the published ApoA5 structure. Two DNA segments carrying exon 2, exon 3, and exon 4 of ApoA5 of the first patient with the ApoA5 polymorphism c.553G>T were amplified by PCR, using genomic DNA as template. Primer pairs 1 (5'-AAGGAAGGGGTAACAGG-ATTT-3') and 2 (5'-CAACTCACGCACGAAGCACAA-3') for exons 2 and 3; and 3 (5'-TGCTTCGTGCGTGAGTTGT-T-3') and 4 (5'-GACAGCAGCCCCCTTGGTG-3') for exon 4 were used. PCR was performed in reaction volumes of 100 μl containing 2.5 U Taq polymerase (Perkin-Elmer Cetus). Amplification was performed by initial denaturation at 96°C for 10 min; followed by 30 cycles at 96°C for 30 s, 60°C for 30 s, and 72°C for 1 min; and a final extension of

7 min at 72°C.

DNA Sequence Analysis

PCR-amplified DNA products were purified with the GeneClean kit (Bio101, La Jolla, CA, USA), and purified DNA fragments were ligated to pT7Blue-T Vector (Novagen, Madison, WI, USA). Following subcloning, double-stranded DNA was sequenced using the BigDye Terminators sequencing kit and the ABI PRISM 373 automated sequencer (PE Biosystems, Branchburg, NJ, USA). Because 2 different translation initiation sites for human ApoA5 have been reported,^{5,12} the G-to-T substitution of exon 3 (c.553G>T) can be shown as ApoA5 (G185C) or (G182C) by 2 different numbers from the translation initiation sites, or as ApoA5 (G162C) using the number from mature ApoA5 amino acid residues. In this study, we used ApoA5 (G185C) from a translation initiation site reported by Pennacchio et al.¹²

Restriction Fragment Length Polymorphism (RFLP) Analysis

Genomic DNA was amplified by PCR using oligonucleotide primers 5 (5'-CTGAAGCCCTACACGATGGA-3') and 6 (5'-GGAAGAGCTCTTTGAAGCGGC-3') for detection of c.553G>T. The PCR product for c.553G>T was digested by restriction enzyme Eco52I. Genotyping for -1131T>C, c.56C>G, IVS+476G>A, and c.1259T>C was carried out by PCR and restriction enzyme digestion, as described previously.^{4,15} Digested fragments were electrophoresed on 8% polyacrylamide gel for 40 min, and DNA fragments were then stained with ethidium bromide.

Haplotype Inference and Linkage Disequilibrium

Haplotypes were inferred on the basis of the diploid sequence data using the PHASE haplotype inference algorithm, version 2.0.¹⁶ Missing genotypes were imputed in the course of the haplotype estimation procedure. Haplotype data for all independent samples were used to calculate pairwise linkage disequilibrium. Inter-locus estimates of linkage disequilibrium were calculated using the software ARLEQUIN, Version 2.00 (<http://anthro.unige.ch/arlequin>).

Cell Culture Experiment

Human hepatoma (HepG2) cells were maintained in Dulbecco's modified medium (DMEM) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS, USA) and antibiotics (100 U/ml penicillin, and 100 μg/ml streptomycin) at 37°C in a humidified atmosphere of 5% CO₂. Cell viability was routinely monitored by trypan blue exclusion. In all experiments, the number of dead cells never exceeded 5% of the total number of cells.

Plasmid Constructs and Transient Expression Assay

The pGL3-ApoA5 (-617/+18)-luc construct was created by inserting a double-stranded oligonucleotide that contained KpnI and NheI restriction sites (Takara Bio, Tokyo, Japan), as described previously.¹⁷ Briefly, amplification of the ApoA5 promoter region was accomplished by PCR with primers JCR45 (5'-AGTCGGTACCTCATGGGGCAAA-TCTTACTTTTCGC-3') and JCR95 (5'-AGTCGCTAGCT-CACCTGCTCACGTCTGG-3') (-617 to +18). The 651-bp fragments obtained were subcloned into pT7Blue-T vectors. Genomic subclones were digested with KpnI and NheI, and cloned into pGL3-Basic (Promega). Plasmid phRL-SV40 was also purchased from Promega.

Fig 1. Nucleotide sequence of part of exon 4 of our first patient (A), and polyacrylamide gel electrophoretic patterns of DNA fragments (B). (A) Polymerase chain reaction (PCR)-amplified DNA was sequenced after subcloning into the T7Blue-T vector. Graphic representation of the G-to-T substitution of codon 185. (B) Analysis of the G-to-T substitution at codon 185. PCR products were digested with Eco52I. Lanes 1 and 2 (GG) show 168-bp bands resulting from complete digestion with Eco52I, lane 3 (GT) shows 190-bp and 168-bp bands, indicating loss of an Eco52I site, and lane 4 (TT) shows a 168-bp band indicating loss of both Eco52I sites. M represents the ϕ X174/HincII digest marker.

Fig 2. Schematic representation of the ApoA5 gene and relative single nucleotide polymorphisms (SNPs) positions (arrows). ApoA5 exons are shown in solid boxes. The predicted transcription start site is depicted by a bent arrow, and the relative position of the promoter and the start and stop codons are shown.

Table 1 Clinical Profiles of Subject Groups

	Normolipidemic	Hypertriglyceridemic
n	119	95
M/F	80/39	76/19
Age (years)	58±14	56±12
TC (mg/dl)	171±20	219±55**
TG (mg/dl)	82±27	343±306**
HDL-C (mg/dl)	47±8.6	39±12**
ApoA-I (mg/dl)	124±20	123±27
ApoA-II (mg/dl)	27±5.1	31±7.8
Apo B (mg/dl)	91±23	126±34**
ApoE (mg/dl)	3.6±1.3	6.3±4.1**

**p<0.01.

TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein-cholesterol; Apo, apolipoprotein.

Generation of Fusion Proteins and Transfection

Expression vectors pSG5-ApoA5 were cloned and prepared from genomic DNA of HepG2 cells. The ApoA5 (G185C) mutant was created using the QuikChange site-directed mutagenesis kit (Stratagene). In mock transfection control samples, the pEGFP (enhanced green fluorescent protein) vector (Clontech) was used. The ApoA5-GFP and ApoA5(G185C)-GFP expression vectors were constructed by fusing the coding sequence of pEGFP to the 3' end of the ApoA5 coding sequence. HepG2 cells were transfected using the Lipofectin reagent according to the manufac-

turer's instructions (Invitrogen, Carlsbad, CA, USA). The efficiency of transfection in each experiment was estimated by comparison to transfection with pEGFP.

Statistical Analysis

All results are reported as means±SD, and all p-values were based on 2-sided tests of significance. Differences of mean plasma lipoprotein levels in individuals from different groups were compared using a 1-way analysis of variance. Genotype distributions and deviation from the Hardy-Weinberg equilibrium were assessed by chi-square analysis.

Results

DNA Sequence Analysis of the ApoA5 Gene

During ApoA5 analysis, we discovered a polymorphism, c.553G>T, in a Japanese patient with HTG (Fig 1A). Homozygous G-to-T substitution was detected in exon 4 from 12 clones of subcloned DNA fragments, resulting in a single amino acid change of Gly to Cys at amino acid residue 185. The rest of the gene coding sequence was normal. The homozygous c.553G>T polymorphism in the proband was confirmed by RFLP with Eco52I (Fig 1B). PCR-amplified DNA (190bp) of ApoA5 obtained from normal subjects was digested with Eco52I, and showed a 168-bp band, indicating an Eco52I site. Digested DNA from the subject with c.553G>T only showed the 190-bp band, which indicated that the subject was homozygous for

Table 4 *ApoA5* Haplotypes and Their Relative Frequencies in Controls and Patients With Hypertriglyceridemia

Haplotype	-1131T>C SNP3	IVS+476G>A SNP2	c.553G>T G185C	c.1259T>C SNP1	Controls	Patients with hypertriglyceridemia
1	T	G	G	T	0.65	0.45
2	C	A	G	C	0.20	0.34
3	C	G	C	T	0.10	0.15
4	T	A	G	T	0.03	0.01
5	T	G	C	T	0.02	0.01
6	C	A	C	C	0	0.03
7	C	G	G	T	0	0.01
8	C	G	G	C	0	0.01

* $p < 0.05$ for patients with hypertriglyceridemia vs controls; p values were obtained using a χ^2 -test.

Fig3. Fenofibric acid induces *ApoA5* gene expression at the transcriptional level in Human hepatoma (HepG2) cells. HepG2 cells were transfected with the pGL3-*ApoA5* plasmid containing the -617 to +18 region of the *ApoA5* promoter with/without the -1131T>C polymorphism. HepG2 cells were incubated for an additional 24h with 300μmol/L fenofibric acid. Firefly luciferase activities were normalized against pRL-SV40 control activities. Values (mean ± SD, n=3) are expressed relative to controls.

sults suggest that the -1131T>C and c.553G>T (G185C) polymorphisms do not exert a direct influence on *ApoA5* expression.

Discussion

We have shown that (1) the -1131T>C, c.1259T>C and c.553G>T polymorphisms are positively associated with HTG in Japanese; (2) the -1131T>C, IVS3+476G>A, c.553G>T, and c.1259T>C polymorphisms are in linkage disequilibrium; and (3) the -1131T>C polymorphism is a stronger determinant of HTG than c.553G>T. Additional in vitro studies showed that the promoter activities of *ApoA5* with/without the -1131T>C polymorphism do not differ, and that *ApoA5*wt-GFP and *ApoA5*(G185C)-GFP similarly localize to cytoplasmic vesicles in HepG2 cells.

The *ApoA5* SNPs have been associated with HTG^{12,13} reduced HDL-cholesterol (-1131T>C, -3A>G and IVS3+476A>G)¹⁸ decreased LDL-cholesterol size (-3A>G and c.1259T>C)¹⁹ and increased numbers of remnant-like particles (-1131T>C and c.56C>G)²⁰ No significant differences have been observed in fasting and postprandial concentrations of serum glucose, insulin, and free fatty acids among subjects with the -1131T>C polymorphism²¹ The -1131T

Fig4. Fluorescence microscopy detection of *ApoA5* expression in cultured human hepatoma (HepG2) cells. Signals from green fluorescent protein (GFP: A), and *ApoA5*-GFP (B), and *ApoA5*(G185C)-GFP fusion proteins (C) in HepG2 cells. Scale bars=50μm.

>C SNP was more frequent in CAD patients²² Both the -1131T>C and c.56C>G SNPs have been associated with cardiovascular events²⁰ but not with coronary artery diameter²³ The rare allele of -1131T>C has also been associated with stroke²⁴

Several SNPs in human *ApoA5* have been detected, with differing frequencies depending on the population analyzed. Common SNPs, including -1131T>C, -3A>G, c.56C>G, IVS3+476G>A, and c.1259T>C, have been widely reported^{20,12} Pennacchio et al showed that there were 3 major haplotypes (ie, *ApoA5**1, *2, and *3) that accounted for 97.6% of haplotypes in a Caucasian population, containing the SNPs -1131T>C, c.-3A>G, c.56C>G, IVS3+476G>A, and c.1259T>C¹² *ApoA5**1 is defined as all the major alleles being present. Because of strong linkage disequilibrium between these SNPs, haplotype *ApoA5**2 can be defined as the occurrence of the minor allele at -1131T>C, c.-3A>G, IVS3+476A, or c.1259T>C, whereas *ApoA5**3 is defined as the c.56G allele. Therefore, 2 independent *ApoA5* SNPs (c.56C>G and -1131T>C) can be analyzed in association studies as indicators of the corresponding haplotypes. However, compared with Caucasians, in Turks, Singaporeans, and African-Americans the *ApoA5* haplotype structure is more complex^{18,25} The -1131T>C poly-

morphism is in strong, but incomplete, linkage disequilibrium with the 3 other SNPs in Turks and Singaporeans;¹⁸ and the c.1259T>C polymorphism is very rare in African-Americans (0.001%)²⁵ Our results indicate that –1131T>C, IVS3+476G>A, c.553G>T, and c.1259T>C polymorphisms in Japanese are in linkage disequilibrium, and that c.56C>G is absent or very rare. The c.553G>T polymorphism, with an allelic frequency of 4.2%, was significantly associated with increased triglyceride levels in a Chinese population,¹⁴ but was very rare in a Turkish population (0.6% allelic frequency), and extremely rare or absent in Caucasians.^{12,26} Nabika et al reported that the calculated frequency of the –1131C allele was 0.34²⁷ In the present study, we showed that the –1131T>C and c.553T>C polymorphisms of *ApoA5* were in strong linkage disequilibrium, and the allelic frequencies of –1131T>C (0.315) and c.553G>T (0.105) in the normolipidemic Japanese subjects were relatively high. The c.56C>G polymorphism consists of a non-synonymous substitution, changing codon 19 from serine to tryptophan (S19W); however, we could not find the c.56C>G polymorphism in this Japanese population. Univariate analyses showed that the allelic frequencies of –1131T>C (0.511) and c.553G>T (0.205) in subjects with HTG were significantly higher than in normolipidemic subjects. Because *ApoA5* polymorphisms have been associated with high plasma levels of triglyceride, SNP-associated increases may reflect impaired function of *ApoA5*. The –1131T>C polymorphism is located in the promoter region, and may alter *ApoA5* expression. However, in our luciferase reporter gene assay, both the –1131T and –1131C promoter constructs with fenofibric acid showed a significant increase in luciferase expression level (Fig 3). Prieur et al¹⁷ and Talmud et al²⁸ also concluded on a lack of functionality of –1131T>C. Therefore, we conclude that a –1131T>C change within the promoter does not affect promoter activity. Talmud et al¹⁵ demonstrated that the –1131T>C polymorphism of *ApoA5* was within a linkage disequilibrium block of approximately 50 kb (up to –482C>T of the *ApoC3* gene), which was strongly associated with plasma triglyceride level. The triglyceride-raising effect of –1131T>C might reflect the linkage disequilibrium between *ApoA5* –1131C and the triglyceride-raising *ApoC3* –482T allele.

Because *ApoA5* displays high affinity, low elasticity, and slow binding kinetics at hydrophobic interfaces,²⁹ it has been postulated that it might retard triglyceride-rich particle assembly in the liver and inhibit secretion of VLDL. Secretion of *ApoA5* by COS1 cells transfected with *ApoA5* complementary DNA was found to be greatly reduced, compared with other human plasma proteins, as a result of retention of *ApoA5* in the endoplasmic reticulum.²⁹ Adenovirus-mediated hepatic *ApoA5* expression in mice decreased the VLDL-triglyceride production rate in a dose-dependent manner, without an effect on VLDL particle numbers, suggesting that *ApoA5* affected lipidation of apolipoprotein B during VLDL assembly.⁹ Results from *ApoA5*(Q148X) carriers and the adenovirus-mediated gene transfer of *ApoA5* into wild-type mice showed that lipoprotein lipase activation by *ApoA5* might play an important role in its function and in repression of the VLDL production rate.⁶ The G185C substitution within human *ApoA5* is adjacent to a typical proline-directed amphipathic α -helix (residues 190–211). The G185C variation might form an intramolecular disulfide bond with Cys227 of human *ApoA5*, which is highly conserved, resulting in a conformational change in the

tertiary structure of the *ApoA5* variant. However, in the present study GFP-positive particles, endosomes, were discernible in both wild-type *ApoA5* and *ApoA5*(G185C)-transfected HepG2 cells (Fig 4). These results suggest that *ApoA5*(G185C) and the wild type were, at least, similarly expressed and assembled in the endosomes of HepG2 cells. The biological consequence of the G185C polymorphism deserves further investigation. Although in vitro studies have their limitations, the –1131T>C polymorphism did not affect promoter activity, as estimated by the luciferase assay, and *ApoA5*wt -GFP and *ApoA5*(G185C)-GFP were similarly expressed in the endosomes.

In conclusion, *ApoA5* –1131T>C and c.553G>T polymorphisms were associated with triglyceride level in Japanese. The strong linkage disequilibrium between the –1131T>C and c.553G>T polymorphisms was identified in our haplotype analysis. Promoter activities of *ApoA5* with the common or mutated allele were not different in HepG2 cells. Further studies are needed to establish the mechanisms of action associated with the presence of these allelic variations.

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