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

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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 CAD2; 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 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 regeneration5; Triglyceride levels are 4-fold higher in ApoA5 knockout mice and significantly lower in transgenic mice6 or adenovirus-treated mice expressing human ApoA5 than in wild-type mice.5 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) triglycerides9.

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/C3A4 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 al10 and is known to be followed by the second ATG codon, 9 nucleotides downstream, as reported by van der Vliet et al.11 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 (Mr) 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,
ApoA5 Polymorphisms and Hypertriglyceridemia

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). 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.

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 (391 IU/L) and alanine aminotransferase (361 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<0.0001) 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-ml samples of peripheral blood obtained from all subjects. Oligodeoxynucleotide primers used for amplification and sequencing were designed 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'-AAAGGACGCTCACGGATT-3') and 2 (5'-CAACTCACCGACAGACACACA-3') for exons 2 and 3; and 3 (5'-TGGCCTGCTACGGCTGCTG-3') and 4 (5'-GACAGAAGGCCCCTGTTGTTG-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, 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.

**Restriction Fragment Length Polymorphism (RFLP) Analysis**

Genomic DNA was amplified by PCR using oligonucleotide primers 5 (5'-CTGAGGCTCAGGGATGA-3') and 6 (5'-GGAGAGGCTCTTTTGAAGCGGC-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. 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 (IRH 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 Nhel restriction sites (Takara Bio, Tokyo, Japan), as described previously. Briefly, amplification of the ApoA5 promoter region was accomplished by PCR with primers JCR45 (5'-AGTCGGTACCATGGGGCAAA-3') and JCR95 (5'-AGTCCTAGCTCCCTTGAGGGA-3') (−617 to +18). The 651-bp fragments obtained were subcloned into pT7Blue-T vectors. Genomic subclones were digested with KpnI and Nhel, and cloned into pGL3-Basic (Promega). Plasmid phRL-SV40 was also purchased from Promega.
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 manufacturer'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 loss of an Eco52I site, and lane 4 (TT) shows a 168-bp band indicating loss of both Eco52I sites.

Table 1 Clinical Profiles of Subject Groups

<table>
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<tr>
<th></th>
<th>Normolipidemic</th>
<th>Hypertriglyceridemic</th>
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<tbody>
<tr>
<td>n</td>
<td>119</td>
<td>95</td>
</tr>
<tr>
<td>M/F</td>
<td>80/39</td>
<td>76/19</td>
</tr>
<tr>
<td>Age (years)</td>
<td>58±14</td>
<td>56±12</td>
</tr>
<tr>
<td>TC (mg/dl)</td>
<td>171±20</td>
<td>219±55**</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>82±27</td>
<td>343±360**</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>47±8.6</td>
<td>39±12**</td>
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<tr>
<td>ApoA-I (mg/dl)</td>
<td>12±20</td>
<td>12±27</td>
</tr>
<tr>
<td>ApoA-II (mg/dl)</td>
<td>27±5.1</td>
<td>31±7.8</td>
</tr>
<tr>
<td>Apo B (mg/dl)</td>
<td>9±23</td>
<td>12±34**</td>
</tr>
<tr>
<td>ApoE (mg/dl)</td>
<td>3.6±1.3</td>
<td>6.3±4.1**</td>
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</tbody>
</table>

*p<0.01.

TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein-cholesterol; Apo, apolipoprotein.
ApoA5 Polymorphisms and Hypertriglyceridemia

The defect. Because c.553G>T was detected in other subjects, the variant was defined as a polymorphism.

Frequencies of ApoA5 Polymorphisms

The ApoA5 genomic structure and the locations of the SNPs used to define each haplotype are shown in Fig 2. Frequencies of the 5 polymorphisms, –1131T>C, c.56C>G, IVS3+476G>A, c.553G>T, and c.1259T>C, were determined in 95 Japanese patients with HTG and 119 unrelated normolipidemic subjects. Polymorphisms were determined using restriction patterns of enzymes. Plasma concentrations of lipids and lipoproteins in both groups are shown in Table 1. Genotype distributions for these polymorphisms were in Hardy-Weinberg equilibrium. As shown in Table 2, the allelic frequencies of –1131T-to-C and c.1259T-to-C polymorphisms in patients with HTG (0.511 and 0.363) were significantly higher than those of the control subjects (0.315 and 0.210, respectively). Frequency of the T allele of c.553G>T in patients with HTG (0.205) was also significantly higher than that (0.105) of normolipidemic subjects (Table 2). The c.56C>G (S19W) polymorphism was not observed in our Japanese population. No association was observed between the polymorphisms and plasma concentrations of calculated low-density lipoprotein (LDL)-cholesterol, HDL-cholesterol, apoAI, apoAII, apoB or apoE in this study (data not shown). As shown in Table 3, the other 4 polymorphic sites were in strong linkage disequilibrium. Distributions of the 4 markers vs genotype in the hypertriglyceridemic and normolipidemic subjects are given in Table 4. There were significant differences between the 2 groups in the overall distribution. Five haplotypes (haplotypes 2, 3, 6, 7, and 8) containing the C allele of –1131T>C were related to HTG among the 8 detected haplotypes.

Functional Studies of Promoter –1131T>C and ApoA5 (G185C)

In order to compare the –1131T>C polymorphism of the human ApoA5 promoter, constructs from –617 to +18 were transfiently transfected into HepG2 cells. HepG2 cells were incubated with/without fenofibrate for 24h. Promoter activities of ApoA5 with/without the –1131T>C polymorphism were estimated by a luciferase assay. We observed that fenofibrate strongly upregulated the activity of both –1131T and –1131C promoters, with over 100% increase when transfected cells were treated with 300 μmol/L fenofibric acid (Fig 3). We constructed plasmids expressing GFP ApoA5 (ie, ApoA5wt-GFP and ApoA5(G185C)-GFP). To assess the function of these ApoA5-GFP fusion proteins, transfected HepG2 cells were imaged by confocal microscopy. ApoA5wt-GFP was localized to cytoplasmic vesicles, previously shown to be early endosomes, and ApoA5(G185C)-GFP also localized to the cytoplasmic vesicles (Fig 4). These re-
results 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 ApoA5wt-GFP and ApoA5(G185C)-GFP similarly localize to cytoplasmic vesicles in HepG2 cells. The ApoA5 SNPs have been associated with HTG\(^2\),\(^3\) reduced HDL-cholesterol (–1131T>C, –3A>G and IVS3+476A>G),\(^3\) decreased LDL-cholesterol size (–3A>G and c.1259T>C),\(^1\) and increased numbers of remnant-like particles (–1131T>C and c.56C>G).\(^2\) 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 polymorphism.\(^2\) The –1131T>C SNP was more frequent in CAD patients.\(^2\) Both the –1131T>C and c.56C>G SNPs have been associated with cardiovascular events\(^2\),\(^1\) but not with coronary artery diameter.\(^3\) The rare allele of –1131T>C has also been associated with stroke.\(^2\)

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.\(^2\),\(^1\) 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>C.\(^1\) 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, c.56C>G, IVS3+476G>A, and c.1259T>C.\(^1\) 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, c.56C>G, IVS3+476G>A, and 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.\(^1\),\(^2\) The –1131T>C poly-

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

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>–1131T&gt;C</th>
<th>IVS+476G&gt;A</th>
<th>c.553G&gt;T</th>
<th>c.1259T&gt;C</th>
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<tbody>
<tr>
<td>SNP3</td>
<td>SNP2</td>
<td>SNP1</td>
<td>SNP4</td>
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<td>Controls</td>
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<td>Patients</td>
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<td></td>
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<tr>
<td>8</td>
<td>C</td>
<td>G</td>
<td>G</td>
<td>0</td>
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*p<0.05 for patients with hypertriglyceridemia vs controls; p values were obtained using a \( \chi^2 \)-test.

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**Fig 3.** 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 24 h with 300 \( \mu \)mol/L fenofibric acid. Firefly luciferase activities were normalized against phRL-SV40 control activities. Values (mean ± SD, n=3) are expressed relative to controls.

**Fig 4.** 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=50 \( \mu \)m.
ApoA5 Polymorphisms and Hypertriglyceridemia

The ApoA5 gene 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 ApoA5wt -GF 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.

Acknowledgment

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References


