A novel genetic variant in the apolipoprotein A5 gene is associated with hypertriglyceridemia

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The apolipoprotein A5 gene (APOA5) has been shown to play an important role in determining plasma triglyceride concentrations in humans. We describe here a novel variant, c.553G>T, in the apolipoprotein A5 gene that is associated with hypertriglyceridemia. In contrast to some other polymorphisms, which occur in non-coding regions of the gene, this variant occurs within the coding region and causes the change of amino acid sequence (a substitution of a cysteine for a glycine residue). The minor allele frequencies were 0.042 and 0.27 (P < 0.001) for control and hypertriglyceridemic patients, respectively. The serum triglyceride level was significantly different among the genotypic groups (G/G 92.5 ± 37.8 mg/dl, G/T 106.6 ± 34.8 mg/dl, T/T 183.0 mg/dl, P = 0.014) in control subjects. Multiple logistic regression revealed individuals carrying the minor allele had age, gender and BMI (body mass index)-adjusted odds ratio of 11.73 (95% confidence interval of 6.617–20.793; P < 0.0001) for developing hypertriglyceridemia in comparison to individuals without that allele. These findings suggest the possible use of c.553G>T polymorphisms in APOA5 as prognostic indicators for hypertriglyceridemia susceptibility in Chinese.

INTRODUCTION

The role of elevated triglycerides as a risk factor of coronary heart disease remains controversial. However, emerging evidence points to an association between elevated plasma triglycerides and coronary heart disease (1–3). Hypertriglyceridemia is a common metabolic disorder in the general population. Although it can be caused by many factors, a relatively large number of individuals have a genetic tendency, but the genes responsible for variation in triglyceride levels have not been fully elucidated. Lipoprotein lipase (LPL) and the variation of the recognition site for Sst1 within the 3'-untranslated region of apolipoprotein C-III have consistently shown an association with a variation in plasma triglycerides. In addition to the Sst1 (3238C>G) polymorphism in the apolipoprotein C-III gene, variation of −482C>T within the insulin-responsive element in the promoter and 1100C>T in exon 3 is strongly associated with differences in plasma triglyceride levels (4–8).

Transgenic mice overexpressing human apolipoprotein A5 decreased plasma triglyceride concentrations to one-third of those in control mice; conversely, knockout mice lacking APOA5 had four times as much plasma triglycerides as controls (9). A minor haplotype of APOA5 (1259C, IVS3+476A and −1131C) and another APOA5 haplotype (1259T, IVS3+476G, 56G and −1131T) which was independently associated with high plasma triglyceride levels in African-American, Hispanic, Caucasians and Japanese were reported (7,9–13). The report mentioned above is suggestive of a role for APOA5 in hypertriglyceridemia.

The current study was undertaken to explore the association between the sequence variations in APOA5 and hypertriglyceridemia in humans. DNA sequencing was used to screen the coding region of APOA5 for DNA sequence variations in both hypertriglyceridemic and normal individuals, and the polymorphisms identified were tested for their frequencies between these two groups. Our data indicate that individuals carrying the 553T allele had an odds ratio of 11.73 for developing hypertriglyceridemia in comparison to individuals without that allele. These findings suggest the possible use of c.553G>T polymorphisms in APOA5 as prognostic indicators for hypertriglyceridemia susceptibility.
RESULTS

DNA sequencing

Biochemical characteristics of the hypertriglyceridemic and control subjects are summarized in Table 1. No statistically significant differences were found between gender and low-density lipoprotein (LDL) cholesterol levels. The hypertriglyceridemic subjects were characterized by an increased age, BMI and elevated serum triglyceride and total cholesterol levels as compared to control subjects, although HDL cholesterol concentrations were somewhat lower in the patient group.

We sequenced exons 2, 3 and 4 of APOA5 and revealed three novel single-nucleotide polymorphisms (SNPs). The first one showed a G→A transition at nucleotide position 457 of exon 4 (c.457G>A), which resulted in a substitution of a methionine for a valine (GTG to ATG) at amino acid residue 153 (V153M).

The second one revealed a G→T mutation at the 553rd nucleotide of the same exon (c.553G>T), which resulted in a substitution of a cysteine for a glycine residue (GGC to TGC) (c.553G>T).

The third polymorphic site was located at the 1177th nucleotide in the 3′-untranslated region of the exon 4, revealing a G→T transition (c.1177G>T). For further study, we have also analyzed two other variations within exons 4 and intron 3 which have been described: a T→C substitution at the 1259th nucleotide (c.1259T>C; SNP1) and a G→A substitution at the 476th nucleotide (IVS3+476G>A; SNP2) (9).

Association studies

We also found significant associations between serum triglyceride levels and the three polymorphisms (c.553G>T, c.1259T>C and IVS3+476G>A), but not the c.457G>A or the c.1177C>T polymorphic sites in normal people (Table 7). The minor allele of each of these three polymorphisms (553T, 1259C and IVS3+476A) was associated with higher serum triglyceride levels. Moreover, serum triglyceride levels were higher in individuals homozygous for the minor alleles compared with individuals homozygous for the major allele. No association of polymorphisms with serum apolipoprotein CIII levels was observed (data not shown).

Allele frequency, linkage disequilibrium and haplotypes analysis

Table 2 shows the genotyping results. Although c.457G>A and c.1177C>T genotype frequencies did not differ between the hypertriglyceridemic and control groups, differences in c.553G>T, c.1259T>C and IVS3+476G>A genotype frequencies were statistically significant (P < 0.001). Likewise, while allele frequencies of c.553G>T, c.1259T>C and IVS3+476G>A obtained by gene counting showed significant differences between these two groups (P < 0.001), the allele frequencies of c.457G>A and c.1177C>T were similar between groups (Table 3).

The pair-wise measure of linkage disequilibrium was calculated for all combinations of SNPs as previously described (14). The linkage disequilibrium amongst the variants in normal subjects is shown in Table 4A. Strong linkage disequilibrium was found in c.553G>T with other variants, and IVS3+476G>A with c.457G>A, c.553G>T and c.1177C>T variants. The c.1259T>C seemed to have least linkage disequilibrium with both c.457G>A and c.1177C>T variants. The phenomena were more apparent in all subjects (Table 4B).

There were 64 possible haplotypes derived from all polymorphic sites in both hypertriglyceridemic patients and control. Common haplotypes and their relative frequencies are shown in Table 5. Haploype 2 is distinguished from the common haplotype 1 by two nucleotide substitutions (IVS3+476G>A and c.1259T>C) and was previously shown to be associated with increased plasma triglyceride concentrations (9). Haplotype 3 is distinguished from the common haplotype by the substitution of T for G at nucleotide c.553. The frequency of haplotype 3 was significantly higher in hypertriglyceridemia than in control subjects (P < 0.0001). We made three hypotheses as H0 (no association among five markers), H1 (marker–marker but not marker–disease associations), and H2 (both marker–marker and marker–disease associations). By likelihood ratio chi-square test, Table 6 shows the significance testing results. For H1 vs H0, the chi-square value was highly significant, indicating the five marker loci are in disequilibrium with each other. For testing for markers and disease association, the chi-square value for H2 vs H1 was 150.78—still highly significant (P < 0.0001). Thus the results clearly demonstrate strong evidence for linkage disequilibrium between the disease and the polymorphic sites.
DISCUSSION

Hypertriglyceridemia may be due to either overproduction or accumulation of chylomicrons, very-low-density lipoproteins (VLDL) in the circulation. Chylomicron accumulation is generally the result of impaired lipoprotein input, while accumulation of VLDL is usually the consequence of excess lipoprotein input and/or impaired removal. Both chylomicrons and VLDL are converted to remnant lipoproteins at the endothelial surface through the lipolytic action of LPL and hepatic lipase (HL). To have effective metabolism and remodeling of the triglyceride-rich lipoproteins, a number of different apolipoproteins and functionally effective LDL receptor-related protein and LDL receptors, in addition to those enzymes mentioned above, are involved in the process of triglyceride metabolism. Although hypertriglyceridemia is a common metabolic disorder, the causes are not well understood. A number of studies have shown that, in addition to the environmental factors, genetic implication may play a role in the vulnerability to hypertriglyceridemia. Primary hypertriglyceridemia has been associated with lipoprotein lipase deficiency, apolipoprotein CII deficiency or HL deficiency (15–17). Variation within and around the apolipoprotein CIII gene has been reported to be associated with elevated lipid levels and
In particular, the SstI polymorphism in the 3'-untranslated region of the apolipoprotein CIII gene has been consistently associated with hypertriglyceridemia (19–22). Polymorphisms in the promoter region of apolipoprotein CIII have been associated with hypertriglyceridemia in most studies of Caucasians (4,22–26). However, other studies have found conflicting results (27–29). Waterworth et al. (5) reported that there was a strong association between variation in APOC3 and triglyceride levels. In their study, all 3238G, 1100T and 7482T alleles were associated with raised triglyceride levels, and the triglyceride-raising effect of the 3238C>G and 7482C>T sites appeared to depend on smoking status. Talmud et al. (10) also reported that haplotypes associated with high triglyceride levels carried the rare allele of the APOC3 482T, in combination with 1100T and 3238G, were common. They also reported that APOA5 c.56G and −1131C men had 52 and 40% higher triglyceride levels compared with common allele homozygotes, respectively, and their effects were independent and additive.

Pennacchio et al. (9) reported that a minor haplotype of APOA5 (1259C, IVS3 + 476A and −1131T) was associated with a 20–30% elevation in plasma triglyceride levels in Caucasian men and women. They also identified another APOA5 haplotype (1259T, IVS3 + 476G, 56G and −1131T) which was independently associated with high plasma triglyceride levels in African-American, Hispanic and Caucasians (11). While polymorphism in APOA5 −1131T>C had a significant independent effect on the serum triglyceride level in Japanese (12), this association was not significant in a population-based Spanish control group (13). This result indicates that the influence of polymorphism in the APOA5 on serum triglyceride level is different in different ethnicities.
In this study, we have characterized the association between a novel genetic variant in APOA5 and hypertriglyceridemia. In comparing the sequence of APOA5 between hypertriglyceridemia patients and normal controls, in addition to the four polymorphisms that have been reported (9), three novel polymorphic sites were observed. The minor allele frequency in normal subjects for c.1259T>C and IVS3 +476G>A was 17.0 and 17.1%, respectively. These frequencies were nearly 2-fold higher than those in Caucasians (9). This indicates that different ethnicity might entail different polymorphism. Although both c.457G>A and c.1177C>T polymorphisms were not significantly different between the hypertriglyceridemia and controls, the minor allele of c.553G>T was estimated to be 6.4-fold more common in hypertriglyceridemic subjects than in normal controls. The frequency of haplotype 3, which carried the minor allele of c.553G>T, was significantly higher in hypertriglyceridemia than in control subjects (P < 0.0001). This distinct variant (c.553G>T) has not been reported before. The association of the minor allele of c.553G>T with hypertriglyceridemia is independent of both c.1259T>C and IVS3 +476G>A. Because previous data have associated the apolipoprotein CIII locus with extremely high plasma triglyceride levels in humans (30), the effect of minor allele of c.553G>T on triglycerides may be mediated through apolipoprotein CIII. However, that no association of the c.553G>T polymorphism with serum apolipoprotein CIII levels was found in this study suggests another mechanism behind this effect. Although the minor allele of c.553G>T polymorphism is a powerful predictor for hypertriglyceridemia, the exact cause for such an association is unknown. Because the c.553G>T polymorphic site is located in the translated region of the APOA5, affecting the amino acid residue 185 causing a substitution of a cysteine which contains sulfur atom, and easily forming disulfide bond for a glycine residue, this amino acid change may alter the function or regulation of the APOA5 related to hypertriglyceridemia. We noted that the apolipoprotein A5 was present in rat plasma fractions containing high-density lipoprotein particles (31). If the human apolipoprotein A5 behaves similarly to that in rat, then it will function differently from apolipoprotein CIII that is a major component in the triglyceride-rich lipoproteins.

Our data indicate an important role for the c.553G>T polymorphism in APOA5 in serum triglyceride homeostasis. Although previous data have associated the apolipoprotein CIII locus with extremely high plasma triglyceride levels in humans, our study indicates that the APOA5 genomic interval represents an independent influence on this important lipid parameter in human. These results suggest the possible use of APOA5 polymorphisms as prognostic indicators for hypertriglyceridemia susceptibility and the focus on apolipoprotein A5 modulation as a potential strategy to reduce this known cardiovascular disease risk factor.

**MATERIALS AND METHODS**

**Study subjects**

Two-hundred and ninety patients with hypertriglyceridemia were selected for study. Hypertriglyceridemia was diagnosed on the basis of the lipid level (serum triglyceride >400 mg/dl) through the metabolic clinic of National Taiwan University Hospital. Patients with secondary hyperlipoproteinaemia, hypertension, diabetes, taking primary lipid-lowering drugs, or endocrine or metabolic disorders were excluded. The control group consisted of 303 individuals, who were recruited via health check performed at the same hospital. All subjects are Chinese and gave their informed consent before participation. The Medical Ethics Committee of National Taiwan University Hospital approved all protocols.

**DNA sequencing**

DNA from both patients and control subjects was extracted and amplified by PCR technique in a GeneAmp® PCR System (Applied Biosystems Division of Perkin-Elmer Corp.). The primer pairs that yielded the exons 2, 3 and 4 of APOA5 were forward primers 5′-TGAGCCCCCAACAGCTCTGTG-3′, 5′-TGTTTCCCCAGGATTACG-3′, 5′-GCAACTGAAGC-CCTACAC-3′, 5′-TTTCCGCAACGACACTAC-3′ and reverse.
Primers 5'-TTTCTCTGTCCAGCAGCG-3', 5'-TCGCGGTATGGGTGAAAG-3', 5'-CTCAGTCTCTGTGTCAAG-3', 5'-GAGCATTCCCAATGAGCCAC-3', respectively. The PCR product was isolated and excised from 2% agarose gel, purified by PCR Clean Up-M system (Viogene, CA, USA), and sequenced by using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kits version 2.0 on an ABI 377 DNA sequencer (Perkin-Elmer Corp.). The sequencing data were collected by using Data Collection version 2.0 software (Applied Biosystems Division of Perkin-Elmer Corp.), and analyzed with Sequencing Analysis version 3.0 software (Perkin-Elmer Corp.).

Polymorphism detection
Both c.457G > A and c.553G > T are naturally occurring restriction enzyme sites in the exon 4 of APOA5. To analyze these two polymorphic markers, exon 4 was amplified using primers 5'-GCAACTGAAGCCTCACC-3' and 5'-CCTGGC-TATGCGTGGAAGAG-3'. Restriction enzymes were added to the PCR products of exon 4 and resolved on 3% agarose gels. To create the HinfI site for the detection of c.1177C > T variant by PCR and restriction digestion, a single-base change (C to G) was incorporated into the downstream primer 5'-CTCTGAGCCCTCAGGGCTG-3' and the mismatch reverse primer 5'-ATCCAGGGCGCTGACTGGC-3' were used to create an EcoRI restriction site.

Lipid/lipoprotein analyses
The serum total cholesterol, LDL cholesterol (32), HDL cholesterol (33), and triglyceride levels were measured enzymatically on a Hitachi 7400 Analyzer (Hitachi, Japan) using Roche reagents.

Statistical analysis
Frequencies of the alleles were estimated by gene counting. Agreement of genotype frequencies with Hardy–Weinberg equilibrium expectations was tested using χ² goodness-of-fit test. Contingency χ² statistics were used to test differences in allele frequencies between the groups. The clinical characteristics of study subjects were compared by unpaired Student's t-test. The serum triglyceride level difference among every genotype was tested with ANCOVA. Odds ratios were calculated using gender–BMI–genotype interaction. Values, as the ratio statistic test was a chi-square distribution that had degree of freedom as number of parameters.

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