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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 rela tively 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 *Sst*1 within the 3′-untranslated region of apolipoprotein C-III have consistently shown an association with a variation in plasma triglycerides. In addition to the *Sst*1 (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.

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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) at amino acid residue 185 (G185C). The c.457G>A variation affects an *Fsp*I restriction site, whereas the c.553G>T alters the site for *Msp*I. The third polymorphic site was located at the 1177th nucleotide in the 3′-untranslated region of the exon 4, revealing a C→T transition (c.1177C>T). We have also analyzed two other variations within exon 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). To simplify genotyping of the last three polymorphic sites, mismatch primers for PCR amplification were designed to create *Hinf*I, *Eco*O109I and *ApaL*I restriction sites for the last three polymorphisms, respectively. The genotype frequencies of these variable sites were all in Hardy–Weinberg equilibrium.

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 vari-ants. 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. Haplotype 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.

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). After adjustment for age, gender and body mass index (BMI), we observed that individuals having the minor allele of each of these three markers (c.553G > T, c.1259T > C and IVS3 + 476G > A) had a higher risk of hypertriglyceridemia, whereas both c.457G > A and c.1177C > T were not significant predictors of risk (Table 8). Specifically, the minor allele of the c.553G > T marker was the most significantly related to hypertriglyceridemia risk with an odds ratio of 9.325 (95% confidence interval of 5.451–15.954, $P < 0.001$). Multiple logistic regression for best selection model in above genotypes after adjusting age, gender and BMI is shown in Table 9. The minor allele of c.553G > T marker was still the most significantly related to hypertriglyceridemia risk with an odds ratio of 11.73 (95% confidence interval of 6.617–20.793, $P < 0.0001$), however, both c.1259T > C and IVS3 + 476G > A were no longer significant predictors of risk. To eliminate confounding by the haplotype 2 that was previously associated with high plasma triglyceride concentrations (9), individuals who carried this haplotype were excluded. The minor allele of c.553G > T marker was still significantly related to hypertriglyceridemia risk with odds ratio of 10.6 (95% confidence interval of 6.2–18.3, $P < 0.0001$). Since individuals with both c.1259C and IVS3 + 476A allele were excluded, the association between the c.553G > T variant and serum triglyceride levels is independent of the haplotype 2 that was previously shown to be associated with increased plasma triglyceride.

Table 1. Characteristics of study subjects

	Controls	Hypertriglyceridemia	*P*
No.	303	290	
Age (years)	49.7 ± 13.7	54.3 ± 12.5	<0.001
Gender (M/F)	180/123	177/113	0.686
BMI (kg/m^2)	23.2 ± 2.9	26.1 ± 3.5	<0.001
TG[a] (mg/dl)	93.8 ± 37.7	660.8 ± 351.9	<0.001
TC[b] (mg/dl)	174.3 ± 25.9	252.6 ± 75.2	<0.001
HDL-C (mg/dl)	52.5 ± 12.5	39.4 ± 12.6	<0.001
LDL-C (mg/dl)	109.5 ± 19.4	105.6 ± 50.4	0.219

[a]Indicates total triglyceride.
[b]Indicates total cholesterol.
Values are expressed as mean ± SD.

Table 2. Comparison of genotype frequencies of the five polymorphisms in the apolipoprotein A5 gene between control and hypertriglyceridemic group

		Controls (*n*)	Hypertriglyceridemia (*n*)	*P*
c.457G > A	G/G	0.801 (238)	0.812 (233)	0.394
	G/A	0.192 (57)	0.188 (54)	
	A/A	0.007 (2)	0 (0)	
c.553G > T	T/T	0.003 (1)	0.087 (25)	<0.001
	G/T	0.078 (23)	0.366 (105)	
	G/G	0.919 (273)	0.547 (157)	
c.1177C > T	T/T	0.010 (3)	0 (0)	0.235
	C/T	0.210 (62)	0.209 (60)	
	C/C	0.780 (231)	0.791 (227)	
c.1259T > C (SNP1)	T/T	0.693 (206)	0.453 (130)	<0.001
	T/C	0.273 (81)	0.442 (127)	
	C/C	0.034 (10)	0.105 (30)	
IVS3 + 476G > A (SNP2)	A/A	0.034 (10)	0.118 (34)	<0.001
	G/A	0.275 (81)	0.436 (125)	
	G/G	0.691 (204)	0.446 (128)	

Table 3. Comparison of allele frequencies of the five polymorphisms in the apolipoprotein A5 gene between control and hypertriglyceridemic group

		Controls (*n*)	Hypertriglyceridemia (*n*)	*P*
c.457G > A	G allele	0.897 (533)	0.906 (520)	0.621
	A allele	0.103 (61)	0.094 (54)	
c.553G > T	T allele	0.042 (25)	0.270 (155)	<0.001
	G allele	0.958 (569)	0.730 (419)	
c.1177C > T	T allele	0.115 (68)	0.105 (60)	0.572
	C allele	0.885 (524)	0.895 (514)	
c.1259T > C (SNP1)	T allele	0.830 (493)	0.674 (387)	<0.001
	C allele	0.170 (101)	0.326 (187)	
IVS3 + 476G > A (SNP2)	A allele	0.171 (101)	0.336 (193)	<0.001
	G allele	0.829 (489)	0.664 (381)	

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 consequences 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

Table 4. Linkage disequilibrium between all the variants. Right upper triangle part as D′, and left lower triangle part as *P* values for pair-wise linkage disequilibrium. (**A**) Linkage disequilibrium in normal subjects. (**B**) Linkage disequilibrium in all subjects

	c.457G > A	c.553G > T	c.1177C > T	c.1259T > C	IVS3 + 476G > A
A					
c.457G > A		0.99901	0.98122	0.69765	1.00000
c.553G > T	0.11107		1.00000	1.00000	1.00000
c.1177C > T	<0.000001	0.09656		0.49154	1.00000
c.1259T > C	0.03281	0.05102	0.08353		0.90198
IVS3 + 476G > A	0.00432	0.05030	0.00255	<0.000001	
B					
c.457G > A		1.00000	0.98019	0.89878	1.00000
c.553G > T	0.00033		1.00000	1.00000	1.00000
c.1177C > T	<0.000001	0.00015		0.80333	1.00000
c.1259T > C	0.00002	<0.000001	0.00005		0.95200
IVS3 + 476G > A	<0.000001	<0.000001	<0.000001	<0.000001	

Table 5. Common haplotype derived from all five polymorphic sites using all the genotype data. The six depicted haplotypes account for 98.4% of all haplotypes, none of the other predicted haplotypes had a frequency of greater than 1%. The minor alleles that define the haplotypes are highlighted in bold

Haplotype	Frequency (%)			IVS3 + 476G > A	c.457G > A	c.553G > T	c.1177C > T	c.1259T > C
	All	Normal	Case					
1	47.3	66.8	28.1[a]	G	G	G	C	T
2	23.4	15.3	31.4[a]	**A**	G	G	C	**C**
3	15.4	4.2	27[a]	G	G	**T**	C	T
4	9.4	9.5	9.2[b]	G	**A**	G	**T**	T
5	1.8	1.2	1.9[c]	**A**	G	G	C	T
6	1.1	0.9	0.9[d]	G	G	G	**T**	T

[a]Normal vs case $P < 0.001$.
[b]$P = 0.8779$.
[c]$P = 0.6982$.
[d]$P = 0.9664$.

Table 6. Haplotype estimation. 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)

	Degree of freedom	ln(Likelihood)	Chi-square	*P*
H0: no association	5	−2064.1	H1 vs H0: 1315.83	<0.00001
H1: markers associated, independent of disease	31	−1406.19	H2 vs H0: 1466.61	<0.00001
H2: markers and disease associated	62	−1330.8	H2 vs H1: 150.78	<0.00001

cardiovascular disease (18). In particular, the *Sst*I 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 *APOC*3 and triglyceride levels. In their study, all 3238G, 1100T and −482T alleles were associated with raised triglyceride levels, and the triglyceride-raising effect of the 3238C > G and −482C > 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 *APOC*3 −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 −1131C) 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.

Table 7. Triglyceride levels (mg/dl) according to different SNPs in control group

		Triglyceride (mean±SD)	*P*
c.457G > A	G/G	92.5 ± 38.3	0.466
	G/A	99.5 ± 36.0	
	A/A	96.0 ± 62.0	
c.553G > T	G/G	92.5 ± 37.8	0.014
	G/T	106.6 ± 34.8	
	T/T	183.0	
c.1177C > T	C/C	92.9 ± 39.1	0.558
	C/T	97.2 ± 33.1	
	T/T	110.0 ± 50.2	
c.1259T > C (SNP1)	T/T	88.5 ± 35.4	<0.001
	T/C	104.1 ± 40.8	
	C/C	123.0 ± 39.0	
IVS3 + 476G > A (SNP2)	G/G	88.3 ± 35.4	<0.001
	G/A	103.7 ± 39.8	
	A/A	123.9 ± 41.8	

Table 8. Odds ratios of hypertriglyceridemia and 95% confidence intervals (CI) in relation to the presence of different alleles of apolipoprotein A5 gene after adjusting for age, gender and BMI values

	Variable	Odds ratio (95% CI)	*P*
c.457G > A	(GA and AA)/GG	1.065 (0.648–1.75)	0.8047
c.553G > T	(GT and TT)/GG	9.325 (5.451–15.954)	<0.001
c.1177C > T	(CT and TT)/CC	1.072 (0.665–1.73)	0.7751
c.1259T > C (SNP1)	(TC and CC)/TT	2.486 (1.653–3.74)	<0.001
IVS3+476G > A (SNP2)	(GA and AA)/GG	2.59 (1.717–3.906)	<0.001

Table 9. Multiple logistic regression after adjusting age, gender and BMI

	Variable	Odds ratio (95% CI)	*P*
c.553G > T	(GT and TT)/GG	11.73 (6.617–20.793)	<0.0001
c.1259T > C	(TC and CC)/TT	1.486 (0.466–4.732)	0.503
IVS3 + 476G > A	(GA and AA)/GG	2.637 (0.824–8.439)	0.1022

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-fo1d 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 hyperlipoproteinemia, 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′-TGAGCCCCAACAGCTCTGTG-3′, 5′-TGGTTCCCCCAGAGGATCAG-3′, 5′-GCAACTGAAGC-CCTACAC-3′, 5′-TTTCCGCCAGGACACCTAC-3′ and reverse

primers 5′-TTTCCTCTGTCCCAGCAGCG-3′, 5′-TCGGCG-TATGGGTGGAAGAG-3′, 5′-CTCAGTCTCCTGGTCGATG-3′, 5′-GAGCATTCCCAAATGAGCAC-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′-GCAACTGAAGCCCTACAC-3′ and 5′-TCGGCG-TATGGGTG GAAGAG-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′-CTCTGA-GCCTCTAGCATGGTT*G*AGT-3′ pairing with the upstream primer 5′-GAGCATTCCCAAATGAGCAC-3′. To detect the IVS3 + 476G > A variation, a forward primer 5′-TGGTTC-CCCCAGAGGATCAG-3′ and the mismatch reverse primer 5′-ATCCAGGCCGTCAGACTGCTAG*G*C-3′ were used to create an *Eco*O109 I restriction site. For the detection of c.1259T > C variation, a mismatch downstream primer 5′-ACCAAAGGGGCTGCTGTCTC*G*TGCA-3′and the upstream primer 5′-GAGCATTCCCAAATGAGCAC-3′ were used to create an *ApaL*I restriction site.

Lipid/lipoprotein analyses

The serum total cholesterol, LDL cholesterol (32), HDL cholesterol (33), and triglyceride levels were measured enzymatically on a Hitachi 7450 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 χ^2 goodness-of-fit test. Contingency χ^2 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 multivariate logistic regression analysis by the SAS program. We calculated the pair-wise linkage disequilibrium, D' values, as the disequilibrium coefficient divided by maximum disequilibrium and respective significance levels (34). For haplotype analyses, there were possible 64 possible haplotypes of these 6 loci (disease + five markers). We estimated the haplotype frequency of five genotype markers by PM (permutation and model-free analysis) and EH (Estimating Haplotypes) programs (35,36). For makers and disease association comparison, 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) were tested. The likelihood ratio statistic test was a chi-square distribution that had degree of freedom as number of parameters.

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