Determination of a T/G Polymorphism at Nucleotide 3206 of the Apolipoprotein C III Gene by Amplification Refractory Mutation System

Michael Y. Tsai,1,3 Naomi Q. Hanson,1 Kenneth R. Copeland,2 Iraj Beheshti,1 and Uttam Garg1

We used the amplification refractory mutation system (ARMS)—a polymerase-chain-reaction-based method—to determine the 3206 T-to-G polymorphism on exon 4 of the apolipoprotein (apo) C III gene. Apo C III is an inhibitor of the enzyme lipoprotein lipase (EC 3.1.1.34). Previous studies have demonstrated that a polymorphism at nucleotide 3175 on exon 4 of this gene is associated with hypertriglyceridemia. We studied 45 hypertriglyceridemic and 46 age-matched controls for the 3206 T-to-G polymorphism. The results showed a significant difference in the distribution of the genotypes with respect to this allele between the hypertriglyceridemic and control individuals. We also determined the presence of the SacI site at nucleotide 3175 in these same individuals and found no significant difference in SacI genotypes between the two groups. This study reaffirms the usefulness of ARMS as a simple, reliable method for detecting mutations and polymorphisms in clinical and epidemiological studies.

Indexing Terms: hypertriglyceridemia/polymerase chain reaction/geneic variants

Hypertriglyceridemia is one of the commonest forms of hyperlipidemia. Although it is still unclear whether high serum triglyceride concentrations represent an independent risk factor, increased triglyceride concentrations are responsible for changes in several lipoproteins, which in turn increase the risk of hypertriglyceridemic individuals to coronary artery disease (1, 2). Three proteins are known to be important in the catabolism of chylomicron and very-low-density lipoprotein (VLDL), two triglyceride-rich lipoproteins: lipoprotein lipase (LPL; EC 3.1.1.34) and apolipoproteins C II and C III (apo C II and III) (3, 4). LPL removes triglycerides from chylomicron and VLDL to form chylomicron and VLDL remnants, respectively. Apo C II is required for the activation of LPL, whereas apo C III modulates this process through its inhibitory effect on LPL. Thus, increased apo C III concentrations may be associated with hypertriglyceridemia—a finding that has recently been demonstrated in a transgenic mouse model (5).

The apo C III gene is composed of 4 exons coding for a mature protein of 79 amino acids. Most of the coding information is contained in exon 4 (6). Several reports suggest the possible association of hypertriglyceridemia with a polymorphic site in the 3′-untranslated region of exon 4 of the apo C III gene (7–9). This polymorphism, a C-to-G substitution at nucleotide 3175 of the human apo C III gene, results in the formation of a SacI restriction enzyme site. Another polymorphism, a T-to-G transition also on the 3′-untranslated sequence of exon 4 at nucleotide 3206, was first reported by Karathanasis et al. (10), but the significance of that polymorphism is currently not known. In this report, we studied whether there is an association between this polymorphism and hypertriglyceridemia.

Amplification refractory mutation system (ARMS), an allele-specific polymerase chain reaction (PCR) method (11–14), has been used to identify point mutations in many systems. ARMS is based on the observation that primers used in PCR, when mismatched in the last base at the 3′ end, will not lead to amplification. To detect a single base substitution, two sense primers differing only in a single base at the 3′ end are used. One of the sense primers matches with the more common or wild-type allele, the other with the mutant or polymorphic allele. A common antisense primer is used for both reactions. DNA with either the wild or polymorphic-type sequences is amplified by the matching primer, whereas heterozygous carriers are amplified by both types of primers.

With the ARMS method, we determined the optimal conditions for detecting the T/G polymorphism at nucleotide 3206 of the apo C III gene. We then used this method to compare the frequency of polymorphisms at nucleotide 3206 in normal and hypertriglyceridemic individuals. By using restriction endonuclease digestion, we also compared the frequency of polymorphisms at nucleotide 3175 in the same groups of individuals.

Materials and Methods

Study Population

The study population consisted of individuals being treated for hypertriglyceridemia at the University of Minnesota Heart Disease Prevention Clinic and the Minneapolis Heart Institute. All had serum triglyceride concentrations >2.6 mmol/L. We studied 45 hypertriglyceridemic individuals (40 men, 5 women) ranging in age from 30 to 60 years (mean 48.7 ± 8.5 years).

The control population consisted of 46 University of Minnesota employees (20 men, 26 women) ranging in age from 31 to 67 years (mean 45.8 ± 8.4 years). There was no significant difference in ages between this group

1. Department of Laboratory Medicine and Pathology, University of Minnesota Hospital and Clinic, Minneapolis, MN.
2. Department of Pathology, Duke University Medical Center, Durham, NC.
3. Address correspondence to this author at: Department of Laboratory Medicine and Pathology, University of Minnesota, Box 609 Mayo, 420 Delaware St., Minneapolis, MN 55455. Fax 612-625-6894; E-mail tsai001@maroon.tc.umn.edu.
4. Nonstandard abbreviations: VLDL, very-low-density lipoprotein; LPL, lipoprotein lipase; apo, apolipoprotein; PCR, polymerase chain reaction; ARMS, amplification refractory mutation system; LDL, low-density lipoprotein; HDL, high-density lipoprotein; and ANOVA, analysis of variance.

Received May 23, 1994; accepted September 8, 1994.
and the hypertriglyceridemic group. All controls had serum triglyceride concentrations <2.3 mmol/L.

This study was approved by the Human Studies Committee of the University of Minnesota Institutional Review Board.

DNA Preparation

Genomic DNA was extracted from peripheral leukocytes isolated from EDTA-anticoagulated blood with a commercially available DNA isolation kit (Puregene; Gentra Systems, Minneapolis, MN).

Oligonucleotide Primers

The oligonucleotide primers used for ARMS were synthesized on a DNA synthesizer (Model ABI 394; Applied Biosystems, Foster City, CA) by the Microchemical Facility at the University of Minnesota and purified by HPLC. Since T is the more common base at nucleotide 3206 in our control population, it is defined as the wild genotype. The sequence of the allele-specific sense primer for the wild genotype was 5’CTTGGGTCTCTGCAATCTCCAGGGCCT3’; that for the polymorphic allele was 5’CTTGGGTCTCTGCAATCTCCAGGGGCG3’. The common (antisense) primer was 5’GGGAGGCCAGCATGCCCTGAG3’. The PCR product generated from this reaction is a 115 base-pair (bp) fragment. To prove the reliability and reproducibility of each assay, we used as an internal control primers spanning a 208-bp fragment of exon 3 of the apo C III gene. The sequence of the sense primer was 5’CCGATCCCCCACTCAGCC3’ and that of the antisense primer was 5’GAATGGAGCGAGCTGGAAG3’ (6).

ARMS Analysis

Two PCR reactions were performed for each sample: The sense primer specific for the wild-type allele was used in one and the sense primer specific for the polymorphic allele was used in the other. The common antisense primer, as well as the sense and antisense internal control primers, were added to both reactions. PCR reactions were carried out with 1 μg of genomic DNA and 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT) in a reaction volume of 10 μL, containing 10 mmol/L Tris (pH 8.3), 1.0 mmol/L MgCl₂, 50 mmol/L KCl, 0.2 mmol/L of all four deoxynucleoside triphosphates, and 0.5 μmol/L of each of the five primers. To avoid evaporation, the reaction mixture was overlaid with mineral oil. Reactions were carried out with a Twin Block thermal cycler (Ericomp, San Diego, CA). The amplification cycles were: denaturation at 95°C for 5 min, primer annealing at 62°C for 75 s, and primer extension at 72°C for 120 s; 30 cycles of denaturation at 95°C for 1 min, primer annealing at 62°C for 45 s, and primer extension at 72°C for 75 s; and then one cycle of denaturation at 95°C for 1 min, primer annealing at 62°C for 45 s, and primer extension at 72°C for 7 min.

Detection of Amplification Products

We added 3 μL of Ficoll loading dye (2.5 g/L bromphenol blue and xylene cyanol in 150 g/L Ficoll) to 15 μL of PCR product. The mixture was electrophoresed for 30 min at 150 V on a 3% agarose gel (NuSieve 3:1; FMC Bioproducts, Rockland, ME) containing ethidium bromide. The presence or absence of amplified DNA products was determined by viewing through an ultraviolet transilluminator (Fotodyne, New Berlin, WI). A size marker was included in each gel (φX174 RF DNA HaeIII Frag; Gibco BRL, Gaithersburg, MD) to confirm the size of amplified fragments.

SacI Digestion

A 306-bp DNA fragment from nucleotide 2989 to 3295 of the apo C III human gene (6) was selectively amplified with primers 5’CTTGGGTCTCTGCAATCTCCAGGGCCT3’ (sense) and 5’GGGAGGCCAGCATGCCCTGAG3’ (antisense) by using PCR as described above with the following exceptions: 1.5 mmol/L MgCl₂ was used, primer annealing occurred at 60°C for 45 s, and primer extension occurred at 72°C for 75 s. The fragment (10 μg of DNA) was digested for 60 min at 37°C with 20 U of restriction endonuclease SacI (Promega, Madison, WI) and visualized after electrophoresis on a 2% agarose gel (NuSieve 3:1) containing ethidium bromide.

Apo C III Determination

Apo C III values were determined by using the apo C III plate method (Daichi Pure Chemicals, Tokyo, Japan), based on single radial immunodiffusion. Plasma samples were assayed according to the manufacturer’s instructions.

Laboratory Determinations

Cholesterol, triglycerides, and high-density lipoprotein (HDL)-cholesterol were determined on a centrifugal analyzer (Cobas-Fara; Roche Diagnostic Systems, Montclair, NJ) in the routine clinical chemistry laboratory of the University of Minnesota Hospital and Clinics. For samples with triglyceride <4.5 mmol/L, low-density lipoprotein (LDL) was calculated with the Friedewald formula. For samples with triglyceride >4.5 mmol/L, LDL was determined by the β-quant method (15) as follows. Serum samples were subjected to density-gradient ultracentrifugation. The top VLDL layer was removed after ultracentrifugation at a density of 1.006 kg/L and LDL-cholesterol was estimated as the difference between the total cholesterol of the infranate and the HDL-cholesterol measured after the precipitation of LDL-cholesterol.

Results

Lipid Profiles

A summary of the serum lipid and lipoprotein data on the 46 control and 45 hypertriglyceridemic individuals is shown in Table 1. Triglyceride and cholesterol concentrations were significantly higher, whereas HDL-cholesterol was significantly lower in hypertriglyceridemic individuals than in controls. No significant difference was found in LDL-cholesterol values between the two groups.
Table 1. Serum lipid and lipoprotein profile in control and hypertriglyceridemic individuals.

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD, mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Triglyceride</strong></td>
<td>1.28 ± 0.49</td>
</tr>
<tr>
<td><strong>Cholesterol</strong></td>
<td>4.92 ± 0.86</td>
</tr>
<tr>
<td><strong>HDL-Chol</strong></td>
<td>1.36 ± 0.43</td>
</tr>
<tr>
<td><strong>LDL-Chol</strong></td>
<td>2.96 ± 0.80</td>
</tr>
</tbody>
</table>

Hypertriglyceridemic (n = 45) 5.67 ± 2.61*

* P < 0.001 as compared with respective controls, by Student’s unpaired t-Test.

Table 2. Nucleotide sequences of the three sets of sense primers and of the human apo C III gene.

<table>
<thead>
<tr>
<th>Set</th>
<th>Sense primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Wild-type</td>
<td>5'</td>
</tr>
<tr>
<td></td>
<td>Polymorphic</td>
<td>CCTGCAATTCTCCASGGCTTT 3'</td>
</tr>
<tr>
<td>2</td>
<td>Wild-type</td>
<td>5'</td>
</tr>
<tr>
<td></td>
<td>Polymorphic</td>
<td>CTGGGTCTGTCAATTCTCCASGGCTTT 3'</td>
</tr>
<tr>
<td>3</td>
<td>Wild-type</td>
<td>5'</td>
</tr>
<tr>
<td></td>
<td>Polymorphic</td>
<td>CCTGCAATTCTCCASGGCTTT 3'</td>
</tr>
<tr>
<td></td>
<td>Apo C III fragment</td>
<td>5' . . . CTGGGTCTGTCAATTCTCCASGGCTTT . . . 3'</td>
</tr>
</tbody>
</table>

** Primer Selection**

Table 2 shows the nucleotide sequences of three sets of sense primers used and the normal DNA sequence of the human apo C III gene fragment studied. In ARMS, the degree of refractoriness depends much on the type of mismatch at the 3'-OH-terminal nucleotide and at the nucleotide adjacent to the 3'-terminal end, with purine/purine and pyrimidine/pyrimidine mismatches being more refractory to amplification than purine/pyrimidine mismatches (11-13). With our primers, the mismatch at the 3'-terminal end between the wild-type primer and polymorphic allele was A/G, or purine/purine, whereas that between the polymorphic primer and normal allele was C/T, or pyrimidine/pyrimidine. To study allele specificity of our primers, we generated additional mismatches at the nucleotide adjacent to the 3'-terminal end. In the first set of primers (Table 2), the penultimate base at the 3' end was not altered. In the second set, the penultimate base at the 3' end of both primers was altered to substitute a C for T, generating a template/primer A/C, or purine/pyrimidine, mismatch. In the third set, the penultimate base at the 3' end of both primers was altered to substitute a G for T, creating a template/primer A/G, or purine/purine, mismatch. The first and second sets of primers gave rise to 108- and 115-bp amplification products, respectively. No amplification product, except for the internal control, was visible with the third set of primers. The second set of primers was chosen for use in this study to assure the required specificity.

**ARMS Analysis of Homozygotes and Heterozygotes**

Figure 1 shows the electrophoretic pattern of ARMS analysis of three individuals homozygous for T, homozygous for G, or heterozygous at nucleotide 3206. The T/T homozygote (lanes 2 and 3) and G/G homozygote (lanes 4 and 5) each produced a 115-bp band only in lanes where the respective allele-specific sense primer was used, whereas a G/T heterozygote produced bands with both primers (lanes 6 and 7). The 208-bp internal control band was visible in all lanes.

**Long-Term Performance of ARMS Assay**

DNA samples from G/G, T/T, and G/T patients were assayed, stored refrigerated, and reanalyzed 1 year later. No changes in the original amplification patterns were observed.

**ARMS Analysis of Control and Hypertriglyceridemic Individuals**

Table 3 shows the frequency of the T/T, G/G, and G/T apo C III genotypes with respect to nucleotide 3206 in 46 control and 45 hypertriglyceridemic individuals.

Within the control group we did not find a significant difference in genotypes between men and women. There was an overall significant difference (P < 0.01) in genotyping between control and hypertriglyceridemic individuals. When the data were further analyzed to determine the specific differences, hypertriglyceridemic individuals were significantly lower (P < 0.001) in T/T
Table 3. Apo C III genotypes with respect to nucleotide 3206 for control and hypertriglyceridemic individuals.

<table>
<thead>
<tr>
<th></th>
<th>T/T</th>
<th>G/G</th>
<th>G/T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 46)</td>
<td>20 (43.5)</td>
<td>3 (6.5)</td>
<td>23 (50.0)</td>
</tr>
<tr>
<td>Hypertriglyceridemic (n = 45)</td>
<td>8 (17.7)*</td>
<td>9 (20.0)</td>
<td>28 (62.2)</td>
</tr>
</tbody>
</table>

* Genotypes between control and hypertriglyceridemic individuals are significantly different (P < 0.01) by x² test.

genotypes than controls. No significant differences were found between G/G and G/T genotypes.

The data were also analyzed to determine if there was any association between the three genotypes and cholesterol, triglyceride, HDL-cholesterol, and LDL-cholesterol values. There was no significant difference between T/T, G/G, and G/T genotypes for any of these analytes in either the control or hypertriglyceridemic groups [by one-factor analysis of variance (ANOVA)].

Analysis by SacI Digestion

We determined the frequency of the apo C III genotypes with respect to nucleotide 3175 in the 46 control and 45 hypertriglyceridemic individuals by using SacI restriction endonuclease digestion (Table 4). Individuals not having the SacI site [SacI (-/-)] had only one band of 306 bp after digestion, whereas individuals homozygous for this polymorphism [SacI (+/+)] had two bands of 190-bp and 116-bp products. When the SacI site was present on one allele [SacI (+/-)], two bands of 190 bp and 116 bp were seen in agarose gel in addition to the 306-bp product.

As Table 4 shows, a greater percentage of control individuals did not have the SacI site as compared with the hypertriglyceridemic individuals; however, the difference was not significant. Only one individual (with triglyceride of 4.7 mmol/L) was homozygous for this polymorphism.

The data were further analyzed to determine if there was any association between the three SacI genotypes and concentrations of cholesterol, triglyceride, HDL-cholesterol, and LDL-cholesterol. There was no significant difference between the genotypes for any of these analytes in either the control or hypertriglyceridemic groups (by one-factor ANOVA).

Association of Apo C III Concentrations with 3206 and 3175 Polymorphisms, and with Lipid and Lipoprotein Values

The concentration of apo C III in the hypertriglyceridemic individuals ranged from 114 to 346 mg/L (reference range 74.9 ± 29.7 mg/L). No significant differences were observed between apo C III concentrations in this group and genotypes with respect to nucleotides 3206 or 3175 of apo C III (by one-factor ANOVA). However, a significant positive correlation was observed between apo C III concentrations and cholesterol and triglyceride values (P < 0.01, by regression analysis). There was no significant correlation between apo C III values and HDL-cholesterol or LDL-cholesterol.

Discussion

The association of apo C III concentrations with hypertriglyceridemia has recently been demonstrated in a transgenic mouse model (5). Mutations or polymorphisms that either increase the apo C III concentration or increase the inhibitory effect of apo C III on LPL may be the cause of a subset of individuals with hypertriglyceridemia. Previous studies have demonstrated that a polymorphism involving a C-to-G transversion at nucleotide position 3175 of the human apo C III gene (SacI site) may be associated with hypertriglyceridemia, at least certain ethnic groups (7–9). We are not aware of any published study on the polymorphism at nucleotide 3206 involving a T-to-G transversion of the same gene. We used ARMS to study the frequencies of the two alleles at this position in normal lipemic and hypertriglyceridemic individuals.

ARMS was originally reported as a means to detect mutations in the α1-antitrypsin gene (11). The method has been applied both in our laboratory and in those of others to detect point mutations (11, 13, 14) and identify isoforms such as the apo E alleles (12), although it has not yet received widespread use in clinical laboratories. The key to the success of the ARMS technique lies in the use of appropriately designed sense primers. Here, we studied the polymorphisms at nucleotide 3206 with three different sets of allele-specific sense primers.

ARMS is based on the fact that the 3'-terminal-mismatched primers are refractory to amplification; a second mismatch in the nucleotide next to the 3'-terminal end of the primers has been deliberately introduced to increase refractoriness and ensure specificity. As demonstrated here, when a second A/G (purine/purine) mismatch was introduced at the penultimate base in addition to the purine/purine or pyrimidine/pyrimidine mismatch at the 3'-OH-terminal (as in the third set of primers), the primers did not produce a product with either allele because the additional purine/purine mismatch was too refractory; therefore, if a second mismatch at the penultimate base is introduced for added specificity, a less refractory purine/pyrimidine (as in the second set of primers) or pyrimidine/purine mismatch is the appropriate choice.

In our study of 46 control and 45 hypertriglyceridemic age-matched individuals, there was a significant difference between the two groups with respect to the frequencies of the two alleles at position 3206. In the control group, the T and G alleles were present with a frequency of 68.5% and 31.5%, respectively. In contrast, in the hypertriglyceridemic group the 3206 T allele and 3206 G allele both had a prevalence of ~50%.
We also determined the frequency of the 3175 polymorphism in the same control and hypertriglyceridemic groups, and found no significant differences among the three SacI genotypes in the two groups. This may be because the group we studied was predominantly Caucasian. Tas (7) has shown a strong association between hypertriglyceridemia and the SacI (+) allele in Arabs in Kuwait.

We found no significant difference in either the hypertriglyceridemic or control group between the apo C III genotypes with respect to either nucleotide 3206 or 3175 and the values of cholesterol, triglyceride, HDL-cholesterol, LDL-cholesterol, or apo C III. This may be due to the small number of subjects studied.

While there is a statistically significant enrichment of the 3206 G allele in the hypertriglyceridemic group as compared with the control group, the significance of this finding is not currently known. Also, it is not clear whether homozygosity for the G-to-T substitution at 3175 allele is required for the individual's predisposition to hypertriglyceridemia. Like the SacI allele, which involves a polymorphic site at nucleotide 3175, the polymorphism at 3206 resides in the 3'-end untranslated region of exon 4 of the apo C III gene. Whether this polymorphism may be directly involved in the regulation of apo C III gene or is a linkage marker to genes that predispose individuals to hypertriglyceridemia requires further study. As in the case of the SacI allele, it is possible that the association of the polymorphism with hypertriglyceridemia may vary in different ethnic populations, and additional dietary factors may increase or decrease this association.

In conclusion, we have successfully used ARMS to study the second known polymorphism in exon 4 of the apo C III gene, thus confirming the usefulness and ease with which this PCR application can be used for the screening of known mutations and polymorphisms. The association of the polymorphism at nucleotide 3206 with hypertriglyceridemia needs confirmation in large epidemiological studies.

References