Detection of three separate DNA polymorphisms in the human lipoprotein lipase gene by gene amplification and restriction endonuclease digestion

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Abstract A rapid detection method was developed for DNA polymorphisms in the human lipoprotein lipase (LPL) gene. The examined polymorphisms include an A-C transversion in the 5'-region of intron 3, a T-G transversion that occurs within a Hind III site of intron 8, and the previously described C-T transition that causes a Pvu II polymorphism in intron 6. Gene fragments encompassing each polymorphic site were amplified by the polymerase chain reaction (PCR) and digested with an appropriate restriction enzyme whose recognition site was either naturally affected by the polymorphism or artificially created with a mismatched PCR-primer. According to the digestion profiles, genotypes were unambiguously distinguished. With this method, respective allelic frequencies were determined for 50 or 70 normal subjects. The procedure will facilitate LPL genotyping in the large population.

Lipoprotein lipase (LPL) is a major determinant of plasma lipoprotein profiles because it affects all classes of lipoprotein particles. The action of LPL is essential not only for the hydrolysis of triglycerides in chylomicrons and very low density lipoproteins (VLDL) but also for the maturation of high density lipoproteins (HDL) and low density lipoproteins (LDL) (1, 2). Human LPL is a glycoprotein of 448 amino acids in its mature form (3), and the corresponding gene has a span of 30 kilobases (kb) comprising 10 exons (4–6). Several DNA polymorphisms that generate restriction fragment length polymorphisms (RFLP) have been identified in the human LPL gene. These include polymorphisms identified with Bam HI (7), Pvu II (7, 8), Hind III (9), Bst NI (10), Bst I (11), Bgl II (12), and Xba I (13).

DNA polymorphism is a useful marker to analyze disorders with genetic backgrounds, even though the genetic cause of the disease has not been elucidated. A number of DNA polymorphisms have been used to examine their possible linkage with a hereditary predisposition to common polygenic disorders such as dyslipidemia (14, 15) and diabetes mellitus (16). Along the same lines, some trials have been carried out to explore associations between LPL gene polymorphisms and lipoprotein phenotypes. The results provided evidence of an association of the genotypes identified by the Pvu II RFLP with plasma triglyceride levels (17) and another association of the genotypes revealed by the Hind III RFLP with plasma HDL-cholesterol concentrations (13). To confirm those observations conclusively, however, further studies among a larger population will be needed.

The DNA polymorphisms at the LPL gene locus have been detected only with the Southern blot procedure, because detailed DNA sequences at and around the polymorphic sites have been unavailable. This conventional procedure requires considerable time, labor, and skill as well as radiolabeled LPL gene probes. Therefore, the technique is inappropriate for a large scale study in an ordinary laboratory. Previously, we and others have identified the DNA sequence encompassing the Pvu II poly-

Abbreviations: LPL, lipoprotein lipase; PCR, polymerase chain reaction; VLDL, very low density lipoproteins; HDL, high density lipoproteins; LDL, low density lipoproteins; kb, kilobases; RFLP, restriction fragment length polymorphism; dNTP, deoxynucleoside triphosphate; bp, base pairs.

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TABLE 1. Sequences of the oligonucleotide PCR-primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>A</td>
<td>5'-GGAAATGTATGAGAGTTG-3'</td>
</tr>
<tr>
<td>B</td>
<td>5'-ACACTGTITITTCGCACTAC-3'</td>
</tr>
<tr>
<td>C</td>
<td>5'-GCTTAAATCTCAATTTCAATG-3'</td>
</tr>
<tr>
<td>D</td>
<td>5'-CTTTAGAATGTTGCTCAAGGT-3'</td>
</tr>
<tr>
<td>E</td>
<td>5'-TGAAGCTCAATTTGAAGAGCT-3'</td>
</tr>
<tr>
<td>F</td>
<td>5'-TCAAGGCAATTTGACTAAA-3'</td>
</tr>
</tbody>
</table>

Primer B has a single base mismatch indicated by an asterisk at its 3' end to create an artificial RFLP by PCR.

morphic site in intron 6 (18, 19), and recently three additional RFLPs identified with Hind III, Xba I, and Bam HI have been mapped in intron 8, intron 9, and the 3'-flanking region of the LPL gene, respectively (13).

In this report, we describe a new sequence polymorphism identified in the 5'-region of intron 3, and another sequence polymorphism shown to be responsible for the Hind III RFLP in intron 8. With application of the polymerase chain reaction (PCR) (20), we have developed a simple procedure for the detection of three separate DNA polymorphisms in the human LPL gene.

MATERIALS AND METHODS

Genomic cloning and identification of DNA sequence polymorphisms

Extraction of leukocyte DNA and construction of genomic libraries were carried out as described previously (18, 21). Genomic DNAs were isolated from 70 unrelated subjects. For three of them, genomic libraries were constructed. DNAs from the three subjects were digested with Eco RI and cloned into λgt 10 phage vectors. By screening the libraries with a human LPL cDNA probe (22), four gene fragments that contain exons 3-9 were obtained from each library. Several portions of those gene fragments were subcloned into the M13 vectors mp18 or mp19, and sequenced by the dideoxy method (23) with Sequenase (United States Biochemicals).

Amplification of DNA fragments encompassing each polymorphic site

In the other 67 subjects, gene amplification was performed. To amplify the PCR products comprising the three polymorphic regions, three pairs of oligonucleotide primers were synthesized by the phosphoramidite method with the Applied Biosystems model 392 DNA/RNA synthesizer. The DNA sequences of the oligonucleotide PCR-primers are shown in Table 1. Five hundred ng of each DNA sample was added to a 50-µl reaction mixture of 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM MgCl2, 0.01% gelatin with 0.05 nmol of each of the paired primers, 1.25 units of Taq DNA polymerase (Perkin-Elmer Cetus). The reaction mixture was applied to 30 cycles of PCR (94°C 1 min; 47-52°C 2 min; 72°C 3 min), which was preceded by an additional denaturation (94°C 5 min) and followed by an extension (72°C 7 min). The annealing temperatures used for the amplification of the polymorphic regions in introns 3, 6, and 8 were 47°C, 52°C, and 50°C, respectively.

Restriction enzyme digestion and electrophoretic analysis

One-tenth of the amplified DNA (5 µl) was directly used for digestion with restriction enzymes Mae I, Hind III, or Pvu II (Boehringer Mannheim). Two (Mae I) or ten (Hind III, Pvu II) units of each restriction enzyme were added to a final volume of 20-µl solution containing each amplified DNA and an incubation buffer recommended by the manufacturer's instruction. The reaction mixture was incubated overnight at 50°C (Mae I) or 37°C (Hind III, Pvu II). The cleaved products were analyzed by electrophoresis on a 1.5-2.0% agarose gel followed by staining with ethidium bromide, and were visualized on a UV-transilluminator. The standard mo-
molecular size marker (φX174/Hae III) was also applied to the gels for reference.

**RFLP-creating PCR**

Of the three DNA polymorphisms described in this study, two can naturally affect the restriction sites for Hind III and Pvu II, respectively. The A-C change found at nucleotide position +20 of intron 3, however, neither creates nor abolishes any restriction site recognized by the commercially available restriction enzymes. To develop a simple detection method for the A-C substitution, an RFLP was artificially created at the substitution site by introducing a single base mismatch into the 3' end of an oligonucleotide primer (21, 24). As shown in Fig. 1, an Mae II cutting site was generated specifically in the "A-allele" by using a mismatched downstream primer (primer B: 5'-ACACTGTTTTGGACACATAc-3'; the mismatched nucleotide is underlined) which corresponds to +41- +22 of intron 3. In the presence of an adenine (A) at the polymorphic site (+20), the DNA sequence of the PCR product was modified to contain an Mae II restriction site (ACGT). In contrast, the PCR product derived from the "C-allele" should contain an alternative sequence (CCGT) at the corresponding position.

**RESULTS AND DISCUSSION**

**A-C polymorphism in intron 3**

During sequence analysis of the cloned gene fragments, a single base substitution was found near the 5'-region of intron 3 in the human LPL gene (Fig. 2A). Of the five clones examined, three had an adenine (A) at nucleotide position +20 of intron 3, consistent with the published data (4, 6). The other two clones derived from different subjects, however, carried a cytosine (C) in place of adenine at the position. This finding suggested the possibility that the A-C substitution is a common polymorphism among the population.

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**Fig. 2.** Comparison of DNA sequences around two polymorphic regions. (A) A-C transversion in intron 3. (B) T-G transversion within a Hind III site of intron 8. Gene fragments containing the respective alleles were subcloned into the M13 vectors and sequenced by the dideoxy method. Each panel comparatively shows the paired autoradiograms of the sequencing gels which represent the DNA sequences of the respective alleles. In each case, more common allele is exhibited in the left of the panel. The sequences of the sense strand (A) or anti-sense strand (B) are shown for comparison. The bases substituted in the less common alleles are indicated by an arrow.
To investigate the prevalence of the base substitution in a large population, we developed a simple detection method. Because the nucleotide sequence around the A-C transversion indicated that the base change alone would not generate any informative RFLP (Fig. 3A), another single base change was introduced near the A-C transversion site with a mismatched PCR-primer (Fig. 1). This procedure created an artificial RFLP site that was affected by the A-C substitution. In this case, by using the primer B with a single base mismatch at its 3’-end, an artificial Mae II site was specifically introduced into the “A-alleles” with an adenine base at position +20. As shown in the left panel of Fig. 4, digestion of the amplified DNAs with Mae II unambiguously distinguished the respective genotypes by their own distinctive restriction patterns. Mae II digestion of the amplified DNA from the A-alleles divided the 215 bp products into two fragments of 194 and 21 bp, but a similar digestion did not cleave the amplified products from the C-alleles.

With this detection method, allelic frequency of the A-C transversion was investigated in the alleles of 50 unrelated Japanese subjects. The A-allele was found in 85%, and the C-allele in 15%. Codominant inheritance of this polymorphism was confirmed in one informative family. The four RFLPs mapped previously were all located in the latter half of the human LPL gene (13, 18, 19). The A-C polymorphism in intron 3 is, therefore, the first DNA polymorphism identified in the other half of the gene, and will become a useful DNA marker at the human LPL gene locus.

Hind III polymorphism in intron 8

A Hind III RFLP was first reported by Heinzmann et al. (9), and a statistically significant association has been reported between the Hind III RFLP and lipoprotein phenotypes (13). From the results of Southern blot analysis, the polymorphic Hind III site was supposed to exist within intron 8 (5, 13) and/or in the 3’ flanking region (6). During the sequence analysis of the cloned gene fragments, we found a T-G substitution within a Hind III site of intron 8 (Fig. 2B). Nucleotide sequence around the Hind III site is shown in Fig. 3B. The replacement of a thymine (T) with a guanine (G) base occurred at approximate position +495 of intron 8 and abolished the Hind III site by converting the recognition sequence for Hind III (AAGCTT) into AAGCST.

The elucidation of the sequence around the Hind III site enabled us to establish a simple PCR method for the detection of the T-G transversion. Two oligonucleotide primers were designed to amplify the 715-bp products comprising the Hind III site of intron 8 (Fig. 4, right). Upon digestion of the amplified DNAs with Hind III, the

(A) A-C polymorphism in intron 3

(B) Hind III polymorphism in intron 8

(C) Pvu II polymorphism in intron 6

Fig. 3. DNA sequence of the regions surrounding the three polymorphic sites. Each sequence begins at the first nucleotide of the respective introns. Both bases are aligned where the base substitutions occur. The polymorphic Hind III and Pvu II sites are boxed. The underlined sequences were used to design PCR-primers, and the thymine (T) base marked with an asterisk was replaced during the RFLP-creating PCR with a 3’-mismatched primer.
Fig. 4. PCR detection methods of the three separate DNA polymorphisms. Upper: Schematic representations of the portion of the LPL gene that were amplified by the individual PCR. Exons and introns are represented by hatched bars and by horizontal lines, respectively. The pairs of primers used for PCR are indicated by the arrows, and the positions of both normal and artificial restriction sites by the vertical lines. Numbers indicate the sizes of the fragments generated by the respective digestions. Each diagram accompanies the sequence of the paired primers used for individual PCR-amplification. To create an artificial Mae II site, a single base mismatch indicated by an asterisk was introduced into the 3' end of the primer B.

Middle: Electrophoretic analysis of the amplified DNAs were performed on agarose gels after digestion of the DNAs with an appropriate restriction enzyme. The respective genotypes were determined according to the digestion profiles and were represented above each lane. The sizes of restriction fragments were estimated by comparison with the standard molecular size marker (pX174/Hae III) applied in the left lanes. Bottom: Schematic representation of the human LPL gene structure and the location of the three polymorphic sites. Restriction profiles directly indicated their respective genotypes.

The Hind III RFLP revealed by Southern blot analysis generates two separate alleles designated H1 and H2, which were characterized by the presence of the bands of 17.5 kb and 8.7 kb, respectively (9, 21). To confirm the identity of the Hind III polymorphism discussed here with the one revealed by Southern blot analysis, we performed the PCR genotyping in 20 normal subjects whose genotypes had been determined previously by Southern blot analysis (21). The results of PCR were completely consistent with those of Southern blot analysis, indicating that the polymorphism in the middle of intron 8 is truly causative of the Hind III RFLP that generated the H1 and H2 alleles. In 70 independent Japanese subjects, allelic frequencies of the T-allele (H2) and the G-allele (H1) were 0.73 and 0.27, respectively.

The T/T genotype was found in only 2 of the 70 Japanese examined. The genotype is therefore less common in Japanese populations as reported previously by Gotoda et al. (7), and an association has been observed between the genotypes and the plasma triglyceride concentrations (17). Previously two independent studies showed that the polymorphism is caused by a C-T transition within a Pvu II site of intron 6 (18, 19). Because both the Hind III and Pvu II polymorphisms occur in the middle of introns, it seems unlikely that these polymorphisms are causative of the observed alterations in lipoprotein profiles.

To develop a PCR detection method, two oligonucleotide primers were synthesized to amplify the products comprising the polymorphic Pvu II site (Fig. 3C). Upon digestion with Pvu II, the restriction patterns accurately showed the respective genotypes (Fig. 4, middle). With this method, allelic frequencies were studied in 70 Japanese subjects. The frequencies of the C- and T-alleles were 0.76 and 0.24, respectively.

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