SIMPLE NON-RADIOACTIVE METHODS OF ANALYSIS OF POLYMORPHIC MARKERS FLANKING HUMAN APOLIPOPROTEIN C-III GENE.

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SUMMARY

We describe two rapid non-radioactive methods for the analysis of polymorphic markers in the flanking region of the human apolipoprotein CIII gene. The polymorphic markers comprise previously described variable sites located upstream from the coding region of the gene (C-641→A, G-630→A, T-625→deletion, C-482→T, and T-455→C) and a polymorphic *SstI/SacI* site in the apoC-III 3' untranslated region. The first method is allele-specific amplification (ASA) with primers complementary to the normal ("wild-type") allele or to the variable ("mutant") allele at their 3' ends. The other is allele-specific oligonucleotide hybridization (ASO hybridization) with pairs of probes labeled by digoxigenin. Comparison with sequencing data showed that both methods are reliable for polymorphism analysis.

KEY WORDS: Apolipoprotein C-III Polymorphism Promoter Polymerase chain reaction
Genotype analysis

INTRODUCTION

Apolipoprotein C-III (apoC-III) is a major protein constituent of triglyceride-rich lipoproteins and is synthesized predominantly in the liver. *In vitro* studies have shown that apoC-III inhibits the hydrolysis of lipids by lipoprotein lipase (Brown and Baginsky, 1972) an enzyme participating in the clearance of triglyceride-rich lipoproteins. Several independent investigations including overexpression of apoC-III in transgenic mice demonstrated a causal role of apoC-III in the development of hypertriglyceridemia (Schonfeld *et al.*, 1979; Malmendier *et al.*, 1989; Ito *et al.*, 1990). A polymorphic *SstI* site (S2 allele) in the apoC-III 3' untranslated region as well as several polymorphic sites in the 5' region are associated with elevated levels of plasma triglycerides (Rees *et al.*, 1983; Tas, 1989; Dammerman *et al.*, 1993). Hypertriglyceridemia (HTG) is etiologically heterogeneous and the role of apoC-III in different types of HTG requires further study. For this purpose a rapid, safe and reliable method is required to determine apoC-III polymorphic markers. Analysis of these, as well as other polymorphic markers, was previously conducted using radioactive probes. To analyze the relevance of polymorphic sites in the flanking regions of the apoC-III gene to the elevation of triglycerides, we developed two non-radioactive methods which can be used both in population and clinical studies.

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MATERIALS AND METHODS

Samples for DNA isolation

DNA was isolated from peripheral blood leukocytes of subjects with different types of lipid metabolism disorders. Genomic DNA was extracted from frozen buffy coats by the salting out procedure (Miller *et al.*, 1988).

Oligonucleotides

Oligonucleotides (Table I and Figure 1) were synthesized using a Cyclone Plus DNA synthesizer with reagents from the Milligen Biosearch Division (Burlington, MA) and purified by thin layer chromatography. The purity of the oligonucleotide was verified by electrophoresis in a denaturing 20% polyacrylamide gel.

Polymerase Chain Reaction (PCR) conditions

Two types of PCR were performed: i) PCR of the sequence from position -699 to -188 was carried out in order to amplify promoter fragments for subsequent analysis by ASO hybridization and sequencing. The amplification was performed in a DNA Thermal Cycler (Perkin Elmer, Cetus) according to the method of Emi and coworkers (Emi *et al.*, 1988) using primers 1 and 2 described by Dammerman *et al.*, 1993 (Table 1). The size of the PCR products was determined by electrophoresis in a 10% denaturing polyacrylamide gel. ii) PCR for Allele-Specific Amplification (ASA) was conducted in order to identify variable bases both in the promoter region and in 3'-flanking untranslated region of the apoC-III gene. Two alternative primers were used to detect a particular mutation, one that is perfectly matched to the "wild type" (1/1) allele at its 3'-end ("wild-type" alternative primer), while the other was non-complementary to the "wild type" allele at 3'-end but formed a perfect duplex with the "mutant" sequence (2/2) (Table 1, primers 3 - 12, Fig. 1). The conditions of PCR are described in (Postnikov *et al.*, 1993). The following combinations of primers were used: site -625/-630/-641 — primers 3 or 4 with primer 2; site -482 — primers 5 or 6 with primer 9; site -455 — primers 7 or 8 with primer 1; *SstI* site — primers 10 or 11 with primer 12.

Oligonucleotide labeling for ASO-Hybridization

Oligonucleotides 13 - 22 were labeled at the 3' end by incorporation of digoxigenin-labeled nucleotide and detected by immunochemiluminescence using a high-affinity sheep antidigoxigenin Fab-fragment (Boehringer Mannheim "DIG Nucleic Acid Detection Kit" and "LumiPhosTM 530"). The labeling reaction was carried out with terminal transferase and stopped by adding glycogen. Labeled oligonucleotides were precipitated by LiCl/ethanol, washed with 70% ethanol (v/v), dried under vacuum, and redissolved in distilled water (Boehringer Mannheim "DIG Nucleic Acid Detection Kit" and "LumiPhosTM 530"). Prehybridization and hybridization of digoxigenin-labeled oligonucleotides were performed as described for P³²-labeled probes (Dammerman *et al.*, 1993).

Nucleotide sequence of PCR products was determined by automated Taq

DyeDeoxyTM Terminator Cycle Sequencing (Applied Biosystems) with one unlabeled oligonucleotide as a primer. PCR products were purified by electrophoresis in 1% agarose with subsequent purification on QIAEX columns (Qiagen Inc., Chatsworth,

Table 1.

	<u>strand</u>	<u>localization</u>	<u>wt</u> <u>or</u> <u>mutant</u>	<u>used for</u>
I. Primers used for PCR, Allele-Specific Amplification and Sequencing				
1. 5'-GGCAAAGGCCTCGGGCTCTGAGCGCCTT-3'	S	-699 - -671	wt	PCR
2. 5'-GCCAGCCCTGCAGCCCAGATGAGCTCAGGA-3'	AS	-217- -188	wt	PCR
3. 5'-ACT <u>C</u> AGGGGGAGGC <u>G</u> GCGG <u>T</u> -3'	S	-644 - -625	wt	ASA -625/-630/-641
4. 5'-ACT <u>A</u> AGGGGGAGGC <u>A</u> GCGG <u>G</u> -3'	S	-644 - -625	m	ASA -625/-630/-641
5. 5'-GCCTTGCCGGAGCCACTGATGCC <u>C</u> -3'	S	-482 - 505	wt	ASA -482
6. 5'-GCCTTGCCGGAGCCACTGATGCC <u>T</u> -3'	S	-482 - 505	m	ASA -482
7. 5'-GGGTGGCTTGGGCTGGGGG <u>A</u> -3'	AS	-436 - -455	wt	ASA -455
8. 5'-GGGTGGCTTGGGCTGGGGG <u>G</u> -3'	AS	-436 - -455	m	ASA -455
9. 5'-CTTCCTAGCTGACTGGCTCC-3'	AS	-247 - -266	wt	ASA -482
10. 5'-ACCTGCCTATCCATCCTGCC <u>G</u> -3'	S	3156-3175*	m (S2)	ASA <i>SstI</i>
11. 5'-ACCTGCCTATCCATCCTGCC <u>C</u> -3'	S	3156-3175*	wt (S1)	ASA <i>SstI</i>
12. 5'-GAGTCTGTCCAGTGCCCAACC-3'	AS	3386-3405*	wt	ASA <i>SstI</i>
II. Probes used for ASO-hybridization (Dammerman <i>et al.</i> , 1993)				
13. 5'-TCCCCCT <u>G</u> AGTGTAG-3'	AS	-634- -648	wt	-641
14. 5'-CTACACT <u>A</u> AGGGGGA-3'	S	-634- -648	m	-641
15. 5'-CCGCC <u>C</u> GCCTCCC-3'	AS	-632 - -644	wt	-630
16. 5'-GGGAGGC <u>A</u> GCGG-3'	S	-632 - -644	m	-630
17. 5'-GCGG <u>T</u> GGGGCAC-3'	S	-629 - -618	wt	-625
18. 5'-GCGGGGGGGCACA-3'	S	-629 - -617	m	-625
19. 5'-TGATGCCCGGTCTTC-3'	S	-489 - -475	wt	-482
20. 5'-TGATGCC <u>T</u> GGTCTTC-3'	S	-489 - -475	m	-482
21. 5'-CTGGGGG <u>A</u> TGTTTGG-3'	AS	-448 - -462	wt	-455
22. 5' <u>C</u> TGGGGGG <u>G</u> TGTTTGG-3'	AS	-448 - -462	m	-455

*numbering of basis in 3' -untranslated region is given as in (Protter *et al.*, 1984), in 5' - promoter region according to (Dammerman *et al.*, 1993). Variable bases are shown in bold and underlined.

CA). The sequencing reaction was carried out in a thermal cycler with purified PCR product (150-250 ng) as template, one of the primers (sense or antisense, 1 pmole) and dideoxynucleotides labeled with different fluorescent dyes under conditions of linear amplification of the reaction product as recommended by the manufacturer (Applied Biosystems. TaqDyeDeoxy™ Terminator Cycle Sequencing Kit, Part #901497).

RESULTS AND DISCUSSION

Identification of polymorphic genotype markers by ASO-hybridization

When PCR-products, obtained from the amplification of genomic DNA with primers 1 and 2, were hybridized with either "wild type" or "mutant" (see Table I, section II) digoxigenin-labeled probes one of the following patterns was observed: i) One of the alternative probes ("wild type" or "mutant"; definition of "wild type" and "mutant" is described in Dammerman *et al.*, 1993) gave an intense signal (Fig. 2, 1a and 3b) while the other one produced little or no signal (Fig. 2, 1b and 3a). These samples were

Figure 1. Localization of primers for PCR and probes for ASO-hybridization on apoC-III gene map.
* primer or probe specific to a "mutant" sequence.

Figure 2. Results of genotyping of -482 marker by ASO-hybridization with digoxigenin labeled probes. Hybridization with probe 272 specific to "wild type" sequence (a) and with probe 273 specific to "mutant" sequence (b). DNA isolated from individual with 1/1 genotype for this marker (1), 1/2 genotype (2) and 2/2 genotype (3).

Figure 3. Results of genotyping of -641/-630/-625 (a), -482 (b), -455 (c) and *SstI* (d) markers by ASA. DNA was isolated from individuals with 1/1, 1/2 and 2/2 genotype and used for PCR with pairs of primers obtained for the corresponding marker. 1, 3 and 5 – PCR with “wild type” alternative primer; 2, 4 and 6 – with “mutant” alternative primer. Right lane is 1 kb ladder.

considered homozygous for the marker (1/1 or 2/2 depending on which of the two probes gave a signal). ii) Both of the probes gave a signal and the intensity of each signal was roughly half of the intensity observed in homozygous samples (Fig. 2, 2a and 2b). These samples were considered heterozygous (1/2) for the marker which was analyzed. Hybridization results for other markers looked similar (not shown).

The classification of the genotypes for all five markers studied (-641, -630, -625, -482 and -455) was highly reliable and the sequences of PCR products used for both ASO and ASA were confirmed by automated sequencing.

Identification of polymorphic markers by ASA

The results of ASA for different markers are presented in Fig. 3. Since the genotype at site -625 predicts, in the majority of cases, the genotypes at sites -641 and -630 (Dammerman *et al.*, 1993 and our own preliminary results based on ASO-hybridization)

we have used a pair of primers 3/4 containing all three variable bases either in the "wild type" (primer 3) or "mutant" form (primer 4). In addition to the variable markers analyzed by ASO-hybridization we used primers capable of differentiating the *SstI* polymorphic marker located in the 3'-untranslated region.

Forty DNA samples typed by ASO-hybridization were subsequently analyzed by ASA and for all of them the initial typing was confirmed. Furthermore, the sequence analysis of nine DNA samples (three DNA samples for each genotype, i.e. 1/1, 1/2 and 2/2) also confirmed the results obtained by ASO-hybridization and ASA.

Comparison of two methods

Each of the two methods described for polymorphism analysis has some advantages and disadvantages. While ASO-hybridization is more time-consuming at preliminary stages (e.g., probe labeling, purification of PCR-products, etc.) it is convenient at the last step because many samples can be analyzed simultaneously by the dot-blot procedure. The method is especially convenient for the analysis of multiple markers located in close proximity (usually within 1 kb distance) on one chromosome since the same PCR product can be used for such analysis. Hence, ASO-hybridization may be superior for epidemiological studies when a large number of samples can be analyzed at the same time and/or for the analysis of closely located multiple markers.

The main advantage of ASA is that it is a one step direct and quick method that allows genotyping using genomic DNA. However, sometimes it is difficult to eliminate non-specific extra bands which may appear in PCR products in addition to the main band (see for example Fig. 3c, lanes 2 and 3). Though these extra bands usually do not prevent the correct genotyping it is desirable to eliminate them or reduce their relative amount in order to increase the reliability of results.

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