note on methodology

Genotyping of apolipoprotein A-IV by digestion of amplified DNA with restriction endonuclease Fnu4HI: use of a tailored primer to abolish additional recognition sites during the gene amplification

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Summary Apolipoprotein A-IV (apoA-IV) is involved in the metabolism of chylomicrons and high density lipoproteins. It displays genetic polymorphism due to two co-dominant alleles apoA-IV^1 and apoA-IV^2. The mutation that causes the polymorphism is a G to T substitution in the third base of codon 360 in the apoA-IV^2 allele which results in a glutamine (Gln) to histidine (His) change of amino acid 360. This substitution leads to the abolition of a recognition site for the restriction enzyme Fnu4HI. Part of the third exon of the apoA-IV gene is amplified by the polymerase chain reaction (PCR) using a tailored primer, which abolishes the downstream recognition sites during the DNA amplification. The PCR products are digested with the restriction enzyme Fnu4HI and electrophoresed on a polyacrylamide gel. The apoA-IV genotypes are determined after staining with either ethidium bromide or silver. To validate the method, we determined the inheritance of the apoA-IV alleles in a three-generation kindred of 8 subjects and analyzed amplified DNA of 32 subjects of different apoA-IV phenotypes with this method. The results were compared to those obtained from isoelectric focusing and immunoblotting. In all cases studied, the two methods gave concordant results. —**Tenkanen, H.** Genotyping of apolipoprotein A-IV by digestion of amplified DNA with restriction endonuclease Fnu4HI: use of a tailored primer to abolish additional recognition sites during the gene amplification. *J. Lipid Res.* 1991. **32:** 545–549.

Supplementary key words genetic polymorphism • polymerase chain reaction • restriction enzyme analysis

Apolipoprotein A-IV (apoA-IV) is a major component of newly synthesized chylomicrons (1, 2) but is dissociated from them soon after they reach the blood stream. In human plasma, the majority of apoA-IV is present in the lipoprotein-deficient fraction (3–5). The physiological function of apoA-IV is unknown although it has been proposed to be of importance for the metabolism of chylomicrons and HDL (6), to activate a plasma factor that converts HDL subclasses (7), and to promote cholesterol efflux from cells to lipoproteins and thereby to participate in reverse cholesterol transport (8). It has also been demonstrated that apoA-IV, at least in vitro, can activate lecithin:cholesterol acyltransferase (9, 10).

Genetic polymorphism of apoA-IV has been demonstrated using two-dimensional electrophoresis (11, 12) or isoelectric focusing (IEF) (13–15). Genetic studies are consistent with a single locus with two main co-dominant alleles A-IV^1 and A-IV^2. The polymorphism has been reported to have an effect on plasma cholesterol and triglyceride levels (16, 17) and it may thus influence susceptibility to atherosclerosis. Recently, the mutation causing this polymorphism has been unraveled (18, 19). It is a point mutation localized to codon 360 where a single G to T substitution in the third base of amino acid 360 leads to a glutamine (Gln) to histidine (His) substitution. This substitution results in the loss of a Fnu4HI enzyme restriction site and is therefore a potential target for restriction enzyme analysis. However, the use of this restriction enzyme for screening the polymorphism from amplified DNA has not been considered feasible because there are two additional cleavage sites for this enzyme, 9 and 12 nucleotides further downstream, in both alleles of the apoA-IV gene. The restriction fragments generated are therefore short, which makes genotyping impossible.

To overcome these problems we have developed a new method. The use of a tailored primer in the polymerase chain reaction changes the downstream cleavage sites for Fnu4HI and results in products that makes the use of this enzyme feasible for apoA-IV typing.

MATERIALS AND METHODS

ApoA-IV phenotyping

Phenotype analysis was performed by immunoblotting after isoelectric focusing of serum (15).

DNA preparation

DNA was isolated from 5 ml of EDTA blood from subjects belonging to different apoA-IV phenotypes (20).

Amplification of DNA

Genomic DNA (0.2 μg) isolated from apoA-IV 1-1, 1-2, and 2-2 subjects was amplified by the polymerase chain reaction (21). The primers were chosen according to the published apoA-IV gene structure (22), I: 5'-CCT GAG GGA CAA GGT CAA CTC-3' (nucleotides 2285–2305), II: 5'-CAC CTG CTC CTG CTA* CTG CTC C-3' (2411–2390). Primers for PCR were synthesized on an Applied Biosystems model 381A synthesizer (23). The PCR was performed in a reaction mixture containing 0.5 μM of primers, 0.2 mM each of dATP, dCTP, dGTP, and

Abbreviations: apoA-IV, apolipoprotein A-IV; Gln, glutamine; His, histidine; HDL, high density lipoproteins; IEF, isoelectric focusing; PCR, polymerase chain reaction; bp, base pairs.

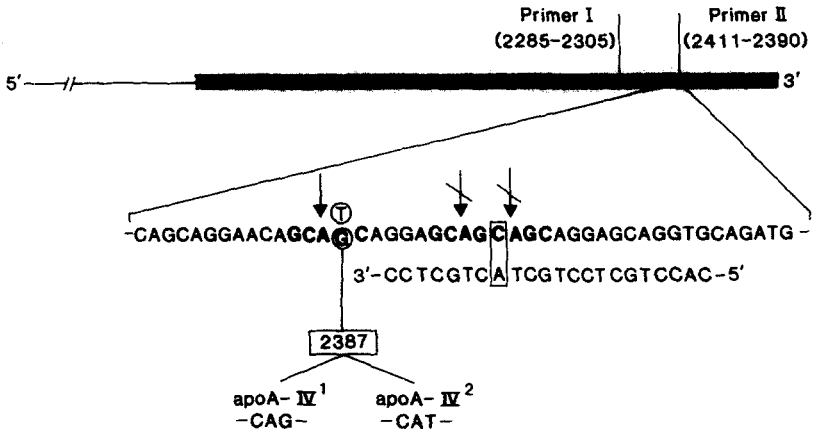

Fig. 1. Schematic presentation of the exon 3 of the apoA-IV gene. In the upper part of the diagram the positions of the primers used to amplify part of exon 3 (2285-2411) by the polymerase chain reaction are indicated. In the lower part an enlarged region of exon 3, adjacent to the polymorphic site located at nucleotide 2387, is presented. In the apoA-IV^1 allele the three recognition sites for the Fnu4H1 restriction enzyme (GCNGC) are indicated in boldface letters. In the tailored Primer II the modification, an A is inserted instead of a G, which leads to the abolishment of the two downstream recognition sites is indicated by a square. Thus, after amplification of the apoA-IV^1 allele, there is only one Fnu4HI restriction site left while the two downstream recognition sites have disappeared (crossed arrows). The product obtained after amplification of the apoA-IV^2 allele using the tailored primer is without recognition sites for the Fnu4H1 enzyme.

dTTP, 20 mM Tris- HCl, pH 8.8, 1.5 mM $MgCl_2$, 15 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 0.1 mg/ml gelatin, and 2 units of *Thermus aquaticus* (Taq) DNA polymerase (Perkin Elmer Cetus Inc.), in a final volume of 100 μl. The cycles of denaturation (1 min at 95°C), annealing (1 min at 60°C), and elongation (1 min at 72°C) were repeated 25 times.

Digestion with Fnu4HI

A 15-μl aliquot of each PCR reaction mixture was used without further purification for analysis. Incubation buffer (New England BioLabs) and distilled water up to a final volume of 30 μl were added. Samples were digested with 3 units of Fnu4HI restriction enzyme (New England Biolabs) for 3 h at 37°C.

Analysis of restriction fragments

After treatment with restriction enzyme, the digested DNAs were loaded onto a 12% polyacrylamide gel (1.5 mm thick × 25 cm long) and electrophoresed for 80 min, 200 V, 4°C. After electrophoresis the gel was stained either with ethidium bromide (24) or silver (25).

RESULTS

The mutation causing the common apoA-IV polymorphism results in disappearance of a restriction site for the Fnu4H1 restriction enzyme (18, 19). However, recognition sites for this enzyme are also present 9 and 12 nucleotides downstream. We amplified part of exon 3 of the apoA-IV gene using a tailored PCR primer which abolishes the downstream recognition sites for the Fnu4HI restriction endonuclease.

The unmodified primer (primer I, 2285-2305) starts 102 nucleotides upstream from the mutation point at nucleotide 2387 (**Fig. 1**). This sequence does not have recognition sites for the Fnu4H1 enzyme. In the tailored primer (primer II, 2411-2390), 5'-CAC CTG CTC CTG CTA* CTG CTC C-3', an A is inserted at the 2404 position instead of a G that would be the nucleotide corresponding to the sequence of apoA-IV (Fig. 1). Incorporation of this sequence into the apoA-IV DNA during amplification leads to the disappearance of the two Fnu4H1 restriction sites present downstream from the point mutation at nucleotide 2387. The amount and quality of the PCR products were not affected by the use of a modified primer (data not shown). After 25 PCR cycles about 1 μg of amplified DNA was obtained. The PCR products were digested with the Fnu4H1 restriction enzyme without prior purification of the amplified fragments. **Fig. 2** shows a 12% polyacrylamide gel on which the digested PCR products from individual probands belonging to different apoA-IV phenotypes have been electrophoresed and thereafter stained with ethidium bromide. Upon electrophoresis of the amplified DNA from subjects homozygous for the apoA-IV^1 allele (Fig. 2, lane 1), one main 101 base pair (bp) band corresponding to nucleotides 2285-2385 is visible. The digested amplified DNA from subjects homozygous for the apoA-IV^2 allele again resulted in one main 127 bp band corresponding to nucleotides 2285-2411 (Fig. 2, lane 2). In subjects heterozygous for the two apoA-IV alleles, both bands were visible (Fig. 2, lane 3). From this banding pattern the

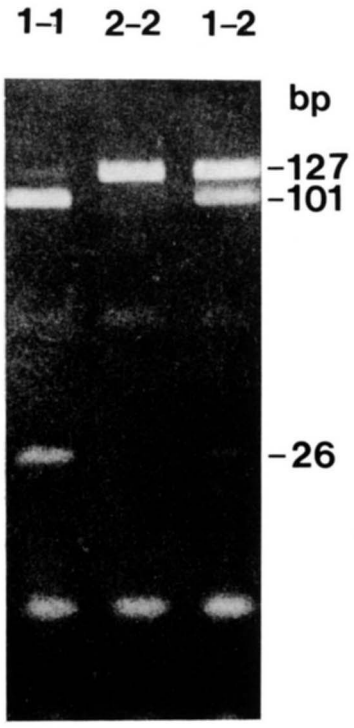

Fig. 2. The common apoA-IV polymorphism detected by Fnu4HI enzyme restriction fragments after polymerase chain reaction on ethidium-bromide stained polyacrylamide gel. Polyacrylamide gel electrophoresis of digested amplified DNA from subjects with the apoA-IV phenotypes 1-1 (lane 1), 2-2 (lane 2), and 1-2 (lane 3). A subject homozygous for the apoA-IV^1 allele (lane 1) is defined by a 101 bp fragment, a subject homozygous for apoA-IV^2 allele (lane 2) by a 127 bp fragment, and a subject heterozygous for the apoA-IV alleles by both fragments (lane 3).

apoA-IV phenotypes can easily be determined. In addition to these strong bands, a weak 26 bp band, derived from digestion of the amplified 127 bp fragment of apoA-IV 1-1 and 2-1 subjects, could also be seen. An alternative method to visualize the fragments is to stain the gel with silver (**Fig. 3**). Silver staining can also follow the ethidium bromide treatment.

To validate the method, the DNAs from a kindred consisting of three generations were amplified and digested with Fnu4H1 (**Fig. 4**). It is evident that the results obtained by isoelectric focusing, sequence analysis of amplified DNAs (19), and by the present method all are in accord. Also, the results from analysis of DNA from 32 subjects (15 apoA-IV 1-1, 14 apoA-IV 2-1, 3 apoA-IV 2-2) using this method were in accord with those obtained by isoelectric focusing and immunoblotting.

DISCUSSION

This report describes a new method for the determination of the alleles of the human apoA-IV polymorphism using tailored DNA amplification of part of the gene followed by cleavage with the restriction enzyme Fnu4H1.

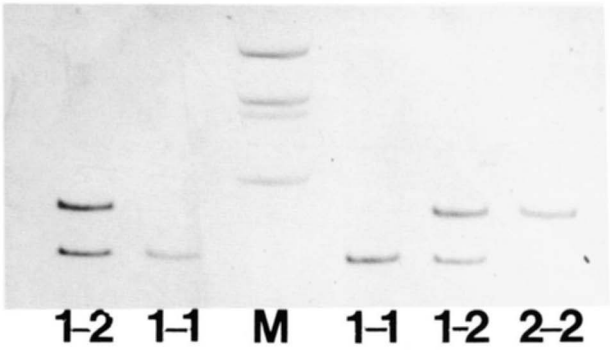

Fig. 3. Visualization by silver staining of Fnu4HI enzyme restriction fragments of amplified DNA from subjects with different apoA-IV phenotypes. The digested amplified DNAs subjected to polyacrylamide gel electrophoresis were from subjects with the apoA-IV phenotypes 1-2 (lane 1), 1-1 (lane 2), 1-1 (lane 4), 1-2 (lane 5), and 2-2 (lane 6). The sizes of the molecular weight marker (lane 3) from the bottom are: 154, 220, 234, 298 bp.

Modified PCR primers have been used to create new restriction sites especially for cloning purposes. In this work a modified PCR primer was used to abolish restriction sites. The tailored primer used in the present study eliminates the two adjacent Fnu4H1 restriction sites during the amplification and makes the use of this enzyme feasible for restriction enzyme analysis of the product. To validate the method, the apoA-IV genotypes of 40 subjects determined by this method were compared to those obtained by isoelectric focusing and immunoblotting. It was evident in all these cases that the G to T substitution at nucleotide 2387, causing the disappearance of the Fnu4H1 enzyme restriction site, in the apoA-IV^2 allele is the sole cause for the difference in charge detected by isoelectric focusing.

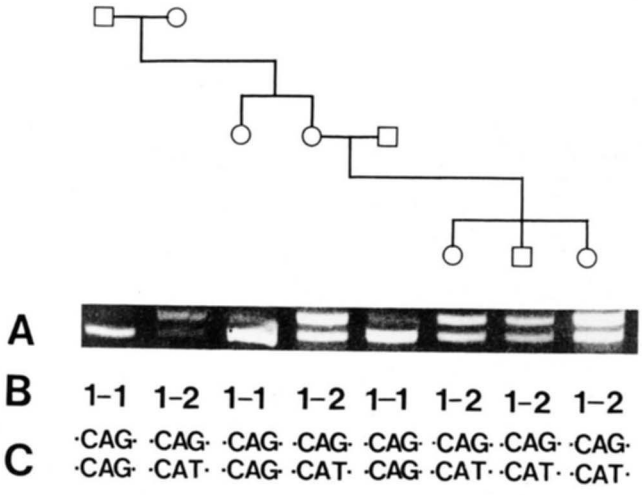

Fig. 4. The inheritance of apoA-IV alleles in a three generations kindred. The banding pattern obtained of Fnu4H1 digested amplified DNA from the eight subjects on eithidium bromide-stained polyacrylamide gel (A) is compared to results obtained by isoelectric focusing (B) and direct sequencing of amplified DNA (C).

Recently, the genetic basis behind isoproteins apoA-IV-0 and apoA-IV-3 has been resolved (26). In the apoA-IV^3 allele, a G to A substitution in the first base of codon 230 causes a glutamic acid to lysine change in the mature protein. This allele cannot be detected by the method presented but it does not disturb the typing of the common alleles either. In the apoA-IV^0 allele there is an insertion of 12 nucleotides (GAA CAG CAG CAG) in the carboxy-terminal region of the apoA-IV gene between nucleotides 2390 and 2391 which in the mature protein leads to a Glu-Gln-Gln-Gln insertion between residues 361 and 362. As the nucleotide preceding nucleotide 2391 also in this variant is a G, the tailored primer (Primer II, 2411–2390) should be able to use this variant as a template. The inserted sequence contains an additional Fnu4H1 restriction site. This would lead to the formation of an additional 12 bp fragment in the digests of amplified DNA of phenotype apoA-IV 1-0 type. A fragment of this size is difficult to observe on the polyacrylamide gel. In order to detect the apoA-IV^0 mutant, the PCR products should also be electrophoresed without digestion as the addition of 12 bp to a 127 bp fragment can easily be detected on a 12% polyacrylamide gel. In subjects with the apoA-IV 2-0 phenotype, the 113 bp Fnu4H1 restriction fragment corresponding to the apoA-IV^0 allele would be detected.

According to recent reports the allelic proteins apoA-IV 1-1 and apoA-IV 2-2 behave differently in activating LCAT (19, 27). Thus the apo-A-IV polymorphism may influence reverse cholesterol transport and thereby play a role in atherogenesis. In this regard, a DNA-based screening method for the apoA-IV polymorphisms should be of help in elucidating the mechanism that underlies this phenomenon.

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