Determination of Apolipoprotein E Genotypes by Single-Strand Conformational Polymorphism

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We used single-strand conformational polymorphism (SSCP) to determine apolipoprotein E (Apo E) genotypes in 47 individuals. A 295-base-pair (bp) DNA fragment coding for amino acid residues 80–178 of the Apo E protein gave distinct patterns for the three alleles. When we used SSCP to determine the Apo E polymorphism of five individuals whose phenotyping results differed from those of genotyping, the SSCP results agreed with the genotyping results obtained by the PCR-based amplification refractory mutation system (ARMS). Because most of the reported rare alleles of the Apo E gene involve mutations of amino acid residues in positions 120–160, our SSCP method is useful for determining rare as well as common alleles.

Indexing terms: electrophoresis, polyacrylamide • gene mutations • lipids

Apolipoprotein E (Apo E) functions as a ligand for the receptor-mediated uptake and clearance of chylomicrons and very-low-density lipoprotein (VLDL) remnants by the liver (1).5 Three common alleles of the Apo E gene, ε4, ε3, and ε2, code for the E4, E3, and E2 isoforms of the proteins, respectively. Consequently, six phenotypes, three homozygous and three heterozygous, are present in the general population. The three isoforms differ from each other at one or both of the amino acid residues at positions 112 and 158 of the protein. Isoform E4 has arginine at positions 112 and 158; E2 has cysteine at positions 112 and 158; and E3, the most common allele, has cysteine at position 112 and arginine at position 158 (2). The receptor-binding region of Apo E is thought to be between amino acid residues 120 and 160. This may explain why Apo E2, which differs from E3 at position 158, has only 1–2% of the receptor-binding activity of E3 (2). The low affinity of Apo E2 for receptor binding is thought to be responsible for type III hyperlipidemia in individuals homozygous for the E2 allele. Phenotyping of Apo E by isoelectric focusing is therefore useful in confirming the diagnosis of this disorder.

Because polymorphism of Apo E is considered one of the most important known genetic factors affecting serum low-density lipoprotein cholesterol concentration (3), Apo E phenotyping has been included in many epidemiological studies and studies on the effects of diet and lipid-lowering drugs on serum lipid profiles. Prior to the sequencing of the Apo E gene, Apo E isoforms had been determined most frequently either by isoelectric focusing of isolated VLDL followed by nonspecific staining or by isoelectric focusing of serum followed by immunoblotting with serum containing specific antibody to Apo E. With the availability of the Apo E gene sequence and the recognition of the specific single-base substitutions responsible for the three common Apo E alleles (4), several genotyping methods based on the polymerase chain reaction (PCR) have been developed (5–7).

In general, interpretation of Apo E phenotyping results is ambiguous because the sialic acid residues on the Apo E protein cause the isoforms to migrate as multiple bands. Glycation of the Apo E protein, particularly in diabetic individuals, further complicates interpretation. PCR-based methods generally give easily interpretable results, but because these methods specifically examine residues 112 and 158, they can potentially misclassify rare Apo E mutants with substitutions at other amino acid residues (8). A recent report has addressed the relatively large percentage (15%) of discrepancies between genotyping and phenotyping results (9). Single-strand conformational polymorphism (SSCP) analysis is generally used to screen for unknown mutations in DNA fragments up to several hundred bases in length. After dissociation of the double-stranded PCR product, each of the two single-stranded fragments assumes a folded conformation. Changes in conformation occur in one or both strands when the DNA sequence is altered, as in the case of a single-base substitution. The alteration in conformation can then be detected as changes in electrophoretic mobilities in polyacrylamide gel electrophoresis. In the current study, we used SSCP for Apo E genotyping and examined the cause of discrepant results between genotyping by the amplification refractory mutation system (ARMS) and phenotyping by isoelectric focusing.

Materials and Methods

DNA Preparation

We isolated DNA from peripheral blood by using a commercially available kit from Puregene (Gentra Systems, Minneapolis, MN). Blood was initially obtained from 20 individuals who were participants in several
studies on the effects of drugs and diet on serum lipid profiles. All individuals were characterized with respect to Apo E phenotype by the various laboratories taking part in the different studies. DNA was also isolated from 27 additional individuals for comparing Apo E genotyping by ARMS and SSCP. This study was approved by the Human Study Committee of the University of Minnesota Institutional Review Board.

Apo E Phenotyping

Apo E phenotyping of all individuals was done by isoelectric focusing of VLDL followed by Coomassie Blue staining. For four of the five individuals whose results from phenotyping did not agree with genotyping, Apo E phenotyping was repeated in our own laboratory by the method described by Warnick et al. (10).

ARMS Analysis of Apo E Genotype

We performed ARMS analysis with primer sequences according to the method described by Wenham et al. (7). Briefly, we set up four PCR reactions with the four different ARMS sense primers and a single common antisense primer. The amplification cycles were as follows: denaturation at 95 °C for 5 min, primer annealing at 58 °C for 30 s, and primer extension at 65 °C for 1 min. This was followed by 24 cycles of denaturation at 95 °C for 1 min, primer annealing at 58 °C for 30 s, and primer extension at 65 °C for 1 min. Reactions were carried out in an Ericomp Twin Block Thermal Cycler (Ericomp, San Diego, CA). The amplified DNA products were subjected to 2% agarose gel electrophoresis (containing ethidium bromide) and viewed under an ultraviolet transilluminator.

PCR for Apo E Fragments Used in SSCP

We used three different primer sets to amplify three different PCR fragments. For the oligonucleotide primers used in primer set 1 the sequences were 5′GAAACACTGACCCGTTGCCCAG3′ (sense) and 5′GGAATGGCGCCTAGGGCGCGCTC3′ (antisense). This set encompasses the sequence from nucleotide 3649 to 3943 (codon 80–178) and should generate a 295-bp product. Primer sets 2 and 3 produce shorter fragments. In primer set 2 we used the sense primer from set 1 and 5′TCATCGGCATCCGGAGGAGCCG3′ as the antisense primer. This set should generate a 224-bp product (nucleotides 3649–3872, codon 80–154). In primer set 3 we used 5′TGCGATACCGCGCCAGGTTGCG3′ (sense) and the antisense primer from set 1, generating a 186-bp fragment (nucleotides 3758–3943, codon 116–178). All three 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 total volume of 100 μL, containing 10 mM Tris (pH 8.3), 1.0 mM MgCl₂, 50 mM KCl, 0.02 μmol of each of the four deoxynucleoside triphosphates, 10 μL of dimethyl sulfoxide, and 20 pmol each of the primers. The reaction mix was overlaid with mineral oil. Amplification cycles were as follows: 30 cycles of denaturation at 95 °C for 1 min, primer annealing at 65 °C for 30 s, and primer extension at 70 °C for 75 s. This was followed by one cycle of denaturation at 95 °C for 1 min, primer annealing at 65 °C for 30 s, and primer extension at 70 °C for 7 min. PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide.

SSCP Analysis

SSCP was carried out with a Mini-Protean II (Bio-Rad, Richmond, CA) vertical gel. PCR product (8 μL) was lyophilized and reconstituted with 4 μL of water, mixed with 2 μL of formamide and 2 μL of loading dye (2.5 g/L bromphenol blue, 2.5 g/L xylene cyanole, and 300 mM/L glycerol). This mixture was denatured for 5 min at 95 °C and cooled rapidly at 4 °C before loading onto a polyacrylamide gel. Electrophoresis was performed in a Tris–borate–EDTA buffer (pH 8.0). The gel concentrations and running conditions were as follows: 150 g/L polyacrylamide, 16 h, room temperature, 45 V (PCR primer set 1); 150 g/L polyacrylamide/100 mM/L glycerol, 2.5 h, 4 °C, 200 V (PCR primer set 2); and 150 g/L polyacrylamide, 2.5 h, 4 °C, 200 V (PCR primer set 3). After electrophoresis, gels were stained with a Bio-Rad silver staining kit.

Results

Figure 1A shows our SSCP results for the 295-bp fragments obtained with primer set 1. All three homozygous genotypes showed two intensely stained bands representing the major conformers of the two single strands. Minor conformers also appeared as lightly stained bands. The major slow-moving band of the e2
e3, and e4 alleles had similar mobilities. However, of the faster-moving strands, the fragment coded by the e3 allele moved fastest (F1), followed by that of e2 (F2), and the DNA of e4 allele (F3). The differences in the mobility of the major fast-moving strands in the three alleles allowed for identification of the three homozygous as well as the three heterozygous states.

We repeated Apo E phenotyping in our own laboratory on DNA samples from four of the five individuals whose original phenotyping results did not agree with those of genotyping by ARMS. For two individuals the results obtained by phenotyping (3/3, 2/3) again did not agree with those obtained by genotyping (2/3, 3/3, respectively). Plasma from the fifth patient was not available in sufficient quantities for phenotyping confirmation. Figure 1B shows that for these five individuals the results from SSCP analysis were in agreement with those obtained from genotyping by the ARMS method. Apo E genotyping was done on a total of 47 individuals by both ARMS and SSCP with use of primer set 1. The results obtained by these two methods agreed completely in all 47 individuals (three 2/2, four 2/3, one 2/4, seventeen 3/3, sixteen 3/4, and six 4/4).

Figures 2A and B show SSCP patterns of DNA fragments amplified from primer set 2 (nucleotides 3649–3872, codon 80–154) and primer set 3 (nucleotides 3758–3943, codon 116–178). As might be expected, the fragment amplified from primer set 2, which includes the coding sequence for amino acid 112, showed similar migration patterns for DNA coded by e3 and e2 alleles. In contrast, Apo E4 was characterized by the presence of band B, and E3 and E2 were characterized by the presence of band A (Figure 2A). The fragment amplified from primer set 3 contained the coding sequence for amino acid 158 and showed different migration patterns between the allele coded by e2 and those coded by e3 or e4. The DNA fragment coded by the e2 allele showed two major bands, whereas e3 and e4 showed three bands. The E2 genotype was characterized by the presence of a faster moving band (band D), whereas the E3 and E4 genotypes were characterized by the presence of two slower moving bands (bands A, B) (Figure 2B). A band with intermediate mobility (band C) was present in all genotypes.

Discussion

SSCP is a recently introduced method for detecting single-base substitutions, small insertions, and small deletions in DNA fragments up to several hundred bases in length (11). This technique has been used for the detection of mutations in several disorders such as phenylketonuria (12), ornithine transcarbamylase deficiency (13), and ornithine δ-amino transferase deficiency (14).

We demonstrated that SSCP can be used successfully to distinguish the three common Apo E alleles that differ from each other by either one or two single-base substitutions by using a 295-bp fragment that contains both sites of substitution. In addition, we used shorter fragments containing sequences coding for either amino acid residues 112 or 158, and demonstrated that they can be used to distinguish E3 from E4 and E3 from E2, respectively. Although it is impractical and unnecessary to use these shorter fragments for routine analysis, these experiments further demonstrate the usefulness of SSCP in detecting single-base substitutions. For routine studies, only primer set 1 need be used.

The current study was initiated because we found a large number of discrepancies (5/20, or 25%) between the results of genotyping by using the ARMS method and results of phenotyping obtained from different laboratories. When phenotyping was repeated in our own laboratory for four of the five available samples, two of the four did not agree with the genotyping results. Because ARMS examines specific single-base substitutions, error may result when one is assigning genotypes to relatively uncommon alleles involving mutations at amino acid residues at positions other than 112 or 158. Most of those rare mutants reported to date are in the region of the Apo E gene coding for residues 120–160 (8). Unlike ARMS, which enables one to distinguish known mutations at specific sites from the wild type by using primers in PCR reactions specifically matching either the wild or mutant allele, one can use SSCP to detect single-base substitutions in the entire sequence of the PCR product.

SSCP analysis was useful for screening for the six genotypes of Apo E polymorphism. Analysis of five individuals whose genotypes determined by ARMS differed from phenotypes obtained by isoelectric focusing showed that SSCP and ARMS results were in agreement. We used a fragment of 295 bp that covers the sequences coding for amino acid residues 80–178 of the Apo E gene. The migration patterns of all five individuals with discrepant ARMS and phenotyping results corresponded to the three known Apo E alleles. Although SSCP is not always 100% sensitive, previous studies in our laboratory with 10 patients with ornithine transcarbamylase deficiency demonstrated that in

![Fig. 2. SSCP analysis of DNA fragment from primer set 2 (A), and from primer set 3 (B)](image-url)

(A) Note the different migration patterns of E3 and E2 vs E4. Band B characterizes E4; band A is present in both E3 and E2. In lane 4, an E3/E4 heterozygote is characterized by the presence of both band A and band B. Lane 1, E2/E2; lane 2, E2/E3; lane 3, E3/E3; lane 4, E3/E4; lane 5, E4/E4. (B) Note the different migration patterns of E2 vs E3 and E4. Band D characterizes E2; bands A and B are present in E3 and E4. Band C is common to all three alleles. Lane 1, E2/E2; lane 2, E2/E3; lane 3, E3/E3; lane 4, E3/E4; lane 5, E4/E4.

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the DNA of each affected patient, only one of the 10 exons showed an altered SSCP pattern. Also, direct DNA sequencing confirmed the existence of mutations in exons with altered SSCP patterns (13, 15). Thus, both our own data and those of others (11–14) show that SSCP is highly sensitive and the possibility that the five discrepant results between the ARMS and phenotyping methods were due to rare mutations would be unlikely. We also tried another screening method—denaturing gradient gel electrophoresis (16). In our hands, this method was more difficult than SSCP and did not give us satisfactory resolution of the different Apo E genotypes.

In summary, we demonstrated that SSCP is a useful method for determining Apo E genotypes. In addition to determining the three common alleles (E2, E3, E4), SSCP can also potentially detect the more rare mutations. However, more studies are required to determine the usefulness of this method in screening for the rare mutations of Apo E.

References