Use of Polymerase Chain Reaction to Detect Heterozygous Familial Hypercholesterolemia

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We used a modification of the polymerase chain reaction (PCR), involving two pairs of oligonucleotide primers, to detect a mutation in the low-density lipoprotein (LDL) receptor gene, commonly occurring among patients with familial hypercholesterolemia (FH) in Finland. This mutation, called FH-Helsinki, involves a large (about 9500 base pairs, bp) deletion in the LDL receptor gene extending from intron 15 to exon 18. For the PCR, one pair of primers was designed to cover both sides of the deletion in its immediate vicinity. In the presence of the deletion, the primers were brought close enough to each other to allow the amplification and electrophoretic detection of a 300-bp amplification product. In the absence of the deletion, no amplification occurred and this band accordingly was not visible in the gel. To render the interpretation of the results unequivocal, we designed a second pair of oligonucleotide primers. This pair of primers allowed another amplification product (158 bp) to appear in samples containing a normal exon 17, i.e., in DNA specimens from healthy subjects and FH heterozygotes with or without the FH-Helsinki deletion. The technique is easy to perform, avoids the use of radioactive reagents, and is applicable to the detection of any extensive DNA deletion.

Additional Keyphrases: gene probes · low-density lipoprotein receptors · electrophoresis, agarose

A specific deletion in the low-density lipoprotein (LDL) receptor gene, designated FH-Helsinki, is found in at least one-third of Finnish patients heterozygous for familial hypercholesterolemia (FH) (1, 2). The 9.5-kb (kb = kilobase pairs) deletion leads to loss of exons 16 and 17 and part of exon 18. The mutation appears to give rise to a phenotype in which internalization of LDL particles by the cells is defective (2). The high prevalence of the deletion justifies the development of a convenient assay for detecting the mutation by using DNA techniques. The deletion can be identified by analysis of genomic DNA, with use of a conventional Southern blotting technique with radioactive probes (1).

Here we present another method for detecting the FH-Helsinki deletion, by using the polymerase chain reaction (PCR) (3). PCR allows rapid visual detection of DNA deletions in ethidium bromide-stained agarose or polyacrylamide gels (4, 5). Small deletions, from a few to several hundreds of base pairs (bp), can be identified in this way by means of the length differences between the amplified DNA fragments from controls and patients. However, longer deletions are likewise amenable to PCR detection. Thus, if the amplification primers are chosen to represent DNA stretches flanking the two ends of the deletion, the deletion brings the primers close enough to allow DNA amplification; in the absence of the deletion, this technique fails to show any bands, the upper limit for PCR amplification being in the range of 2000 bp (5). To avoid the problem of a false-negative result, we have designed two pairs of amplification primers and used a "duplex" PCR reaction (5, 7) to be able to verify with certainty the presence or absence of the FH-Helsinki mutation.

Materials and Methods

The samples for PCR were obtained from nine patients heterozygous for FH and from normal controls. The diagnosis of FH was based on typical clinical features, including a total serum cholesterol concentration >9.5 mmol/L, the presence of tendon xanthomas, and a positive family history (at least one first-degree family member with hypercholesterolemia), and was confirmed by establishing a...
functional defect in the LDL receptor activity in cultured skin fibroblasts from the patients (2).

Template DNA was prepared either by extracting genomic DNA by a standard technique involving digestion with proteinase K, phenol extraction, and ethanol precipitation (3), or by obtaining DNA from lysed leukocytes. Cell lysis was performed by incubating about 100,000 leukocytes in 100 μL of distilled water at 95 °C for 5 min (6). Before cell lysis, erythrocytes were removed by sedimenting them with the aid of dextran (median molecular mass 500 kDa; Pharmacia, Uppeala, Sweden), 6 g/L, for 30–50 min at room temperature.

About 500 ng of extracted DNA or DNA from 10 μL of cell lysate was used as a template DNA for PCR. Similar results were obtained with DNA samples prepared with the two different methods. The oligonucleotide primers from both sides of the deletion were designed according to the published LDL receptor cDNA sequence (9); the sense primer (a) was derived from exon 15 and the antisense primer (a′) from exon 18 (Figure 1). "Control primers" (b) and (b′) were derived from exon 17 (Figure 1).

The PCR reaction was performed according to the method described by Seiki et al. (3). The PCR mixture (100 μL) contained the template DNA and, per liter, 0.6 μmol of each oligonucleotide primer and 200 μmol each of dATP, dCTP, dGTP, and dTTP in "Taq" buffer (containing, per liter, 1.5 mmol of MgCl₂, 50 mmol of KCl, 10 mmol of Tris, pH 8.3, and 0.1 g of gelatin). After an initial denaturation step for 10 min at 95 °C, 1 U of the "Taq" polymerase enzyme (Cetus Corp., Berkeley, CA) was added, the mixture overlaid with 100 μL of mineral oil, and the amplification reaction performed as follows: 1.5 min at 95 °C for denaturation, 1.2 min at 56 °C for primer annealing, and 2 min at 72 °C for extension. The last (30th) extension reaction at 72 °C was prolonged to 10 min. Amplification cycles were carried out by using a programmable heater block (Techno, Cambridge Ltd., Cambridge, U.K.). After amplification, we applied 20 μL of the amplification product to a 2% agarose gel containing ethidium bromide and ran it for 1.5 h at 100 V.

For Southern blot analysis, 10-μg DNA samples were digested with the restriction enzyme Pvu II, electrophoresed in 0.5% agarose, transferred to nitrocellulose filters, and hybridized with an LDL receptor cDNA probe covering the exons 11 to 17 (I). These conditions reveal a restriction fragment length polymorphism in normal subjects (the presence of 16-kb or 14-kb fragments, or both), whereas patients with the FH-Helsinki mutation display a unique 11-kb fragment (J).

Results

Four of the nine FH patients had the FH-Helsinki deletion, as determined by the standard Southern blotting technique, and five had another, as yet unidentified, mutation of the LDL receptor gene (Figure 2A). The FH-Helsinki deletion brings the primers (a) and (a′) as close as 250 bp to each other, resulting in an amplified fragment spanning 300 bp (two 25-bp primers and 250 bp between them). These 300-bp fragments were visible in ethidium bromide-stained agarose gels whenever the FH-Helsinki mutation was present (Figure 2B, lanes 2, 4, 5, and 7).

In the absence of the FH-Helsinki mutation, i.e., in subjects without FH and in FH patients with LDL receptor gene mutations other than the FH-Helsinki type, the distance between the primers (a) and (a′) is about 9.5 kb. This distance should be long enough to permit an amplification of the spanning region between (a) and (a′). Data for five FH patients without the FH-Helsinki mutation are illustrated in Figure 2B (lanes 1, 3, 6, 8, and 9). As expected, no fragments in the 300-bp size class were visible in these lanes. For these patients, the lack of any amplified bands would have made the interpretation of results equivocal. To ensure that the amplification reaction operates correctly even in samples from FH patients without the FH-Helsinki mutation and in samples from normal individuals, we added to the reaction mixtures primers derived from exon 17, (b) and (b′) (Figure 1), capable of yielding an amplification product of 158 bp. Generation of this 158-bp fragment was demonstrated in all samples, whether positive or negative for the FH-Helsinki mutation (Figure 2B, lanes 1–9), including those from healthy controls (data not shown).

Discussion

The PCR is very useful for detecting mutations of genes with known DNA sequences. Identification of single-base changes usually requires hybridization of amplified and filter-immobilized DNA with allele-specific oligonucleotide probes (10–12) or direct sequencing of the amplification product (13–15). If a single nucleotide change creates or eliminates a restriction enzyme cleavage site, direct analysis of the mutation becomes feasible by electrophoretic analysis of the enzyme-digested DNA-amplificate covering this restriction site (16). These types of techniques have been used to set up DNA diagnostic methods for two mutations of the LDL receptor gene very prevalent in South Africaners (17).
of primers, corresponding to closely spaced intradeletional DNA stretches, would identify the normal allele. The presence or absence of the FH-Helsinki mutation was correctly identified in every case examined, as compared with the results of the standard Southern blot technique. The principle of the technique described here should also be applicable to the diagnosis of FH among French Canadians, in whom about two-thirds of the FH patients are characterized by a large (>10 kb) deletion of the LDL receptor gene (18).

In summary, our data indicate that a genetic disease characterized by a constant large DNA deletion may be diagnosed by a very convenient "duplex" PCR technique based on two pairs of oligonucleotide primers. This method is cheap, rapid, and easy to perform and circumvents the use of radioisotopes.

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References
Convenient Screening for Hemoglobin Variants by Using the Diamat HPLC System

Thomas Delahanty

Hemolysates from 102 different patients previously assessed for the presence of hemoglobin variants by cellulose acetate electrophoresis were reanalyzed with the Diamat, a microprocessor-controlled step-gradient HPLC technique designed to determine glycohemoglobin (Hgb A1c). This pool contained 81 abnormal specimens, each with one of seven different variant abnormalities. In all cases the HPLC technique correctly detected the presence or absence of a variant. The common S trait gave a large peak immediately after Hgb A, and the SS and SC variants gave clearly abnormal patterns, caused in part by the absence of Hgb A. The pattern from EE variants was distinguished by the appearance of the glycated fraction Hgb E1c, which is eluted at 4.7 instead of 4.2 min, whereas the AE pattern had two glycated peaks. Hemoglobins F, “fast”, S, and C were discerned by the presence of a major peak at 3, 5.2, 6.5, and 7.5 min, respectively. The findings suggest that the Diamat is a convenient tool to detect and help diagnose variants in a screening pool.

Additional Keyphrases: chromatography, cation-exchange, glycohemoglobin

The clinical benefits of a rapid screening method for hemoglobin (Hgb) variants in infants is well recognized (e.g., 1). However, the popular electrophoretic techniques involving cellulose acetate or agarose (2) are relatively labor-intensive and often difficult to interpret. Isoelectric focusing is the method of choice for mass screening (3), but requires special training and equipment. Although solubility, open-column, and immunoassay methods directed at specific variants can also be used (4,5), there is a need for a convenient method that will detect the presence of any variant in a screening pool. Earlier workers have shown that Hgb variants can be resolved by HPLC, but the equipment used is not readily available in clinical laboratories (6). I describe here how a commercially available technique for detecting glycated Hgb A1c (7) can be used to detect and help diagnose Hgb variants in a clinical setting by using two different operating modes. The quantification of Hgb A1c by a modified Diamat system has also been reported by Bio-Rad personnel (8).

Materials and Methods

Hemolysates of washed erythrocytes (EDTA-treated specimens) were electrophoresed according to instructions by Helena Laboratories (Beaumont, TX 77704). This cellulose acetate method (Super Z 5442) is capable of detecting the presence of the Hgb variants S, F, C, E, or “fast,” but does not distinguish between C and E. To differentiate C and E, citrate agar electrophoresis (9) was used (Titan IV, Helena; and Paragon Acid Hb, Beckman, Brea, CA 92621).

All specimens were also subjected to HPLC in the Diamat system (Bio-Rad, Hercules, CA 94547). This instrument is designed to automatically detect the relative abundance of glycated Hgb (% of total Hgb) in the blood of diabetics, with a throughput of seven samples per hour. After a simple dilution, samples are loaded in a refrigerated 48-well holder and analysis is initiated, with no further technologist intervention required. Sample components are separated by step-gradient elution of phosphates buffers matched to a specific cation-exchange column, and detected by measuring the absorbance of the effluent at 415 and 690 nm. To achieve precise retention times (Rt), one fine-tunes the Rt of the Bio-Rad A1c standard by vernier adjustment. Data reduction and chromatogram production are controlled by a microprocessor. Although the analysis routinely produces a chromatogram with a 10% scale (standard mode), for variant confirmation the scale can be converted to 100% by a simple program that extends the analysis time and allows the major peaks to be clearly delineated. The Rt and relative areas of all peaks are always reported, regardless of the scale.

Results

Standard Scale (10%)

Figure 1A depicts a typical HPLC chromatogram for glycated hemoglobin obtained when healthy patients’ blood (n = 21) was analyzed on the Diamat. Only minor peaks were eluted before the A1c peak (Rt = 4.2 min); the major peak was that for nonglycated Hgb A (A0, Rt = 6.1 min). By