Rapid Detection of 3500Q and 3531 Mutations and *MspI* Polymorphism in Exon 26 at the Apolipoprotein B Gene

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Several environmental and genetic factors are associated with high levels of cholesterol. Hypercholesterolemia is the main phenotype of Familial Defective Apolipoprotein B and Familial Hypercholesterolemia that are caused by mutations at the apolipoprotein (apo) B and LDL receptor genes, respectively. Identification of the specific genetic alteration associated with hypercholesterolemia is an important issue in clinical diagnosis of high risk for CAD. Apo B gene mutations and polymorphisms are usually screened by SSCP, DGGE, and heteroduplex, which must be confirmed by DNA sequencing or by direct detection using PCR techniques. In this study, we have optimized a PCR-RFLP procedure for identification of 3500Q and 3531 mutations and *MspI* polymorphism at the apo B gene. The technique

can be performed in a single reaction, using the restriction endonuclease *MspI* for simultaneous detection of 3500Q mutation and *MspI* polymorphism, and *NsiI* for detection of 3531 mutation. The procedure was validated by analysis of control DNA samples from individuals carrying these mutations. Screening of 186 Brazilian hypercholesterolemic individuals showed that the frequency of the M-allele (7.8%) of *MspI* polymorphism was similar to that found in other individuals with CAD. However, neither 3500Q nor 3531 mutations were detected in this group. In conclusion, this procedure is simple and rapid, being easily introduced in clinical laboratories for direct detection of the more frequent mutations at the apo B gene associated with hypercholesterolemia. J. Clin. Lab. Anal. 15:35–39, 2001. © 2001 Wiley-Liss, Inc.

Key words: apolipoprotein B gene; hypercholesterolemia; FDB mutations; DNA polymorphism; genetic markers

INTRODUCTION

Coronary artery disease (CAD) is a multifactor disease (1). Several genetic factors, including variations in genetic loci responsible for lipoprotein structure and metabolism, and low density lipoprotein receptor (LDLR) may contribute to the development of CAD. Some of these genetic variations are associated with increased levels of serum lipids and, therefore, can be associated with high risk for CAD (2–7).

High cholesterol levels have been associated with mutations at the LDLR gene that lead to the Familial Hypercholesterolemia (FH) (8). However, hypercholesterolemia is also observed in the presence of mutations at the apolipoprotein B (apo B) gene, which are responsible for the Familial Defective Apolipoprotein B (FDB). The apo B 3500Q mutation was the first described alteration at the apo B gene associated with FDB, which changes glutamine to arginine at position 3500 in apo B protein. In addition, other genetic forms of FDB have been described: CGG-to-TGG change in codon 3500 (Arg₃₅₀₀→Trp), that is denominated 3500W, and CGC-to-TGC change in codon 3531 (Arg₃₅₃₁→Cys) (9,10). Recently, another mutation, in codon 3405 (Glu₃₄₀₅→Gln), has

also been associated to FDB (11). These mutations affect the functional domain of the apo B apolipoprotein, which reduces the LDLR binding affinity (12,13).

Considering the phenotypic similarity between FH and FDB, the accurate clinical diagnosis requires additional functional and genetic tests. The differential diagnosis based on functional tests requires expensive, laborious, and time-consuming methods performed by clinical laboratories.

Despite the rising applications of the nucleic acid-based diagnosis, the screening for a large number of mutations affecting both LDLR and apo B genes is not yet affordable for routine laboratories.

Mutations at the apo B gene can be screened by several methods, such as SSCP, DGGE, or heteroduplex analysis.

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These methods must be confirmed by DNA sequencing or by direct detection using polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (PCR-RFLP) (9), electrochemiluminescence detection (14), amplification refractory mutation system (ARMS), or mutagenically separated PCR (MS-PCR) (14–19). DGGE is highly efficient and simple to perform, but it has a low throughput and requires specialized equipment to be performed (20). SSCP analysis is useful for screening of mutations, but it is very laborious, because it has many variables to be optimized (size of PCR product, temperature, and other gel-electrophoresis features). Heteroduplex analysis has a high throughput, but it does not allow discrimination between homozygous wild type and mutated DNA (15). Also, these techniques require DNA sequencing for identification of the specific mutation, which is laborious and expensive. On the other hand, the available routine techniques, such as PCR RFLP (9), PCR with electrochemiluminescence detection (14), or allele specific oligonucleotide (ASO) (18), usually are able to identify a single mutation or polymorphism. Therefore, the detection of specific mutations and polymorphisms can be improved by using methods that allow the identification of various alterations in a single test.

We propose here the optimization of a procedure for screening of two mutations and one polymorphism at the exon 26 of the apo B gene, using the PCR-RFLP genotyping, that is rapid, simple, and improves the genetic identification of FDB in hypercholesterolemic individuals.

MATERIALS AND METHODS

Subjects

Blood samples were collected from 186 individuals—at a number of diagnostic centers in São Paulo City—with severe hypercholesterolemia (mean cholesterol 8.42 ± 1.97 mmol/L) after an overnight fast. The ethical committees of the involved institutions approved the study protocol, and informed consent was obtained from each participant. The control group (CG) consisted of 100 white unrelated normolipidemic individuals from São Paulo City.

Lipid Measurements

Total cholesterol (TC) and triglycerides (TG) were measured by enzymatic colorimetric tests (21,22). High-density lipoprotein cholesterol (HDL-C) concentrations were determined by enzymatic assay after phosphotungstic acid and magnesium precipitation (23). Low-density lipoprotein cholesterol (LDL-C) was calculated according to Friedewald's formula and very-low-density lipoprotein cholesterol (VLDL-C) was calculated using the formula $TG/5$ (24).

DNA Isolation and Amplification by PCR

Genomic DNA was extracted from leukocytes by a salt-in-out procedure modified in our laboratory (25). A region

of 465 bp in the exon 26 of the apo B gene was amplified by PCR using primers flanking the nucleotides 10,628 to 11,092. For this proposal, we have used the sense oligonucleotide, described by Hansen et al. (9), that creates an *MspI* endonuclease cleavage site, allowing the identification of apo B 3500Q mutation and the anti-sense oligonucleotide described by Pullinger et al. (26). This PCR product is larger than those previously proposed by Hansen et al. (9) and allow concomitant identification of 3500Q and 3531 mutation and *MspI* polymorphism at apo B gene. The PCR reaction was performed in a total volume of 100 μ L, containing 10 μ L of amplification buffer (50 mM KCl, 4 mM $MgCl_2$, 10 mM Tris-HCl pH 9.0), 10 pmol of each primer, 200 μ M of each nucleotide (dATP, dCTP, dGTP, and dTTP), 2U of *Taq* DNA polymerase (Amersham Pharmacia Biotech do Brasil, Brazil) and 0.5 μ g of genomic DNA.

The PCR was carried out in a thermal cycler, mod. 2400 Perkin-Elmer Inc. (Norwalk, CA) with a hot start at 98°C for 5 min, denaturation at 95°C for 1 min, annealing at 58°C for 1 min, and extension was optimized to 72°C for 2 min. The number of cycles was reduced to 30, when compared to the technique described by Hansen et al. (9). These modifications in the PCR reduced the total amplification time.

Restriction Genotyping

Detection of 3500Q mutation and *MspI* polymorphism was carried out by digestion of the PCR-amplified product with *MspI*. The 3531 apo B mutation was identified by the restriction with *NsiI*, at the position 122 of the amplified product.

Enzymatic digestions were carried out at 37°C for 4 hours, in a total volume of 15 μ L, using 5 U of restriction endonuclease. PCR and restriction products were identified on 3 and 1.5% agarose gel electrophoresis for 3500Q mutation/*MspI* polymorphism and 3531 mutation, respectively. DNA restriction fragments were visualized on a standard UV transilluminator, after ethidium bromide staining.

The described technique was performed using DNA samples from patients with the apo B-3500Q and apo B-3531 mutations, kindly provided by Dr. Guey-Jen Lee-Chen (Department of Biology, National Taiwan Normal University, Taipei, China), Dr. Henrik Nissen (Department of Clinical Chemistry, Odense University Hospital, Denmark), Dr. Clive Pullinger (Cardiovascular Research Institute, San Francisco, CA), Dr. Peter Hansen (Department of Clinical Biochemistry, University Hospital of Aarhus, Denmark), and Dr. Anne Tybjerg Hansen (Department of Clinical Biochemistry, Herlev Ringvej, Denmark). The 3500Q and 3531 mutations in these DNA samples were previously detected by SSCP or DGGE and confirmed by DNA sequencing (10,20,26). The 186 hypercholesterolemic individuals were screened for 3500Q and 3531 apo B mutations and *MspI* polymorphism. In previous studies, these patients were also genotyped for *XbaI*, *Ins/Del*, and 3'HVR apo B polymorphisms (27) and for *Avall*, *HincII*, and *PvuII* LDLR polymorphisms (3,4).

Homozygous samples (with the presence of the cutting site) for *MspI* polymorphism were used to control the restriction reactions. In addition, 10% of the PCR and restriction reactions were repeated randomly.

Statistical Analysis

The allelic frequency and genotype distribution for *MspI* polymorphic site were estimated by gene counting. Differences in genotype distribution were obtained by chi-square (χ^2) -test. A value of $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The aim of this study was to optimize a procedure for detection of two mutations (3500Q and 3531) and one polymorphism (*MspI*) at the apo B gene, which are associated to high cholesterol levels in several populations.

The cleavage reaction shows that the *MspI* restriction endonuclease can recognize two sites in the PCR product: at position 29, when the 3500Q mutation is absent, and at position 362 when the M+ allele of the *MspI* polymorphism is present. Therefore, for this particular genetic profile, *MspI* restriction endonuclease reaction produces three fragments (29, 103, and 333 bp). The identification of 3500Q mutation and *MspI* genotypes can be achieved by analysis of the fragment sizes provided in Table 1.

Figure 1 shows the electrophoretic profile of individuals carrying different *MspI* genotypes in presence or absence of the 3500Q mutation. As is demonstrated, the presence of the fragments 333, 362, 436, and 465 bp allows the discrimination among the *MspI* genotypes and 3500Q mutation. For this reason, the visualization of the 29- and 103-bp fragments are not necessary for identification of apo B gene alterations. Besides that, the electrophoretic profile on agarose gel has shown to be easy and accurate for identification of the different genotypes of the *MspI* polymorphism and the 3500Q apo B mutation on agarose gel.

As demonstrated in Fig. 2 the enzymatic restriction of the PCR product with *NsiI* generate a 343-bp fragment in the presence of 3531 mutation at apo B gene.

Analysis of the *MspI* polymorphism showed that the fre-

Fig. 1. Three percent agarose gel electrophoresis stained with ethidium bromide of PCR amplification and *MspI* restriction products of apo B gene. Lane 1, heterozygous for 3500Q mutation and M+M+ genotype; lanes 2 and 3, absence of 3500Q mutation and M+M+ genotype; lane 4, absence of 3500Q mutation and M+M- genotype; lane 5, absence of 3500Q mutation and M-M- genotype; lane 6, PCR product; lane M, 100-bp DNA ladder.

quency of the M+ allele was significantly higher in both, hypercholesterolemic (92.2%) and controls (87.0%). Despite of the lower frequency of M- allele (7.8%) in the hypercholesterolemic group, it is comparable (χ^2 test, $P = NS$) to that found in other individuals with CAD from Russia (10.2%) (2) and the United States (10.2%) (28). However the frequency

Fig. 2. One and one-half percent agarose gel electrophoresis stained with ethidium bromide of PCR amplification and *NsiI* restriction products of apo B gene. Lane 1, PCR product; lane 2, heterozygous for 3531 mutation; lanes 3-5, absence of 3531 mutation; lane M, 100-bp DNA ladder.

TABLE 1. Pattern of restriction fragments for the different genotypes of *MspI* polymorphism and 3500Q mutation at the apo B-100 gene

3500Q mutation	<i>MspI</i> genotype		
	M+M+	M+M-	M-M-
Homozygous for mutation	362 and 103 bp	464, 362, and 103 bp	465 bp
Heterozygous for mutation	362, 33, 103, and 29 bp	465, 436, 362, 333, 103, and 29 bp	465, 436, and 29 bp
Absence of mutation	333, 103, and 29 bp	436, 333, 103, and 29 bp	436 and 29 bp

of the M- allele is higher than that found in Chinese CAD patients (0.00) (29) and lower than the one found in hypercholesterolemic Norwegian (16%) (30). The analysis of lipid parameters of the hypercholesterolemic individuals did not show association between *MspI* genotypes and total cholesterol and LDL-C serum levels. However, M- allele and M+M- genotype has been associated with hypercholesterolemic phenotype in some studies (30,31).

Even though we did not find 3500Q and 3531 mutations in the hypercholesterolemic group, when we used the positive DNA controls, this technique has shown to be very accurate. The procedure described here can be used for screening of the more frequent mutations at the functional domain of the apo B gene. Therefore, it enables the investigation of the hypercholesterolemia related to FDB or its association with *MspI* polymorphism, as it has been demonstrated previously (30,31).

Moreover, this procedure is less laborious and it does not require confirmatory tests, as do other screening genetic tests—SSCP, DGGE, and heteroduplex. Also, the proposed procedure has higher discrimination power than other methods, such as ARMS and MS-PCR (18,19), used for identification of specific mutations at the apo B gene.

The procedure described in this study is simple, rapid, and does not require specialized equipment, allowing the identification of two mutations and one polymorphic site at the apo B gene in a single assay. With these characteristics, we could introduce this method in clinical laboratory routines for FDB screening and *MspI* genotyping; this would be quite useful in the management of the hypercholesterolemia as well as in epidemiological studies.

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