


Identification of Two LDL-Receptor Mutations Causing Familial Hypercholesterolemia in Indian Subjects by a Simplified Rapid PCR-Heteroduplex Method, Tester F, Ashavaid,1 Alfaj A. Kondkar,1 and Kappiareth G. Nair2 (Research Laboratory, P. D. Hinduja National Hospital & Medical Research Center, Mumbai 400 016, India; * author for correspondence: fax 91-22-4449151, e-mail dr_tashavaid@hindujahospital.com)

The molecular analysis of familial hypercholesterolemia (FH), an autosomal dominant disease caused by a multitude of LDL receptor (LDLR) gene mutations, is complicated by mutational heterogeneity of the disease in the majority of population studied to date (1). Exceptions occur where the frequencies of specific mutations are increased in a population because of founder effects, or where a mutation has been introduced on many occasions into a small isolated community (2–4). The prevalence of FH in India is not known. In addition, to the best of our knowledge there are no published reports on systematic analyses of mutations underlying FH in India. However, to date 10 different LDLR mutations in immigrants from India have been reported in the literature (5). Most of these mutations have been reported in exons 3, 4, 9, and 14 among Indians settled in South Africa, which suggests an increased frequency of FH in India (5). More than 80% of the 150,000 Indians who immigrated to South Africa between 1860 and 1911 originated from diverse areas in India (6). This group has remained isolated as a whole and also within different communities, primarily as a result of religious and cultural practices. This probably indicates that the increased incidence of mutations is attributable to multiple entries of defective LDLR genes into the Indian population of South Africa from India and that the group in South Africa probably represents the incidence in the Indian subcontinent.

The current study was thus undertaken to identify four previously reported LDLR gene point mutations in Indian hypercholesterolemic patients with clinical features of FH. The subjects were screened for mutations W66G (7), E207K (7), E387K (8), and P664L (9) in exons 3, 4, 9, and 14, respectively, which are those most reported among Indians in South Africa. In an attempt to identify other mutations causing FH among Indian subgroups in the same exons by heteroduplex (HDX) analysis, we further modified the technique and applied this approach for rapid detection of mutations in the LDLR gene.

Genomic DNA was extracted from blood samples of an apparently healthy control group and hypercholesterolemic patients with clinical features of FH (10) to detect mutations in exons 3, 4, 9, and 14 of the LDLR gene, using a salting out method (11). The exons were PCR amplified (12) using specific oligonucleotide primers (Table 1) and conditions as described elsewhere (7–9). The known point mutations were detected by restriction enzyme digestion of the amplified DNA as described previously (7–9). HDX analysis was performed with slight modification (13) of the known techniques (14, 15). The amplified products (5 μL) from both controls and patients were mixed and subjected to denaturation and renaturation cycles. Denaturation and renaturation were performed in a programmed PCR thermal cycler (model 480; Perkin-Elmer) at 95°C for 8 min, followed by 1 min each at 90, 80, and 70°C, and so forth until room temperature (20°C) was reached. The renatured samples were diluted with equal volumes of gel loading buffer (950 mL/L formamide, 20 mmol/L EDTA, 0.5 g/L xylene cyanol, and 0.5 g/L bromphenol blue) and electrophoresed through low cross-linking 10% denaturing polyacrylamide gels (160 × 180 × 1 mm) supplemented with 75 g/L urea. The low cross-linking (1%) protocol was followed for 10% polyacrylamide gel. Electrophoresis was performed with 1× Tris-borate-EDTA buffer at 200 V and 23 mA for 3–5 h at room temperature, stained with 0.5 mg/L ethidium bromide in 0.6× Tris-borate-EDTA buffer for 30 min, and visualized under ultraviolet light.

### Table 1. Primers for PCR amplification.

<table>
<thead>
<tr>
<th>Region</th>
<th>Primer sequence (5′→3′)</th>
<th>Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 3</td>
<td>TACAGGTCGACGTCATCTTCCTGCb</td>
<td>380</td>
</tr>
<tr>
<td>Exon 4</td>
<td>CCCCAAGACTGTCTCCCAGGACGAa</td>
<td>342</td>
</tr>
<tr>
<td>Exon 9</td>
<td>CTGCCGACCGCTGCCCGACCb</td>
<td>350</td>
</tr>
<tr>
<td>Exon 14</td>
<td>AATGTGAGCAGCTCCTGCGCTATCGb</td>
<td>390</td>
</tr>
</tbody>
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* Sense.

b Antisense.
DNA samples from 25 hypercholesterolemic patients and equal numbers of healthy controls were screened for mutations in exons 3, 4, 9, and 14 of the LDLR gene. The previously reported point mutations were absent in the samples analyzed. Using HDX analysis, we detected two patients showing an abnormal HDX pattern, one each in exon 3 and exon 4, indicating sequence variation (Fig. 1A). Sequencing reactions revealed the presence of single nucleotide “G” insertions at position 242 in exon 3 (Fig. 1B) and position 397 in exon 4 (Fig. 1C) compared with the LDLR cDNA sequence (16). Both the 242insG and 397insG mutations are predicted to cause translational frameshift, encoding unstable truncated LDLR proteins because of premature termination codons created downstream of the mutations. The mutant proteins are likely to be degraded intracellularly and are therefore pathogenic.

Fig. 1. PCR-HDX (A) and sequence (B and C) analysis of LDLR gene. (A), lanes 1, 3, and 8 represent wild-type control samples for exons 3, 14, and 4, respectively. Lanes 4, 6, and 7 are mutation-positive control samples for exon 14 (496 bp; lane 4) and exon 4 (431 bp; lanes 6 and 7), respectively. HDX patterns in lanes 2 and 5 were observed in exon 3 and exon 4, respectively. M, molecular weight marker αX174 HaeIII digest. The fragment size is indicated in bp. (B and C), sequence analysis of samples with HDX bands in exon 3 (B) and exon 4 (C) show single nucleotide “G” insertions, indicated by arrows.
A study by Kotze et al. (5) has indicated that CpG dinucleotides may be mutational hotspots among Asian Indians settled in South Africa. However, our study indicates mutational heterogeneity among Indian subgroups, which is not surprising considering the different ethnic groups present in the heterogeneous Indian population.

In addition to the two previously unpublished insertion mutations that we detected, the usefulness of this technique to detect minor changes was tested using mutation-positive control samples for exon 3 (R57C), exon 4 (E207K and D200Y), exon 9 (E387K), and exon 14 (P664L) of the LDLR gene (kindly provided by Dr. M.J. Kotze (Department of Human Genetics, Faculty of Medicine, University of Stellenbosch, Tygerberg, South Africa), Dr. A Soutar (MRC Lipoprotein Team, Clinical Science Centre, Royal Postgraduate Medical School, Hammersmith Hospital, London, United Kingdom), and Dr. A Minnich (Clinical Research Institute of Montreal, Montreal, Quebec, Canada)). It remains to be seen whether all possible base pair changes could be detected by the method described, but of the five known point mutations that we have tested (relatively large PCR fragments), four (fragments up to 496-bp) were easily detectable using this technique, whereas one (550-bp fragment in exon 9) was not.

The use of low cross-linked polyacrylamide gels supplemented with urea (9, 17) previously has been demonstrated to be highly sensitive in detecting single-base substitutions as HDXs. Furthermore, we also observed that the mixing of control and mutant DNA samples and an extra denaturing/renaturing step increases the chances of HDX formation between the nonhomologous DNA strands during cross-annealing, making them more apparent and easily visualized with reduced electrophoresis time. The mixing of control and mutant DNA can also facilitate formation of HDXs in homozygous mutant alleles where HDXs do not arise during amplification cycles.

In conclusion, this simplified nonradioactive PCR-HDX analysis gives reliable results with simple procedures within a short time. The method requires only small amounts of PCR-amplified DNA, easy to handle because radioactivity is not involved, and readily resolves differences between mutant and control DNA. Accordingly, we expect that it could be the preferred initial method of screening and could complement other techniques for detecting minor gene mutations.

References

Does Iron Concentration in a Liver Needle Biopsy Accurately Reflect Hepatic Iron Burden in β-Thalassemia? Guido Crisponi,1* Rosanno Ambu,2 Franco Cristiani,1 Gabriella Mancosu,2 Valeria Marina Nurchi,1 Rosalba Pinna,1 and Gavino Faa2 (1 Dipartimento di Chimica Inorganica ed Analitica, Università di Cagliari, Complesso Universitario di Monserrato, 09042 Monserrato-Cagliari, Italy; 2 Dipartimento di Citomorfologia, Divisione di Anatomia Patologica, Università di Cagliari, Via Ospedale 60, 09124 Cagliari, Italy; * author for correspondence: fax 39-0706754478, e-mail crisponi@unicaita.it)

β-Thalassemia major is an autosomal recessive disease characterized by absent or decreased synthesis of the β-globin gene (1). Thalassemic children, estimated at 100 000 worldwide, are affected by chronic anemia and need regular blood transfusion (2). Because of the limited capacity of iron excretion in humans, the iron in transfused red cells accumulates in the body. The liver, heart, and pancreas are the target organs of iron-induced injury; therefore, the major pathological manifestations observed in β-thalassemia major are chronic liver disease, evolving to cirrhosis, and dilative cardiomyopathy, both characterized by severe iron deposition (3, 4). The dangerous effects of iron excess can be managed by administration of chelators capable of removing iron from transferrin, fer-