Molecular Genetic Diagnosis of a Family with Hypercholesterolemia by a Mismatched PCR-RFLP Method for Genotyping Single Base Substitution of the LDL Receptor Gene

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SUMMARY

Plasma lipid and lipoprotein levels reflect in part the influence of relevant genetic loci. Defects at some of these loci account for specific types of dyslipoproteinemia occurring with regularity among family members. In the course of familial investigations of coronary artery disease, we identified a family in which several members were affected with elevated low density lipoprotein (LDL) cholesterol levels. To study the genetic defects responsible for plasma lipoprotein abnormality in this pedigree, we developed a simple method for genotyping a single base substitution that does not affect a restriction recognition enzyme site in exon 10 of the LDL receptor gene. Using our mismatched PCR method, this G->A substitution at nucleotide 1413 could be genotyped in the form of a biallelic restriction fragment length polymorphism (RFLP) after digestion with restriction enzyme Hpa II. Linkage analysis using this molecular method demonstrated that the defect at the LDL receptor locus is responsible for elevated LDL cholesterol phenotype observed in this family by segregation of defective alleles at the LDL receptor locus with the disease (peak decimal logarithm of odds score > 3.0). (Jpn Heart J 1998; 39: 681–686)

Key words: LDL receptor, Familial hypercholesterolemia, Mismatched PCR, Restriction fragment length polymorphism, Genotyping

HETEROZYGOUS familial hypercholesterolemia (FH) is caused by the inheritance of a single copy of a defective low density lipoprotein (LDL)
receptor gene. It is recognized clinically by striking elevations of LDL cholesterol (about twofold above normal or >95th percentile) affecting both children and adults. Affected families display bimodally distributed LDL cholesterol levels consistent with an autosomal dominant trait. Typically, there is a striking family history of early coronary disease. In general, only LDL cholesterol levels are elevated, and the finding of elevated triglycerides or other lipid abnormalities may bring into question the diagnosis of FH and may suggest other inherited primary lipid disorders such as familial combined hyperlipidemia or secondary lipid disorders.

Although mutations in the LDL receptor gene are known to cause FH, it is not always a straightforward process to find them in each suspected family. The LDL receptor gene is large and complex in structure; it spans 45 kb and consists of 18 exons and 17 introns. In addition, no mutational hot spot has been identified and mutations are rarely recurrent in Caucasians. In this situation, restriction fragment length polymorphisms (RFLPs) are a useful tool for linkage studies and haplotype association of FH in families and populations. Investigators can follow a gene's inheritance by studying the pattern of allelic bands within the family of interest. If a RFLP marker appears consistently with the disease in affected family members, then DNA variation at the marker locus is said to be linked with the disease. Once linkage with a genetic locus is established, secondary influences on the disease such as other modifier genes, diet or exercise can be examined.

We developed a mismatched PCR-RFLP method for genotyping one of the single base substitutions previously described as SSCP variants that does not affect restriction enzyme recognition sequences within the LDL receptor gene. We applied this method for genetic analysis and diagnosis of a family suspected to have FH.

**MATERIALS AND METHODS**

Family B was ascertained from a proband who had early myocardial infarction and hyperlipoproteinemia at age 42. Blood samples collected after 12–16 hours of fasting were prepared from family members. Lipid and lipoprotein concentrations were measured by a procedure previously described. In brief, plasma cholesterol and triglyceride concentrations were assayed enzymatically with Baker reagents on an Encore II Autoanalyzer (Baker Instrument Corp., Allentown, PA, USA.). Concentrations of HDL cholesterol were determined by the MgCl₂-dextran precipitation method. Plasma lipoproteins (in 200 μl EDTA plasma) were centrifuged in a Beckman TL-100 tabletop ultracentrifuge for 4 hours at 60,000 rpm at room temperature and thereafter separated into top
(VLDL) and bottom (LDL plus HDL) fractions by tube slicing. LDL cholesterol was determined by subtracting the HDL cholesterol concentration from LDL plus HDL cholesterol.

Genomic DNA was extracted from lymphocytes of family members. PCR was carried out using 20 ng of genomic DNA, 10 mM of Tris-HCl (pH 8.4), 50 mM of KCl, 1.5 mM of MgCl₂, 0.01% of gelatin, 200 µM of dNTPs, 2.5 pmol each of mismatched primer on one side and normal primer on the other side, sense 0.25 units of Taq polymerase in a volume of 10 µl. The sequence of the mismatched PCR primer was 10F Hap II (5'-CTATGACACCGTCATCAGCC-3'). The sequence of the anti-sense primer was 10R (5'-TCAGCGTCGTGGA-TACGCAC-3'). Cycle conditions were 94°C for 4 min, then 30 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec, with a final extension step of 5 min at 72°C, in a Gene Amp PCR 9600 System (Perkin Elmer Cetus, CA). PCR products were mixed with 10 µl of 2× reaction mixture containing 2 U of restriction enzyme Hpa II, incubated at 37°C for one hour, electrophoresed in 6% polyacrylamide gels and DNA visualized by ethidium-bromide staining. Allele frequency was calculated among 48 healthy individuals. SSCP analysis was carried out in the same DNA samples in order to compare the results of the two procedures.

Analysis of the relation among lipid measurements and genotypes at the LDL receptor locus was performed with the LINKAGE computer program. Carrier status was assigned by risk calculations using LDL cholesterol concentration and genotype at the LDL receptor locus for any subject with a probability in excess of 0.95, as determined by complex segregation analysis.

**RESULTS**

During a search of DNA sequence variation within the LDL receptor gene by direct sequencing, we found that the proband (No. II-3) of family B is heterozygous for Guanine and Adenine at nucleotide position 1413 in the exon 10 of the LDL receptor gene. Although this polymorphism was described previously as an SSCP variant,5) a more reliable method such as PCR-RFLP is obviously needed for unambiguous genotyping in order to use it as a tool for segregation and linkage analysis.

To transform the G1413A substitution into a PCR-RFLP format, a mismatch was introduced to 5' primer at the last nucleotide of its 3' end which creates a Hpa II site for 1413G (allele 2) but not for 1413A (allele 1), since the G1413A polymorphism originally did not affect any restriction site. Figure 1a shows the results of mismatched PCR and Hpa II digestion. The sequence containing 1413G (allele 2) was cleavable by Hpa II and digestion produced 175 bp
Figure 1. Genotyping of G1413A by the mismatched PCR-RFLP method (a), in comparison with typing by the SSCP method (b). A panel of the same individuals were genotyped by both methods for comparison.

and 29 bp fragments, whereas the sequence containing 1413A (allele 1) was uncleavable by Hpa II and digestion produced a single 204 bp fragment. The SSCP patterns of the same individuals are shown below for comparison (Figure 1b). The allele frequencies for 1413G (allele 2) and 1413A (allele 1) were 0.37 and 0.63, respectively, with an observed heterozygosity of 0.49.

The truncated pedigree structure of family B is shown in Figure 2 with ID number, age at diagnosis, plasma total cholesterol, total triglyceride, HDL cholesterol, LDL cholesterol, and RFLP genotypes are shown beneath the symbol of each member. Allele 2 (175 bp) band cosegregated with the elevated levels of LDL cholesterol.

Figure 2. Genotyping of G1413A in a family with hypercholesterolemia. Affected individuals having LDL cholesterol levels > 95th percentile are shown as filled symbols. Shaded symbol; N.D. ID number, total cholesterol, total triglyceride, HDL cholesterol, LDL cholesterol, RFLP genotypes are shown beneath the symbol of each member. Allele 2 (175 bp) band cosegregated with the elevated levels of LDL cholesterol.
terol, LDL cholesterol levels and LDL receptor genotypes. Eleven members of this branch of the family had elevated levels of LDL cholesterol. Affected members having LDL cholesterol above the 95th percentile are shown as filled symbols. The members were genotyped with the mismatched PCR-RFLP method and genotypes, either allele 1 or allele 2, are shown beneath the symbols. Allele 2 cosegregates with elevated LDL cholesterol without exception in this pedigree. Linkage analysis showed that the genotype represented by allele 2 is linked to the high LDL cholesterol phenotype with a peak decimal logarithm of odds score of > 3, with no recombination. In other words, the odds favoring linkage between familial hypercholesterolemia and the “allele 2” genotype exceed 1,000 : 1. DNA variation at the LDL receptor gene locus is significantly linked with the disease. All affected family members studied carry the same mutant allele of the LDL receptor gene. Therefore, even from the small nuclear family shown, the results are consistent with the LDL receptor gene as being causative for the high LDL phenotype in this family. The genotyping by mismatched PCR-RFLP was useful in the genetic diagnosis of FH in this family.

**DISCUSSION**

We identified a Guanine-Adenine polymorphism at nucleotide 1413 of the LDL receptor gene, and used this as a genetic marker in a form of mismatched PCR-RFLP to show the cosegregation of high LDL cholesterol phenotype and a specific allele of the LDL receptor locus.

The G1413A substitution was previously described as a variant detectable by SSCP analysis. Although SSCP is an efficient method with which to search for new sequence variations in a portion of a gene, it is not always a reliable tool for unambiguous genotyping because the results are strongly influenced by subtle changes in denaturation and electrophoresis conditions, such as buffer composition, running voltage and duration, and gel temperature. Once the variation is found by SSCP analysis, a PCR-RFLP method is usually employed to replace SSCP for an unambiguous and consistent allele detection of the three genotypes present in biallelic polymorphic systems. The comparison of genotyping data between mismatched PCR-RFLP and SSCP presented in Figure 1 for G1413A polymorphism support the fact that this is a practical choice.

Some single base pair substitutions do not affect the restriction enzyme recognition sequence, and thus, PCR-RFLP is not applicable as it is. This inconvenience can now be overcome by the use of mismatched primers in PCR to incorporate an artificial restriction enzyme recognition site into the polymorphic sequence. This treatment transforms almost any single base substitution into a PCR-RFLP style polymorphism. In the present study, we used mismatch PCR to
incorporate a cytosine in place of adenine at a nucleotide two base pairs upstream of the polymorphic G1413A site. This resulted in the creation of a Hpa II restriction site at the polymorphic G1413A site and successful genotyping by restriction enzyme digestion.

The lipoprotein phenotype of inherited hyperlipoproteinemia is often modified by diet, lifestyle and medications. Clinicians often encounter equivocal phenotypes that are difficult to diagnose on pure clinical grounds. As genetic markers can strengthen an equivocal clinical diagnosis, it is hoped that genetic markers will assist decision-making in diagnosis, therapy and prognosis.8,9) The present study illustrates the potential usefulness of a new genetic marker in the diagnosis of FH. More affected family members can now be studied with markers to extend and establish a linkage with the LDL receptor gene.

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REFERENCES