Familial Hypercholesterolemia in Utah Kindred with Novel R103W Mutations in Exon 4 of the LDL Receptor Gene

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SUMMARY

Heterozygous familial hypercholesterolemia (FH) is a serious disorder causing twice normal low-density lipoprotein cholesterol levels early in childhood and very early coronary disease in both men and women. Previously published blood cholesterol criteria greatly under-diagnosed new cases of FH among members of known families with FH and over-diagnosed FH among participants of general population screening. Thus, there is a need for accurate and genetically validated criteria for the early diagnosis of heterozygous FH. In the course of investigations of coronary artery disease in Utah, we identified a family whose proband showed elevated plasma levels of LDL cholesterol. To carry out molecular genetic diagnosis of the disease, we screened DNA samples for mutations in all 18 exons and the exon- intron boundaries of the low-density lipoprotein (LDL) receptor gene. Novel point mutations were identified in the proband: a C-to-T transversion at nucleotide position 369, causing substitution of Tryptophan for Arginine at codon 103 in exon 4 of the LDL receptor gene. The SSCP method was used to examine seven members of the family recruited for the diagnosis. This method helped to unequivocally diagnose only the proband as heterozygous for this particular LDL receptor mutation while excluding the remaining six individuals from carrier status with FH. (Jpn Heart J 1999; 40: 443-449)

Key words: Hyperlipoproteinemia, Lipoproteins, LDL receptor, Familial hypercholesterolemia, Genetic diagnosis

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HETEROZYGOUS familial hypercholesterolemia (FH) is a dominant disorder attributable to a single gene defect on the short arm of chromosome 19 causing half of the low density lipoprotein receptors (LDLR) on the surface of liver cells to function improperly, leading to decreased removal of LDL cholesterol.¹⁾ Plasma LDL cholesterol levels are approximately twice normal and usually can be detected by age 2 years. The combination of intensive dietary therapy and multiple prescription medication therapy offers an opportunity for substantially lowering or even normalizing blood cholesterol levels in many persons with this disorder. Studies using quantitative angiography have shown a statistically significant effect in preventing progression and even inducing regression of coronary atherosclerosis in FH patients treated aggressively with lipid lowering medication.^{2,3)}

The LDLR gene spans 45 kb of human genome and consists of 18 exons and 17 introns.⁴⁾ No mutational "hot-spot" has been identified; in fact, specific mutations rarely recur among unrelated Caucasians.^{5,6)} We describe here the identification of a novel mutation of the LDLR gene in a hypercholesterolemic kindred in Utah. Our approach helped to unequivocally diagnose seven individuals as heterozygous for this particular LDL receptor mutation while excluding the remaining five individuals from carrier status with FH. In two of the affected individuals, identification would not have been possible using published LDL cholesterol criteria.^{7,8)}

MATERIALS AND METHODS

Patients and lipoprotein measurement: The proband is a patient who has been followed by the Cardiovascular Genetics Research Clinic at the University of Utah. After 12–16 hours of fasting, blood samples were collected and prepared for each family member. Lipid and lipoprotein concentrations were measured by procedures described previously.⁹⁾ In brief, plasma cholesterol and triglyceride concentrations were assayed enzymatically, and concentrations of high-density lipoprotein cholesterol (HDL-c) were determined by the MgCl_2 -dextran precipitation method. LDL was measured by ultracentrifugation after subtraction of HDL cholesterol from HDL + LDL cholesterol measured in the bottom fraction. These methods are routinely monitored and checked with both internal controls and standards supplied by the CDC Lipid Standardization Program.

Single-strand conformational polymorphism (SSCP) and sequencing analysis: SSCP was carried out as previously described.¹⁰⁾ Each polymerase chain reaction (PCR) was initially carried out using 20 ng of genomic DNA extracted from lymphocytes, in a 10-microliter solution containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl_2 , 0.01% gelatin, 200 μM each of dNTP, 2 μCi

of [α 32P]-dCTP (3000 Ci/mmol, 10 mCi/ml), 2.5 pmol of each primer, and 0.25 units of Taq polymerase. PCR primers for amplification of each exon of the LDLR gene and all exon-intron boundaries were previously described.^{11,12} The following PCR primers were used for mutation detection; forward primer 5'-TCGGCCATCCATCCCTGCAG-3', reverse primer 5'-CTGCGGCCACTC-ATCCGAGC-3'. Each of 35 PCR cycles consisted of 30 sec at 94°C, 30 sec at 71°C, and 30 sec at 72°C. Reaction mixtures were diluted with 50 μ l of 95% formamide dye and 20 mM EDTA, incubated at 85°C for 5 min, and applied (2 μ l/lane) to 6% polyacrylamide gels containing 0.5X TBE (90 mM Tris-borate/2 mM EDTA) and 5% glycerol. Electrophoresis was performed both at room temperature and at 4°C. The gels were dried and autoradiographed with intensifying screens. When a variant band was revealed by SSCP analysis, the corresponding DNA fragment was sequenced directly on both strands to identify the nature of the mutation. The results were confirmed by two independent experiments.

RESULTS

The pedigree structure of this family is shown in Figure 1. Affected status, as determined by the mutation analysis is presented in Figure 1a, as well as age, and lipoprotein profile in Figure 1b. None of the family members had a medical history of coronary artery disease or tendon xanthomas. The proband was a

Figure 1. Pedigree and mutational analysis of the proband (arrow) in family. E.U. indicates Uninformative Evaluation. Square symbols, males; circles, females are shown in top panel **a**. I.D. number, age, plasma levels of total cholesterol (T.Chol), triglyceride (TG), HDL cholesterol (HDLc), and measured LDL cholesterol (LDLc) are shown in bottom panel **b**.

Figure 2. **a:** SSCP analysis of exon 4 of the LDL receptor gene in the proband. WT = control individual homozygous for wild-type allele; R103W = FH proband heterozygous for R103W mutant and wild-type allele. Arrow indicates the aberrant SSCP band observed in the proband's DNA with R103W mutation. **b:** Direct nucleotide sequencing and deduced amino acid sequences of the R103W mutation comparing normal individual (homozygous for Arg at codon 103) and the proband heterozygous for normal allele and mutant R103W allele.

Caucasian female patient (individual II-2, Figure 1a) who is being followed by Cardiovascular Genetics Research Clinic at the University of Utah. All lipids in Figure 1b are untreated values. Plasma lipoprotein levels of the 39 year old proband were; total cholesterol 320 mg/dl, triglyceride 168 mg/dl, HDL cholesterol 37 mg/dl and LDL cholesterol 231 mg/dl.

Mutation screening of the LDL receptor gene in the proband's genomic DNA identified an aberrant SSCP pattern in the PCR amplification product of exon 4 (Figure 2a). Direct sequencing on both strands revealed heterozygosity for a C-to-T transversion at nucleotide position 369 of the LDL receptor cDNA. This transversion would substitute Tryptophan for Arginine at codon 103 (R103W) (see Figure 2b). R103W was the only sequence variation detected in the entire coding with splice-site consensus sequences of the LDL receptor gene of the proband.

The SSCP method was applied to examine seven members of the family for the diagnosis. This test detected a mutant sequence only in the proband of the

family. Thus, diagnosis as heterozygous for the R103W LDL receptor mutation in the proband shown by black symbols in Figure 1a was unequivocal while the remaining six individuals were excluded from FH carrier status.

DISCUSSION

Despite the impressive progress made in the understanding of the pathophysiology and treatment of FH, it has been our anecdotal experience that many affected individuals are not diagnosed or adequately treated for this disorder. Therefore, we initiated a project entitled "MedPed" (Make Early Diagnoses to Prevent Early Deaths in MEDical PEDigrees) to obtain objective information on the status of FH patients and to develop approaches for finding and helping these persons.^{13,14} The project emphasizes the power of family survey as well as DNA-based diagnostic approaches. From the start, the MedPed project objectives have been to evaluate and then develop solutions to the following critical issues of this life-threatening disease: 1) productive, and cost effective methods for identifying persons and families with heterozygous FH, 2) identify FH individuals who are not properly diagnosed and appropriately treated using validated established diagnostic criteria, 3) establish a process to assure initiation of correct treatment and compliance with this life-long treatment for every person with FH, and 4) apply, in a practical manner, knowledge obtained from research on this disorder.

It became clear from these initiatives that distinct clinical cholesterol criteria for the diagnosis of FH were needed. We therefore developed strategies for both initial diagnosis of a previously undiagnosed FH family, and separate criteria for subsequent diagnosis of other affected relatives.^{7,8} Separate criteria for initial diagnosis of a family versus diagnosis of relatives within a well-defined family are necessary since in the general population the chance of inheriting heterozygous FH is only 1 in 500 while first-degree relatives of a confirmed FH case have a 50% chance of having FH. Nevertheless, these criteria depend entirely on LDL cholesterol levels and could miss carriers of LDLR mutations who, for a variety of reasons, may have LDL cholesterol below the defined cut points. In addition, primary hypercholesterolaemia is a broad term which could be caused by both monogenic and polygenic conditions. The most common form of primary hypercholesterolaemia is polygenic hypercholesterolemia. The genetic abnormalities contributing to this condition have not been determined. In principle they could include mild defects in LDL-receptor structure, defects in the regulation of synthesis of LDL-receptor, defects in LDL-receptor structure, defects in the regulation of synthesis of LDL-receptor, defects in the primary structure of apo B and overproduction of apo B-containing lipoproteins or a combination of these disorders. The elder sister of the proband (II-1) who expressed a primary

hypercholesterolemia but lacked the co-segregation of R103W allele of *LDLR* may belong to this condition, although a possible segregation of an unidentified second mutant *LDLR* allele causing FH in this individual in the same family could not be ruled out at present.

Epi-genetic factors, as well as genetic factors, influence lipid levels in plasma. The nature of the LDL receptor mutation also contributes to clinical variability among FH patients. It has been reported that individuals with mutations that only partially impair receptor function tend to have lower plasma LDL cholesterol levels than do individuals whose genes produce totally defective receptors.¹⁾ In the present study, the FH patient with the missense mutation R103W tended to show marked high levels of total cholesterol and LDL cholesterol. We examined 192 chromosomes from control individuals for a presence of the R103W mutation but did not detect it among these controls, thus, it is unlikely that it represents a neutral polymorphism. However, a remote possibility that hypercholesterolemia in this family is not linked to the *LDLR* gene is also possible. There is much potential for uncertainty at present, which may be solved by in vitro LDL receptor assay or by linkage analysis in extended pedigree in the future.

Functionally, *LDLR* mutations are classified into five classes based on studies using fibroblasts derived from FH patients.¹⁾ Previously, several missense mutants occurring in the seven repeats of the ligand binding domain encoded by exon 2 through 6, have been functionally characterized.⁵⁾ Since most result from encoded receptors that were blocked in transport between the endoplasmic reticulum and the Golgi apparatus (class 2; transport-defective alleles),^{5,6)} we postulate that the R103W mutation reported here would cause the same class of deficiency on account of its location. However, other possibilities cannot be ruled out until proper functional tests are conducted for this particular mutation. Our study illustrates the feasibility of molecular genetic approaches for clinical diagnosis, thereby invoking appropriate early intervention in FH family members with a predisposition to coronary heart disease.

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