
HUMAN
GENETICS

Four New Mutations and Two Polymorphic Variants of the Low-Density Lipoprotein Receptor Gene in Familial Hypercholesterolemia Patients from St. Petersburg

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Received February 13, 2001

Abstract— In a collection of DNA samples from 100 unrelated patients with clinical features of familial hypercholesterolemia (FH), a search for mutations of exons 4 and 10 of the low-density lipoprotein (LDL) receptor gene was performed using heteroduplex and single-strand conformational polymorphism (SSCP) analyses followed by sequencing of amplified DNA fragments. Four new mutations of the LDL receptor gene were identified: C146R (c.499 T > C), A130P (c.451 G > C), G128G (c.477 T > C), and C188Y (c.626 G > A). Mutation A130P was assigned to the same chromosome with allele variant 447C. Two polymorphic sites in exon 10 of the LDL receptor gene (1413G/A and 1545C/T) were found in the Russian population for the first time. Based on the data obtained, familial hypercholesterolemia was confirmed in seven patients.

INTRODUCTION

Familial hypercholesterolemia (FH) belongs to the widespread monogenic human disorders. The incidence of a heterozygous form of this autosomal dominant disease is 1 : 500. Clinical symptoms of the disease are an atherosclerotic affection and, as a consequence, a coronary failure and myocardial infarctions at a relatively early age. Biochemically, FH is characterized by type IIa hyperlipidemia. In FH patients, a high blood cholesterol level (relative to the population means of 5.5 mM) is caused by quantitative deficiency or dysfunction of specific low-density lipoprotein (LDL) receptor [1]. Mutations in the LDL receptor gene cause various abnormalities of processing and cyclic transformations (ligand binding, internalization of receptor with ligand, dissociation of ligand from receptor in the acid medium of lysosomes, and ligand resetting to cell surface) of the receptor protein. Different populations are characterized by their own diversity of LDL receptor mutations, and their detection is necessary for preclinical DNA diagnostics of FH, genetic counseling in families of patients, and the subsequent pharmacotherapy.

In Russia, the first studies of FH started in 1987 at the Institute for Experimental Medicine, Russian Academy of Medical Sciences. Recently, a collection of DNA samples has been obtained from 100 St. Petersburg probands with clinical features of FH. Earlier, seven mutations in the LDL receptor gene were found in some of the probands [2–6]. In view of the growth of the collection of DNA samples, we continued the

search for genetic abnormalities and described polymorphic variants and new missense mutations in exons 4 and 10 of this gene.

MATERIALS AND METHODS

The choice of patients with clinical features of FH was carried out as described earlier [7]. DNA of blood leukocytes of patients was isolated using the method described by Kunkel *et al.* [8]. Separate amplification of the LDL receptor gene exons was conducted using PCR with oligonucleotide primers (Sibenzim) suggested by Hobbs *et al.* [9]. In view of considerable length of exons studied, primers were arranged as follows: 5' and 3' ends (designated below as A and B) were amplified separately and were interlaced with one another. The amplified fragments A and B of exon 4 (4A and 4B) had a length of 355 and 267 bp, respectively. The length of the fragments A and B of exon 10 (10A and 10B, respectively) were 202 and 162 bp, respectively. The PCR mixture (30 µl) consisted of the following components: 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 1.5 mM MgCl₂, 200 µM of each of four dNTPs, 0.25 µM of each primer, and 1 EU of *Taq* polymerase [10]. The amount of template DNA was 30 to 50 ng per sample. The optimized temperature profile of PCR included the initial cycle of denaturation of DNA template for 5 min at 95°C and the following 30 cycles: 95°C, 1 min; 59°C, 1 min; and 72°C, 1 min. After completion of these cycles, samples were left for 9 min at 72°C. We used a “Cyclotemp-2” thermal cycler (STM, Moscow). The PCR products were separated by electrophoresis in an 8% polyacrylamide gel (PAG)

† Deceased.

prepared using an acrylamide : *N,N'*-methylenebis(acrylamide) ratio of 29 : 1 in a 1× TBE buffer [11]. Gels were stained with argentic nitrate. The conditions of electrophoresis allowed us to identify heteroduplexes [12]. Single-strand conformational polymorphism (SSCP) of all PCR products was studied in a 12% PAG [13].

Samples, in which we found a change of electrophoretic mobility of single-strand conformation isomers, were sequenced using Sanger's method [14]. For sequencing of double-strand DNA molecules, we used a fmol[®] DNA Cycle Sequencing System kit (Promega) and the corresponding protocol. α -[³³P]dATP was used as a label. The products of sequencing were electrophoresed using a Macrofor unit (LKB-Pharmacia).

In only one case (patient O.), the PCR products of exon 4A of the LDL receptor gene were cloned using a PCR[®]2.1 plasmid vector (Promega) prior to sequencing. This was necessary, because we detected two mutations in the DNA of this patient and needed to determine whether these mutations are in *trans* or *cis* configuration. For ligation of the vector and PCR product, we used a T4 phage DNA ligase (Sibenzim). The clones of *E. coli* DH5 α (*SupE44* Δ *lacU169* (ϕ 80 *lacZ* Δ *M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*), which contained recombinant plasmids, were selected using α -complementation [11]. The total DNA isolated from the cells of the selected clones was used as DNA template for PCR. PCR products were studied using restriction analysis, to detect colonies with the mutant allele of the studied gene.

The following restriction endonucleases were used in the restriction analysis: *RsaI*, *BsuRI* (Sibenzim), *ApaI* (Boehringer Mannheim), and *Cac8I* (New England Biolabs). Restriction was conducted as recommended by the manufacturers.

RESULTS

With the aim of searching mutations in exons 4 and 10 of the LDL receptor gene, we amplified the DNA fragments of interest by PCR. Using the heteroduplex and SSCP analyses followed by sequencing, we found four mutations and two polymorphic variants of the LDL receptor gene.

The radioautograph of the sequencing gel, in which mutation c.499 T > C in exon 4A was identified in patient S., is shown in Fig. 1. This substitution causes the change of codon TGC, which encodes cysteine in position 146 of the amino acid sequence of the LDL receptor, into the arginine-encoding codon CGC. The analysis of the nucleotide sequence of exon 4A, which contains this mutation, revealed a site of specific restriction endonuclease *ApaI* lacking the wild-type DNA. Using restriction analysis, we found this mutation in a sister and a son of this patient (Fig. 2). The presence of the mutation in them is confirmed by the results of restriction analysis presented in the electrophoregram and by clinical data on the cholesterol level.

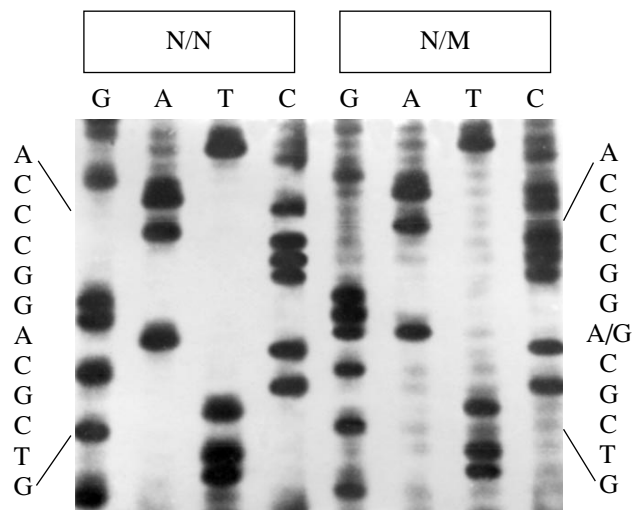


Fig. 1. Radioautograph of the gel separating the products of sequencing of a normal (N/N) exon 4A and a mutant exon containing mutation c.499 T>C (C146R) in a heterozygous state (N/M). The radioautograph shows the results of sequencing of the complementary DNA sequence.

In patient O., sequencing of exon 4A revealed two nucleotide substitutions: c.447 T > C and c.451 G > C. The former does not change the amino acid in position 128 of the amino acid sequence of the LDL receptor (codons GGT and GGC encode glycine). Mutation c.451 G > C causes the change of codon 130 (codon GCC encoding alanine) into the codon for proline (CCC). In the nucleotide sequence of exon 4A, the substitution of T with C in position 447 causes the emergence of a new recognition site for restriction endonuclease *BsuRI*, and the substitution of G with C in position 451 causes the disappearance of recognition sites for restriction endonucleases *Cac8I* and *FauI*. As both substitutions were detected in the sample containing both maternal and paternal alleles of this gene, it remains unclear whether both genetic defects are located in one allele or in different alleles. To answer this question, we divided the alleles by direct cloning of the PCR products of exon 4A in a PCR[®]2.1 plasmid. After amplification of plasmid insertions, the PCR products were consecutively hydrolyzed by restriction endonucleases *BsuRI* and *Cac8I*. We found out that both genetic defects are situated in the same allele of the LDL receptor gene (*cis* configuration); i.e. they were inherited from one parent. This was confirmed by sequencing of the cloned allele (Fig. 3). The change of the nucleotide sequence in position 447 of cDNA can be considered a silent mutation, because it was not detected in other samples of the studied collection. Relatives of patient O. were not available for the study.

In patient Zh., we found mutation c. 626 G > A in exon 4B (Fig. 4). This mutation caused the change of cysteine into tyrosine in position 188. This change of the nucleotide sequence causes the formation of a new recognition site for restriction endonuclease *RsaI*,

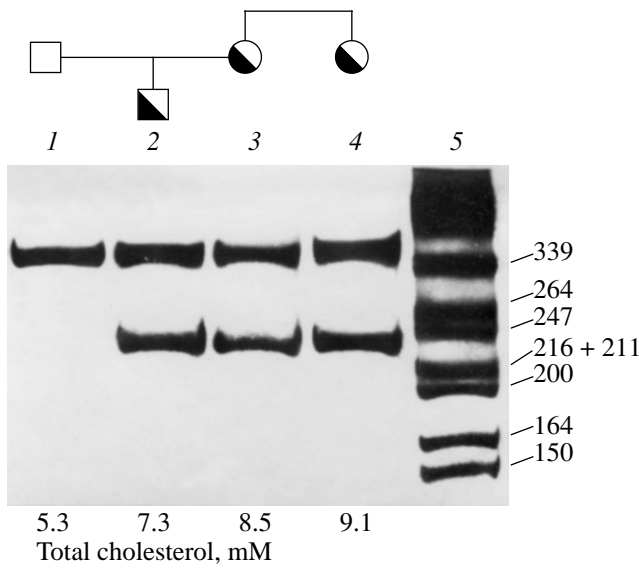


Fig. 2. Inheritance of mutation c.499 T > C (C146R) in the family of patient S. The electrophoregram shows the results of hydrolysis of PCR products of exon 4A of the LDL receptor gene with restriction endonuclease *ApaI* in members of this family: lane 1, amplified exon 4A without mutation; lanes 2, 3, and 4, amplified exon 4A with the mutation in a heterozygous state. In addition to the band of amplified exon 4A (355 bp), additional 235- and 120-bp *ApaI* fragments were formed (the latter not shown); and lane 5, DNA of phage λ hydrolyzed with restriction endonuclease *PstI* (molecular-weight marker). Below the lanes, cholesterol levels are shown for each family member.

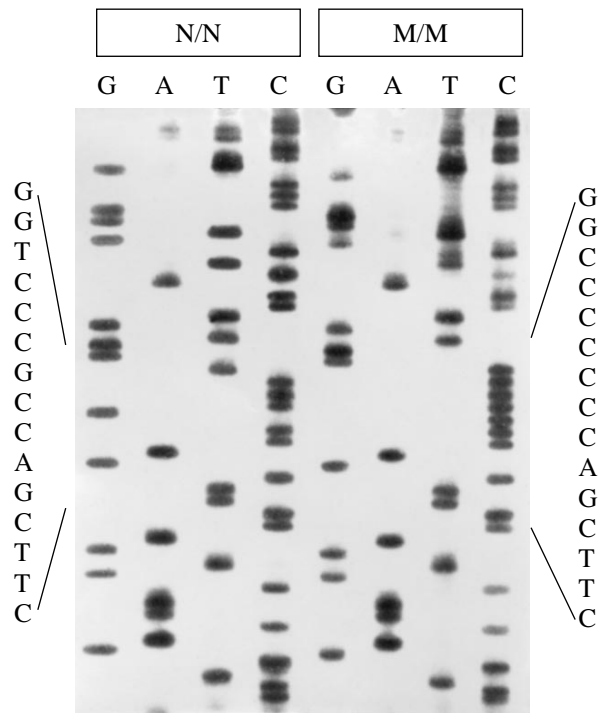


Fig. 3. Radioautograph of the gel separating the products of sequencing of a normal (N/N) exon 4B and a mutant exon containing mutations c.447 T > C (G128G) and c.451 G > C (A130P) in a homozygous state (M/M). The radioautograph shows the results of sequencing of complementary DNA sequence.

which can be used for a quick detection of this mutation. Using restriction analysis, we detected this mutation in a sister and a daughter of patient Zh. (Fig. 5). The data of restriction analysis (see the electrophoregram in Fig. 5) and clinical indices of the cholesterol level confirmed the presence of this mutation in the relatives of this patient.

A rare variant of 1545T polymorphism, 1545C/T, was found in exon 10B in two unrelated patients. It does not cause a change in the amino acid sequence of the LDL receptor, because both codons AAT and AAC code for asparagine. This polymorphism was first found in 1996 by Jensen *et al.* [15], and its description is present in the world mutation database.

Polymorphism 1413G/A was found in exon 10A by SSCP analysis. This polymorphism was first described in 1992 by Warnich *et al.* [16]. In 12 patients studied by these authors, both alleles were found with equal frequency. In our study, the allele frequencies were also close to 50%, (genotypes AA, GG, and GA were found in 24, 27, and 49 patients studied; i.e., the frequency of allele A was 47.3 and 52.7%, respectively).

DISCUSSION

We studied selected samples from the collection of 100 DNA samples from patients with type IIa hyperlip-

idemia. For this study, patients were selected based on clinical features (high cholesterol level, early myocardial infarctions, cardiac ischemia, and tendon xanthomas) and the familial pattern of the disease [7].

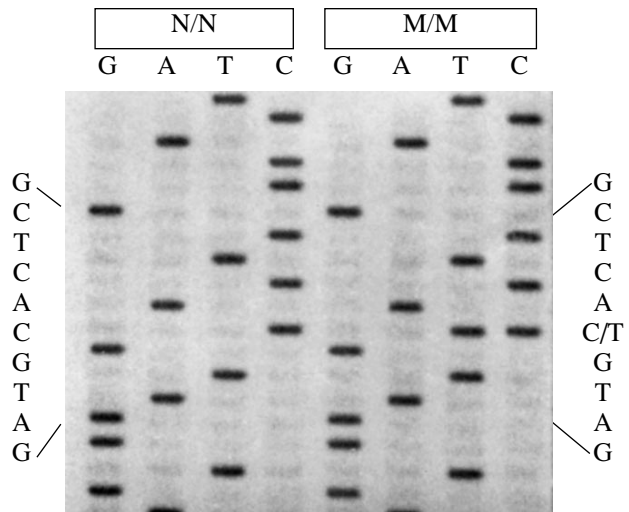


Fig. 4. Radioautograph of the gel separating the products of sequencing of a normal (N/N) exon 4B and a mutant exon containing mutation c.626 G > A (C188Y) in a heterozygous state (N/M). The radioautograph shows the results of sequencing of complementary DNA sequence.

In these patients, we studied exons 4 and 10 of the LDL receptor gene, in which these exons are the most extended. The choice of exon 4 was motivated by the fact that this exon is among the most mutable regions [9]. In addition, exon 4 encodes a major part of the ligand-binding domain; therefore, mutations in this exon most frequently cause a dysfunction of the receptor.

For the primary detection of point mutations, we used heteroduplex and SSSP analyses. Earlier, it was demonstrated that large-scale rearrangements of the LDL receptor gene are not the widespread cause of FH in St. Petersburg [2]. An extended deletion (5 kb) was detected by Southern blotting in only one out of 50 FH patients studied. Hence, most of FH cases from St. Petersburg are caused by point mutations and short deletions which can be detected by the methods used in this study.

Using these methods, we detected four new mutations in exon 4 of the LDL receptor gene (table). Mutations C146R and C188Y appear as replacements of cysteine for arginine and tyrosine, respectively. The significance of these replacements is obvious, because cysteine plays an important part in the formation of disulfide bonds, and their presence define the conformation of functionally active receptor [1]. Moreover, these mutations were found in three family members having clinical symptoms of FH and were not found in the relatives of patients, who had a normal cholesterol level (Figs. 2, 5). The significance of these mutations can only be proved experimentally by conducting expensive studies of binding of labeled LDLs with the mutant receptor protein obtained by gene engineering or by culturing fibroblasts of FH patients. We did not carry out these studies. Mutation A130P causes the change of alanine into proline, which modifies valence angles in the polypeptide chain of the protein, which can also be the reason for the loss of its function. In addition, using the PCR-SSSP analysis, we did not find mutations in

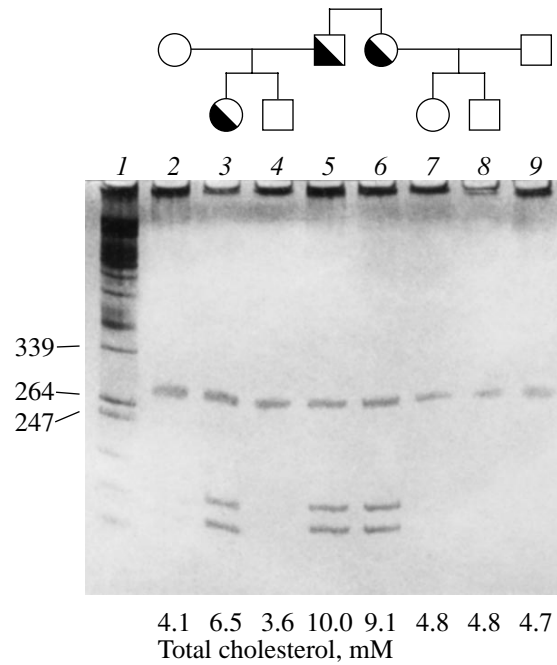


Fig. 5. Inheritance of mutation c.626 G>A (C188Y) in the family of patient Zh. The electrophoregram shows the results of hydrolysis of PCR products of exon 4A of the LDL receptor gene with restriction endonuclease *RsaI* in members of this family: lanes 2, 4, 7, 8, and 9, amplified exon 4B without mutation; lanes 3, 5, and 6, amplified exon 4B with the mutation in a heterozygous state. In addition to the band of the amplified exon (267 bp), additional 141- and 126-bp *RsaI* fragments are present; and lane 1, DNA of phage λ hydrolyzed with restriction endonuclease *PstI* (molecular-weight marker). Below the lanes, cholesterol levels are shown for each family member.

other exons of this gene in the proband with mutation A130P. Based on this, we concluded that, namely, this mutation causes FH. As yet, the silent mutation G128G has only been found linked with mutation A130P.

LDL receptor gene mutations and polymorphic variants identified in this study

Mutation	Change in DNA	Exon	Method of rapid testing	Number of families (number of patients)	Comment
C146R	TGC → CGC c. 499 T > C	4A	Restriction analysis: new <i>Apal</i> recognition site	1 (3)	New
C188Y	TGC → TAC c. 626 G > A	4B	Restriction analysis: new <i>RsaI</i> recognition site	1 (3)	New
A130P	GCC → CCC c. 451 G > C	4A	Restriction analysis: disappearance of <i>Cas8I</i> recognition site	1 (1)	New; assigned to the same allele
G128G	GGT → GGC c. 477 T > C	4A	Restriction analysis: a new <i>BsuRI</i> site	1 (1)	"
R450R	AGG → AGA c. 1413 G > A	10A	SSCP analysis		Frequencies of alleles A and G: 47.3 and 52.7%, respectively; n = 152
N494N	AAC → AAT c. 1545 C > T	10B	SSCP analysis	2 (2)	Frequencies of alleles T and C: 2 and 98%, respectively; n = 100

n is the number of chromosomes studied.

All detected mutations (table) are new, and no data on these mutations are included in the world mutation database. This fact can be explained by high ethnic heterogeneity, high level of migration, and very low inbreeding rate, which is typical of the St. Petersburg population. Based on this, we can suppose a wide diversity of mutations in FH patients from St. Petersburg.

In this study, we detected two polymorphic variants of the LDL receptor gene: 1545C/T and 1413G/A. Earlier, these variants were described abroad [15, 16], but in Russia we found them for the first time. Polymorphisms or neutral mutations does not phenotypically express, but they are suitable genetic markers. Based on genealogical studies, one can trace their inheritance in the successive generations and can study their linkage with each other and with known mutations. This is why polymorphic variants can have diagnostic significance.

This study allowed us to diagnose FH in other seven patients (three probands and four members of their families) based on the DNA analysis. This study does not exhaustively describe the genetic variability of the LDL receptor gene in FH patients from St. Petersburg, and further studies are needed.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research (grant nos. 97-04-48887 and 00-04-48962), Russian State Program "Human Genome" (section no. 4/00), and the Russian Council of Support of the Leading Scientific Schools (grant no. 00-15-97931).

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