Screening for Mutations in Exon 4 of the LDL Receptor Gene in Thai Subjects with Primary Hypercholesterolemia: Detection of a Novel Mutation D151Y by PCR-CFLP

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Abstract

A mutation in low density lipoprotein (LDL) receptor gene causes an autosomal codominant disorder namely familial hypercholesterolemia (FH). Mutations in the LDL receptor gene are very heterogeneous at the DNA levels, occurring in all 18 exons of the gene. However, exon 4 has been found to be the hot spot for mutational events. In this study DNA from 45 Thai subjects with primary hypercholesterolemia was screened for mutations in the hot spot exon 4. The DNA samples were amplified by Polymerase Chain Reaction (PCR) and screened for mutation by Cleavase Fragment Length Polymorphism (CFLP) technique. Identification of mutation was performed by direct sequencing of PCR product. From this screening, one female patient was found to be heterozygous for a novel mutation which was due to a G to T transversion at nucleotide 514. This transversion would change the species-conserved amino acid at codon 151 from charged R group aspatic (GAC) to uncharged R group tyrosine (TAC), termed D151Y. From the same screening strategy, we found that this mutation was absent in 33 healthy normolipidemic subjects. In this index subject, Arg 3500 Gln mutation in apo B-100 gene, causing hypercholesterolemia namely familial defective apo B-100 (FDB), was not found. Therefore, hypercholesterolemia in this index subject was possibly caused by the D151Y mutation in the LDL receptor gene.

Key word : Hypercholesterolemia, LDL Receptor Gene, Mutation

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Mutation in the LDL receptor gene results in impaired removal of apolipoprotein (apo) B- and apo E-containing lipoprotein from circulation and causes familial hypercholesterlemia (FH)[1]. This autosomal co-dominant disorder is characterized by an elevated concentration of plasma LDL frequently giving rise to tendon xanthomas and premature coronary artery disease (CAD)[2]. A similar pattern is seen in individual heterozygous for a defect in apo B-100, namely familial detective apo B (FDB), one of the ligands for the LDL receptor[3]. However, FH is relatively more common. It has been ranked as one of the most common inherited disorders in human. The frequency of FH is approximately 1 in 500 for the heterozygous form and one in million for the homozygous form[2]. Homozygotes are particularly severely affected and often succumbs to CAD before the age of 30 years[4]. More than five hundred mutations at the LDL receptor locus, causing FH, have been described to date (http://www.ucl.ac.uk/fh/muttab.html).

High prevalence of hypercholesterolemia due to elevated serum LDL-cholesterol levels is also found in Thai population [5]. This finding may correspond to the high incidence of CAD in Thais. Nevertheless, the molecular mechanism underlying this lipid disorder and CAD in Thais still receive less attention. We thus attempt to determine whether mutations in LDL receptor gene is also a possible cause of primary hypercholesterolemia in Thais. FH- causing mutations have been found in all exons including the promoter region and immediate intron-exon junction of the gene. In this study we focussed our attention on exon 4 which has been found to be the hot spot for mutations in this gene[6]. Exon 4 of the LDL receptor gene was amplified by PCR from genomic DNA of Thai subjects, 45 primary hypercholesterolemic and 33 normolipidemic individuals. These PCR fragments were analyzed for mutations by CFLP technique. Screening by single strand conformation polymorphism (SSCP) was not feasible because the size of amplified exon 4 fragments (436-bp) were beyond the efficiency of conventional SSCP[7,8]. Of 45 subjects with primary hypercholesterolemia, five DNA samples showed abnormal CFLP patterns, relative to a wild-type DNA. A DNA sample with the abnormal CFLP pattern was selected for subsequent identification by direct sequencing of PCR product. From this screening approach, we describe here a novel missense mutation in the exon 4 of the LDL receptor gene in a Thai patient with primary hypercholesterolemia.

MATERIALS AND METHODS

Subjects

The patient sample consisted of 45 (11 males, 34 females) primary hypercholesterolemia (hyperlippidemia type IIa) attending the Department of Preventive and Social Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, and also a small number from Samutsakorn Hospital, Samutsakorn province. Healthy normolipidemic subjects consisted of 33 individuals (4 males, 29 females), attending Siriraj Hospital for regular clinical check up. These subjects were recruited on the basis of plasma cholesterol levels. The subjects whose plasma total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C), corrected for sex and age, exceeding above the 90th percentile were selected as hypercholesterolemic subjects for this study. The cutoff at 90th percentile was made accordingly as defined by NIH[9]. Patients with secondary hypercholesterolemia were excluded from this study.

Plasma Lipid and Lipoprotein Determinations

Lipid profiles were analyzed in collaboration with the Clinical Laboratory Service Department, Faculty of Medical Technology, Mahidol University, Bangkok. Plasma total cholesterol and triglyceride levels were determined with automation (Hitachi 917 Autoanalyzer). The concentration of plasma HDL-cholesterol was measured after precipitation of LDL and VLDL fractions with dextran sulfate and $MgCl_2$ and plasma LDL- cholesterol level was calculated using the formular of Friedewald *et al* as previously described [9].

Amplification of genomic DNA

Genomic DNA used as template for PCR was extracted from lymphocytes by the Guanidine-HCl method from UCLA tissue typing laboratory [10]. Exon 4 of the LDL receptor gene was amplified by PCR with a pair of oligonucleotide primers, SP61 (5'-TGGTCTCGGCCATCCATCCCTGCAG-3') and SP51 (5'-ACGCCCCGCCCCCACCCTGCCC CGC-3')[11]. The nucleotide sequences of SP61 and SP51 primers were complementary to the sequences flanking exon 4 of the LDL receptor gene. The normal PCR fragment was 436 bp in size. The PCR was performed by the protocol as described by Saiki

et.al.[12] Taq DNA polymerase and dNTPs were commercially supplied (Pharmacia Biotech, Sweden). Target DNA was amplified in 1x reaction buffer provided by the manufacturer. A 100 ml reaction volume contained approximately 100 ng of genomic DNA, 0.2 mM each dNTP, 20 pmol of each oligonucleotide primer, and 2.5 units of Taq DNA polymerase. The reaction mixture was covered with mineral oil, heated at 97°C for 2 min to denature DNA and then subsequently involved 35 cycles of denaturation at 95°C for 1 min, annealing at 68°C for 30 sec and extension at 72°C for 1.5 min. The amplification was performed in a DNA thermal cycler (Gene Amp PCR system 2400, Perkin-Elmer, USA).

The PCR products from DNA of hypercholesterolemic and normolipidemic subjects were used for either CFLP analysis or direct DNA sequencing.

Apo B analysis

The DNA sample of the index subject was analysed for Apo-B 3500 mutation by PCR[13,14].

CFLP analysis

The 436-bp PCR fragment of exon 4 was analysed for nucleotide sequence variation by CFLP technique using a commercial kit, CFLP Power Scan System (Life technologies, USA). In this technique, a PCR fragment is denatured and cooled so that intrastrand secondary "hairpin" structures form. The DNA is then partially digested, specifically on the 5' sides of the hairpins, by thermostable endonuclease, Cleavase I. Sequence variation often affects the secondary and tertiary structure of DNA, thereby altering the Cleavase I sites of the mutant relative to the wild-type sequence. When these digested DNA fragments are electrophoresed on a denaturing polyacrylamide gel, a banding pattern typical of each DNA is displayed. The wild-type DNA used in this study was an exon 4 fragment amplified from genomic DNA of a normolipidemic subject whose sequence data conformed the sequence published for exon 4 of the LDL receptor gene[15].

For CFLP analysis, a PCR product was precipitated in 1/10 volume of 4 M NaCl and 2 volumes of cold absolute ethanol, let stand at -20°C for 1 h, centrifuged at 10,000 g for 15 min, washed twice with 70 per cent ethanol, air dried and resuspended in sterile water. The analysis was performed by the protocol provided by the manufacturer, except that the negative control reactions omitting Cleavase I for each PCR sample were not conducted. This was because during optimization the negative control reactions always yielded undigested PCR fragments which were apparent on the top of the gel. For each analysis, the PCR product was denatured at 95°C 30 s, cooled down to 45°C for one min prior to the incubation at 45°C for 2 min with 6 units of Cleavase I in 1x CFLP reaction buffer and 0.2 mM $MnCl_2$. The reaction was stopped by the stop solution supplied with CFLP kit and analysed by 9 per cent (19:1) denaturing polyacrylamide gel electrophoresis in 0.5xTBE buffer. Electrophoresis was carried out with mighty small vertical slab unit (Hoefer, USA) at constant 100 V, 3 hours at room temperature. The DNA pattern was visualized by silver staining[16,17]. Abnormal banding pattern of any PCR fragment, relative to that of wild type DNA, reflects that mutation exists in that PCR fragment. According to the manufacturer's protocol, the location of mutation can be estimated if an end-labeled primer is used. However, in this study the site of mutation was identified later by DNA sequencing.

Direct sequencing

The 436-bp PCR products were purified by using QIA quick PCR purification kit (Qiagen, Germany) and directly sequenced by the dideoxy nucleotide chain termination method[18], using a commercial kit (Silver Sequence Kit, Promega, USA). SP61 and SP51 were also used as sequencing primers. Sequencing signals were detected by silver staining[16,17].

Computer analysis

The amino acid sequences encoded by exon 4 of the LDL receptor gene in six species (seven members) were retrieved from SWISS-PROT database (http://www.expasy.ch/sprot/sprot.top.html). The software for sequence homology analysis was MultAlin (http://www.toulouse.inra.fr/multalin/cgi-bin/multalin.pl)[19].

RESULTS

DNA sample from 33 normolipidemic subjects and 45 patients with primary hypercholesterolemia were screened for mutations in exon 4 of the LDL receptor gene by PCR –CFLP technique. No abnormal DNA banding patterns relative to the

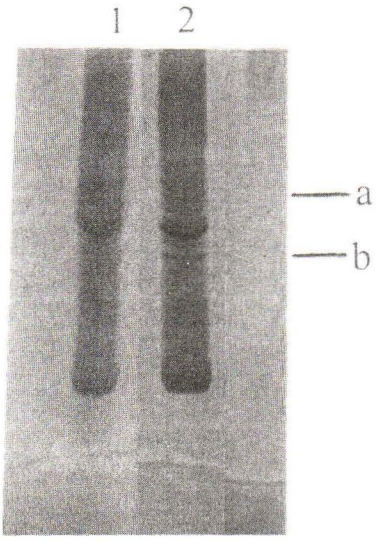

Fig. 1. Screening for mutations in the amplified exon 4 of the LDL receptor gene using the Cleavase Fragment Length Polymorphism (CFLP) technique. Lanes 1 and 2 are the CFLP banding patterns of wild-type and mutant DNA, respectively. The DNA fragments were separated in denaturing polyacrylamide gel and visualized by silver staining. a and b indicate the additional bands apparent only in the mutant DNA, relative to the wild-type DNA.

wild-type DNA was observed in all 33 normolipidemic subjects. However, different CFLP banding patterns relative to the pattern of wild-type DNA were observed in six DNA samples obtained from subjects with primary hypercholesterolemia. One DNA sample with different CFLP pattern, thus presumed as a mutant, was selected for further investigation by direct DNA sequencing. The CFLP patterns of both wild-type and the putative mutant DNA were presented in Fig. 1. The result of direct DNA sequencing, comparing the DNA sequences of the putative mutant with the wild-type DNA, is shown in Fig. 2. The patient in this analysis was heterozygous for a G to T transversion at nucleotide sequence number 514 which would change the amino acid encoded by codon 151 from charged R group aspatic (GAC) to uncharged R group tyrosine (TAC) (see Fig. 2), noted as D151Y. The amino acid encoded by codon151 belongs to the fourth repeat of the ligand binding domain. This region of the ligand binding domain in the LDL receptor gene is evolutionarily conserved as revealed by multiple amino acid sequence alignment. Amino acid sequences of 7 members: hamster (sp, p35950), mouse (sp, P35951), rat (sp, p35952), human (sp, p01130), rabbit (sp, p20063), frog 1 (sp, p99087) and frog 2 (sp, p99088) of the LDL receptor were

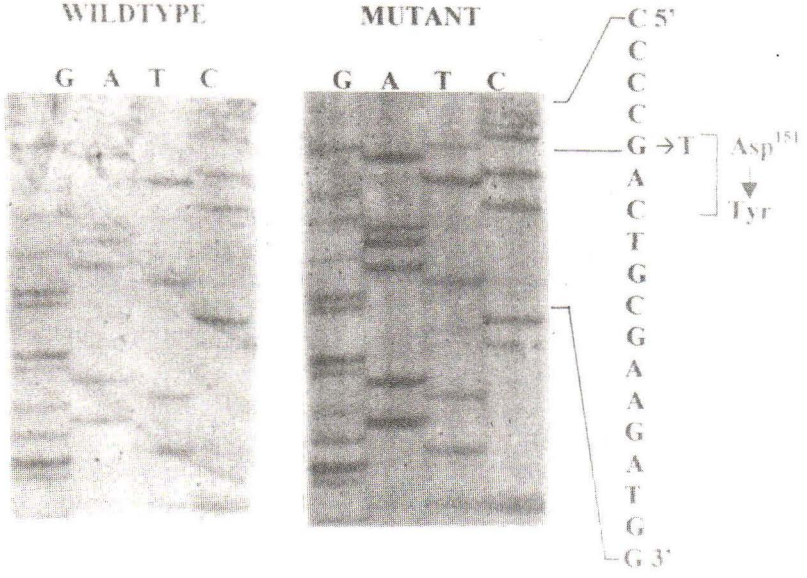

Fig. 2. DNA sequencing of exon 4 of wild-type DNA (left) and mutant DNA (right). The DNA sequences of nucleotides 510-527 in exon 4 are shown for the normal and abnormal CFLP banding patterns. Heterozygosity (G/T) at nucleotide 514, resulting in a substitution of tyrosine for aspartic acid, is indicated

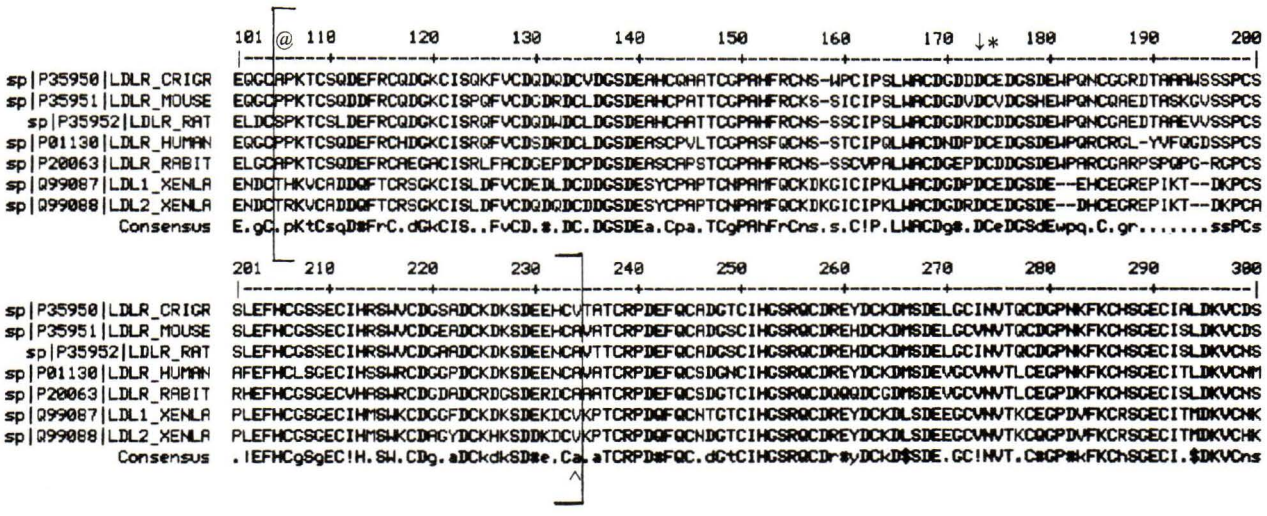

Fig. 3. A multiple amino acid sequence alignment of exon 4 of the LDL receptor gene in six species (7 members). The SWISS-PROT accession number of each sequence is presented in front of the sequence. @ and ^ indicate, respectively, the first (codon 84) and last (codon 211) amino acid of exon 4. The conserved codons 151 and 152 are marked by ↓ and *, respectively. High consensus amino acid are shown in red and low consensus are in blue. In this study, a missense mutation D151Y, i. e., a substitution of tyrosine for aspartic acid at codon 151, was observed in an index subject.

aligned, and the alignment was presented in Fig.3. The SWISS-PROT accession number of each sequence is indicated in parenthesis. Among 129 amino acid residues coding by exon 4 of the LDL receptor gene, 59 amino acid residues are absolutely conserved and the other 6 residues undergo conservative substitution (i.e., substitution where similar physical and chemical properties are conserved)[20]. The amino acid at codon 151 is completely conserved in all members of 6 species (as shown in Fig. 3) and the D151Y mutation is a non-conservative substitution at this completely conserved amino acid residue in the LDL receptor protein.

The index subject is a 53 years old woman. Her untreated lipid levels were 332 mg/dl for plasma total cholesterol, 217 mg/dl for LDL-cholesterol, 84.3 mg/dl for HDL-cholesterol and 159 mg/dl for triglyceride. She has no features of either tendon xanthomas or CAD. Arg 3500 Gln mutation, causing FDB, was not found in this patient.

DISCUSSION

One of the mutation screening methods which has been wildly used is SSCP. This technique has been proved to be a powerful tool to detect mutations in the LDL receptor gene[21]. However, one common limit for SSCP detection is that the size of DNA fragment to be analysed must be approximately 200 bp[7,8]. Due to this limitation of SSCP, CFLP technique was used to analyse nucleotide sequence variation in PCR amplified fragment (exon 4 of LDL receptor gene) of 436 bp in size. PCR fragments ranging in size from 50 bp to 2.7 bp have been successfully analysed by CFLP [22]. From this study, CFLP analysis was proved to be an alternatively powerful mutation screening technique.

In this study, we screened for mutations in the LDL receptor gene in Thai subjects with primary hypercholesterolemia. The screening was also applied for 33 Thai subjects with normolipidemia. Of the 45 DNA samples from hypercholesterolemic

subjects, six of them had different CFLP banding patterns relative to a wild-type DNA pattern. In order to prove that a mutation or nucleotide sequence variation did exist in the PCR fragment with different CFLP banding pattern, one of these six samples was subsequently analysed by DNA sequencing. From this analysis, a single base change, G to T transversion at nucleotide sequence number 514, in codon 151 was detected. This mutation results in a nonconservative substitution of charged R group aspatic acid (GAC) with uncharged R group tyrosine (TAC), i. e., D151Y. The amino acid encoded by codon 151 belongs to the fourth repeat of the ligand binding domain. From the multiple sequence alignment for amino acid sequences of 6 species, this region of ligand binding domain in LDL receptor gene is evolutionarily conserved (see Fig. 3). In particular, it should be noted that the amino acid codon 151 and 152 are absolutely conserved in all 6 species in this alignment. The codon 152 in exon 4 of the LDL receptor gene has been reported previously as a hot spot for point mutations[23]. However, the missense mutation D151Y, has never been described elsewhere.

In one report, the substitution of Ser 156 with Leu in the same fourth repeat of ligand binding domain of the LDL receptor gene slows the transport of the protein to the cell surface[24]. This defective receptor cannot bind LDL, which contains apo B-100, but it does bind β-migrating VLDL, which contains apo E in addition to apo B-100[24]. The serine residue at codon 156 is also completely conserved in the six species as seen from the multiple amino acid sequence alignment (see Fig. 3). The conservation of amino acid residues among related proteins suggests their significance in protein structure and function. D151Y is a nonconservative substitution resulting in charge alteration of an amino acid located in a completely conserved position in LDL receptor sequences. So, it might be expected that the D151Y mutation could result in profound effects on the receptor protein. In this case, the interaction between the negatively charge Asp and the positively charged ligand is an important step in ligand binding and the substitution of Asp for the weakly acidic Tyr possibly impairs the binding of the apo B-100.

From this observation, the D151Y mutation might cause a defect in the LDL receptor protein and therefore, is a causative mutation of hypercholesterolemia in the index subject. The further observations which might reinforce this conclusion are that the D151Y mutation was absent in 33 normolipidemic subjects, as analysed by PCR-CFLP technique, and Arg 3500 Gln mutation, causing FDB, was not found in this index subject. More over, the same group of subjects used in this study was also screened for mutations in exon 9, the second hot spot of the LDL receptor gene. No mutation was observed in this exon in the index subject [11]. However, in order to conclude that D151Y is a disease-causing mutation, coseggregation analysis of this mutation with the hypercholesterolemia phenotype in the pedigree of the index case and *in vitro* expression of the mutant allele must be performed. In addition, the remainder of the gene must be scanned to demonstrate that no other mutation exists in this locus.

The other five samples with different CFLP banding patterns will be also characterized later by DNA sequencing. If mutations really occur in these DNA samples as seen from CFLP analysis, these mutations (in exon 4 of the LDL receptor gene) possibly account for about 13 per cent (5/45) of hypercholesterolemia phenotype in these Thai patients.

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การกลายพันธุ์ D151Y ใน Exon 4 ของยีน low density lipoprotein receptor ในคนไทยที่มีภาวะโคเลสเตอรอลในเลือดสูงแบบปฐมภูมิ

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การกลายพันธุ์ที่เกิดขึ้นในยีน low density lipoprotein (LDL) receptor ก่อให้เกิดโรคพันธุกรรมที่เรียกว่า familial hypercholesterolemia (FH) การกลายพันธุ์ดังกล่าวเกิดขึ้นได้ทั้ง 18 exon ของยีนนี้ แต่ exon 4 เป็น hot spot การศึกษาครั้งนี้เป็นการศึกษาการกลายพันธุ์ของยีน LDL receptor ในบริเวณ exon 4 ในคนไทยที่มีภาวะโคเลสเตอรอลในเลือดสูงแบบปฐมภูมิจำนวน 45 คน โดยใช้เทคนิค PCR-CFLP และ direct DNA sequencing ด้วยเทคนิคดังกล่าวนี้ได้พบผู้ป่วยเพศหญิงหนึ่งคนมีการเปลี่ยนแปลงของเบสแบบ transversion ใน allele ข้างหนึ่งจาก guanine เป็น thymine ที่นิวคลีโอไทด์ลำดับที่ 514 อันจะมีผลทำให้เกิดการ เปลี่ยนแปลงของกรดอะมิโน จาก aspartic acid (GAC) ซึ่งมีประจุไปเป็น tyrosine (TAC) ซึ่งไม่มีประจุ การเปลี่ยนแปลงนี้เกิดขึ้นที่ codon 151 การกลายพันธุ์นี้จึงเรียกว่า D151Y กรดอะมิโนที่ตำแหน่งนี้ไม่มีการเปลี่ยนแปลงเลยในวิวัฒนาการ ดังนั้นการเปลี่ยนแปลงที่เกิดขึ้นนี้อาจมีผลทำให้โครงสร้างและ/หรือหน้าที่ของโปรตีน LDL receptor เสียไปและก่อให้เกิดภาวะโคเลสเตอรอลในเลือดสูงตามมา การกลายพันธุ์ D151Y นี้ไม่พบในคนปกติจำนวน 33 ราย ผู้ป่วยรายนี้ได้รับการตรวจหาการกลายพันธุ์ Arg 3500 Gln ในยีน apo B-100 ด้วย การกลายพันธุ์นี้ก่อให้เกิดโรคพันธุกรรมที่เรียกว่า familial defective apo B-100 (FDB) พบว่าไม่มีการกลายพันธุ์ดังกล่าวในผู้ป่วยรายนี้ ดังนั้น จึงน่าจะเป็นไปได้ว่า ภาวะโคเลสเตอรอลในเลือดสูงของผู้ป่วยรายนี้อาจเกิดจากการกลายพันธุ์ D151Y ในยีน LDL receptor

คำสำคัญ : ภาวะโคเลสเตอรอลในเลือดสูง, ยีน LDL receptor, การกลายพันธุ์

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