Mutation Analysis of Exon 9 of the LDL Receptor Gene in Thai Subjects with Primary Hypercholesterolemia

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Abstract

The low density lipoprotein (LDL) receptor plays an important role in cholesterol homeostasis. A mutation in this gene causes an autosomal codominant disorder, namely familial hypercholesterolemia (FH). In this study, single strand conformation polymorphism (SSCP) analysis was used to screen for mutations in exon 9 of the LDL receptor gene in a group of 45 Thai patients (11 males and 34 females) with primary hypercholesterolemia. The peptide encoded by exon 9 belongs to the epidermal growth factor (EGF) precursor homology domain which is highly conserved in the LDL receptor protein. An abnormal SSCP pattern was observed in one female patient. The same screening strategy was also used to screen DNA samples from 33 normolipidemic subjects. All of these samples showed normal SSCP pattern. By direct DNA sequencing, the underlying mutation in the DNA with abnormal SSCP pattern was identified. The index subject was heterozygous for a T to C transition at nucleotide 1235. This transition would cause a nonconservative substitution of a nonpolar side chain amino acid "methionine" at codon 391, with an uncharged polar side chain amino acid "threonine", note M391T. From multiple amino acid sequence alignment in six species, the amino acid at codon 391 and the others nearby are completely conserved. Such nonconservative substitution of an amino acid residue in a highly conserved region could consequently result in a functional and/or structural defect in the receptor protein. In conclusion, we propose that M391T is likely to be the cause of hypercholesterolemia in this index subject.

Key word : Hypercholesterolemia, LDL Receptor Gene, Mutation

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Hypercholesterolemia has been recognized as a major risk factor for atherosclerosis and coronary heart disease (CHD)[1]. A high prevalence of hypercholesterolemia due to elevated serum LDL-cholesterol levels is also found in the Thai population[2]. This finding may correspond with the high incidence of CHD in Thais. Hypercholesterolemia can be caused by both genetic and environmental factors. However, it is now evident that genetic factors potentially play a more important role in causing an elevation of blood cholesterol. Mutations in genes involving in lipid metabolism have been found to effect lipid phenotypes. Mutations in LDL receptor and apolipoprotein (apo) B-100 genes cause familial hypercholesteolemia (FH) and familial defective apo B-100 (FDB), respectively[3,4]. LDL receptor gene defects are relatively common, occurring in about one in every five hundred persons as the heterozygous form and one in a million as the homozygous form[4,5].

We speculate that a mutation in the LDL receptor gene is a possible cause of primary hypercholesterolemia in Thais. Therefore, in this study we focussed our attention on the LDL receptor gene. The human LDL receptor gene is located on the distal short arm of chromosome 19[6]. Its cDNA was cloned and sequenced in 1984[7]. The product of this gene is a transmembrane receptor protein which removes LDL particles from blood. Defects in this protein result in increased serum cholesterol. So far, more than five hundred mutations at the LDL receptor locus, causing FH, have been reported in several populations (http://www.ucl.ac.uk/fh/muttab.html). The LDL receptor gene consists of 18 exons and 17 introns. Mutations causing FH occur in all exons throughout the gene. However, exon 4 and 9 of this gene have been found to be the first and second hot spot for mutations, respectively[8].

Here, we have screened for mutations in exon 9 at the LDL receptor locus in 45 Thai subjects with primary hypercholesteolemia and 33 subjects with normolipidemia. DNA samples were amplified by polymerase chain reaction (PCR) and screened for mutations by single strand conformation polymorphism (SSCP) technique. Direct sequencing was performed to identify mutations in DNA samples with an abnormal SSCP pattern. In this report we have described a novel missense mutation in exon 9 of the LDL receptor gene in a Thai patient with primary hypercholesterolemia.

MATERIAL AND METHOD

Subjects

The recruitment of most patients and normolipidemic subjects was performed by the Department of Preventive and Social Medicine, Faculty of medicine Siriraj Hospital, Mahidol University, Bangkok and a small number were found from Samutsakhon Hospital, Samutsakhon Province. The subjects were recruited on the basis of plasma cholesterol levels. Subjects were recruited as hypercholesterolemics when their plasma total cholesterol and LDL-cholesterol levels exceeded the 90th percentile, corrected for age and sex, as defined by NIH[9]. Patients with secondary hypercholesterolemia were excluded from this study. The patient sample consisted of 45 individuals with primary hypercholesterolemia (11 males, 34 females). Healthy normolipidemic subjects consisted of 33 individuals (4 males, 29 females).

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 automatically using a 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 described previously[9].

Amplification of genomic DNA

Genomic DNA was prepared from fasting EDTA blood samples by Guanidine-HCl method[10]. DNA samples were amplified by PCR in a DNA thermal cycler (Gene Amp PCR System 2400, Perkin Elmer, USA). The oligonucleotide primers used for amplification of exon 9 were SP70 (5'-CCTGACC TCGCTCCCCGGACCCCCA-3') and SP71 (5'-GGCTGCAGGCAGGGGCGACGCTCAC-3')[11]. The PCR was performed according to a modification of the protocol previously described by Saiki *et al* [12]. Taq DNA polymerase and dNTPs were commercially supplied (Phamacia Biotech, Sweden). In a final volume of 25 ml, the PCR reaction contained genomic DNA, 1x PCR buffer (supplied by the manufacturer as 10x PCR buffer), 5 pmole of each primer, 200 mM of each dNTP, and a unit of Taq DNA polymerase. The PCR reaction comprised

40 cycles of 95°C 1 min for denaturation and 72°C 1 min for extension. The reaction mixture was initially denatured at 95°C for 3 min and finally extended at 72°C for 5 min.

The PCR products from the DNA of patients and normal subjects were used for either SSCP analysis or direct DNA sequencing.

SSCP Analysis

The PCR fragments of exon 9 were analysed for nucleotide sequence variations by SSCP technique(13). A quantity of 3 ml of a PCR product was mixed with 30 ml of 5x formamide dye mix (Bio101, USA). The mixture was denatured by boiling for 5-10 minutes. After denaturation, the mixture was immediately chilled on ice. All samples were loaded onto 6 per cent (49:1) non-denaturing polyacrylamide gel in 0.5x TBE buffer. Electroporesis was carried out with the S2 sequencing electrophoresis apparatus at 40 watts for 1.5 hours at 4°C. After electrophoresis, the DNA patterns were visualized by silver staining method(14,15).

The wild-type DNA for SSCP analysis was obtained from an amplified exon 9 of DNA sample from a normal control subject whose sequence data conformed the sequence published for exon 9 of the LDL receptor gene(7).

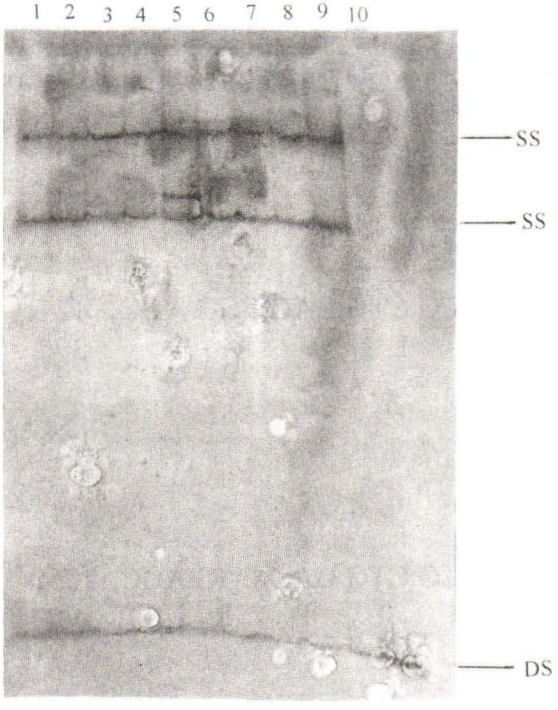

Fig. 1. SSCP analysis of LDL receptor gene. Lanes 1 to 4 are normal SSCP patterns of normolipidemic subjects. Lane 5 is the abnormal SSCP pattern. Lanes 6 to 9 are normal SSCP patterns of high cholesterol level subjects. Lane 10 is a double-stranded DNA pattern. The abnormal SSCP pattern belonged to a 42-year-old woman whose plasma cholesterol and LDL-cholesterol were 357 mg/dl and 279.2 mg/dl, respectively. Single-stranded and double-stranded DNA are indicated as SS and DS.

DNA sequencing of the PCR amplified DNA

The amplified fragment of exon 9 which showed abnormal SSCP pattern was further analysed by automated DNA sequencing on Model 377 DNA sequence, version 2.1.1 (Applied Biosystems Inc., Foster City, CA).

Computer Analysis

The amino acid sequence encoded by exon 9 of the LDL receptor gene in six species 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) (16).

RESULTS

All DNA samples used in this study yielded a 243 bp fragment after amplification. The PCR products from the DNA of patients and normal control subjects revealed the same SSCP patterns as wild-type DNA except for one DNA sample from a female patient. In all SSCP patterns, there were the expected two major single-strand bands plus a more rapidly migrating double strand band. However, an additional "heteroduplex" band between the two single-strand bands was observed in the amplified DNA from the female patient as shown in Fig. 1.

DNA from this index subject was used to amplify the exon 9 fragment and direct sequencing was carried out. From this analysis, the index subject was heterozygous for a T to C transition at nucleotide 1235. The DNA sequence surrounding this transition is shown in Fig. 2. This nucleotide

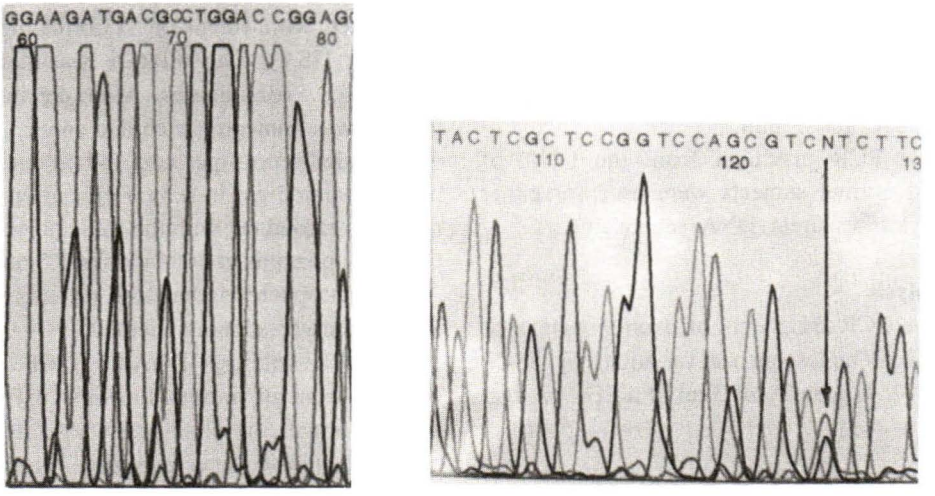

Fig. 2. **DNA sequence of exon 9 of the LDL receptor gene. A PCR fragment of a normolipidemic subject was sequenced by the sense primer (SP70) (A). A PCR fragment with abnormal SSCP pattern was sequenced by the antisense primer (SP71) (B). The subject with the abnormal SSCP pattern is heterozygous for a T to C transition. Since the antisense primer was used for DNA sequencing, the heterozygosity T/C was apparent as A/G (marked with an arrow).**

Normal

1216	CGG	CAC	GAG	GTC	AGG	AAG	ATG	ACG	CTG	GAC	CGG	AGC		1251
385	R	H	E	V	R	K	M	T	L	D	R	S		396

Mutant

							☆							
1216	CGG	CAC	GAG	GTC	AGG	AAG	ACG	ACG	CTG	GAC	CGG	AGC		1251
385	R	H	E	V	R	K	T	T	L	D	R	S		396

Fig. 3. **Comparison of the DNA and amino acid sequence of the wild-type and the mutant exon 9 of the LDL receptor gene. The changed nucleotide is marked with an asterisk.**

changed was predicted to cause the substitution of methionine with threonine at codon 391 in the LDL receptor gene. This is a nonconservative substitution because the amino acid at this position has been changed from an amino acid with nonpolar side chain to another one with uncharged polar side chain. The DNA and amino acid sequence of this exon and the indicated change in the index case is shown in Fig. 3.

Amino acid sequences of exon 9 of the LDL receptor in six species (7 members) were aligned in this study as shown in Fig 4. The SWISS-PROT accession number of each sequence is indicated in parenthesis. The amino acid sequences encoded by

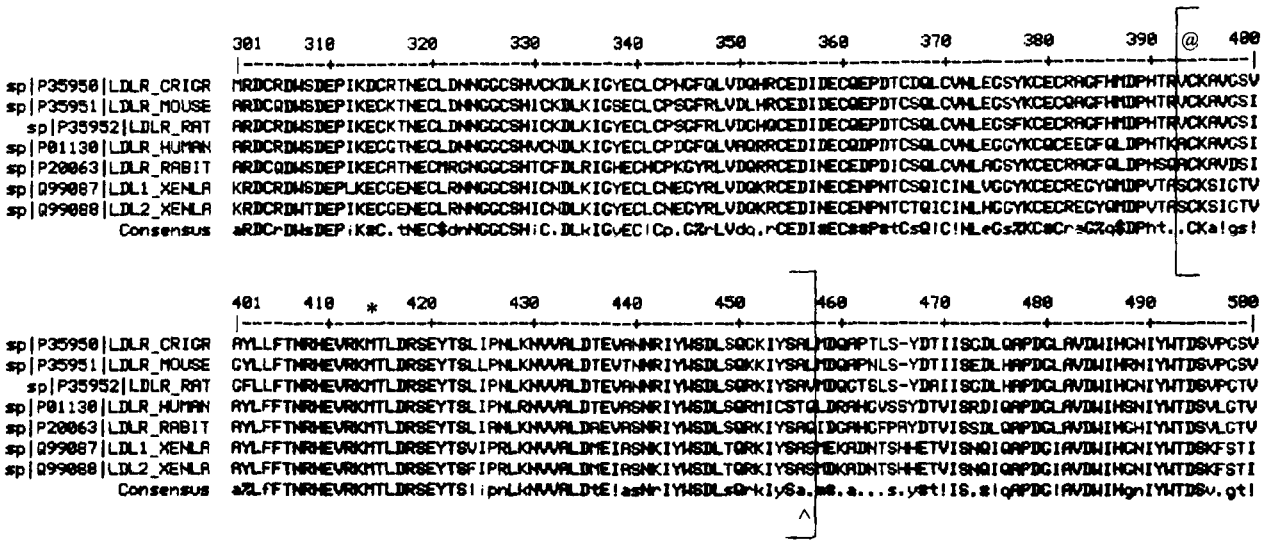

Fig. 4. A multiple amino acid sequence alignment of exon 9 of the LDL receptor gene in six species (7 members). The SWISS-PROT accession number of each sequence is presented in front of sequence. @ and ^ indicate the first (codon 370)and final (codon 456) amino acid of exon 9. The codon 391, where the missense M391T occurred, is marked by *. High and low consensus amino acids are shown in red and blue, respectively.

exon 9 are evolutionarily conserved as revealed by this alignment. Among 65 amino acid residues, coded by exon 9, 41 residues are completely conserved and three residues are in positions with conservative change (i. e., a change where similar physical and chemical properties are conserved). The amino acid at codon 391 and the others nearby are completely conserved in six species (as shown in Fig. 4).

The index subject was a 42 year old women whose untreated lipid levels were 357 mg/dl for total cholesterol, 279.2 mg/dl for LDL-cholesterol, 39 mg/dl for HDL-cholesterol and 194 mg/dl for triglyceride. She has no features of either tendon xanthomas or CAD.

DISCUSSION

As mentioned earlier, a defect in the LDL receptor is a cause of primary hypercholesterolemia, namely FH. Such a defect is in turn caused by a mutation in the LDL receptor gene and is inherited in an autosomal codominant manner[17]. We speculate that a mutation in the LDL receptor gene is a possible cause of primary hypercolesterolemia in Thais so we have searched for mutations in this gene in a group of Thai subjects. In this study, we screened for mutations in exon 9, the second hot spot, of the LDL receptor gene by PCR-SSCP method.

Normal SSCP pattern of the exon 9 was observed in all DNA samples from 33 normolipidemic subjects. This result should indicate that mutation was absent in the exon 9 in these samples. Of the 45 DNA samples from hypercholesterolemic subjects, one abnormal SSCP pattern was apparent in a female patient. The abnormal SSCP pattern was identified by direct DNA sequencing. A single base change, a T (ATG) to C (ACG) transition at nucleotide sequence number 1235 in codon 391 was found in one allele. This heterozygous transition is predicted to cause a nonconservative substitution of a nonpolar side chain amino acid "methionine" in codon 391 with an uncharged polar side chain amino acid "threonine", namely M391T.

This missense mutation occurred in exon 9 which belongs to the highly conserved EGF precursor homology domain of the LDL receptor protein[5]. The multiple amino acid sequence align-

ment performed in this study suggests that the amino acid sequences encoded by exon 9 of LDL receptor gene are evolutionarily conserved. In paticular the amino acid codon 391 is located in the completely conserved sequences in 6 species in this alignment. The conservation of amino acid residues among related proteins should reflect their significance in protein structure and function. Mutation in any of these conserved residues, such as the codon 391 in this case, might disturb the structure and/or function of the protein. Several missense mutations occur in this region, including exon 9, are reported to cause FH. These mutations are, for examples, L393R, D394H, R395G and R395W (http://www.ucl.ac.uk/fh/muttab.html)[8,18]. The EGF-precursor homology domain is required for LDL binding, for acid induced association of ligand and receptor, and for receptor recycling[19]. From the recent database, 40 disease-causing mutations occurring in exon 9 have been reported (http://www.ucl.ac.uk/fh/muttab.html). However, the missense M391T mutation presented in this study has never been reported elsewhere.

The same group of subjects used in this study was also screened for mutations in the hot spot exon 4 and this index subject has no mutation in this exon[20]. Further analyses are required to conclude that M391T is a disease-causing mutation. This mutation should be scanned in several more normal healthy individuals. For the DNA sample from this index subject, the promoter sequence and the rest of the exons of the LDL receptor gene must be scanned to see whether any other mutation exists in this gene or not. Other genes involving in cholesterol metabolism, such as apo B and apo E genes, need to be analysed. Most necessarily, cosegregation analysis of this mutation with the hypercholesterolemia phenotype in the family members of the index subject and *in vitro* expression of the mutant allele must be performed. All of these analyses altogether will be necessary to confirm that M391T is a mutation which causes hypercholesterolemia in the index subject.

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การกลายพันธุ์ M391T ใน exon 9 ของยีน LDL receptor ในคนไทยที่มีภาวะโคเลสเตอรอลในเลือดสูงแบบปฐมภูมิ

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โปรตีน LDL receptor มีบทบาทสำคัญต่อการรักษาระดับโคเลสเตอรอลในเลือด การกลายพันธุ์ของยีน LDL receptor ก่อให้เกิดภาวะโคเลสเตอรอลในเลือดสูง ซึ่งเป็นโรคที่ถ่ายทอดได้ทางพันธุกรรมเรียกว่า Familial hypercholesterolemia (FH) การกลายพันธุ์ในยีน LDL receptor มีความหลากหลายในระดับโมเลกุลเกิดขึ้นได้ในทุก exon แต่การวิจัยนี้ได้เลือกศึกษาเฉพาะ exon 9 เป็นไทด์ของ exon นี้ เป็นส่วนหนึ่งของ epidermal growth factor (EGF) precursor homology domain ซึ่งเป็นส่วนที่มีลำดับของกรดอะมิโนเปลี่ยนแปลงน้อยมากในวิวัฒนาการ จากการตรวจตัวอย่างดีเอ็นเอของผู้ป่วย 45 ราย โดยใช้เทคนิค SSCP พบว่ามีผู้ป่วยหญิง 1 รายที่มี SSCP pattern ผิดปกติ ส่วนตัวอย่างดีเอ็นเอของคนปกติ จำนวน 33 ราย ไม่พบ SSCP pattern ที่ผิดปกติเลย จากการหาลำดับเบสของ exon 9 ในตัวอย่างที่มี SSCP pattern ผิดปกติ ได้พบการกลายพันธ์ M391T การกลายพันธ์นี้มีการเปลี่ยนแปลงของเบสใน allele ข้างหนึ่งตรงนิวคลีโอไทด์ลำดับที่ 1235 ซึ่งเปลี่ยนจาก thymine เป็น cytosine อันจะมีผลทำให้เกิดการเปลี่ยนแปลงแบบ nonconservative substitution ของกรดอะมิโนที่ codon 391 จาก methionine (ATG) ซึ่งไม่มีขั้วไปเป็น threonine (ACG) ซึ่งมีขั้ว กรดอะมิโนที่ตำแหน่งนี้ใน 6 species ไม่มีการเปลี่ยนแปลงเลยในวิวัฒนาการจากการวิเคราะห์ด้วย multiple amino acid sequence alignment การกลายพันธุ์ซึ่งก่อให้เกิดการเปลี่ยนแปลงดังกล่าวนี้อาจมีผลทำให้โครงสร้างและ/หรือหน้าที่ของโปรตีน LDL receptor เสียไป และก่อให้เกิดภาวะโคเลสเตอรอลในเลือดสูงในผู้ป่วยรายนี้

คำสำคัญ : ภาวะโคเลสเตอรอลในเลือดสูง, ยีน LDL receptor, การกลายพันธุ์

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