Screening for Mutations in Exons Encoding the Ligand-Binding Domain of the LDL Receptor Gene Using PCR-CFLP and PCR-SSCP

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Abstract

Primary hypercholesterolemia includes both monogenic disorders and polygenic conditions. Two well defined monogenic disorders are familial hypercholesterolemia (FH) and familial defective apolipoprotein (apo) B-100 (FDB). Both disorders convey high risk of premature coronary artery disease. FH and FDB are caused by mutations in LDL receptor and apo B-100 genes, respectively. In the present study, mutations in both genes in Thai subjects with primary hypercholesterolemia were screened. For apo B-100 gene, a common mutation R3500Q was screened. This mutation was not observed in the patients (n = 45). For LDL receptor gene, mutations in the exons encoding the ligand - binding domain were screened. By PCR-CFLP analysis, 18 abnormal CFLP patterns in exon 4, the hot spot for mutations, were found in patients (n=45). One of the DNA samples with abnormal CFLP patterns was previously identified and reported as a possible disease-causing mutation, namely D151Y. For the other exons, the screening technique was PCR-SSCP. Abnormal SSCP patterns in DNA samples from patients (n=20) were found as follows, two in exon 3, one in exon 5 and another one in exon 6. Further characterization by DNA sequencing and family studies for these abnormal patterns are underway.

Key word : Hypercholesterolemia, LDL Receptor Gene, Mutation, PCR-CFLP, PCR-SSCP

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Mutations of the LDL receptor gene, causing primary hypercholesterolemia, are among the most common genetic changes in humans. The disorder caused by such mutations is known as familial hypercholesterolemia (FH). FH has been considered as one of the most common inherited disorders in humans[1] and FH subjects have a considerably increased risk of premature coronary artery disease (CAD)[2,3]. However, effective lipid lowering agents are now available and therefore, efforts should be undertaken to identify those carrying the disease[3].

FH has previously been diagnosed by the use of clinical criteria: elevation of LDL cholesterol up to twice the normal level, presence of xanthomas and a family history of premature CAD[4]. However, clinical diagnosis of FH is not always unequivocal, especially in young patients in which physical stigmata are often not present[4]. In addition, mutations in another gene, apo B-100, also cause a phenotype undistinguishable from FH[5]. The disorder is known as familial defective apo B-100 (FDB). In these cases, a molecular assay would be desirable for certain diagnosis in families and for genetic counselling. Identification of the underlying mutations in the LDL receptor gene, however, could serve as a more specific test for FH. More than 600 different mutations in the LDL receptor gene causing FH have so far been reported worldwide[4].

It has long been believed that life style and dietary habits of Thai people has become more Westernized and this might promote a risk for both hypercholesterolemia and CAD. However, two novel possible disease-causing mutations in the LDL receptor gene were recently identified in patients with primary hypercholesterolemia[6,7]. This is the first evidence that primary hypercholesterolemia in some of Thai people is likely due to mutations in LDL receptor gene.

Herein, further search for mutations in the exons encoding the ligand-binding domain (exons 2 to 6) of the LDL receptor gene was carried out. The screening for mutations in exon 4 was performed by polymerase chain reaction-cleavase fragment length polymorphism (PCR-CFLP) in 45 patients with primary hypercholesterolemia and 33 normolipidemic subjects. Actually, such screening in exon 4 was already done for this same group of subjects[6]. The reinvestigation was performed to confirm the previous finding. From this reinvestigation, several more abnormal CFLP patterns were observed. Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) was applied for the other exons. The PCR-SSCP screening was conducted in a subset of those subjects who had already been screened for mutations in exon 4 (n = 20 and 19 for patients and normolipidemic subjects, respectively). Two abnormal SSCP patterns were observed in exons 3, one was seen in exon 5 and another one in exon 6. Characterizations of these abnormal patterns are underway.

MATERAIL AND METHOD

Subjects

These subjects were the same group used in the previous studies[6,7]. The patient samples consisted of 45 (11 males, 34 females) primary hypercholesterolemics (hyperlipidemia 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, Samusakorn province. Healthy normolipidemic subjects consisted of 33 individuals (4 males, 29 females), attending Siriraj Hospital for regular clinical check up. These subjects were recruited mainly on the basis of their plasma cholesterol levels. The subjects whose plasma total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C), corrected for sex and age, exceeding the 90th percentile were selected as hypercholesterolemic subjects for this study. The cutoff at 90th percentile was made accordingly as defined by NIH (The National Institue of health)[8]. Patients with secondary hypercholesterolemia were excluded from this study. Subjects with triglyceride levels exceeding 200 mg/dl were also excluded.

Plasma lipid and lipoprotein determinations

Lipid profiles were analyzed by 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 was calculated using the formula of Friedewald et al as previously described[8].

Amplification of genomic DNA

Genomic DNA used as template for PCR was extracted from lymphocytes by the Guanidine-HCl method[9]. The oligonucleotide primers used

Table 1. Nucleotide sequences of primers used for amplification of the exons encoding the ligand-binding domain of the LDL receptor gene[10].

Exon number	Sequence name	Nucleotide Sequences of primers (5' → 3')
2	SP 57	CCTTTCTCCTTTTCCTCTCTCTCAG
	SP 58	AAATGCATATCATGCCCAAACTCAC
3	SP 59	TGACAGTTCAATCCTGTCTCTTCTG
	SP 60	AATAGCAAAGGCAGGGCCACACTTA
4	SP 51	ACGCCCCGCCCCCACCCTGCCCCGC
	SP 61	TGGTCTCGGCCATCCATCCCTGCAG
5	SP 62	CAACACACTCTGTCCTGTTTTCCAG
	SP 63	GGAAAACCAGATGGCCAGCGCTCAC
6	SP 64	TCCTTCCTCTCTCTGGCTCTCACAG
	SP 65	GCAAGCCGCCTGCACCGAGACTCAC

Table 2. Amplification profiles for the exons encoding the ligand-binding domain of the LDL receptor gene.

Exon number	Amplification profiles	Number of cycles
2	95°C 1 min, 47°C 1 min, 72°C 1 min	35
3	95°C 1 min, 68°C 1 min, 72°C 1 min	35
4	97°C 1 min, 68°C 2 min, 72°C 1 min	35
5	95°C 1 min, 68°C 1 min, 72°C 1 min	35
6	95°C 1 min, 72°C 1 min	35

for PCR were synthesized as previously described (10). Their nucleotide sequences are presented in Table 1. The nucleotide sequences of these primers were complementary to the sequences flanking the exons to be amplified. Therefore, the amplicons would include the intron-exon junctions. The PCR was performed by the protocol as described by Saiki et al[11]. Taq DNA Polymerase and dNTPs were commercially supplied by Amersham Pharmacia Biotech (Sweden).

Amplification in a final volume of 25 μl contained genomic DNA, 1x PCR buffer (supplied by the manufacturer as 10xPCR), 0.2 mM each dNTP, 10 pmol of each oligonucleotide primer, and 1 unit of Taq DNA Polymerase. The reaction mixture was covered with mineral oil and subsequently amplified according to the cycling conditions as presented in Table 2. All PCR reactions were denatured at the specified denaturation temperatures for 5 min in the first cycle and extended at 72°C for 7 min in the final cycle. For amplification of exon 4, the reaction mixtures, omitting Taq DNA Polymerase, were denatured by incubating in a boiling water-bath for 5 min, subsequently chilled on ice for 1 min before the addition of the Polymerase enzyme. The amplification was performed in a DNA thermal cycler (Gene Amp PCR system 2400, Perkin-Elmer, USA).

The PCR products from DNA samples of hypercholesterolemic and normolipidemic subjects were subsequently used for CFLP (exon 4) and SSCP (exons 2, 3, 5 and 6) analyses.

Apo B analysis

All DNA samples from hypercholesterolemic subjects used in this study were also analysed for the common R3500Q mutation in the Apo B gene by PCR[12,13].

CFLP analysis

The 436-bp PCR fragment of exon 4 was analysed for sequence variation by CFLP technique using a commercial kit, CFLP Power Scan System (Life Technologies, USA). The method for this analysis was as previously described[6]. The same wild-

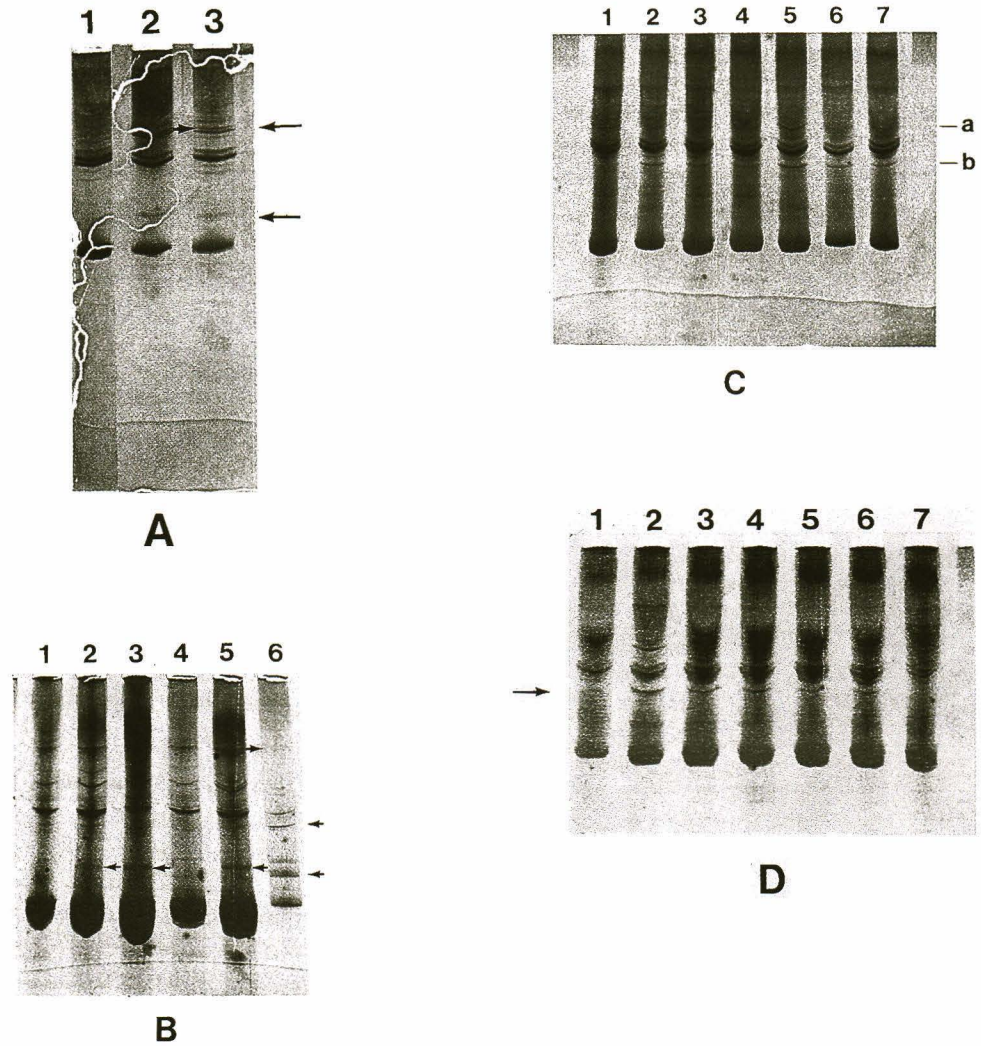

Fig. 1. PCR-CFLP patterns in wild-type and putative mutant DNA. Lane 1 in panels A, B, C and lane 7 in panel D are wild-type DNA obtained from a normolipidemic subject. This wild-type DNA is the same sample as previously reported[6]. Other lanes in all panels are DNA from patients with primary hypercholesterolemia. Panel A: extra bands (marked with arrows) are apparent in putative mutant DNA samples in lane 2 and 3. Panel B: abnormal PCR-CFLP patterns are observed in lanes 2, 3, 5 and 6. Extra bands in each lane are marked with arrows on the right. A missing band, compared with the wild-type CFLP pattern, in lane 6 is marked with an arrow on the left. Panel C : abnormal PCR-CFLP patterns are observed in lanes 2 - 7, extra bands observed in these lanes are marked with an arrow(b). Another extra band observed only in lane 5 is also marked with an arrow (a). Panel D: abnormal PCR-CFLP patterns are observed in lanes 2 - 6. An extra band in these lane is marked with an arrow. Plasma lipid levels of patients with abnormal CFLP patterns are presented in Table 3.

Table 3. Age, sex and plasma lipid levels of patients with abnormal CFLP patterns (presented in Fig. 1).

Panel	Lane No.	Sex	Age (year)	TC (mg/dl)	LDL-C (mg/dl)	TG (mg/dl)	HDL (mg/dl)
A	2	M	53	360	302	109	36
	3	F	59	369	304.4	114	49.8
B	2	M	60	280	191	154	58.2
	3	F	64	331	213	285	61
	5	F	52	286	193.4	223	48
	6	F	34	379	318.8	56	49
C	2	F	65	310	195	136	58.2
	3	F	68	281	193	205	47
	4	M	51	305	226.8	216	35
	5	F	65	318	239.6	1,977	39
	6	F	65	344	267.	128	51
	7	M	53	302	221	215	38
D	2	F	44	318	237.4	231	34.4
	3	F	54	322	217.1	153	84.3
	4	F	75	281	211.6	127	44
	5	F	44	286	219.6	117	43
	6	F	56	287	237.4	47	39.62
	7	M	41	267	214.4	128	27

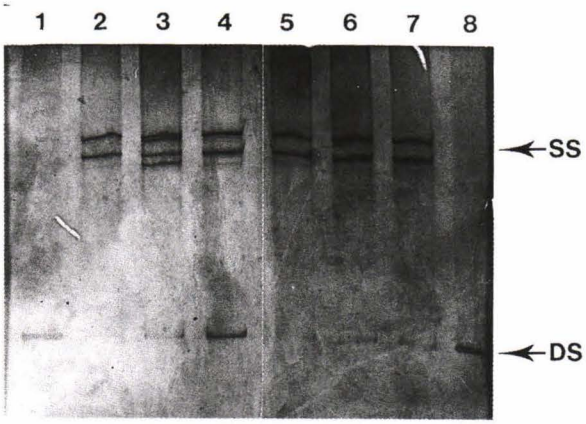

Fig. 2. PCR-minigel SSCP analysis of exon 3 of LDL receptor gene. Lanes 1 and 8 are double-stranded DNA. Lane 2 presents a normal SSCP pattern of a normolipidemic control subject. Lanes 3 to 7 are SSCP patterns of patients with primary hypercholesterolemia condition. Abnormal SSCP pattern is apparent in lane 3. The abnormal SSCP belongs to a 53-year-old man whose plasma lipid levels were 360 mg/dl (TC), 302 mg/dl (LDL-C), 109 mg/dl (TG) and 36 mg/dl (HDL-C). Single-stranded and double-stranded DNA are indicated as SS and DS. Another sample (with similar abnormal SSCP pattern) is not included in this figure.

type DNA sample used for the previous analysis[6] was also used for this analysis. The DNA patterns were visualized by silver staining[14,15]. All analyses were done in duplicate.

SSCP analysis

The PCR fragments of exons 2 (178 bp), 3 (177 bp), 5 (173 bp) and 6 (177 bp) were analysed for sequence variations by SSCP technique[16]. The method for this analysis was similar to that previously described [7]. However, minigel apparatus (10 x 7.3 x 0.075 cm; Protein II apparatus, Biorad, USA) was optimized for replacement of the S2 sequencing apparatus (30 x 40 x 0.04 cm; S2, BRL, USA). The minigel-SSCP was performed with 12 per cent polyacrylamide gel (2% cross link) in 0.5 x TBE buffer. Electrophoresis, with Protein II apparatus, was carried out for 1.5 to 2 hours at 100 volts at 4°C. The DNA patterns were visualized by silver staining[14,15]. All analyses were done in duplicate.

RESULTS

The apoB R3500Q mutation was not observed in all DNA samples from hypercholestero-

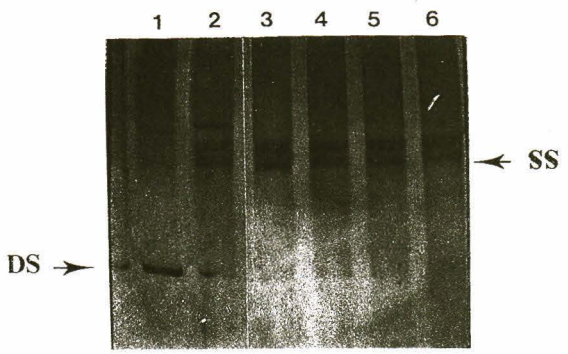

Fig. 3. PCR- minigel SSCP analysis of exon 5 of LDL receptor gene. Lane 1 is double-stranded DNA. Lane 6 presents a normal SSCP pattern of a normolipidemic control subject. Lanes 2 to 5 are SSCP patterns of patients with primary hypercholesterolemia condition. Abnormal SSCP pattern is apparent in lane 2. The abnormal SSCP belongs to a 50-year-old woman whose plasma lipid levels were 287 mg/dl (TC), 223.4 mg/dl (LDL-C), 98 mg/dl (TG) and 44 mg/dl (HDL-C). Single-stranded and double-stranded DNA are indicated as SS and DS.

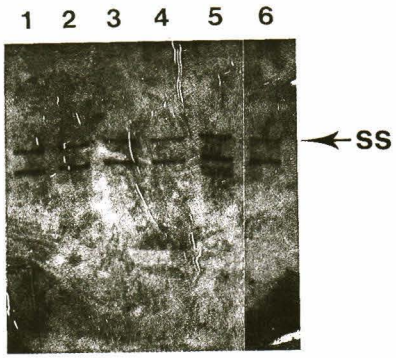

Fig. 4. PCR- minigel SSCP analysis of exon 6 of LDL receptor gene. Lane 6 presents normal SSCP pattern of a normolipidemic control subject. Lane 1 to 5 are SSCP patterns of patients with primary hypercholesterolemia condition. An abnormal SSCP pattern is apparent in lane 5. The abnormal SSCP belongs to a 59-year-old woman whose plasma lipid levels were 369 mg/dl (TC), 304.4 mg/dl (LDL-C), 114 mg/dl (TG) and 41.8 mg/dl (HDL-C). Single-stranded DNA is indicated as SS. Double-stranded DNA is not apparent in this figure.

lemic subjects. For exon 4 screening, 18 abnormal CFLP patterns, relative to wild-type, were observed. Six of these CFLP abnormal patterns were already mentioned of which one was characterized as a possible disease-causing mutation[6]. The results are presented in Fig. 1. Age, sex and lipid levels of patients with abnormal CFLP patterns are shown in Table 3.

DNA samples from 19 normolipidemic subjects and 20 patients with primary hypercholesterolemia were screened for mutations in exons 2, 3, 5 and 6 by PCR-SSCP technique. Two SSCP mobility shifts were observed in amplified exon 3. One mobility shift was also apparent in the amplified exon 5 and another one in the amplified exon 6. The results are presented in Fig. 2, 3 and 4, respectively.

DISCUSSION

Primary hypercholesterolemia, due to an increase of plasma LDL, is a broad term which includes both monogenic disorders and polygenic conditions[17]. There are two well defined monogenic conditions which cause an increased level of plasma LDL, i.e. FH and FDB[17]. In this study, Thai subjects with primary hypercholesterolemia and also in healthy normolipidemic subjects (as control) were searched for mutations in the LDL receptor gene. All patients were also examined for a common mutation, R3500Q, in apo B-100 gene before analysis for mutations in the LDL receptor gene. The R3500Q in the apo B-100 gene was not observed in all DNA samples from this group of patients.

The mutation analyses in the exons encoding the ligand-binding domain of the LDL receptor gene revealed abnormal screening patterns in all exons except exon 2. For the hot spot exon 4, 18 abnormal CFLP patterns were observed in DNA samples from patients (n = 45) but not from normolipidemic subjects (n = 33). Recently, an abnormal CFLP pattern in exon 4 was identified and reported as a possible disease-causing mutation in one of

the patients[6]. Rescreening confirmed the previous results and further revealed the other possible mutations in this hypermutable exon. From PCR-SSCP analyses (20 patients and 19 normal subjects), 4 abnormal SSCP patterns were found, two in exon 3 and one each in exons 5 and 6. Characterization of these abnormal CFLP and SSCP patterns are being carried out. Family analysis for these index subjects together with other studies[18] will be pursued in order to verify that these genetic variations are disease-causing mutations.

The current findings from the present study raise the possibility that genetic factors especially mutations in the LDL receptor gene may underlie the condition of primary hypercholesterolemia in most Thai patients. However, before such a conclusion can be ensured, a large number of primary hypercholesterolemia subjects must be further examined.

It is expected that the present molecular genetic study will uncover genetic factors which underlie primary hypercholesterolemia in Thai populations. Understanding such genetic factors will lead to understanding those nongenetic factors which cause such dyslipidemia in population with Thai ethnic background. Therefore, accurate diagnosis and treatment focusing on the underlying disorder should be possible. If causality is genetic, the inherited nature of the abnormality will make screening of family members informative. Identification of affected family members, who require cholesterol lowering, will reassure non-affected sibs. Preventive measures can start early in life to delay or prevent CAD in those affected family members.

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การตรวจค้นการกลายพันธุ์ของยีน Low density lipoprotein receptor ใน exons ที่เป็นรหัสของ Ligand-binding domain ด้วยวิธี PCR-CFLP และ PCR-SSCP ในคนไทยที่มีภาวะโคเลสเตอรอลในเลือดสูงแบบปฐมภูมิ

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ภาวะโคเลสเตอรอลในเลือดสูงแบบปฐมภูมิเกิดได้จากความผิดปกติทางพันธุกรรมของยีนเพียงยีนเดียวหรือหลายยีนร่วมกัน ภาวะโคเลสเตอรอลในเลือดสูงแบบปฐมภูมิซึ่งเกิดจากความผิดปกติของยีนเพียงยีนเดียวที่รู้จักกันดีเกิดจากความผิดปกติของยีน Low density lipoprotein (LDL) receptor และ apolipoprotein (apo) B-100 ซึ่งทำให้เกิดโรคทางพันธุกรรมที่เรียกว่า familial hypercholesterolemia (FH) และ familial defective apo B-100 (FDB) ตามลำดับ ในการศึกษานี้ได้ตรวจค้นการกลายพันธุ์ของยีนทั้งสองในผู้ป่วยคนไทยที่มีภาวะโคเลสเตอรอลในเลือดสูงแบบปฐมภูมิ ในยีน apo B-100, ได้ตรวจหา R3500Q ซึ่งเป็นการกลายพันธุ์ที่มีโอกาสพบได้บ่อย ปรากฏว่าไม่พบการกลายพันธุ์นี้ในผู้ป่วยกลุ่มนี้ (n = 45) ส่วนยีน LDL receptor นั้นได้ตรวจหาการกลายพันธุ์ใน exons ที่เป็นรหัสของ Ligand-binding domain การกลายพันธุ์ใน exon 4 ได้ตรวจด้วยวิธี PCR-CFLP (n = 45) ปรากฏว่าพบ CFLP pattern ที่ผิดปกติ 18 ราย ซึ่งหนึ่งรายในจำนวนนี้ได้เคยรายงานการกลายพันธุ์ (D151Y) ที่คาดว่าน่าจะเป็นการกลายพันธุ์ซึ่งก่อให้เกิดภาวะโคเลสเตอรอลในเลือดสูงแบบปฐมภูมิในผู้ป่วยคนหนึ่ง ส่วนการกลายพันธุ์ใน exons อื่นนั้นได้ตรวจโดยใช้เทคนิค PCR-SSCP (n = 20) ได้พบ SSCP patterns ที่ผิดปกติ 2 รายใน exon 3, 1 รายใน exon 5 และอีก 1 รายใน exon 6 ขณะนี้กำลังศึกษา CFLP และ SSCP patterns ที่ผิดปกติเหล่านี้ด้วยการหาลำดับเบสที่มีการเปลี่ยนแปลงไป รวมทั้งได้เตรียมการศึกษาการเปลี่ยนแปลงที่พบในครอบครัวของผู้ป่วยเหล่านี้ด้วย

คำสำคัญ : ภาวะโคเลสเตอรอลในเลือดสูง, ยีน LDL receptor, การกลายพันธุ์, PCR-CFLP, PCR-SSCP

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