IDENTIFICATION AND CONFIRMATION OF UNKNOWN MUTATIONS AT LOW DENSITY LIPOPROTEIN RECEPTOR LOCUS

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IDENTIFICATION AND CONFIRMATION OF UNKNOWN MUTATIONS AT THE LDL RECEPTOR LOCUS.

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ABSTRACT

Familial hypercholesterolemia (FH) is caused by a defect in the function of the low-density lipoprotein (LDL) receptor and is inherited in an autosomal dominant pattern. Single strand conformation polymorphism (SSCP) analysis was used to screen for mutations at the LDL receptor locus in Thai subjects with primary hypercholesterolemia. The abnormal SSCP patterns were subjected to direct DNA sequencing and secondary confirmation by examination of the DNA fragment used for sequencing, either by restriction fragment length polymorphism (PCR-RFLP) or allele specific amplification (ASA). Four mutations and one polymorphism, 313+1 G>T, E153K, M391T, S554L and G471A, respectively were found. Three mutations (313+1 G>T, M391T, S554L) were screened in a group of 100 normolipidemic subjects by PCR-RFLP and ASA. From this screening, these mutations were not observed in control DNA samples (n=100). The E153K mutation was observed in a 10 years old Thai boy and his father. The E153K mutation was not present in the boy's mother. It might be a pathogenic mutation causing the hypercholesterolemic condition in the boy and his father. Since the boy has severe hypercholesterolemia, then, boy may be a compound heterozygous FH and has inherited another FH causing mutation from his mother. G471A polymorphism, the abnormal SSCP pattern was observed in five unrelated patients. Two in five of the abnormal SSCP patterns were subjected to sequencing analysis. From amino acid sequence alignments, these mutations cause non-conservative substitution in the relatively conserved region of the protein molecule and thus may consequently disturb the function of the receptor protein. These findings, including the absence of these mutations in normolipidemic subjects, suggest that these mutations are pathogenic and could be the possible cause of the hypercholesterolemic condition in these index subjects.

KEY WORDS : LOW DENSITY LIPOPROTEIN RECEPTOR/ FAMILIAL HYPERCHOLESTEROLEMIA/
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การค้นหาและการยืนยันผลของการกลายพันธ์ในยีน LDL receptor (IDENTIFICATION AND CONFIRMATION OF UNKNOWN MUTATIONS AT THE LDL RECEPTOR LOCUS)

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บทคัดย่อ

Familial hypercholesterolemia เป็นโรคทางพันธุกรรมที่เกิดจากการกลายพันธุ์ในยีน LDL receptor ซึ่งมีผลทำให้การทำงานของ LDL receptor protein ผิดปกติ ส่งผลให้เกิดภาวะโคเลสเตอรอลสูงในกระแสเลือด การศึกษานี้ใช้เทคนิค single strand conformation polymorphism (SSCP) ในการค้นหาการกลายพันธุ์ในคนไทยที่มีภาวะโคเลสเตอรอลสูงแบบปฐมภูมิ ชิ้นDNA ที่พบ Abnormal SSCP pattern ได้ถูกวิเคราะห์หาลำดับเบส โดยใช้เทคนิค automated DNA sequencing การเปลี่ยนแปลงของเบสที่เกิดขึ้นจะยืนยันผลโดยใช้เทคนิค Polymerase chain reaction-restriction fragment length polymorphism(PCR-RFLP)หรือเทคนิค allele specific amplification(ASA) จากการศึกษาครั้งนี้พบว่ามีการกลายพันธุ์เกิดขึ้นทั้งหมด 4 ตำแหน่งและความหลากหลาย 1 ตำแหน่ง ได้แก่ 313+1 G>T, E153K, M391T, S554L และ G1414A ตามลำดับ โดยตำแหน่งที่ 313+1 G>T, E153K, M391T ถูกนำไปค้นหาการกลายพันธุ์ในคนที่มีภาวะโคเลสเตอรอลในระดับปกติจำนวน 100 คน โดยใช้เทคนิค PCR-RFLP และ ASA ผลจากการศึกษาว่าไม่มีการกลายพันธ์เหล่านี้ในคนปกติ สำหรับE153Kการกลายพันธุ์ที่ตำแหน่งนี้ได้ถูกพบในเด็กชายอายุ 10 ขวบและในพ่อของเด็กโดยที่ไม่พบในแม่ การกลายพันธุ์ที่ตำแหน่งนี้จึงน่าจะเป็นสาเหตุของภาวะโคเลสเตอรอลสูงทั้งพ่อและลูกโดยเฉพาะในลูกมีภาวะโคเลสเตอรอลที่สูงมากอาจเป็นcompound heterozygous FH โดยอาจได้รับการถ่ายทอดการกลายพันธุ์อีกตำแหน่งหนึ่งจากแม่ซึ่งมีภาวะโคเลสเตอรอลสูงและมีอาการของโรคหลอดเลือดหัวใจขณะนี้อยู่ในระหว่างขั้นตอนการศึกษา จากการเปรียบเทียบ amino acid ในหลายสายพันธุ์ พบว่าการกลายพันธุ์เหล่านี้เป็นผลให้เกิดการแทนที่ของ amino acid แบบ non-conservative substitution ใน conserved region ของ receptor protein และน่าจะมีผลให้ต่อการทำงานของโปรตีน ส่วน G1414(R471)พบเฉพาะ ในผู้ที่มีภาวะโคเลสเตอรอลสูงทั้งหมด 5 คนโดยที่ทั้ง 5 คนนี้ไม่มีความเกี่ยวข้องกัน ความหลากหลายที่ตำแหน่งนี้มีรายงานแล้วในฐานข้อมูล(NCBI) และน่าจะเป็นสาเหตุที่ทำให้เกิดภาวะโคเลสเตอรอลสูงในผู้ป่วยเหล่านี้

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LISTS OF ABBREVIATIONS

ACAT	= Acyl CoA cholesterol Acyl Transferase
bp	= Base pair
CHD	= Coronary Heart Disease
CETP	= Cholesteryl Ester Transferase Protein
ER	= Endoplasmic Reticulum
EGF	= Epidermal Growth Factor
EDTA	= Ethylenediamine Tetra Acetic Acid
FH	= Familial Hypercholesterolemia
HDL	= High Density Lipoprotein
HMG -CoA	=3-Hydroxy-3-Methylglutaryl CoA reductase
IDL	= Intermediate Density Lipoprotein
kb	= Kilobase
LCAT	= Lecithin Cholesterol Acyltransferase
LDL	= Low Density Lipoprotein
LDLR	= Low Density Lipoprotein Receptor
PCR	= Polymerase Chain Reaction
RFLP	= Restriction Fragment Length Polymorphism
SDS	= Sodium Dodecyl Sulfate
SSCP	= Single Strand Conformation Polymorphism
TC	= Total Cholesterol
VLDL	= Very Low Density Lipoprotein

CHAPTER I
INTRODUCTION

Cardiovascular disease is the major leading cause of untimely death worldwide. The magnitude of the problem related to atherosclerotic vascular disease demands significant attention by both the research and therapeutic communities in clinical medicine. At present, there continues to be significant controversy regarding the etiology and methods for preventing the development and progression of atherosclerotic vascular disease. Epidemiological data has demonstrated correlations between certain types of hyperlipidemia, smoking, diabetes, lifestyles and family history as predisposing factors(1, 2). However, it has been well established that the long-term hypercholesterolemia plays a key role in the development of atherosclerosis. Hypercholesterolemia is a high blood cholesterol condition(3). The excess cholesterol in the circulation promotes formation of bulky plaques in arterial wall. The cholesterol in atherosclerotic plaques is mostly derived from particles called LDL particles(4). As a result, the plaques gradually reduce the caliber of the artery and blood flow. This reason may cause endothelial injury and lead to myocardial infarction or sudden death (5).

Recently, a number of experimental studies were carried out to identify and evaluate both genetic and nongenetic determinants of atherosclerosis, including hypercholesterolemia. They have clearly shown that the increase in plasma LDL (low-density lipoprotein) cholesterol is frequently due to genetic alterations at the gene locus specifying the formation of the LDL receptor(6). The LDL receptor gene removes cholesterol-carrying LDL in blood circulation by a process of receptor-mediated endocytosis. Mutations in the LDL receptor gene result in “familial hypercholesterolemia”(FH). FH is a common autosomal dominant disorder that affects around one in 500 individuals in the heterozygous form. The receptor defect reduces

the catabolism of LDL by around 50% and results in an approximately two-fold elevation in plasma LDL cholesterol. The excess plasma LDL-C deposits in tendons and arterial walls, forming tendon xanthomas and atherosclerosic plaques. Homozygous FH is much less common but more severe, occurring with a frequency of approximately one in a million and resulting in plasma LDL-C levels four-to five-fold higher than normal. FH homozygotes are frequently died of myocardial infraction by the second decade(7).

The LDL receptor gene is located on the short arm of chromosome 19. It consists of 18 exons and 17 introns that span 45 kb. The gene encodes a single-chain transmembrane polypeptides of 839 amino acids, which consists of five functional domains(8). Well over 900 different LDL receptor mutations have been identified, ranging from single nucleotide substitution to large deletion, and the frequency of these mutations varies considerably in different populations(9). The mutations have been divided into five classes based upon their phenotypic effects on receptor functioning, viz. null receptor protein, transported-defective, binding-defective, internalization-defective and recycling-defective (7).

Many molecular biology techniques have provided greater understanding of the relationship between its genetic variations and their functional significance. However, not all DNA sequence variation is disease-causing mutations. The analytical criteria for confirmation of pathogenic mutation have been described by Day *et al*. as followings (10)

The criteria for analysis must be conducted according to Day *et al.*

1. Sequencing comparison always with at least one control template.
2. Recognition of the combination of a pathogenomic SSCP profile and a particular sequence variation where the same SSCP is apparent in more than one proband.
3. Sequencing of the opposite strand.
4. Secondary confirmation by examination of the PCR product used for sequencing, either by restriction digestion (where the variation creates or destroys a restriction site), or by the allele-specific oligonucleotide binding.
5. The sequence variation must be screened in a number of normolipidermic subjects (n=100) to assure that the mutation is found only in index subject.
6. Composite protein and nucleotide multiple alignment, annotate with previously described mutations and polymorphisms.
7. Functional analysis at the cellular level.

In our previous study, PCR-SSCP and sequencing techniques were used to identify mutations and polymorphisms in LDL-receptor gene in DNA samples obtained from Thai subjects with primary hypercholesterolemia(11-13). The variations in DNA sequences in the LDL-receptor gene observed in the previous and this study are 313+1 G>T, E153K, M391T, S554L and G471A

To assure that sequence variations observed in our study are possibly disease-related mutations, this thesis research performed conformation analysis according to the criteria as described by Day et al.Both wild types (as control) and putative mutant DNAs (as seen from abnormal SSCP patterns) were subjected to DNA sequence analysis. The DNA sequence analyses were performed for both sense and antisense DNA strands. The secondary confirmations for these DNA sequence variations were performed by using PCR-RFLP or allele specific amplification (ASA) techniques. Also, these techniques have been used to screen for these sequence variations in control DNA samples (n=100). Protein and nucleotide sequences alignments as well as annotation with previously described mutations and polymorphisms have been undertaken to see whether these observed variations are disease-associated mutations. For family analysis, families of two mutations, E153K and M391T were accessible and partial analysis was made in this study. However, functional analysis of these sequence variations has not been performed yet since the settings of such analysis are considerably expensive. In parallel to these analyses, unknown mutations of the LDL receptor gene will be further identified in a number of hypercholesterolemic cases both with and without coronary artery disease.

Objectives

1. To identify mutations in the LDL receptor gene by Polymerase Chain Reaction-Single Strand Conformation Polymorphism (PCR-SSCP) and DNA sequencing techniques.
2. To confirm the identified mutations in the LDL receptor gene by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) or Allele Specific Amplification (ASA).

CHAPTER II
LITERATURE REVIEW

1. Lipoproteins

Since cholesterol is so highly insoluble in aqueous solution, it cannot circulate freely in plasma. Instead, it must be transported in molecular complexes called lipoprotein. These particles contain both lipid and proteins. Several classes of lipoproteins are involved in transport of cholesterol and triglycerides. The structure and function of the various lipoproteins are briefed in the following sections (14).

1.1 Lipoprotein structure

The basic structures of all lipoproteins are similar; they contain a core of neutral lipids consisting of cholesterol ester and triglycerides, a surface coat of more polar lipids such as unesterlified cholesterol and phospholipids and apoproteins (figure2.1) (15). The surface coat, whose lipids provide a covering structure that resembles the typical plasma membrane of cells, serves as an interface between the aqueous plasma and the inner nonpolar lipid core. This polar surface thus makes possible the transport of the highly insoluble cholesterol ester and triglycerides in plasma(16). In addition, each class of lipoprotein has a specific function, determined by its point of synthesis, lipid composition and apolipoprotein content. At least nine different apolipoproteins are found in the lipoproteins of human plasma (Table 2.1). These protein components act as signals, targeting lipoproteins to specific tissues or activating enzymes that act on the lipoprotein (17).

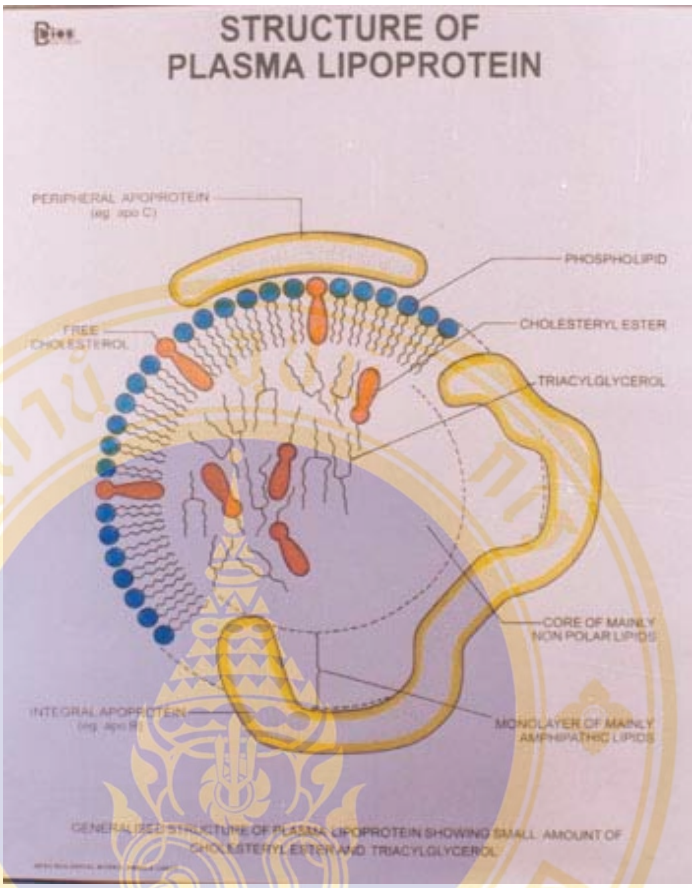

Figure 2.1 The structure of lipoprotein (15)

Table 2.1 Apolipoproteins of the Human Plasma Lipoproteins (17)

Apolipoprotein	Molecular weight	Lipoprotein association	Function (if known)
ApoA-I	28,331	HDL	Activates LCAT; interacts with ABC transporter
ApoA-II	17,380	HDL	
ApoA-IV	44,000	Chylomicrons, HDL	
ApoB-48	240,000	Chylomicrons	
ApoB-100	513,000	VLDL, LDL	Binds to LDL receptor
ApoC-I	7,000	VLDL, HDL	
ApoC-II	8,837	Chylomicrons, VLDL, HDL	Activates lipoprotein lipase
ApoC-III	8,751	Chylomicrons, VLDL, HDL	Inhibits lipoprotein lipase
ApoD	32,000	HDL	
ApoE	34,145	Chylomicrons, VLDL, HDL	Triggers clearance of VLDL and chylomicron remnants

1.2 Classification of lipoprotein

The five major classes of lipoprotein are named either by their density or by electrophoretic mobility (16).

Chylomicrons are very large particles (>70nm) secreted into the intestinal lymphatic by small intestine mucosal cells. About 90% of their mass is triglycerides, which, together with cholesteryl ester, forms the core of the particle. Very low-density lipoproteins (VLDL) are also large particles (25-70 nm), which are secreted into the blood stream by hapatocytes, and probably by intestinal mucosal cells. Only 50-60% of their mass is triglycerides as they contain relatively more cholesteryl ester than Chylomicrons. Intermediate-density lipoproteins (IDL) are produced by the catabolism ofVLDL. Their lipid composition is intermediate between that of VLDL and Low density lipoprotein (LDL), which are derived by catabolism of IDL. LDL carried about 75% of total cholesterol in human plasma. Its major functions appear to be the provision of cholesterol for many extrahepatic tissues. Finally, high-density lipoproteins (HDL) comprise several components derived from various sources: liver, intestine, other lipoproteins and other tissues(18). Transport of excess cholesterol from tissues to the liver where it can be excreted either as biliary cholesterol or, after conversion, as bile acids. In addition to the use of techniques depending on their density, lipoproteins may be separated according to their electrophoretic properties. This technique electrophoresis separates lipoproteins into alpha- (∝), pre beta- (β) and beta- (β) migrating particles. The most negatively charged particles (∝-migrating) travel the fastest. From the electrophoretic mobility, VLDL are pre-beta migrating, IDL migrate between VLDL and LDL. LDL are beta migrating and HDL migrate in the alpha (∝) position. Chylomicrons also have alpha (∝) migration(19). The electrophoretic mobility of these lipoproteins are shown in figure2.2(20). The biochemical characteristics and their concentration in fasting plasma are presented in table2.2.

Table 2.2 Major Classes of human Plasma Lipoproteins(21)

Lipoprotein	Density (g/mL)	Protein	Phospholipids	Free Cholesterol	Cholesteryl ester	Triglycerides
Chylomicrons	<1.006	2	9	1	3	85
VLDL	0.95-1.006	10	18	7	12	50
LDL	1.006-1.063	23	20	8	37	10
HDL	1.063-1.210	55	24	2	15	4

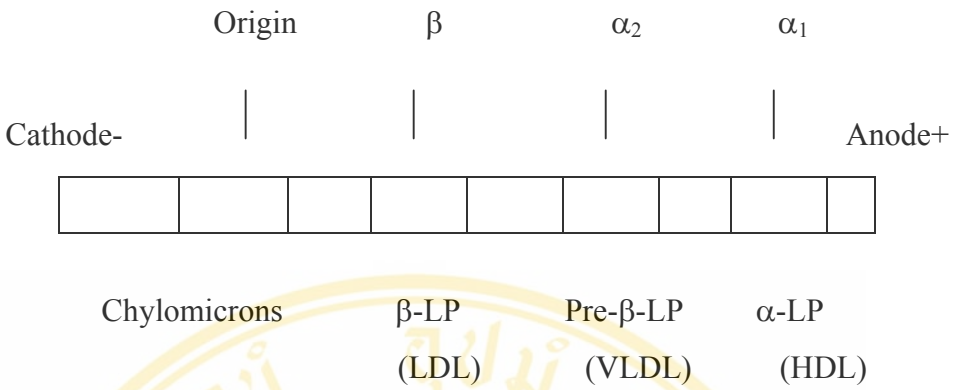

Figure 2.2 Migration of plasma lipoproteins on electrophoresis (20)

1.3 Lipoprotein metabolism

Lipoprotein metabolism may be through of as three interconnected and interdependent cycles. The first is exogenous lipid pathway, which deals with the transport and utilization of dietary lipids. Secondly, the endogenous pathway that deals with the transport and utilization of lipid produced in the liver. Thirdly, the reverse cholesterol pathway serves to return cholesterol from peripheral tissues to the liver where it can be excreted in the bile (22). All three pathways are centered on the liver, which is the key organ in lipoprotein metabolism (23).

1.3.1 Exogenous lipid pathway

Before ingested triglycerides are able to absorb through the intestinal wall, they must be converted from insoluble macroscopic fat particles to finely dispersed microscopic micelles. Storage bile salts, synthesized from cholesterol in the liver released from the gallbladder into the small intestine for digestion of fatty meal. These amphipathic compounds act as biological detergents, converting dietary fats into mixed micelles of bile salts and triglycerides. Micelle formation enormously increases the fraction of lipid molecules accessible to the action of water-soluble lipases in the intestine and lipase action converts triacylglycerols to monoacylglycerols, diacylglycerols, free fatty acids and glycerol. These products of lipase action diffuse into the intestinal mucosa where they are reconverted to triglycerides and packaged

with dietary cholesterol and specific proteins into lipoprotein aggregates called Chylomicrons. Chylomicrons which contain apoC-II move from the intestinal mucosal into the lymphatic system and blood stream to tissues. In the capillaries of these tissues, the extracellular enzyme lipoprotein lipase activated by apoC-II hydrolyzes triglycerides to fatty acid and glycerol. Fatty acids and glycerols are taken up by cells in the target tissues (21).

After the core triglycerides have been removed. The remainder of Chylomicrons reenters the circulation, called chylomicron remnant particles. Chylomicron remnants depleted of most of their triglycerides but still containing cholesteryl ester and apolipoprotein, travel in the blood to the liver where they are taken up via endocytosis mediated by receptors for their apolipoprotein. In the liver they release cholesterol and are degraded in lysosomes. In addition, when the diet contains more fatty acids than are needed, the liver converts them to triglycerides and also distributes to other tissues by the endogenous pathway (21).

1.3.2 Endogenous pathway

Excess carbohydrate in the diet can also be converted into triglycerides in the liver and packaged with specific apolipoproteins in VLDL. VLDL contains triglycerides, cholesterol and cholesteryl ester, as well as apoB-100, apoC-I, apoC-II, apoC-III and apoE. These lipoproteins are transported in the blood from the liver to muscle and adipose tissues, where activation of lipoprotein lipase by apoC-II causes the release of free fatty acids from triglycerides of the VLDL. Adipocytes take up these fatty acids and store in intracellular lipid droplets. The loss of triglycerides converts some VLDL to VLDL remnants. VLDL remnants can have two fates. Normally, 60-70% of VLDL remnants are removed directly from the circulation by hapatocytes. The uptake is receptor-mediated and depends on the presence of apoE in VLDL remnants. Normally, 30-40% of all VLDLs remnants are converted to LDL. LDL particles are very rich in cholesterol and cholesteryl esters and containing apoB-100, which is recognized by specific surface receptor proteins called LDL receptor. After bindings, the LDL particles are internalized by receptor-mediated endocytosis and transferred to lysosomes, where they are degraded (16). Their cholesteryl esters are hydrolyzed. The resulting free cholesterol can be used for membrane biosynthesis,

conversion to steroid hormones or bile acids and as a regulatory molecule that suppress the synthesis of LDL receptors (18).

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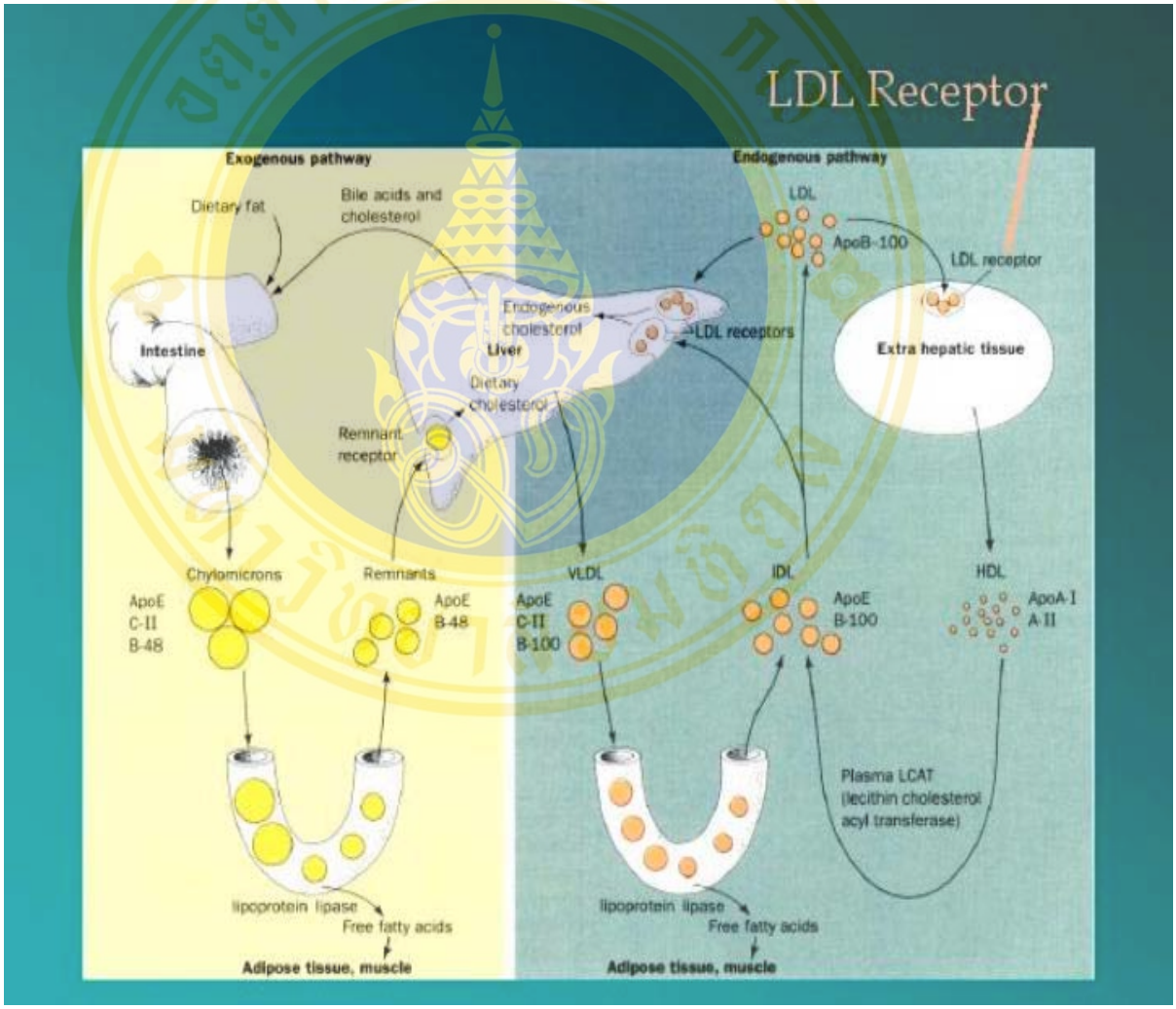


Figure 2.3 Exogenous pathway and Endogenous pathway of lipoprotein metabolism (71)

1.3.3 Reverse cholesterol transport pathway

Cholesterol can only be degraded and excreted by the liver. Therefore, excess cholesterol from peripheral tissues must be transported back to the liver, known as reverse cholesterol transport pathway. High-density lipoprotein (HDL) plays key role in this pathway. There are two mechanisms for reverse cholesterol transport. In the first, unesterified cholesterols in the surface membranes of cells is transferred to HDL and through the esterification by LCAT (lecithin-cholesterol acyl transferase) to yield lysolecithin and cholesteryl ester. A portion of HDL cholesterol ester is shuttled to VLDL, IDL and LDL by CETP (cholesteryl ester transferprotein). The cholesteryl ester in VLDL can be returned to the liver by direct uptake of VLDL remnants or after conversion to LDL. By the second mechanism, whole HDL particles possibly may be taken up in the liver by receptor-mediated endocytosis (16).

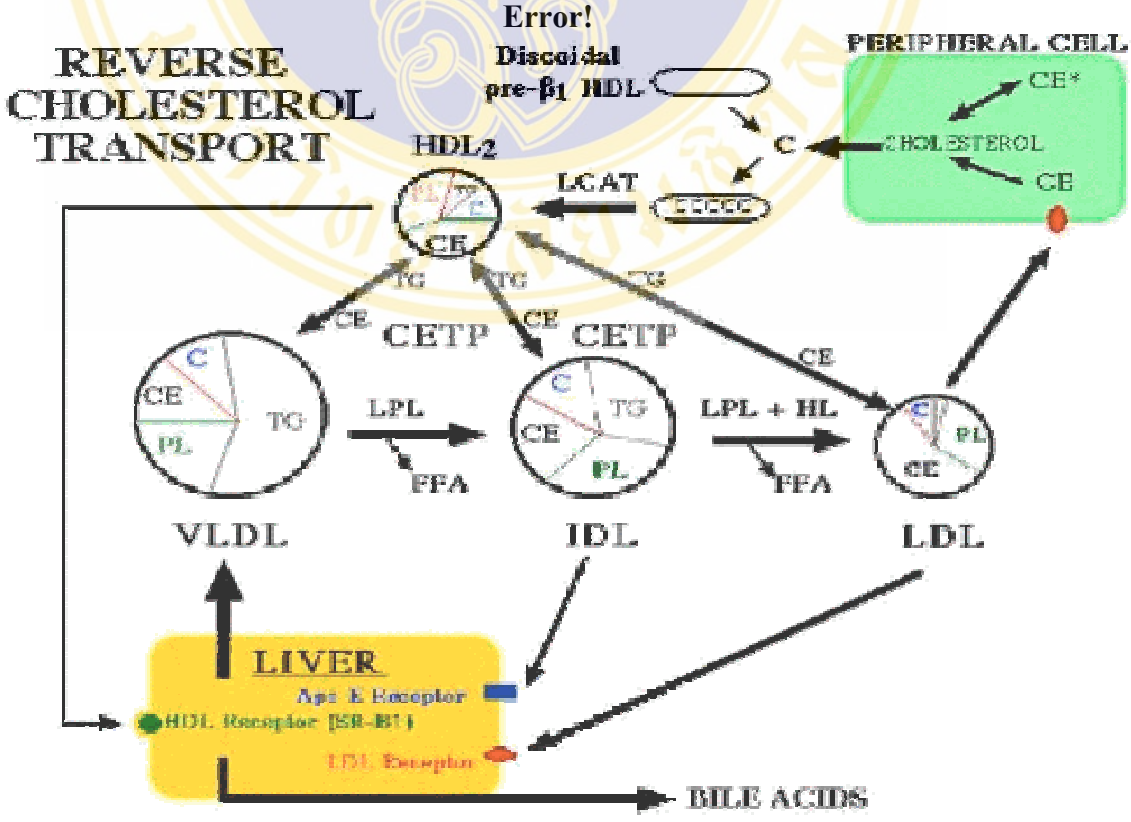


Figure 2.4 Reverse cholesterol transport pathway (72)

1.4 Hyperlipoproteinemia

Primary hyperlipoproteinemias are conditions in which there is increased plasma cholesterol and/or triglycerides, for which there is no other obvious cause (16,24). They are usually classified according to the WHO modification of the original classification of Fredrickson *et al* (25). This classification has divided hyperlipoproteinemia into five phenotypes according to which of lipoproteins is increased. This classification of hyperlipoproteinemia is presented in table 2.3. A many primary hyperlipoproteinemias have a genetic base, a genetic system of classification has also been proposed (table 2.4). Type I hyperlipoproteinemia is characterized by severe chylomicronemia. The diagnosis is established by the finding of very high triglycerides in fasting plasma. Occasionally subjects whose basic phenotype is a type I pattern may show a type V pattern.

Type II hyperlipoproteinemia or familial Hypercholesterolemia. This phenotype represents an increase in LDL. Type II is subdivided in IIa and IIb. Type IIa refers to an increase in lipoprotein restricted to LDL, where as type IIb is marked by increase in LDL and VLDL.

Type III hyperlipoproteinemia, also called dysbetalipoproteinemia is characterized by an accumulation of chylomicron remnants and VLDL remnants. The condition is determined genetically through the gene for apoE. In most cases apoE appears to have defective binding to the remnant receptors.

Type IV hyperlipoproteinemia or hyper-pre-betahyperlipoproteinemia. This phenotype refers to an increase in VLDL level and hypertriglyceridaemia. Causes of type IV hyperlipoproteinemia are multiple-genetic, other disease or dietary.

Type V hyperlipoproteinemia. This phenotype is due to the presence in plasma of large amount of both chylomicron and VLDL. A secondary type V hyperlipoproteinemia is sometimes found as a complication in diabetes mellitus, in alcoholics, patients with glycogen storage disease and in the nephrotic syndrome (16).

Table 2.3 Classification of primary hyperlipoproteinemias (23).

Type	Lipoprotein elevated	Lipid elevation
I	Chylomicrons	Triglycerides
IIa	LDL	Cholesterol
IIb	LDL and VLDL	Triglycerides and cholesterol
III	Chylomicrons remnants and VLDL remnants	Triglycerides and cholesterol
IV	VLDL	Triglycerides
V	Chylomicrons and VLDL	Triglycerides

Table 2.4 Genetic classification (16).

	Fredrickson Type
1. Recessive apoliprotein disorder	
(i) Apoprotein CII deficiency	I, V
(ii) Familial dysbetalipoproteinemia	III
2. Dominant receptor mutations	IIa
3. Recessive enzyme mutations	
(i) Familial lipoprotein lipase deficiency	I, V
(ii) Familial LCAT deficiency	-
4. Possible monogenic disorders	
(i) Familial hypertriglyceridemia	IV (mild form) V (severe form)
(ii) Familial multiple-type hyperlipoproteinemia	IV, IIb
5. Polygenic disorders	
(i) Hypercholesterolemia	IIa
(ii)Hypertriglyceridemia	IV

2. Hypercholesterolemia

Hypercholesterolemia is a condition of high plasma cholesterol level and may occur with a normal or high plasma triglycerides level. For most people, an increase in total cholesterol concentration is due to high levels of LDL-cholesterol. Four factors appear to contribute to the development of high serum cholesterol level: ageing, diet, genetic and secondary causes. Two general mechanisms are responsible for increased LDL levels. The first is a decrease in LDL-receptor activity, leading to delayed clearance of both LDL and VLDL remnants. Moreover, the delay in clearance results in a greater conversion of VLDL remnants to LDL, which further raises LDL level. The second abnormality is an overproduction of lipoprotein which in turn tends to raise concentration of all along the lipoprotein cascade (16).

Primary hypercholesterolemias are the elevation of plasma low-density lipoprotein (LDL) cholesterol that is not secondary to environmental, dietary or other underlying disease. Primary hypercholesterolemia is relatively common in plasma LDL-cholesterol and frequently due to genetic alteration at the gene locus specifying (LDLR). This condition has been associated with the development of atherosclerosis and premature cardiovascular disease (24). Recently, a number of experimental studies were clearly shown that the genetic alterations of the LDL receptor gene play important role in defective catabolism of LDL, leading to familial hypercholesterolemia (16).

3. Familial hypercholesterolemia

Familial hypercholesterolemia (FH) is a classical monogenic disorder associated with primary hypercholesterolemia. FH is an autosomal inheritance disorder of lipid metabolism mainly caused by defect in LDL receptor gene(26). The untreated disorder leads to severe hypercholesterolemia, premature cardiovascular disease and untimely death. Consequently, the life expectancy of FH patients is reduced by 10-20 years. Unfortunately, the disorder is often diagnosed after cardiovascular system becomes evident and precious time for reducing the cardiovascular burden by efficacious lipid lowering drug treatment is lost (27).

Familial Hypercholesterolemia is characterized clinically by elevation in the concentration of low-density lipoprotein (LDL) cholesterol, tendon xanthomata(28).

FH occurs clinically in two forms. FH heterozygote inherit one mutant LDL receptor allele, manifest a 2-3 folds elevation in plasma LDL cholesterol and typically develop premature coronary heart disease after age 35 based on the estimated population frequency of carrier of 1 in 500(29). FH homozygotes inherit two mutant LDL receptor alleles and have a more severe clinical manifestation. Their plasma concentrations of LDL cholesterol are elevated 6-8 folds and they often die of myocardial infraction during the first two decades of life(30). Some phenotypic FH homozygotes inherit two identical mutant LDL receptor alleles (true homozygote), while others inherit two different mutant alleles (compound heterozygotes) (31). A second monogenic disorder associated with primary Hypercholesterolemia is familial defective apolipoprotein B-100 (FDB). This condition results from the presence of LDL particles, which bind poorly to LDL receptor because of a mutation in apoB-100 (32, 33). However, this thesis research is focused especially on the defect of LDL receptor gene.

4. Low density lipoprotein (LDL) receptor

4.1 Protein structure

The LDL receptor gene is located on the distal short arm of chromosome 19 (13.1-13.3). It spans 45 kb, comprises of 18 exons and 17 introns and encodes a 5.3 kb mRNA (figure 2.5)(34). The amino acid sequence as deduced from the nucleotide sequence revealed that the receptor was synthesized as a cell surface glycoprotein of 860 amino acids (7,35).

The structure of the mature protein is well established, as shown in figure 2.6. Exon1 encodes 21 amino acids constitute a typical hydrophobic signal sequence that is cleaved from the protein prior to its appearance on the cell surface, leaving an 839 amino acid mature protein with five recognizable domains (16).

The first domain of the mature receptor contains the binding site for apolipoprotein B and E. It is encoded by exon 2-6. This domain consists of 300 amino acid residues, which is assembled from multiple repeats of 40 residues each. Each repeat has six cysteine residues, all of which are involved in disulfide bonds. At the COOH-terminal end of each repeat, there is a negatively charged amino acid, Asp-X-Ser-Asp-Glu, that is important for ligand binding(36).

The second domain of the human LDL receptor is encoded by exons 7-14. This domain encodes a region that shares sequence identity to the human epidermal growth factor (EGF) precursor gene. It comprises approximately 400 amino acids, cysteine-rich growth factor repeats (designated A, B and C). The A and B repeats (encoded by exons 7 and 8) contiguous and separated from the C repeat (encoded by exon14) by a 280 amino acids sequence that contains five copies of a conserved motif, Tyr-Try-Thr-Asp (YWTD) repeated once each 40-60 amino acids. This domain is required for the dissociation of lipoproteins from the receptor in the endosome during receptor recycling(37). It also serves to position the ligand binding domains so that it can bind LDL on the cell surface.

The third domain is encoded by exon 15. This domain comprises 58 amino acids that are enriched in serine and threonine residues, which serve as attachment sites for O-linked sugar chains. Absence of this exon has no significant functional consequence in cultured hamster fibroblasts. The fourth domain is encoded by exon 16 and the 5' end of exon 17. This domain comprises a 22 amino acid stretch of hydrophobic residues bordered by charged residues and is believed to anchor the receptor in the cell surface by spanning the membrane.The fifth domain is encoded by 3' of exon17 and the 5' end of exon18. This domain is 50 amino acids that make up the cytoplasmic domain. This domain is important for the localization of the receptor in endocytosis (coated pit) on the cell surface.(38-41)

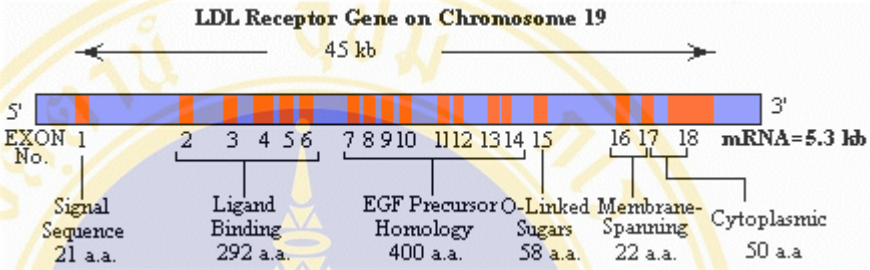


Figure 2.5 The structure of LDL receptor gene (73)

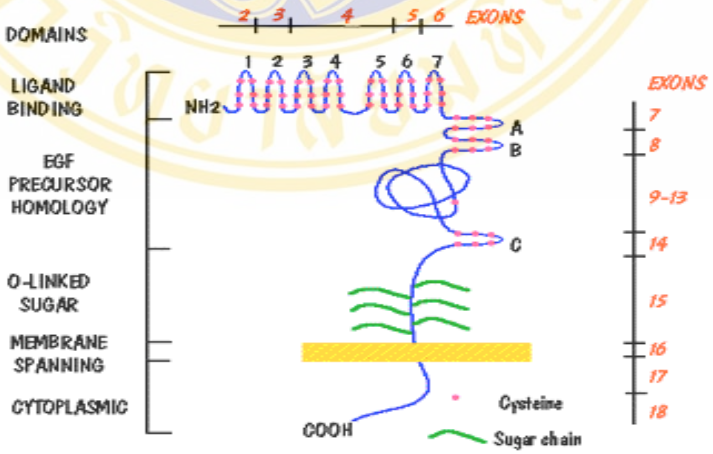


Figure 2.6 The structure of LDL receptor protein(38)

4.2 Function

The low-density lipoprotein (LDL) receptor is a transmembrane cellular protein that is pivotal in cholesterol homeostasis (figure 2.6). AT the cell surface, the LDL receptor recognizes cluster of positive-charged basic amino acids (lysine, arginine, histidine) located with apoB-100, the signal protein component of LDL, and apoE, a protein associated with IDL (17). LDL binds to its receptor and is transported into the cell via receptor-mediated endocytosis. The intracellular LDL-derived cholesterol down regulates both the LDL receptor gene and genes encoding enzymes (such as HMG-CoA reductase) in the cholesterol biosynthetic pathway (31).

The LDL receptor is synthesized in the rough endoplasmic reticulum. Within 1 hour after its synthesis, the mature LDL receptor appears on the cell surface where it binds lipoprotein particle (figure 2.7).(42) The LDL receptor mediates uptake and degradation of its bound ligand by an endocytotic pathway. The ligand receptor complexes cluster in coated pits, specialized domains of a structural protein called clathrin. The coated pits pinch off from the surface to form coated vesicles, which mature into early endocytotic vesicles by losing their clathrin coat. After further maturation and fusion, late endosomes are formed that maintain an acidic environment because of the action of membrane-associated protein pump. The lower pH of endosomes promotes dissociation of LDL from its receptor and directs unoccupied receptor back to the cell surface where they can rebind additional LDL particles and initiate another round of endocytosis. By this recycling process, an LDL receptor molecule can be reutilized several hundred times before being degraded, making one round trip every 10 minutes. The LDL particles that dissociate in endosome are delivered to lysosomes when endosomal and lysosomal membranes fuse. Once within lysosomes, the LDL apolipoprotein is degraded to amino acids and its cholesteryl esters are hydrolyzed to produce unesterified cholesterol. The cholesterol liberated by lysosomal hydrolysis of LDL is translocated across the lysosomal membrane into the microsomal compartment of the cell where it mediates several metabolic processes. It suppresses synthesis of the LDL receptor and enzymes of sterol biosynthetic pathway to shut off further delivery and production of cholesterol.

These regulatory processes allow cells to obtain enough cholesterol for membrane synthesis and steroidgenesis, and they protect cells from overaccumulation of cholesterol, which can damage membranes when in excess. As an additional protection, excess lysosomal-derived cholesterol is esterified by the microsomal enzyme acyl CoA cholesterol acyltransferase (ACAT) and stored as lipid droplets within the cell. Overaccumulation of cholesterol within cells also activates the HDL pathway that promotes excretion of excess cholesterol from cells (21).

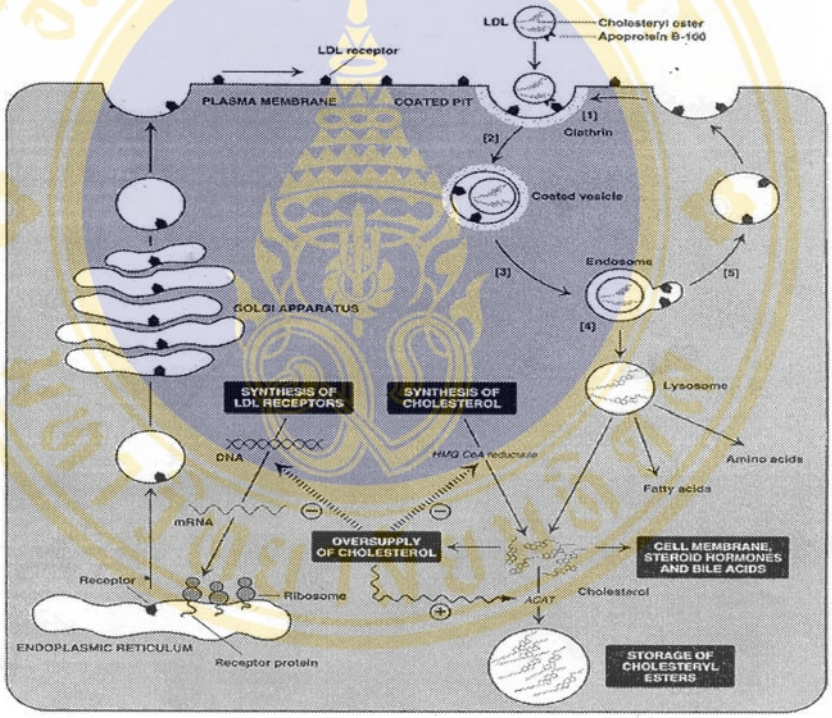


Figure 2.7 LDL receptor metabolisms (74)

4.3 Classification of mutations

Mutations in the low-density lipoprotein receptor gene cause familial hypercholesterolemia (FH). The LDL receptor mutations have been classified into 5 classes based on biosynthesis and function (figure 2.8).(Brown and Goldstein,18-986; Hobbs et al.1990)

Class 1 mutations fail to produce immunoprecipitable LDL receptor protein. The most frequent types of class 1 mutations are nonsense and frameshift mutations, and these are randomly distributed among the exons.

Class 2 mutation contains alleles coding for proteins exhibiting an inhibition of intracellular transport from the endoplasmic reticulum to the golgi apparatus, either completely class 2A or partially class 2B. Class 2A mutations block receptor, while class 2B mutations produce proteins that are transported at a detectable, but markedly reduce rate. Most of class 2 mutations are located in the EGF precursor homology domain. This is the most highly conserved domain in the receptor protein, and its structure is easily disrupted if only a single amino acid is changed. Of nine alleles that produce a complete block in transportion of the receptor protein (class2A), eight are located in this domain.

Class 3 mutations produce proteins that are transported to the cell surface, but fail to bind LDL normally (binding-defective alleles). Most are caused by inframe insertions or deletions in ligand binding domain or EGF precursor homology domain. Deletion of the cysteine-rich repeats results in decreased binding of LDL, which binds receptor via its single apoB-100.

Class 4 mutations encode receptors that move to cell surface and bind LDL normally, but are unable to cluster in clathrin-coated pits and do not internalize LDL (internalization-defective alleles).

Finally, class 5 mutations encode receptors that bind and internalize ligands in coated pit, but fail to release the ligand in the endosome and thus do not recycle to the cell surface (recycling-defective alleles). Most of mutations are found in the EGF precursor homology domain, especially in the YWTD repeat. This domain mediates acid-dependent dissociation of receptor and ligand in the endosome, an event that is essential for receptor recycling. If the ligand is not released from the receptor in the

endosome, the receptor-ligand complex is degraded and the receptor fails to be recycled to the cell surface. (29,38,40)

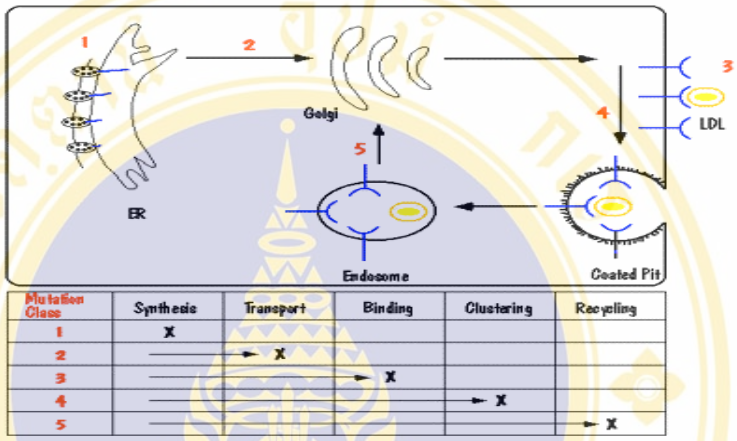


Figure 2.8 Classification of LDL receptor mutations based on abnormal function of the mutant protein. (38)

To date 920 mutations in LDL receptor gene have been worldwide described associated with FH, at cDNA level, most mutations (840) are point mutations. These mutations distributed as follows: 610 substitutions, 121 small deletions, 53 small insertions or duplication. At the genomic level, there are also 57 intronic substitutions of which 37 (65%) affect consensus splice sequence. At the protein level mutations, 103 nonsense mutations, 139 premature stop codon and 35 in frame amino acid insertions or deletions. Major rearrangements account for 10% of all mutations. Only 7 mutations (1%) have been found in the promoter (9). There are certain mutations in the LDL receptor gene which have been seen more frequently in specific parts of the world or within isolated ethnic groups, such as French Canadians and Afrikaners. In the Afrikaner population, FH is very common; heterozygotes are found with a frequency of more than 1 in 100 while homozygotes are 1 in 30,000(42).

However, only few studies in Thai have to date characterized mutations of the LDL receptor gene. Knowledge of the mutations in the LDL receptor gene which

predominate in certain regions help to confirm the clinical diagnosis and also to identify younger affected member in a known family or a new FH proband. Furthermore, the presymptomatic diagnosis and the early therapeutic intervention could prevent premature atherosclerosis.

CHAPTER III

MATERIALS AND METHODS

MATERIALS

1. Subjects

The selection of subjects for this study was mainly based on the plasma cholesterol levels with normal triglycerides level (less than 200 mg/dl). Patients with secondary hypercholesterolemia causes, such as diabetes mellitus and hypothyroidism were excluded. All DNA samples were extracted by the Guanidine-HCl method (UCLA 1993). There were 170 subjects used in this study. These subjects were divided into two groups. One hundred were normal control subjects. Seventy were primary hypercholesterolemia subjects. Total cholesterol and LDL cholesterol level of primary hypercholesterolemia subjects exceeded 290 mg/dl, 190 mg/dl respectively (Broome register).

The Department of Preventive Medicine, Department of Pediatrics, Siriraj Hospital, Mahidol University and Navy Hospital, Bangkok, Thailand kindly provided these subjects.

2. Oligonucleotide Primers

The nineteen pairs of oligonucleotide primers used in this study are shown in Table 3.1. All of oligonucleotide primers used in this study were as described by Litersdorf et al. and Hobbs et al. according to the published LDL receptor sequences and were synthesized and purified by QIAGEN Operon (43).

Table 3.1 Oligonucleotide primers for amplification all 18 exons of LDL receptor gene

Exon	Primer	sequence
2	SP57/58	5'CCTTTCTCCTTTTCCTCTCTCTCAG 3'
		5' AAAATAAATGCATATCATGCCCAAA3'
3	SP59/60	5'TGACAGTTCAATCCTGTCTCTTCTG 3'
		5'AATAGCAAAGGCAGGGCCACACT 3'
4	SP4.1/4.2	5' TGCAGCCCCCCAAGACGTGCT 3'
		5' CGCAGTTTTCCTCGTCAGAT 3'
5	SP62/63	5'CAACACACTCTGTCCTGTTTTCCAG 3'
		5' GGAAAACCAGATGGCCAGCGCTCAC 3'
6	SP64/65	5'TCCTTCCTCTCTCTGGCTCTCACAG 3'
		5'GCAAGCCGCCTGCACCGAGACTCAC 3'
7	SP66/67	5'AGTCTGCATCCCTGGCCCTGCGCAG 3'
		5' AGGCTCAGTCCACCGGGGAATCAC 3'
8	SP68/69	5'CCAAGCCTCTTTCTCTCTCTTCCA3'
		5' CCACCCGCCGCCTTCCCGTGC 3'
9	SP70/71	5'CCTGACCTCGCTCCCCGGACCCCCA 3'
		5' GGCTGCAGGCAGGGGCGACGCTCAC 3
10	SP72/73	5'ATGCCCTTCTCTCCTCCTGCCTCAG 3'
		5'AGCCCTCAGCGTCGTGGATACGCAC 3'
11	SP74/75	5'CAGCTATTCTCTGTCCTCCCACCAG 3'
		5' TGGCTGGGACGGCTGTCCTGCGAAC 3'
12	SP76/77	5'TCTCCTTATCCACTTGTGTGTCTAG 3'
		5' CTTCGATCTCGTACGTAAGCCACAC 3'
13	SP78/79	5' GTCATCTTCCTTGCTGCCTGTTTAG 3'
		5' GTTTCCACAAGGAGGTTTCAAGGTT 3'

Table 3.1 Oligonucleotide primers for amplification all 18 exons of LDL receptor gene. (cont)

Exon	Primer	Sequence
14	SP80/81	5'CCTGACTCCGCTTCTTCTGCCCCAG 3' 5' ACGCAGAAACAAGGCGTGTGCCACA 3'
15	FH15/35	5'GAAGGGCCTGCAGGCACGTGGCACT 3' 5' GTGTGGTGGCGGGCCCAGTCTTTAC 3'
16	SP84/85	5' CCTCACTCTTGCTTCTCTCCTGCAG 3' 5' CGCTGGGGGACCGGCCCGCGCTTAC3'
17	SP86/87	5' TGACAGAGCGTGCCTCTCCCTACAG 3' 5' TGGCTTTCTAGAGAGGGTCACACTC 3'
18	FH19/38	5' TCCGCTGTTTACCATTTGTTGGCAG 3' 5' AATAAAACAAGGCCGGCGAGGTCTC 3'
Promotor	PP1/PP2	5' GAGTGGGAATCAGAGCTTCACGGGT3' 5' CCACGTCATTTACAGCATTTCAATG3'

3. Enzymes

All of the enzymes used in this study were molecular biology grade.

Table 3.2 List of enzymes and Restriction enzymes

Enzymes	Sources
Lysozyme	BioBasic Inc., Canada
Proteinase K	Invitrogen
Taq DNA polymerase	ITS (spain)
EcoRI	BioLabs (USA)
RsaI	Fermentas
TaqI	Fermentas

4. Chemical substances

Chemical substances	**Molecular weight**	**Source**
Acrylamide (C_3H_5NO)	71.08	Fluka, Switzerland
Acetic acid (CH_3COOH)	105.00	Merck, Germany
Absolute ethanol (C_2H_2OH)	92.14	Merck, Germany
Agarose		BMA, USA
Ammonium persulfate ($(NH_4))_2S_2O_8$)	228.20	USB, USA
Boric acid (H_3BO_3)	61.85	Merck, Germany
Bromophenol blue		USB, USA
Deionized Formamide	45.04	BioBasic Inc.,Canada
Deoxynucleotide triphophate(dNTPs)		Pharmacia Biotech,USA
DL-Dithiothreitol (DTT, $C_4H_{10}O_2S_2$)	154.25	BioBasic Inc.,Canada
Dimethyl Sulfoxide (DMSO,$(CH_3)_2SO$	372.24	BioBasic Inc.,Canada
Ethanol (C_2H_5OH)	46.0	Merck, Germany
Ethidium bromide ($C_{21}H_{20}N_3Br$)	394.31	BioBasic Inc.,Canada

Chemical substances	Molecular weight	Source
Ethylenediaminetetracetic (EDTA) ($C_{10}H_{14}N_2Na_2O_8.2H_2O$)	78.13	Merck, Germany
37%Formaldehyde (CH_2O)	30.0	Merck, Germany
Glycerol ($HOCH_2CH(OH)CH_2OH$)		Merck, Germany
Guanidine-HCl ($(NH_2)_2–C=NH.HCl$	95.53	Sigma, USA
IGEPAL		Sigma, USA
Isopropyl-β-D-Thiogalactopyranoside (IPTG, $C_9H_{18}O_5S$)	238.31	BioBasicInc.,Canada
Ladder marker		Fermentas, Germany
Methanol (CH_3OH)	32.04	Merck, Germany
Mineral oil		Sigma, USA
N,N-Methylene-bis-acrylamide ($C_7H_{10}N_2O_2$)	154.20	Sigma, USA
N, N, N', N'-Tetramethyl-ethylenediamine (TEMED) ($C_6H_6N_2$) 116.20	116.21	BioBasicInc.,Canada
Polyethylene-20 Sorbitan minolaurate		Research Organics, USA
QIAGEN PCR purification kit		QIAGEN
Silver nitrate ($AgNO_3$)	169.87	Merck, Germany
Sodium carbonate (Na_2CO_3)	105.99	Merck, Germany
Sodium dodecyl sulphate (SDS, $C_{12}H_{25}O_4SNa$)	288.40	Sigma, USA
Tris (Hydroxymethyl-aminomethane) ($C_4H_{11}NO_3$)	121.1	Research Organics, USA
Sodium hydroxide (NaOH)	40.0	Merck, Germany
Xylene Cyanol	554.6	Research Organics, USA

5. Instruments

Instruments	Source
Analytical balance	Scaltec
Autoclave	Hirayana, Japan
Hettich zentrifugen	Germany
DNA Thermal Cycles (Gene Amp PCR System 2400)	Perkin Elmer, USA
Horizontal agarose gel electrophoresis set	MupidII, Japan
Hotplate Stirrer SM22	Stuart Scientific, UK
Hot air oven	Memmert, Germany
Incubator	Heraeus, Germany
Microcentrifuge	Heraeus Sepatech, USA
Protein II electrophoresis set	BIO-RAD, USA
Pipette	Pipetteman, Biohit, USA
Polaroid camera	Photodye, USA
Power supply	BIO-RAD, USA
UV-transluminator	Fotodyne, USA
Refrigerator	Sharp, Japan
Vortex	IKA Work Inc., USA

METHODS

1. Determination of lipid profiles

Lipid profiles were analyzed by the Department of Clinical Pathology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok Thailand and Navy Hospital, Bangkok Thailand. Whole blood (5-10 ml) was taken after 12-14 hours of fasting. Plasma cholesterol, HDL cholesterol and triglyceride levels were determined with automation. Plasma LDL level was calculated using the formula described by *Friedewald et al.* The experimental design for this study is shown in figure 2.6

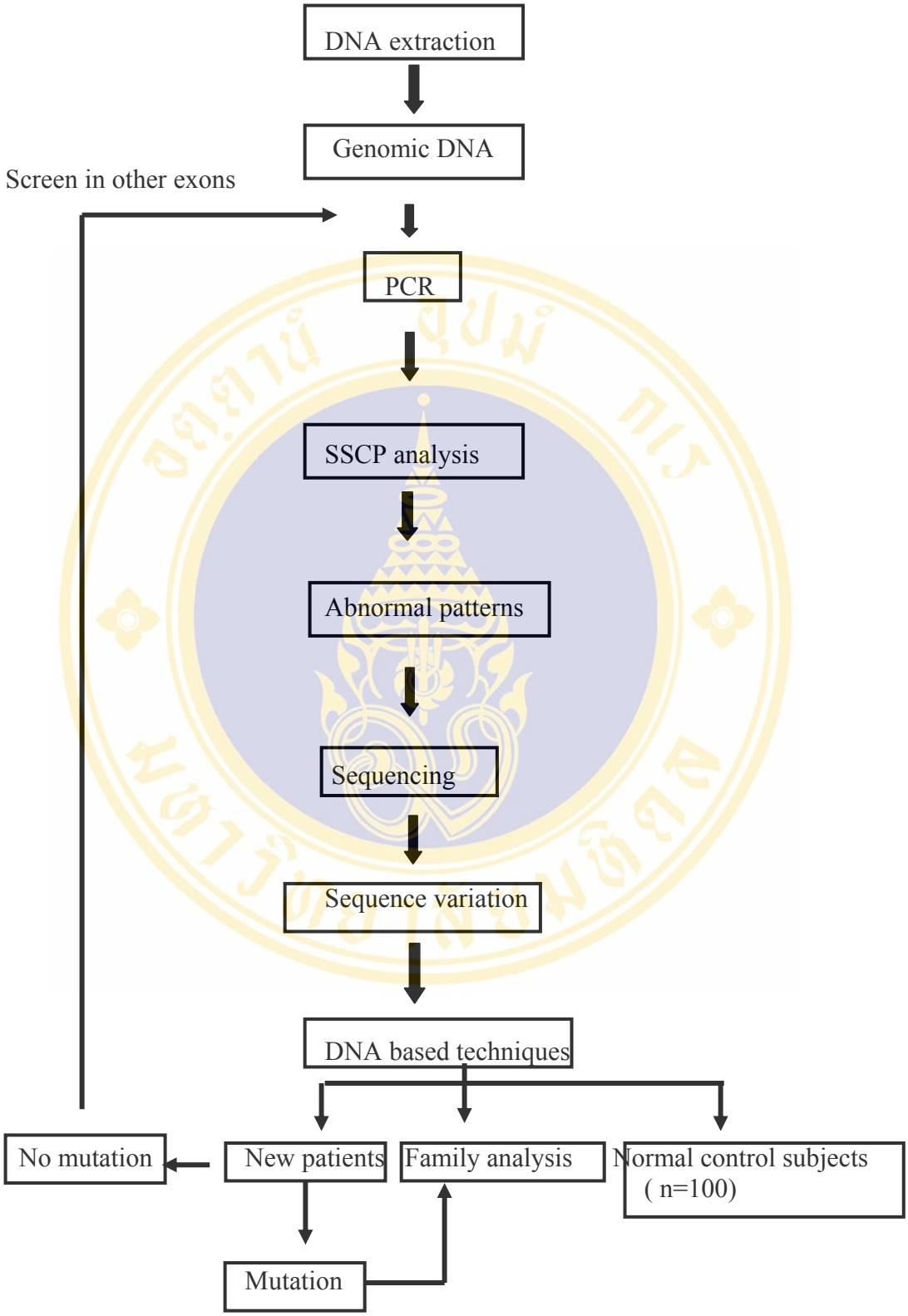


Figure3.1 Schematic of experimental design for identification mutation of LDL receptor gene

2. Genomic DNA extraction

2.1 Reagents preparation

a. Solution A

6.35 g of NH_4CL, 1.33 g of EDTA and 0.92 g of Trisma base were weighted and dissolved with 800 ml of distilled water. The pH of the solution was adjusted to 7.2 and the volume was adjusted to 1 Liter. The solution was autoclaved. Working solution A was prepared by diluting solution A with 2 volume of ddH_2O before use.

b. Proteinase K

0.002 g Proteinase K was weighed and dissolved with 2 ml of ddH_2O and mixed. The mixed solution was aliquoted into 1.5 ml eppendrof tubes. The solution was stored at -20°C until use.

c.10% SDS

10 g of SDS was weighed and dissolved with ddH_2O. The solution was stirred and incubated at 56°C until the solution was homogeneous and the solution was adjusted to 100 ml until dissolved. This solution was used with no need to be autoclaved.

d. 1 M Tris-HCl

60.6 g of Tris-HCl was weighed and dissolved with ddH_2O. Approximately 13 ml of 6 M HCl was added to the solution to adjust pH to 7.6. The volume of the solution was adjusted to 500 ml. The solution was sterilized by autoclaving.

e. Guanidine-HCl

72.0 g of Guanidine-HCl was weighed and 1 M Tris-HCl was added to adjust the pH to 7.6. The volume of the solution was adjusted to 100 ml with ddH_2O. The solution was filtered by 0.22 μm Nalgene filter.

f. 0.5 M EDTA pH 8.0

93.0 g of EDTA disodium salt was weighed and dissolved with approximately 400 ml of ddH_2O. 10.0 g of NaOH pellets was added. The pH of the solution was adjusted to 8.0 with 10 N NaOH. The volume of the solution was adjusted to 500 ml. The solution was sterilized by autoclaving.

g. 5% EDTA pH 7.4

25 g of EDTA disodium salt was weighed and dissolved in 400 ml of ddH_2O. The pH of the solution was adjusted to 7.4 with 10 N NaOH. The volume of the solution was adjusted to 500 ml. The solution was sterilized by autoclaving.

h. TE 10-1 buffer

10 ml of 1 M Tris-HCl pH 7.6 and 2 ml 0.5 EDTA pH 8.0 were diluted in distilled water the volume was adjusted to 1 Liter. The solution was sterilized by autoclaving.

2.2 Procedure

Ten milliliters of whole blood were collected into 50 ml capped tube which contained 400 µl of 5% EDTA (blood anti-coagulant). Two volume of working solution A was added and mixed by vortexing and left for 10 minutes at room temperature. The mixture was centrifuged at 3000x g for 10 minutes. After centrifugation, the supernatant was discarded and 2 volumes of working solution A was added again and previous step was repeated until the supernatant was clear but not more than three times. The supernatant was discarded and white blood cell was packed at the bottom of tube. 80 µl of 10 mg/ml of proteinase K was added and mixed with transferred pipette. 800 µl of distilled water was added and resuspended further. 300 µl of 10% SDS was added, gently mixed and the air-bubble was avoided. The mixture was incubated at 37°C overnight. After, the O/N incubation 300 µl of Guanidine-HCl was added and gently mixed. After mixing, the solution was incubated at 68-70 °C for 3-4 hours. The mixture was centrifuged at 3000x g for 10 minutes at 4°C and only the above clear solution was reincubated at 68-70 °C for 30 minutes or until the pellet looks like dark green jelly. The mixture was centrifuged again at 3000x g for 10 minutes. The supernatant was transferred to a new 50 ml capped tube and precipitated with 5 ml of cold absolute ethanol. The tube was gently rock back and forth until cotton-like strand of DNA appeared and then stored at -20°C and transferred pack DNA into 1.5 ml eppendroff tubes. The pack DNA was washed in 70% ethanol. The genomic DNA was suspended in 150-200µl of TE 10-1 buffer and stored at -20°C until use. The genomic DNA was verified by agarose gel electrophoresis.

3. Polymerase Chain Reaction (PCR)

3.1 Principle

Polymerase chain reaction (PCR) is a powerful technique for amplification of DNA via reaction carried out entirely *in vitro*. Essentially, DNA polymerase is used for repeated replication of a defined segment of DNA. The numbers of DNA molecules increase exponentially, doubling with each round of replication. The region of DNA to be amplified is flanked by two sequences used to prime DNA synthesis. The starting double stranded DNA is heated to separate the strands and then cooled to allow primers (usually oligonucleotide of 20 to 25 bases) to bind to each of DNA strand. *Taq DNA polymerase* from *Thermus aquaticus* and deoxynucleoside triphosphates are then added(44, 45), and primers are selectively replicated a definite segment of DNA (figure 3.1). This process is repeated for multiple cycles(46, 47).

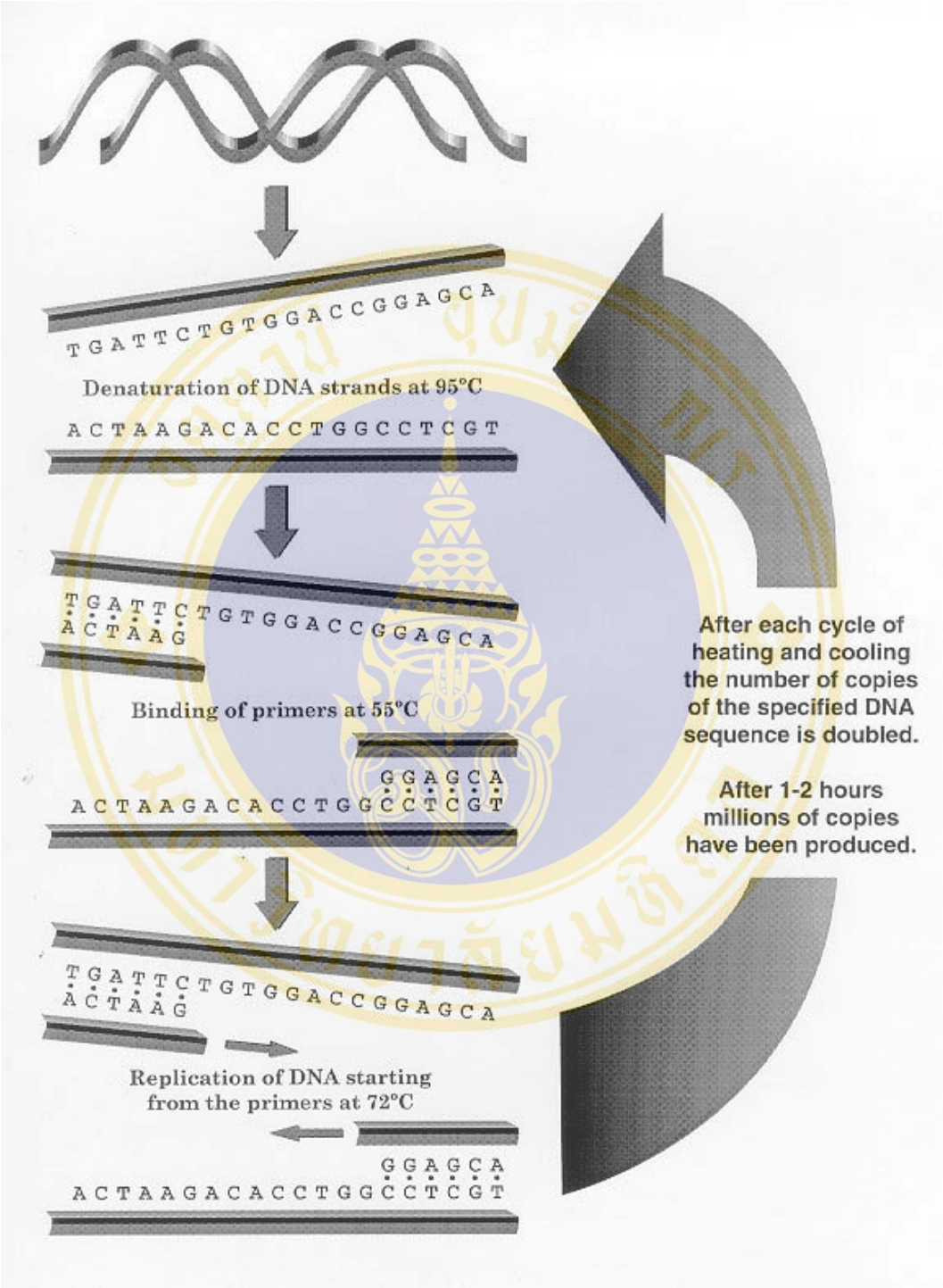


Figure 3.2 Amplification of DNA using the polymerase chain reaction (75)

3.2 Reagents

a. 10X buffer

200 ul of 1 M Tris-HCl (pH 8.4), 15 ul of 1 M $MgCl_2$, 500 ul of 1M KCl and 10 ul of Tween 20 were mixed and the volume was then made up to 1,000 ul with autoclaved sterile water for injection.

b.10 mM dNTPs

10 ul of each 100 mM dNTP were mixed and the mixture was diluted to 100 ul with autoclaved sterile water for injection.

c.1 mM dNTPs

10 ul of each 10 mM dNTP were mixed and the mixture was diluted to 100 ul with autoclaved sterile water for injection.

d. *Taq* DNA polymerase.

Taq DNA polymerase used for PCR-RFLP and PCR-SSCP was expressed from a recombinant plasmid according to the protocol of Pluthero *et al*. as described in method 10. Commercial Taq DNA polymerase from ITS was used to confirm the SSCP results and to perform DNA sequencing.

3.3 Procedure

Polymerase chain reaction was used to amplify exon 1 to exon 18 of the low-density lipoprotein receptor gene. The sense and antisense oligonucleotide primers of each exon are show in Table 3.1. The amplification reaction of each exon was performed in a final volume of 25 µl, in 0.2ml thin-wall microtubes, which contained genomic DNA, 10xPCR buffer, 10 pmole of each primer, 200 µM of each dNTP, and 0.5 unit of *Taq* DNA polymerase. The reaction mixtures were overlaid with a drop of mineral oil. PCR reactions of all exons were firstly denatured at 95°C for 5 minutes and finally extended at 72°C for 5 minutes. The amplification profile are shown in Table 3.3. The cycling condition of each exon was performed for 35 cycles. All reactions were stopped by chilling at 4°C. The PCR products of each exon were subjected to 2% agarose gel electrophoresis.

Table 3.3 The PCR conditions of all 18 exons of LDL receptor gene

Exon	PCR condition (35 cycles)	PCR SIZE (bp.)
1		
2	95 °C 1 min 68°C 1 min 72°C 1 min	178
3	95 °C 1 min 58°C 1 min 72°C 1 min	187
4	95 °C 1 min 54°C 1 min 72°C 1 min	383
5	95 °C 1 min 68°C 1 min 72°C 1 min	183
6	95 °C 1 min 68°C 1 min 72°C 1 min	177
7	95 °C 1 min 72°C 1 min 72°C 1 min	170
8	95 °C 1 min 68°C 1 min 72°C 1 min	176
9	95 °C 1 min 72°C 1 min 72°C 1 min	223
10	95 °C 1 min 72°C 1 min 72°C 1 min	278
11	95 °C 1 min 72°C 1 min 72°C 1 min	173
12	95 °C 1 min 58°C 1 min 72°C 1 min	190
13	95 °C 1 min 53°C 1 min 72°C 1 min	219
14	95 °C 1 min 72°C 1 min 72°C 1 min	204

Table 3.3 The PCR conditions of all 18 exons of LDL receptor gene (cont)

Exon	PCR condition (35 cycles)	PCR SIZE (bp.)
15	95 °C 1 min 68°C 1 min 72°C1 min	246
16	95 °C 1 min 58°C 1 min 72°C1 min	128
17	95 °C 1 min 58°C 1 min 72°C1 min	120
18	95 °C 1 min 68°C 1 min 72°C1 min	135

4. Agarose gel electrophoresis for analysis of DNA fragments

4.1 Principle

Gel electrophoresis is used to separate DNA molecules according to their sizes. DNA molecules carry negative charges. Whenever DNA are placed in an electric field, they will migrate towards the positive pole. The factors that affect the rate of DNA migration in the gel are molecular size of DNA, gel concentration, conformation of DNA, applied voltage and electrophoresis buffer(49).

4.2 Reagents preparation

a. 10x TBE buffer

108 g of Trisma base, 55 g of Boric acid and 9.3 g of EDTA disodium salt were weighed and dissolved with distilled water. The volume of the solution was adjusted to 1 Liter and mixed.

b. 2 % agarose gel

2 g of agarose was weighed and dissolved in 100 ml of 1x TBE buffer. The mixture was heated. The melting gel was poured into an electrophoresis chamber set with a comb inserted and left to polymerize at room temperature for 1 hour.

c. Ethidium bromide solution

1 g of ethidium bromide was weighed and dissolved in 100 ml of water.

4.3 Procedure

Agarose gel electrophoresis is the most common method for analyzing PCR products. According to this method, 5µl of PCR products of each exon were pipetted and mixed with 2µl of loading buffer. The mixtures were loaded in each well on 2 % agarose gel and electrophoresed for 15-20 minutes at 100 volts. After electrophoresis, the PCR products were stained with ethidium bromide solution for 1-2 minutes and visualized under ultraviolet light. The size of each exon was estimated by comparison with 100 bp DNA marker under the same condition.

5. Single strand conformation polymorphism (SSCP)

5.1Principle

Single strand conformation polymorphism (SSCP) analysis was first described by Orita *et al* in 1983(50). SSCP is widely used for mutation detection because of its simplicity and versatility. In this method, a segment to be searched for a mutation is amplified by PCR from genomic DNA, denatured, and separated by electrophoresis in non-denaturing polyacrylamide gel. Nucleotides change of the single-stranded mutant DNA is believed to be the reason for the mobility shift (Figur3.2)(51). Conformation of single-stranded DNA is determined by intramolecular interactions, which can change depending on physical conditions such as temperature and ionic environment. Accordingly, separation of mutant DNA in SSCP varies depending on the conditions of electrophoresis. Although empirical rules on good separation of sequence variants are emerging, whether a certain mutation can be detected in a given condition is not predictable. However, sensitivity of PCR-SSCP is generally believed to be high if the fragments are short. The optimal length of a single strand seems to be approximately between 150 to 200 nucleotides(52).

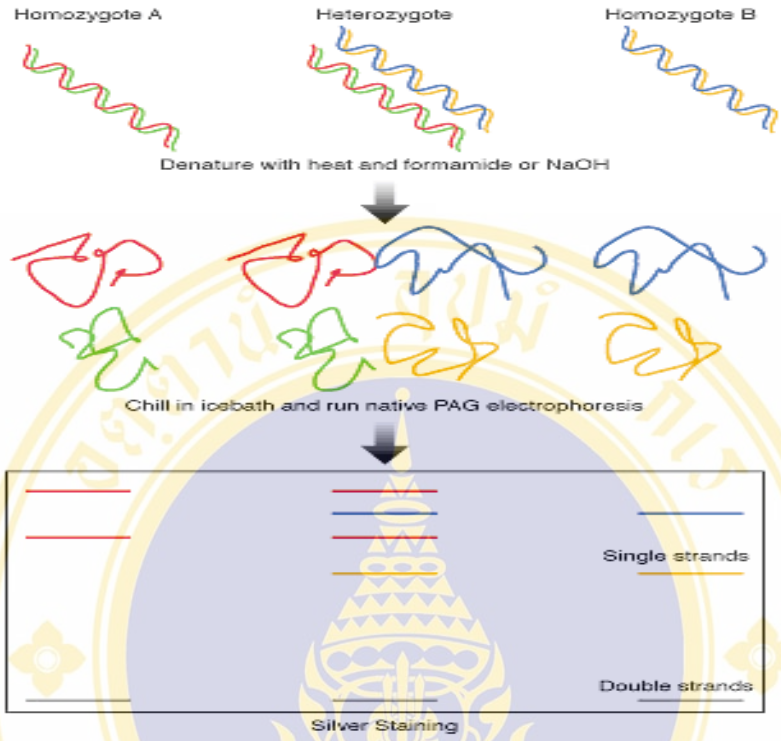


Figure 3.3 The diagram of single strand conformation polymorphism (SSCP) (34)

5.2 Reagents preparation

a. 50% (49:1) acrylamide stock solution 49 g of acrylamide and 1 g of N'N'-methylene-bis-acrylamide were weighed and dissolved in 100 ml sterile distilled water for injection. The solution was filtered with whatman paper no.1 and stored at 4°C.

b. Formamide dye

200 µl of Bromophenol blue-Xylene cyanol FF solution , 800 deionized formamide, 200 ul of 0.5 M EDTA were aliquoted. The solution was homogeneously mixed.

c. 20% w/v Ammonium persulfate

0.2 g of Ammonium persulfate was weighed and dissolved in 1 ml of distilled water. The solution was freshly prepared, kept away from light and stored at 4°C.

d.Temed

5.3 Preparation of the glass plates

The glass plates were cleaned with water and a detergent, then rinsed throughly with deionized water to remove detergent residues. Two of the glass plates were cleaned with 95% ethanol.Side spacers were placed on two sides of the inner side of the shorter glass plate. For the sandwich preparation, the longer plate was carefully placed on the shorter plate.

5.4 Preparation of Non- denaturing polyacrylamide gel

The polyacrylamide gel solution was the reagents shown in Table3.4.Then the total volume was adjusted to 10 ml with sterilized miliQ water. 70 ul of 20% ammonium persulfate and 6 ul of TEMED were added and mixed gently. The polyacrylmide gel solution mixture was poured between the minigel glass plates until the sandwiched glass plate was full. A comb was placed in the assembled gel sandwich. The gel was left to polymerize for at least 30 minutes at room temperature. Gel loading buffer and 1 ul of PCR products were mixed together. Then the mixtures were loaded in each well of acrylamide gel. An electric current of 100 volts was applied for 2 to 3 hours to determine DNA fragments. The PCR product sizes were estimated by comparison with 100 bp. DNA ladder marker. After electrophoresis, the gel was stained with silver stain method and the gel was wrapped by cellophane, air dried and collected as such.

Table 3.4 The mixture of Non-denaturing polyacrylmide gels

Percentage of gel	50%acrylamide stock solution (49:1;w/v) (ml)	10X TBE (ml)	MiliQ water (ml)
8%T, 2%C	1.6	0.5	7.9
10%T, 2%C	2.0	0.5	7.5
12%T. 2%C	2.4	0.5	7.1

5.5 Procedure

A volume of 1 µl of PCR product obtained from amplification of each exon of The LDLR gene was mixed with 10µl of formamide dye. The mixture was denatured by heat for 10 minutes. After denatured, the mixture was immediately chilled on ice. All samples were loading onto a non-denaturing polyacrylamide gel in 0.5x TBE buffer. Electrophoresis was carried out with the Protein II electrophoresis apparatus at 80 volt for 1-3 hours at 4°C(53). After electrophoresis, the silver staining method was used to visualize the DNA fragments.

5.6 Polymerase Chain Reaction-Multiplex Single Strand Conformation Polymorphism (PCR-MP SSCP)

Each exon or DNA fragment under analysis was amplified separately. These amplified products were then pooled and loaded on 4-20 % gradient gel in the same lane. The PCR amplified products were pooling in 1 ul of each exons. A volume of 0.5 ul of pooled PCR amplified products were taken into a single tube that contained 20 ul of 10X loading dye. The mixture was denatured by heat for 10-15 minutes. After denaturation, the mixture was immediately chilled on ice. The samples were loaded on gel in a single lane of 4-20% non-denaturing gradient polyacrylamide gel in 0.5XTBE buffer. Electrophoresis was carried out with the Hoefer apparatus at 100 volts for 8 hours at 4°C. After electrophoresis, the DNA fragments were visualized by the silver staining method.

6. Silver staining method

6.1 Principle

Many silver staining methods and their variations have been applied in biology since the latter years of the nineteenth century. It is thought that, as a result of electron exchange with the reducer, the silver method strongly catalyses the deposition of fresh silver metal from ionic silver in solution. Under this condition, formaldehyde is a reducer in an alkaline sodium carbonate solution. It reduces silver ions from silver solution to black silver at a higher pH and low temperature.(54, 55)

6.2 Reagents preparation

Reagent for silver staining method comprised three solutions, fix and stop solution (10% glacial acetic acid), staining solution and developing solution.

a. Fix and stop solution (10% glacial acetic acid)

100 ml of glacial acetic acid was added to 900 ml of ddH_2O.

b. Staining solution

2 g of silver nitrate was weighed and homogeneously dissolved in 1 ml of sterile water for injection.

c. Developing solution

30 g of sodium carbonate was weighed and dissolved in 1 L of milli Q water. The solution was chilled to 4°C in the refrigerator. Immediately before use, 1.5 ml of 37% formaldehyde was added.

6.3Procedure

After electrophoresis, the glass plates were carefully separated using a plastic wedge. The gel was placed in a plastic tray and covered with fix solution and agitated well for 30 minutes or until the tracking dyes were no longer visible. Fixative was saved for later use in terminating the development reaction. The gel was rinsed three times in deionized water with two minutes of agitation each time. After the gel was completely rinsed, it was transferred to 1,000 ml of staining solution and was agitated for 30 minutes. The gel was removed from the staining solution and rinsed for no longer than 5-10 seconds to remove an excess of silver nitrate in milliQ water. The gel was then placed in a chilled tray containing cold developing solution and was agitated again. As soon as the bands were clearly resolved, the fixative solution from the first step was poured directly into the developer to stop reaction, and the mixture was agitated for 3 min. The gel was rinsed twice in distilled water. The gel was covered with cellophone wrap. The gel was dried at room temperature for 1 day.

7. DNA sequencing

7.1Principle

Direct DNA sequencing will be used for characterizing DNA sequence variation, i.e., polymorphism or mutation by the method as described by Sanger *et al*.1997. In this method, the dideoxynucleotide triphosphate (ddNTP) analogs are used to interrupt DNA synthesis (figure 3.3). When the dNTP is replaced by the ddNTP, strand elongation is stopped after the analog is added because it lacks the 3'hydroxyl group needed for next step (figure 3.4).

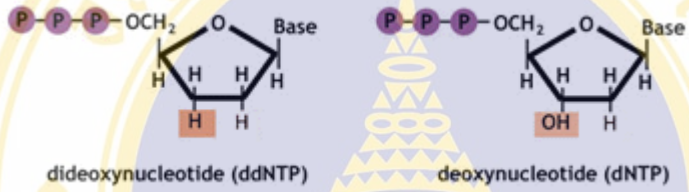


Figure 3.4 The structure of dideoxynucleoside triphosphate

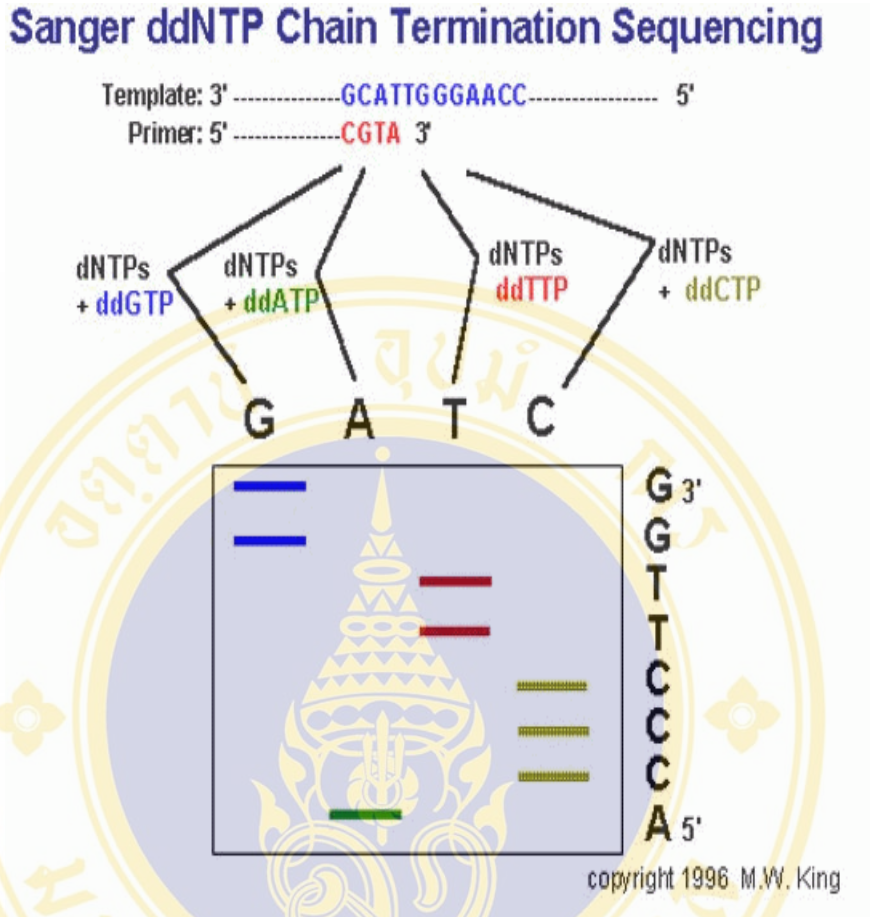


Figure 3.5 The Sanger DNA sequencing method.

7.2 Procedure

The different pattern of each exon from SSCP technique was sequenced by automated DNA sequencing (Applied Biosystems). The DNA sequence analyses were performed for both sense and antisense DNA strands.

8. PCR-RFLP

(Polymerase Chain Reaction- Restriction Fragment Length Polymorphism)

8.1 Principle

This technique is used for detecting mutation or polymorphism, which creates or destroys a restriction site. The mutated fragment with such a restriction site is digested with a restriction enzyme. The restricted DNA fragments will be separated by gel electrophoresis. In case that the mutation site is not recognized by any restriction enzyme, the restriction site cut will be designed and introduced into the PCR-amplified fragment (figure 3.5). The difference in genotypes will be visualized by etidium bromide. This technique will be used to confirm the result from DNA sequence analysis and to search for the identified mutation in family members of case, other hypercholesterolemic patients and normal subjects(56).

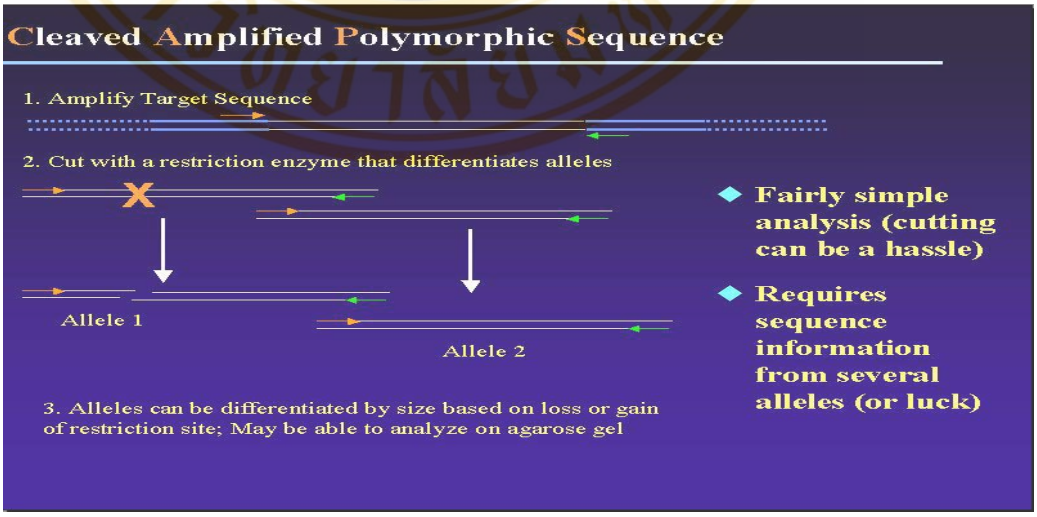


Figure 3.6 Principle of restriction fragment length polymorphism.(77)

8.2 Enzymes and Reagents

Enzymes used for digesting PCR products of exon 3 and exon 11 were RsaI and TaqI restriction enzymes, respectively. The other reagents were sterile water and 10X buffer (330mM Tris-acetate, 100 mM magnesium acetate, 660 mM potassium acetate and1 mg/ml BSA)which was supplied together with its enzyme.

8.3 Procedure

The digestion was performed in a final reaction volume of 20 ul. The reaction mixture contained 10 ul of PCR product, 2 ul of 10X buffer and 1 ul of restriction enzyme. The reaction volume was adjusted to 20 ul with sterile water. The mixture was incubated at 37°C overnight for RsaI restriction enzyme and 68°C overnight for TaqI restriction enzyme. After digestion, the digested samples were fractionated on 12 % polyacrylamide gel electrophoresis.

9. Allele Specific Amplification (ASA)

9.1 Principle

Allele specific PCR was designed to amplify a specific DNA sequence while excluding the possibility of amplifying other alleles. Based on the requirement for precise base matching between the 3' end of a PCR primer and the target DNA. The crucial dependence of correct base pairing at extreme 3'end of bound primers has allowed methods to be developed which permits distinction between alleles that differ at just a single nucleotide. In the popular ARMS (amplification refractory mutation system) method, primer are designed with their 3' end nucleotides designed to base-pair with the variable nucleotide which distinguishes the two alleles, and with the remaining primer sequence designed to be complementary to the sequence immediately adjacent to the variable nucleotide. Under suitable experimental conditions amplification will not take place where the 3' end nucleotide is not perfectly base-paired thereby distinguishing the two alleles (figure 3.6).

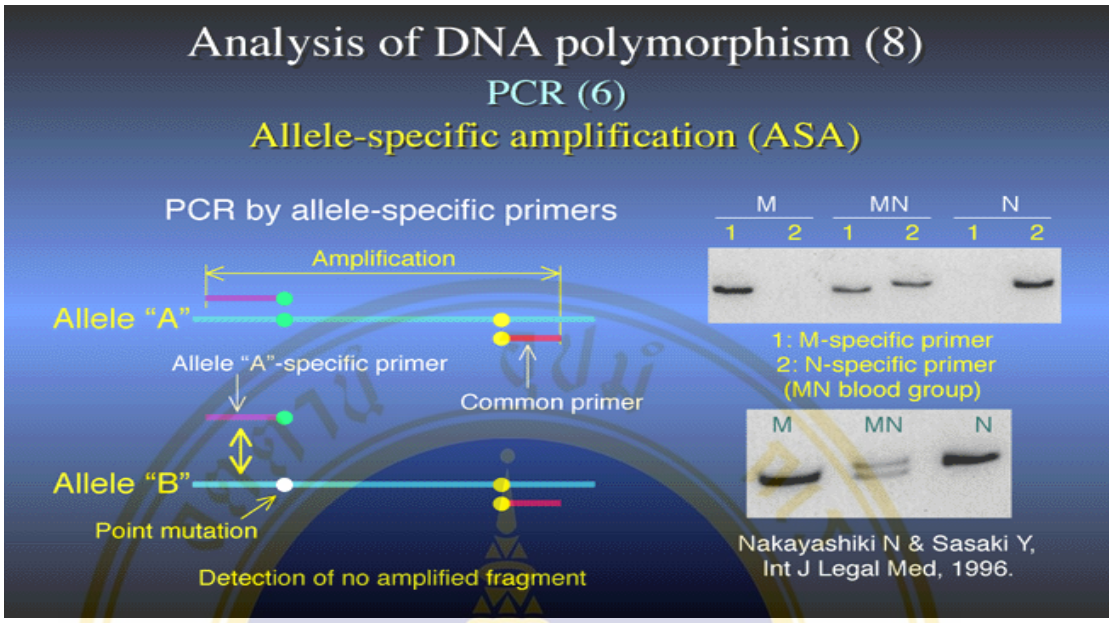


Figure 3.7 Principle of allele-specific amplification (76)

9.2 Reagents

Reagents used for DNA amplification were mentioned above in method 3.

9.3 Procedure

The DNA region at the 3'end of exon 9 of LDL receptor gene, containing the M391T mutation was amplified under the condition at 95°C: 1 min, 73°C: 40 sec. Two forward primers were used to differentiate (underlined) mutant allele from normal allele, i.e., E9MC (5'CCGGCACGAGGTCAGGAAGAC3') and E9NT (5'CCGGCACGAGGTCAGGAAGAT3'), respectively(58). Amplification reaction was made in duplicate for each sample using either E9MC or E9NT with a reverse primer SP 71A. In each ASA reaction, exon 10 of LDL receptor gene was used as positive control. The ASA reaction was performed in a final volume of 25 ul, in 0.2 ml thin- wall microtubes, which contained genomic DNA, 10X PCR buffer, 10 pmole of each primer, 10 mmole of each dNTP and a unit of *Taq* DNA polymerase. The cycling condition of each exon was performed for 35 cycles. All reactions were stopped by chilling at 4°C. The PCR products were subjected to 2% agarose gel electrophoresis.

10. Transformation and expression of p*Taq* DNA polymerase

Taq DNA polymerase was prepared in house in order to economize the screening process. All PCR fragments for SSCP screening were amplified by our in house *Taq* DNA polymerase. The commercial *Taq* DNA polymerase was used for the preparation of PCR templates for DNA sequencing.

10.1 Reagent preparation

a. LB broth

2.5 g of Bacto-tryptone, 1.25 g of yeast-extract, 1.25 g of sodium chloride (NaCl) were weighed and dissolved in 250 ml of distilled waster. This broth was sterilized by autoclaving.

b. LB agar

2.5 g of Bacto-tryphone, 1.25 g of yeast-extract, 1.25 g of sodium chloride (NaCl) and 3.75 g of agar were weighed. 250 ml of distilled water was added and heated until no granule of agar remained visible. The media was sterilized by autoclaving.

c.10 mg/ml Amplicilin stock

0.1 g of amplicilin was weighed and dissolved in 1 ml of autoclaved dH_2O.

d.1 M Glucose

1.8 g of glucose was weighed and dissolved in 10 ml of autoclaved dH_2O. The solution was filtered by 0.2 um Nalgene filter and kept at 4°C.

e.100 mM PMSF

0.0174 of PMSF was weighed and dissolved in 1ml of absolute ethanol. The solution was stored at -20°C until use.

f.1 M DTT

0.1543 g of DTT was weighed and dissolved in 1 ml autoclaved dH_2O.

g.10 mM DTT

10 ul of 1 M DTT stock was diluted to 1000 ul by sterilized dH_2O.

h. 1 M KCl

7.455 g of KCl was weighed and dissolved in dH_2O. The volume of solution was adjusted to 100 ml. The solution was sterilized by autoclaving.

i. Buffer A solution

2 ml of 1 M Tris-HCl pH 8.0, 2 ml of 1 M Glucose and 80 ul of 0.5M

EDTA were aliquoted. The volume was adjusted to 40 ml with autoclaved dH_2O.

j. Prelysis buffer

0.002 g of lyzosome was weighed and dissolved in 5 ml of buffer A.

k. Lysis buffer

50 ul of 1 M Tris-HCl pH 8.0, 250 ul of 1 M KCl, 10 ul of 0.5 M EDTA, 50 ul of 10 mM PMSF, 25 ul of Tween20 and 25 ul of IGEPAL were mixed together. The volume was adjusted to 5 ml with autoclaved dH_2O.

l. 50 mM $CaCl_2$ and 10 mM Tris-HCl (pH 8.0) solution

500 ul of 1 M $CaCl_2$ and 100 ul of 1 M Tris-HCl, pH 8.0 were mixed. The volume was adjusted to 10 ml with autoclaved dH_2O.

m. p*Taq* recombinant plasmid and *E.coli* (DH $_{5\alpha}$)

The p*Taq* recombinant plasmid has been aviable in our laboratory *E.coli* DH $_{5\alpha}$ strain was purchased commercially.

10.2 Preparation of fresh competent *E. coil* Cells

20 ul of frozen stock of *E.coli* (DH $_{5\alpha}$) was inoculated into 2 ml LB broth and then incubated overnight at 37 °C with shaking. 500 ul of the culture was inoculated to 50 ml of new LB broth and shaked at 37°C about 2-3 hours to an OD_{550} of approximately 0.5. 3 ml of inoculum was aliquoted into foncal tube, chilled on ice for 10 minutes and then centrifuged at 2000 rpm for 5 minutes at 4°C. The supernatant was discarded and the packed cells were resuspended in 1.5 ml of cold 50 mM $CaCl_2$, 10 mM Tris-HCl (pH 8.0) solution. The mixture was left stand on ice for 15 minutes and then centrifuged at 2000 rpm at 4°C for 5 minutes. After the supernatant was discarded the cell pellet was resuspended in 200 ul of cold 50 mM $CaCl_2$, 10 mM Tris-HCl pH 8.0 solution. The mixture was then transferred to new 1.5 ml eppendorf tube.

10.3 Transformation

5 ul of recombinant p*Taq* plasmid was mixed with 200 ul of the component *E.coli.* cells. The mixture was incubated on ice bath for 30 minutes, heated shock at 42°C for exactly 2 minutes and left stand on ice for 1 minute. 1 ml of LB broth with 100 mg/l amplicilin was added to the transformed cells and incubated for 1 hour at

37°C. After incubation, the suspension was centrifuged at 5000 rpm for 1 minute. 900 ul of supernatant was discard. The cell pellet was gently vortexed and spreaded on LB agar with 100 mg/l amplicilin.

10.4 Gene Expression

A single colony of transformed *E.coli* was inoculated to 2 ml LB broth with amplicilin (100 mg/l) and incubated at 37°C overnight with shaking. 400 ul of inoculum was subcultured into 40 ml LB broth with amplicilin (100 mg/l) and incubated at 37°C with shaking until the OD_{550} is 0.2. The inoculum was kept on ice to prevent overgrowth until use. 1 ml of culture was aliquoted and used for dissolving 0.005 g IPTG. The culture with IPTG was taken back to the flask and then incubated at 37°C for exactly 12 hours with shaking. 10 ml of the culture was aliquoted in foncal tube, chilled on ice bath and centrifuged for 5 minutes at 2000 rpm 4°C. The supernatant was discarded. The cell pellet was resuspended and vortexed in 5 ml buffer A. The mixture was centrifuged, for 5 minutes at 2000 rpm, 4°C and then supernatant was discard. The cell pellet was resuspended with 0.5 ml of prelysis buffer, mixed gently and left stand for 15 minutes at room temperature. 0.5 ml of lysis buffer was added, mixed gently and transferred to new glass tube. The mixture was incubated at 75°C for 1 hour. After incubation, the mixture was transferred to 1.5 ml eppendrof tube and placed on ice bath for 5 minutes. The mixture was centrifuged at 12,000 rpm for 15 minutes. The supernatant was transferred to new 1.5 eppendorf tube and then added with 100 ul of 10 mM DTT into 1 ml of lysate. The *Taq*DNA polymerase activity was assayed by PCR reaction.

CHAPTER IV

RESULTS

1. 313+1 G>T mutation in intron 3 (IVS 3+1 G>T)

1.1 Single strand conformation polymorphism (SSCP) analysis.

The PCR product of exon 3 was screened for mutation using multiplex SSCP (figure 4.1 A). The amplified exon 3 products with mobility shift in the multiplex assay were reanalyzed individually for confirmation. (figure 4.1 B). The results revealed mobility shift in amplified exon 3 of the LDL receptor gene in two DNA samples obtained from two unrelated patients with primary hypercholesterolemia.

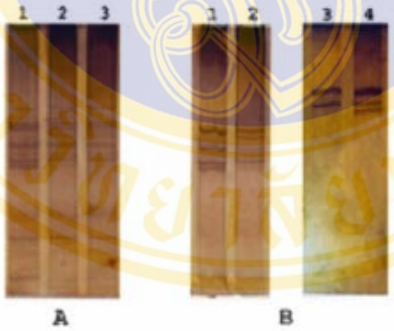


Figure 4.1 SSCP analysis

(A) Multiplex SSCP patterns of exons3, 16 and 17 of the LDL receptor gene in 4-20% gradient polyacrylamide gel. Lane1 was control DNA sample. Lanes 2 and 3 were hypercholesterolemic DNA samples.

(B) SSCP patterns of exon 3 of the LDL receptor gene. Lanes 1 and 3 were control DNA sample. Lanes 2 and 4 were two hypercholesterolemic DNA samples corresponding to the multiplex samples in lanes 2 and 3 in (A). The SSCP gel for lanes 1 and 2 was 4-20% gradient polyacrylamide gel. The SSCP gel for lanes 3 and 4 was 8% polyacrylamide gel.

Table 4.1 The corresponding cholesterol profiles of different SSCP patterns in exon 3.

Lane	Gender	Age	Total-cholesterol (mg /dl)	LDL-cholesterol (mg /dl)
2(B)	Male	53	360	302
4(B)	Female	59	307	220

1.2 DNA sequencing analysis of abnormal SSCP pattern of exon 3

The different SSCP pattern of exon 3 of the LDL receptor gene was subjected to automate DNA sequencing for both sense and antisense DNA strands. The nucleotide sequences of normal and abnormal patterns are shown in figure 4.3.

The index subjects were heterozygous for the G to T transversion at position 1 of intron 3. The GT dinucleotide at 5' end of an intron is highly conserved serving as part of the consensus recognition signal for mRNA splicing in vertebrate genes. The DNA sequence of this exon and an indicated change in the index cases are shown in figure 4.2.

Normal GAGCAAGGCTGTCGTAAGTGTGGCCCT…….

|___ Exon 3_____ |_____Intron 3_____……

Mutant GAGCAAGGCTGTCTTAAGTGTGGCCCT……..

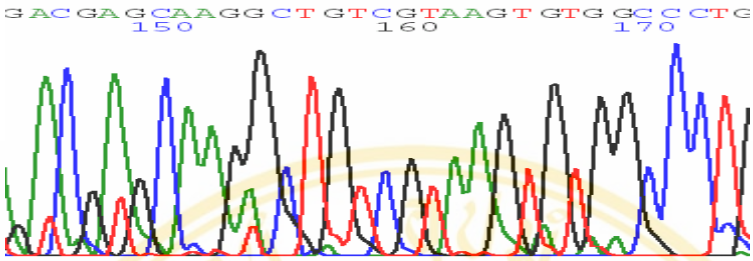
|___ Exon 3_____ |_____Intron 3_____……

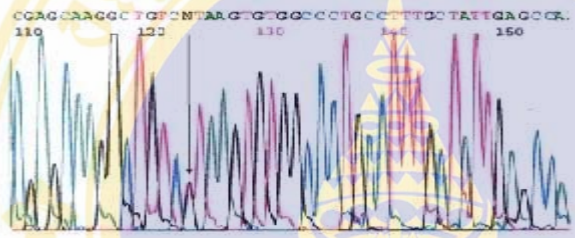
Figure 4.2 Comparison of DNA sequences in exon 3 in the LDL receptor gene of normal and the index subjects. The nucleotide changed is marked by an underline.

(A)

(B)

(C)





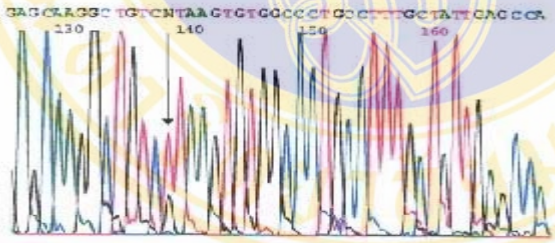


Figure 4.3 Partial automated DNA sequence of exon 3 of the LDL receptor gene. PCR fragments of this exon obtained from wildtype DNA and putative mutant DNAs (as seen from abnormal SSCP pattern) sequenced by sense primer (SP 59 A). (A) Nucleotide sequence of a normal control subject. (B) and (C) Nucleotide sequences showing the mutation 313+1 G>T from the first (B) and second (C) hypercholesterolemic patients. Arrow indicates the variant sequence (N), i.e., the heterozygous substitution of G to T in the first nucleotide in intron 3. The DNA sequences presented in this picture were obtained from Bio Service Unit (BSU).

1.3 PCR- RFLP analysis of the 313+1 G>T mutation

The 313+1 G>T mutation in intron 3 of the LDL receptor gene was confirmed by PCR-RFLP analysis. The DNA fragments were amplified by the sense primer (SP59A) and the antisense primer (IN3M1T) 5'TAGCAAAGGCAGGGCCACACGTA3'. The underlined base G was introduced into the antisense primer (replacing the authentic base " T" at the position +4 in intron 3) in order to create a *RsaI* site (GT/AC) in the PCR product of normal allele (figure 4.4 A). The PCR condition for this amplification is shown in figure 4.4 B

The DNA samples under analysis were amplified with the specified primer, subjected to *RsaI* digestion, electrophoresed in 12% polyacrylamide gel and visualized with ethidium bromide staining. The fragment size of the amplified exon 3 product is 172 bp and the size of the apparent *RsaI* digested fragment is 148 bp. (figure 4.5)

(A)

Primer SP59A >

5' | ----------Exon 3---| ----------Intron 3---------- | 3'

GTCGTC | **G**TAAGTGTGGCCCTGCCTTTGCTA normal sequence

| ATGCACACCGGGACGGAAACGAT IN3M1T primer

| ----------148 bp----------| ----------24 bp----------|

|----------172 bp---------- ----------|

B)

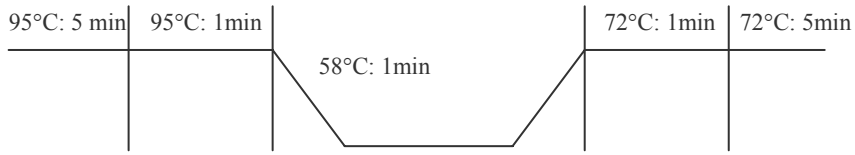


Figure 4.4 The assay for the 313+1 G>T mutation of the LDL receptor gene.

(A) Schematic picture of the primer-introduced restriction analysis of the mutation. The deliberate mismatch close to the 3' end of the primer IN3M1T is indicated by single underlining and the mutation point (G>T) is indicated by a bold letter

(B) The PCR condition for amplification of PCR-RFLP analysis.

Figure 4.5 12 % PAGE analyses of DNA samples amplified with primer SP59A and IN3M1T. Lane 1, molecular size marker (100 bp ladder). Lane 2, undigested DNA sample from control subject. Lane 3, *RsaI*-digested DNA sample from control subject. Lanes 4, 5, and 6, *RsaI*-digested DNA samples from the index subjects. Note Lane 6, the mutation in this patient was observed during PCR-RFLP analysis performed in DNA samples of new patient prior to SSCP screening. Plasma total cholesterol and LDL cholesterol in this patient (female, 37 years old) was 449 mg/dl, 373.3 mg/dl, respectively.

From PAGE analysis, DNA from Patients in lanes 4,5,6 have digested with enzyme *RsaI*

2. E153K mutation

2.1 DNA sequencing analysis of exon 4

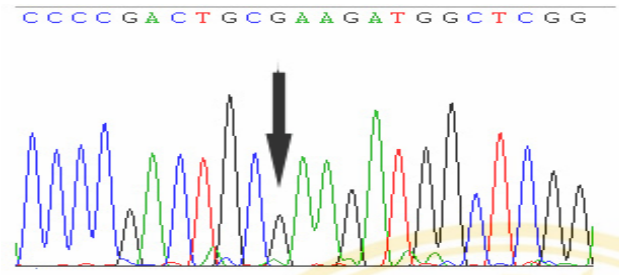
The PCR fragment of exon 4 of the LDLR gene was subjected to automate DNA sequencing. The DNA sequence change of exon 4 and an indicated change the index case are shown in figure 4.6. The nucleotide sequences of normal and abnormal SSCP patterns are shown in figure 4.7.

Normal 499 TGC GAC AAC GAC CCC GAC TGC **G**AA GAT GGC TCG 531
146 C D G D P D C E D G S 156

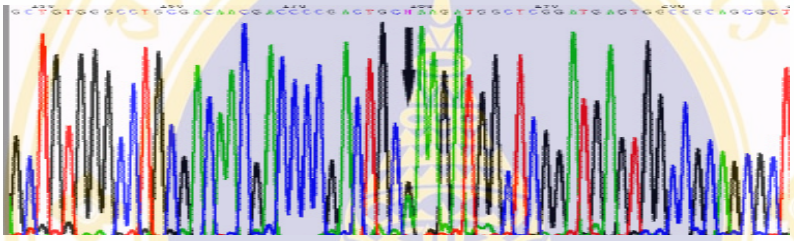
Mutant 499 TGC GAC AAC GAC CCC GAC TGC **A**AA GAT GGC TCG 531
146 C D G D P D C K D G S 156

Figure 4.6 Comparison of DNA sequences in exon 4 of the LDL receptor gene between a normal control subject and the index case. The nucleotide changed is marked by an underline

(A)



(B)



(C)

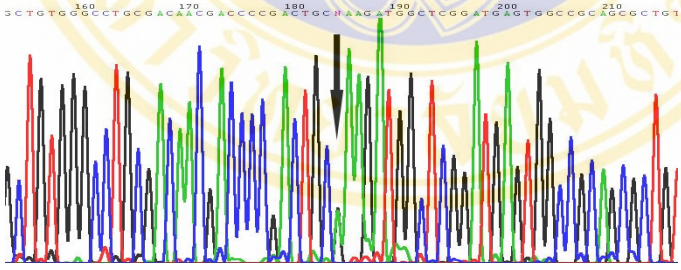


Figure 4.7 Partial automated DNA sequences of exon 4 of the LDL receptor gene. PCR product of normal control subject and the index subject with primary hypercholesterolemia subjects were sequenced by forward primer (SP4.1). (A) The Nucleotide sequence of normal control subject. (B) and (C) The nucleotide sequence of the index subjects.

These subjects are heterozygous for a G to A transversion at nucleotide 494 (indicated with N). DNA samples obtained from two related patients. From figure 4.7 (B) and (C), The subject is a 10 years old Thai boy who has presented severe hypercholesterolemic phenotype (total cholesterol 396 mg/dl, LDL-cholesterol 336 mg/dl) with normal plasma triglyceride level (114 mg/dl) and a minimal plaque apparent at both carotid ateries. His parents also have already hypercholesterolemic condition, respectively.

This nucleotide change was predicted to cause the substitution of a Lysine for Glutamate at codon 153 in the LDL receptor gene. The amino acid at this position has been changed from an amino acid with negatively charged side chain to amino acid with positively charged side chain.

2.2 Amino acid sequence alignment of E153K mutation

Amino acid sequence alignment analyses of E153K in six species (7 members) were aligned from MultAlin (http://www.toulouse.inra.fr/multalin/cgi-bin/multalin.pl) (figure 4.8). From this alignment, it is conceivable that this mutation would cause nonconservative amino acid substitution in relatively conserved region of the LDL receptor protein.

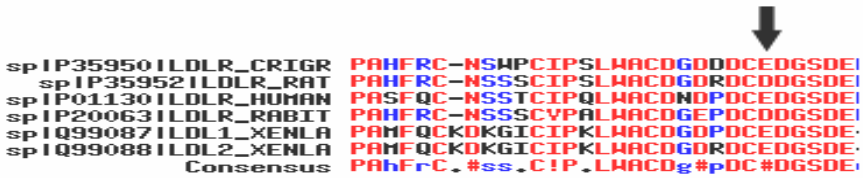


Figure 4.8 Amino acid sequence alignment analysis of E153K in six species
The Swiss-port accession number of each sequence is presented in front of sequence. The codon 153, where the missense E153K occurred, is marked by ⬇ .

2.3 Family analysis

This study presents a 10 years old Thai boy who has presented severe hypercholesterolemic phenotype (total cholesterol 396 mg/dl, LDL-cholesterol 336 mg/dl) with normal plasma triglyceride level (114 mg/dl) and minimal plaques apparent at both carotid ateries. Both of his parents also have hypercholesterolemic phenotype. In addition, his mother manifested myocardial infarction at the age of 46.

From this analysis, the R3500Q mutation was not observed in this family. So, the hypercholesterolemia condition in this family may be due to any mutation in the LDL receptor gene. From DNA sequence analysis, a mutation E153K was observed at the hot spot exon 4 in the boy and his father. This non-conservative amino acid substitution occurs in the relatively conserved region of the LDL receptor molecule and thus may consequently disturb the structure and/or function of the receptor protein. The E153K mutation was not present in the boy's mother. It might be a pathogenic mutation causing the hypercholesterolemic condition in the boy and his father. We then speculate that the boy may be a compound heterozygous FH and has inherited another FH causing mutation from his mother. Now we are examining the remaining exons and found G361S mutation in exon 8 of LDL receptor which should be observed in both the boy and his mother. However, this mutation is under confirmation. The family analysis of this family are shown in figure 4.9

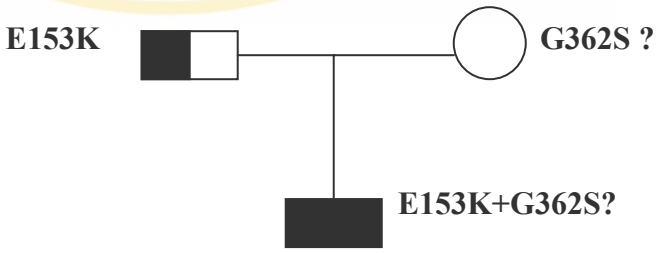


Figure 4.9 The family analysis of E153K

3. M391T mutation in exon 9 of the LDL receptor gene

3.1 Single strand conformation polymorphism (SSCP) analysis.

The PCR products of exon 9 were analyzed for nucleotide sequence variation by SSCP technique. A different pattern of fragment was seen as presented in figure 4.10

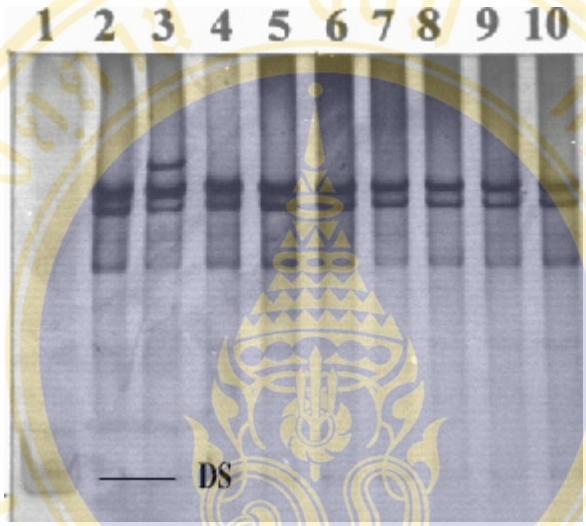


Figure 4.10 SSCP analysis of exon 9 of the LDL receptor gene. Lane 1, double strand DNA. Lane 2, DNA from normal control subject. Lanes 3-10, DNA from subjects with primary hypercholesterolemia. Lane 3, abnormal mobility shift in SSCP pattern.

The different SSCP pattern belonged to a 42 years old woman, whose plasma cholesterol and LDL- cholesterol were 357 mg/dl and 279 mg/dl, respectively.

3.2 DNA sequencing analysis of abnormal SSCP pattern of exon 9

The different SSCP pattern of exon 9 of the LDL receptor gene was amplified and subjected to automate DNA sequencing. The DNA sequences of this exon and an indicated change in the index case are shown in figure 4.11.

The automated nucleotide sequences of PCR fragments with normal and different SSCP patterns are shown in figure 4.12.

Normal

1222 CGG CAC GAG GTC AGG AAG ATG ACG CTG GAC CGG AGC 1257

385 R H E V R K M T L D R S 396

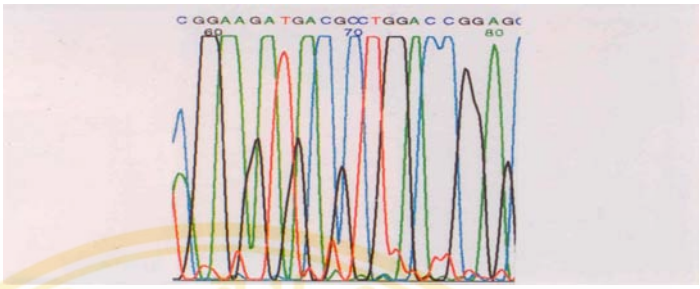
Mutant

1222 CGG CAC GAG GTC AGG AAG ACG ACG CTG GAC CGG AGC 1257

385 R H E V R K T T L D R S 396

Figure 4.11 Comparison of DNA sequences in exon 9 of the LDL receptor gene between a normal control subject and the index case. The nucleotide changed is marked by an underline.

(A)



(B)

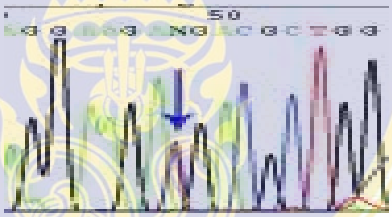


Figure 4.12 Partial automated DNA sequences of exon 9 of the LDL receptor gene. PCR product of normal control subject and the index subject with abnormal SSCP pattern were sequenced by forward primer (SP70). (A) The Nucleotide sequence of normal control subject. (B) The nucleotide sequence of the index subject with abnormal SSCP pattern.

The subject with different SSCP pattern is heterozygous for a T to C transition at nucleotide 1235 (indicated with N). This nucleotide change was predicted to cause the substitution of a methionine for threonine at codon391 in the LDL receptor gene. The amino acid at this position has been changed from an amino acid with nonpolar side chain (Methionine) to uncharged polar side chain (Threonine).

3.3 Allele specific amplification (ASA) analysis of M391T mutation

Secondary confirmation for M391T mutation was performed by ASA (allele specific amplification) technique. The PCR product size of 155 bp was amplified by same reverse primer (SP71 A) and different forward primers (E9MC and E9NT) in each sample. Two forward primers were used to differentiate mutant allele from normal allele are shown in figure 4.13. The PCR condition for amplification of ASA analysis is shown in figure 4.14. DNA of patient who has phenotype T/C was amplified by both forward primers (E9MC and E9NT) where as DNA of normal control (T/T) was amplified only by E9NT primer. In this ASA reaction, exon 10 (278 bp) of LDL receptor gene was used as positive control (figure 4.15)

Mutant primer (E9MC) 5'CCGGCACGAGGTCAGGAAGAC3'
Normal primer (E9NT) 5'CCGGCACGAGGTCAGGAAGAT3'

Figure 4.13 Two forward primers were used to differentiate mutant allele from normal allele. Mutant primer is E9MC and normal primer is E9NT. The differentiation of both primers indicated with an underlining.

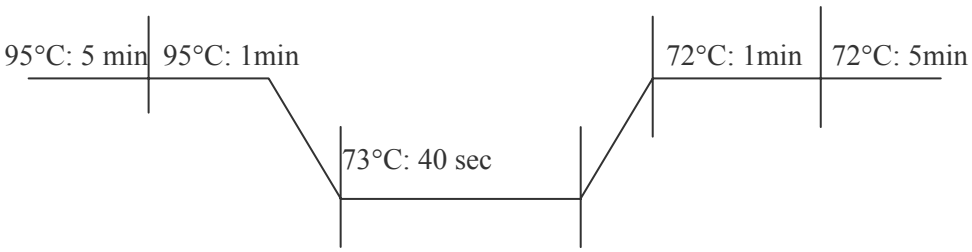


Figure 4.14 The PCR condition for amplification of ASA analysis.

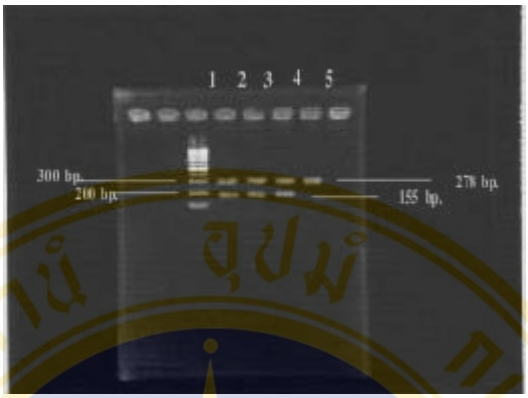


Figure 4.15 Allele specific amplification analysis.
The upper bands (278 bp) are internal control. Lane 1, molecular size 100 bp markers. Lanes 2 and 3, DNA sample from the index subject amplified with different reverse primers, E9MC and E9NT, respectively. Lanes 4 and 5, DNA sample from a control subject amplified with different reverse primers, E9NT and E9MC, respectively.

The ASA analysis revealed that DNA of patient who has phenotype T/C was amplified by both forward primers (E9MC and E9NT) where as DNA of normal control (T/T) was amplified only by E9NT primer. This result thus confirmed the DNA sequencing result that the patient is heterozygous for a T to C transition at nucleotide 1235. This ASA analysis was screened in 70 of primary hypercholesterolemia patients and 100 normal control subjects

3.3 Amino acid sequence alignment analysis of M391T mutation

Amino acid sequence alignment analyses of M391T in six species (7 members) were aligned from MultAlin (http://www.toulouse.inra.fr/multalin/cgi-bin/multalin.pl) (figure 4.16). From this alignment, it is conceivable that this mutation would cause

nonconservative amino acid substitution in relatively conserved region of the LDL receptor protein.

Figure 4.16 A multiple sequence alignment of amino acids correspondingly encoded by exon 9 in six species (7 members). The Swiss-port accession number of the each sequence is presented in front of relevant sequence. The codon 391, where the missense M391T occurred, is marked by ⬇ . At codon 391 in six species are represent to Methionine.

3.4 Family studies

FH patient with M391T mutation in LDL receptor gene was a single woman who has two older brothers. Her parents were dead many years ago. Her mother was recorded as having coronary artery disease with severe stenosis in more than one vessel. One of her brother has the condition of high plasma cholesterol level as same as the index subject. However, the family of this brother is inaccessible for our study at present. The other brother and his daughters apparently have normal blood cholesterol levels (table 4.2). The DNA samples obtained from this family were screened for the M391T mutation by the ASA analysis described above. The screening indicated that the M391T was not inherited in this family. The screening of M391T mutation in this family are shown in figure 4.17

Table 4.2 Lipid profile of family members of index subject with M391T mutation in LDL receptor gene

Number	Sex	Age	Total cholesterol (mg/dl)	LDL cholesterol (mg/dl)
1(II)	Male	--------	----------	----------
2(II)	Male	58	181	117.4
3(II)	Female	42	357	297
1(III)	Female	20	135	72.2
2(III)	Female	14	152	92.8

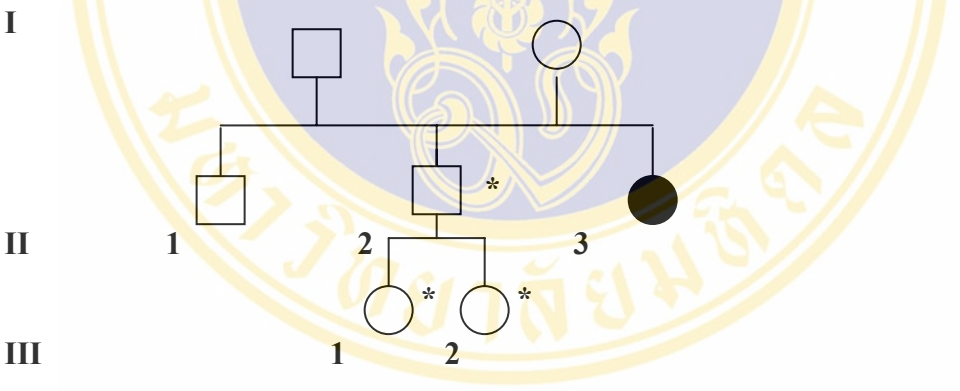


Figure 4.17. The family analysis of M391T mutation. Circles and squares represent females and males, respectively. Full- filled circles is proband with M391T mutation. Symbols with a diagonal star (*****) represent persons who are screened with M391T mutation.

4. S554L mutation in exon 11 of the LDL receptor gene

4.1 Single strand conformation polymorphism (SSCP) analysis

The PCR products of exon 11 were analyzed for sequence variation by SSCP technique. A different SSCP pattern was seen in figure 4.18

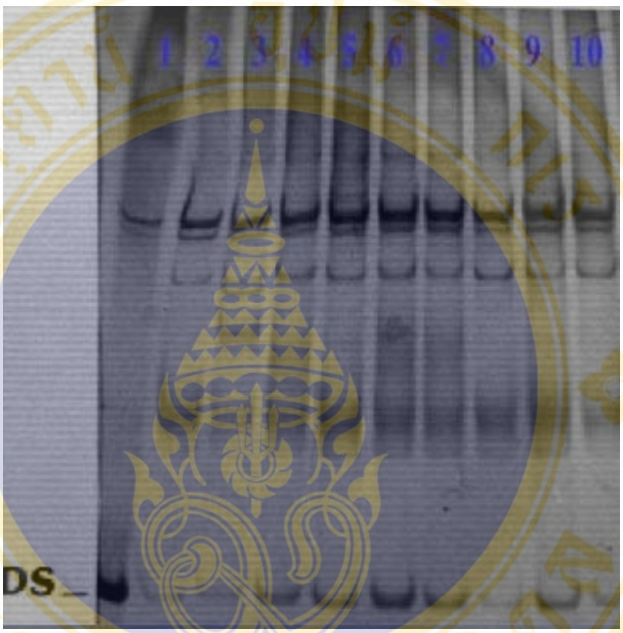


Figure 4.18 SSCP analysis of exon11 of the LDL receptor gene. Lane 1, Double-stranded DNA. Lane 2, DNA from normal control subject. Lanes 3-10, DNA from patients with primary hypercholesterolemia. Lane 3, abnormal mobility shift in SSCP pattern.

The abnormal mobility shift in SSCP pattern was observed in a 50 years- old woman whose plasma cholesterol and LDL cholesterol were 287mg/dl and 223 mg/dl, respectively.

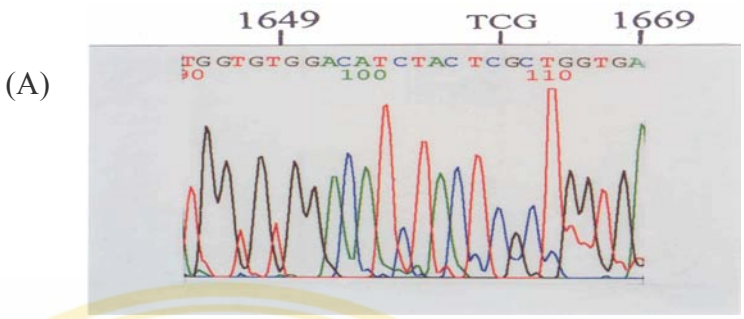
4.2 DNA sequencing analysis of the abnormal SSCP pattern of exon 11

The abnormal SSCP pattern of exon11 of the LDL receptor gene was amplified and subjected to automated DNA sequencing. The DNA sequences of this exon and indicated change in the index case are shown in figure 4.19 The nucleotide sequences of the PCR fragment with normal and different SSCP patterns are shown in figure 4.20

Normal 1651 GAC ATC TAC TCG CTG GTG ACT GAA AAC AAT 1680
551 D I Y S L V T E N I 560

Mutant 1651 GAC ATC TAC TTG CTG GTG ACT GAA AAC AAT 1680
551 D I Y L L V T E N I 560

Figure 4.19. Comparison of DNA sequences in exon 11 of the LDL receptor gene between a normal control subject and the index case. The nucleotide changed is marked by an underline.



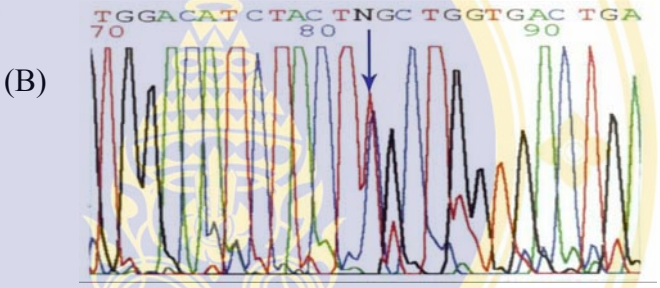


Figure 4.20 Partial automated DNA sequence of exon 11 of the LDL receptor gene. PCR products of normal control subject and the index subject with different SSCP pattern were sequenced by the both primer (SP 74) and antisense primer (SP75). This figure is the DNA sequence as read by sense primer. (A) The nucleotide sequence of PCR fragment from normal control subject. (B) The nucleotide sequence of PCR fragment with abnormal SSCP pattern.

The subject with different SSCP pattern was heterozygous for C to T transition at nucleotide 1661. This nucleotide changed was predicted to cause the substitution of Serine to Leucine at codon 554 of the LDL receptor gene. The amino acid at this position has been changed from an amino acid with uncharged polar side chain to nonpolar aliphatic side chain.

4.3 PCR-RFLP analysis of S554L mutation

Secondary confirm the S554L mutation was performed by PCR-RFLP technique. An artificial *TaqI* site (T/CGA) was introduced into the PCR product of non-mutated LDL receptor allele using a forward primer (SP74) and deliberate mismatch primer E11M1698T: 5 TGAATGTTTTCAGTCACCATC'3'. The latter having a mismatch (underlined) compared to the normal LDL receptor cDNA sequence.

Amplification products were subjected to *TaqI* digestion and analyzed by electrophoresis on 12% polyacrylamide gels (PAGE) with ethidium bromide staining. This assay confirmed the nature of the mutation in the proband. The PCR product size was 121 bp. The fragment sizes of normal allele digested with *TaqI* were 99 bp and 22 bp. (figure 4.21)

Figure 4.21 PAGE analysis of DNA samples amplified with the primer pair SP74 and E11M1698T to confirm S554L mutation. Lane 1, molecular size100 bp marker. Lane 2, undigested sample from Normal control subject. Lane 3, *TaqI*-digested sample from normal control subject. Lane 4, *TaqI*-digested sample from the index subject.

PCR-RFLP analysis confirmed that this patient is heterozygous for C to T transition at nucleotide 1661. This technique was screened in 70 of primary hypercholesterolemia patients and 100 normal control subjects.

4.4 Amino acid sequence alignment of S554L mutation

Amino acid sequence alignment analyses of M391T in six species (7 members) were aligned (figure 4.22). From this alignment, it is conceivable that this mutation

would cause nonconservative amino acid substitution in conserved regions of the LDL receptor protein.

Figure 4.22 Amino acid sequence alignment analysis of M391T in six species (7 members). The Swiss-port accession number of each sequence is presented in front of sequence(59). The codon 554, where the missense S554L occurred, is marked by ⬇

5. G1414A (R471) polymorphism

5.1 Single strand conformation polymorphism (SSCP) analysis

The PCR products of exon 10 were analyzed for sequence variation by SSCP technique. Two different SSCP patterns were seen in figure 4.23

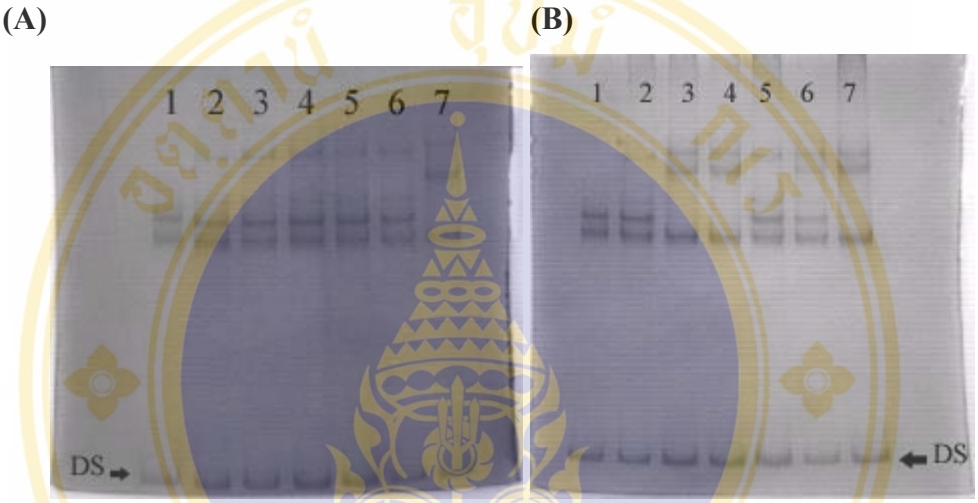


Figure 4.23 SSCP analysis of exon10 of the LDL receptor gene. (A) Lane 1, Double-stranded DNA. Lane 2, DNA from normal control subject. Lanes 3-7, DNA from patients with primary hypercholesterolemia. Lane 7, abnormal mobility shift in SSCP pattern. (B) Lane 1, Double- stranded DNA. Lane 2, DNA from normal control subject. Lanes 3-7, DNA from patients with primary hypercholesterolemia. Five samples revealed abnormal mobility shift in SSCP pattern. Mobility shifts in lanes 7(in

A) and lanes 3,4,7 (in B) were identical. Mobility shift in lane 6 (in B) was unique (different from the other four abnormal patterns).

The same abnormal SSCP patterns of exon 10 were observed in five patients with primary hypercholesterolemia.

5.2 DNA sequencing analysis of the abnormal SSCP pattern of exon 10

Two DNA samples with abnormal SSCP patterns of exon 10 [samples in lanes 7(A) and lanes 7 (in B) in figure 4.23] of the LDL receptor gene were amplified and subjected to automated DNA sequencing. The DNA sequences of this exon and indicated change in the index case are shown in figure 4.24 The nucleotide sequences of the PCR fragment with abnormal SSCP patterns are shown in figure 4.25

Normal 1393 TAT GAC ACC GTC ATC AGC AGG GAC ATC CAG GCC 1426

Mutant 1393 TAT GAC ACC GTC ATC AGC AGA GAC ATC CAG GCC 1426

Figure 4.24 Comparison of DNA sequences in exon 10 of the LDL receptor gene between a normal control subject and the index case. The nucleotide changed is marked by an underline.

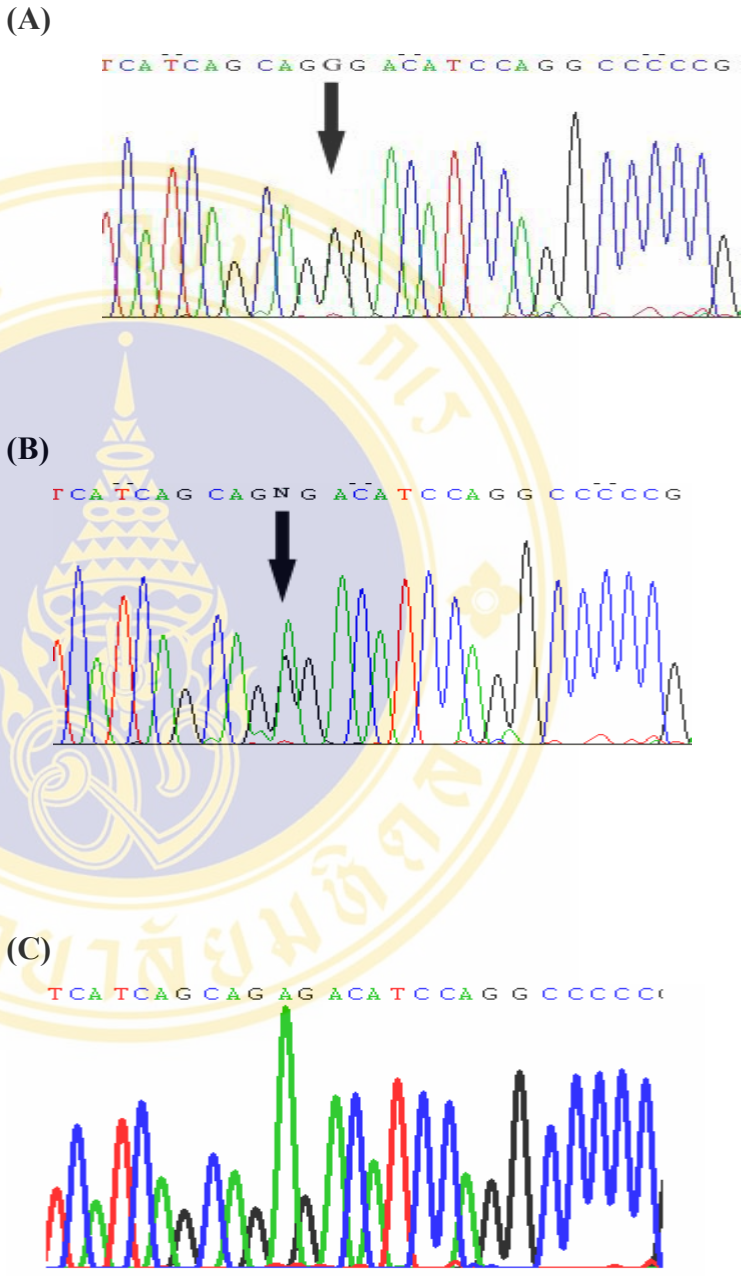


Figure 4.25 Partial automated DNA sequence of exon 10 of the LDL receptor gene. PCR products of normal control subject and the index subjects with different SSCP pattern were sequenced by both sense primer (SP 72) and antisense primer (SP73). This figure is the DNA sequence as read by sense primer. (A) The nucleotide sequence of PCR fragments with normal SSCP pattern. (B) and (C) The nucleotide sequence of PCR fragments with abnormal SSCP pattern.

The subjects with different SSCP patterns were heterozygous (figure 4.25 B) and homozygous (figure 4.25C) for G to A transition at nucleotide 1414. This nucleotide change did not cause amino acid substitution (ariginine to arginine) at codon 471 of the LDL receptor gene.

CHAPTER V
DISCUSSION

Since the elucidation of the cholesterol pathway by Brown and Goldstein, many studies have addressed the relation between mutation causing FH and the resulting phenotypic expression of the disorder(60). It has been frequently assumed that FH, the consequence of mutation in LDL receptor gene, is a major form of primary hypercholesterolemia in many populations. In order to survey mutations leading to FH in Thai population, 70 primary hypercholesterolemia patients were examined at the LDL receptor gene locus. The identification for point mutations and small structure alterations in unrelated patients with primary hypercholesterolemia was primarily done by PCR-SSCP technique. The mutations underlying the SSCP positive amplicons were subsequently characterized by DNA sequencing. The mutations observed via DNA sequencing analysis were confirmed by the PCR-based methods which were developed for each mutation. These PCR-based methods then were used to screen for the particular mutation in the family members of the patients, other patients as well as normal control subjects (n=100).

SSCP analysis is technically a simple method for detecting sequence variation in many genes. It has been reported to have a sensitivity of 75-85%, suggesting that a mutation may have been missed for technical reasons in possibly 15-25% of patients. In some studies, the sensitivity of SSCP analyses is lower than 75%(61, 62). Many factors can influence the sensitivity of SSCP analysis. The sensitivity of the method depends on the length of the amplified fragments, the electrophoretic conditions and the extent of cross-linking concentration of acrylamide(63). To eliminate these factors in our experiments, SSCP conditions have been varied and repeated about 2-3 times in each sample. The SSCP positive amplicons were sequenced by automated DNA sequencing which is very highly sensitive and rapidly to reveal the nucleotide sequences in target DNA fragments.

The PCR-based methods are widely used to detect a known or identified point mutation. In this study, PCR-RFLP and ASA analysis were developed to confirm the PCR-SSCP and DNA sequencing identified mutations. The sensitivity and specificity of these methods are close to 100% and are very cost-effective, however, duplication or even triplication of such analysis is necessary for confirmation in our study.

The results from our study provide three novel mutations (E153K, M391T, S554L), one previously reported mutation (313+1 G>T) and one common polymorphism (G1414A). All of these mutations are point mutation. However, not all mutations cause FH. Therefore, family analysis and screening of these identified mutations in 100 DNA samples from control subjects have been performed to assure that these mutations are possibly FH-causing mutations according to criteria of Day.*et.al.* In the family analysis, it is a pity that this assay was not successful during the time course of this thesis. This was due to many factors such as inaccessibility to family members of proband. The screening of these mutations in 100 normolipidemic subjects with the corresponding secondary confirmation techniques revealed that none of these mutations were observed in normal DNA subjects. To see whether these mutations were common among primary hypercholesterolemic patients, the screening was made in the 70 unrelated DNA samples obtained from patients under this study. Such screening should help determine the prevalence of the identified mutations in our patients.

5.1 A splice site mutation

313+1 G>T mutation

SSCP analysis of exon 3 and flanking intron sequences of genomic DNA from two hypercholesterolemic patients showed an identical mobility shift in a multiplex analysis as well as individual exon 3 analysis. Genomic sequencing revealed the wildtype sequence in exon 3 and one point mutation, a G to T transversion, in the first base of the splice donor consensus GT dinucleotide of intron

3. During the PCR-RFLP analysis, a DNA sample from an apparently unrelated female patient recruited after the SSCP analysis also revealed the presence of this mutation. The GT dinucleotide at the 5' end of an intron is highly conserved serving as part of the consensus GT (A/G) AGT recognition signal for mRNA splicing in vertebrate genes(64). Mutations involving the invariant GT dinucleotide at the 5' end of intron have been found to cause several genetic diseases in human including FH.

The 313+1 G>T mutation was previously reported in a Danish FH patient(65). This article reported that this mutation could result in exon 3 skipping and consequently, with a deletion of amino acids in ligand binding domain of the LDL receptor protein. This mutation is the first known pathogenic mutation of the LDL receptor gene observed in Thai patients and these patients should be considered as FH patients. From this preliminary analysis, it could be roughly estimated that the frequency of 313+1 G>T mutation in our sample in 4.3 % (3 in 70). 2 in 3 of these heterozygous FH patients were observed by SSCP analysis. Another FH heterozygous patient was observed by the screening with PCR-RFLP analysis.

This 313+1 G>T mutation was detected in 3 unrelated probands in which no other LDL receptor gene mutation were apparent in SSCP analysis throughout the gene. The common R3500Q mutation in apoB-100 gene was not observed at all in these patients. In addition, this 313+1 G>T mutation did not exist in 100 normal control subjects as assayed by PCR-RFLP technique. These analytical informations thus favor the hypothesis that this mutation have functional effect and should be causative for FH in our hypercholesterolemic patients.

5.2 Missense mutation

E153K mutation

From this analysis, the R3500Q mutation was not observed in this family. So, the hypercholesterolemia condition in this family may be due to any mutation in the LDL receptor gene. From DNA sequence analysis, the heterozygous G to A transition at nucleotide 494 was found in the boy and his father. The nucleotide change was predicted to cause the substitution of a Lysine for Glutamate at codon 153 in the LDL receptor gene. The amino acid at this position has been changed from an acidic amino acid to a basic amino acid.

The mutation E153K was observed at the hot spot exon 4 in the boy and his father. This non-conservative amino acid substitution occurs in the relatively conserved region of the LDL receptor molecule and thus may consequently disturb the structure and/or function of the receptor protein. The E153K mutation was not present in the boy's mother. It might be a pathogenic mutation causing the hypercholesterolemic condition in the boy and his father. Since the boy has severe hypercholesterolemia, we then speculate that the boy may be a compound heterozygous FH and has inherited another FH-causing mutation from his mother. Now we are examining the remaining exons of the LDL-receptor gene to identify the second mutation which should be observed in both the boy and his mother. About one in a million people have two mutatnt alleles of the LDL receptor gene (10). Atherosclerosis is often manifested with ischemic heart disease or vascular involvement occurring in the second decade in such homozygous patient (35). Death may occur before 20 years of age (67). Apart from monitoring lipid levels, it is not clear as to what therapeutic targets should be set in this younger age group. Careful long-term follow-up of this family is essential. Aggressive lipid lowering therapy in children with homozygous FH is needed to prevent early onset atherosclerosis and often includes lipid aphaeresis and/or liver transplantation(67).

5.3 M391T and S554L mutation

SSCP analysis of the exons 9 and 11 were performed in all DNA samples from 70 Thai primary hypercholesterolemic subjects. Abnormal SSCP patterns were apparent in both exons. The abnormal SSCP patterns were identified by direct DNA sequencing. From DNA sequencing, the abnormal SSCP pattern in exon 9 was due to a heterozygous transition of T to C at nucleotide sequence number 1235, where as in exon 11, it was due to a heterozygous transition of C to T at nucleotide 1661.

The transition in exon 9 would cause a nonconservative substitution of Threonine (uncharged polar side chain) for Methionine (nonpolar side chain) at codon 391 in the LDL receptor gene, namely M391T mutation. The transition in exon 11 would also cause a nonconservative substitution of Leucine (nonpolar aliphatic side chain) for Serine (uncharged polar side chain) at codon 554 in the LDL receptor gene, namely S554L mutation.

Secondary confirmation for M391T mutation was performed by allele specific amplification (ASA) technique, this methodology is suitable when the point mutation does not introduce a restriction site, where as the S554L mutation in exon 11 was performed by PCR-RFLP. These assays confirmed the nature of the mutation in the proband.

In exon 9, the M391T missense mutation belongs to the highly conserved EGF precursor homology domain of the LDL receptor protein. The multiple amino acid sequence alignment performed in this study suggests that the amino acid sequences encoded by exon 9 of LDL receptor gene are evolutionarily conserved. In particular the amino acid codon 391 is located in the completely conserved sequence 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)(68). Mutations in this domain are associated with impaired transport of LDL receptor from the rough endoplasmic reticulum to the coated pits on the liver cell surface. The recycling of internalized receptors can also be disturbed. These mutations result in a normal sized LDL receptor precursor, which is processed more slowly and degraded more rapidly. From the recent database, many disease-causing mutations occurring in exon 9 have been reported. However, the missense mutation M391T mutation presented in this study has never been reported elsewhere. Secondary confirmation for M391T mutation was performed by ASA (allele specific amplification) technique. This ASA analysis confirmed that this patient is heterozygous transition of T to C at nucleotide 1235 in exon 9 of LDL receptor gene. Three members of this proband's family were tested for M391T mutation using ASA analysis. The analysis indicated that only the proband had the M391T mutation. This result was reasonable because these family members did not have hypercholesterolemic condition. However, it is expected that the other family member(s) with hypercholesterolemic condition will be analyzed later.

In exon 11, the S554L missense mutation occurred in EGF precursor homology domain of LDL receptor gene. From multiple amino acid sequence alignment in six species, this mutation would also cause nonconservative amino acid substitution in conserved regions of the receptor protein. Such substitution should possibly disturb the LDL receptor's function as same as M391T. From a database (http://www.ucl.ac.uk/fh/muttab.html), about thirty missense mutations have been reported in exon 11. For instance, twenty-three percentage (23%) of Greek FH patients were reported to possess 1641 G>A mutation located in exon 11. For another example, the transition (GGT>GAT) at codon 528 in exon 11 was found in many Southern Italy's families (69). The confirmation of S554L mutation by PCR-RFLP analysis assured that this patient possessed a heterozygous transition of C to T at nucleotide 1661 in exon 11 of LDL receptor gene. The screening of S554L in family members of this index subject was not accomplished because of the residential movement of this family.

Both of these mutations (M391T and S554L) were neither observed in 100 control DNA samples nor in other primary hypercholesterolemic subjects, as screened by ASA and PCR-RFLP method, respectively. This result should probably suggest that these mutations are rare among patients and the absence of these mutations in normolipidemic subjects should suggest that they are potentially pathogenic and should be the possible cause of the hypercholesterolemic phenotype in these patients.

5.4 G1414A (R471) Polymorphism

SSCP patterns of the exon 10 were analyzed in the same set of DNA samples from Thai primary hypercholesterolemic subjects. Five patients apparently presented abnormal SSCP patterns in this exon. Two abnormal SSCP patterns were identified by direct DNA sequencing. From DNA sequencing, the abnormal SSCP patterns in exon 10 were due to transversion of G to A at nucleotide sequence number 1414. This nucleotide alteration did not change amino acid (ariginine to arginine) at codon 471 of the LDL receptor gene. G1414A (R471) polymorphism has been reported in NCBI database. Several studies have reported that many polymorphisms in the LDL receptor gene are associated with difference in plasma lipid levels(70). In this study, this polymorphism was observed only with the hypercholesterolemic condition (5 in 70 patients) and this polymorphism might be associated with the hypercholesterolemic

condition in our people. However, further study comparing allele frequency of this polymorphism in normolipidemic subjects and hypercholesterolemic patients should be necessarily undertaken for such a conclusion.

The DNA samples which showed mobility shift and base changes as reported above were also screened for mutation and polymorphism in the other exons of the LDL receptor gene and also for the apo B R3500Q mutation, with the exception of the promoter region. No mobility shift was observed in the other exons of LDL receptor gene in these DNA samples. The apoB R3500Q mutation was not present in these patients, either. These results should altogether suggest that these observed mutations were potentially pathogenic and might thus cause the hypercholesterolemic condition in our patients.

CHAPTER VI

CONCLUSION

1. One novel mutation, E153K is found in a Thai boy and his father.
2. One common polymorphism G1414A (R471) is observed only in hypercholesterolemic subjects (5 in 70 patients).
3. The PCR-RFLP analyses were performed to confirm the DNA sequence data of the 313+1 G>T mutation in intron 3 and S554L mutation in exon 11.The PCR-RFLP analyses were also used to screen for these mutations in normolipidemic subjects (n=100) so as to exclude the possibility of existence of these putative pathogenic mutations in normal control subjects.
4. Allele specific amplification (ASA) analysis was performed to confirm the DNA sequence data of the M391T mutation in exon 9. This ASA analysis was also used to screen for the presence of M391T in normolipidemic subjects (n=100) as to exclude the possibility of the existence of this mutation in normal control subjects.

Table 6.1 Conclusion of mutations and polymorphism in Thai primary hypercholesterolemic subjects in this analysis.

Exon	Change in nucleotide sequence	Chang in amino acid sequence	Mutation name
3	IVS3+1 G>T	----------	313+1(G>T)
4	GAA>AAA	Glu/Lys	E153K
9	ATG>ACG	Met/Thr	M391T
11	TCG/ACG	Ser/Leu	S554L
10	AGG/AGA (G1414A)	Polymorphism	R471

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