IDENTIFICATION OF SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs) OF CHOLESTERYL ESTER TRANSFER PROTEIN (CETP) GENE IN THAI POPULATION

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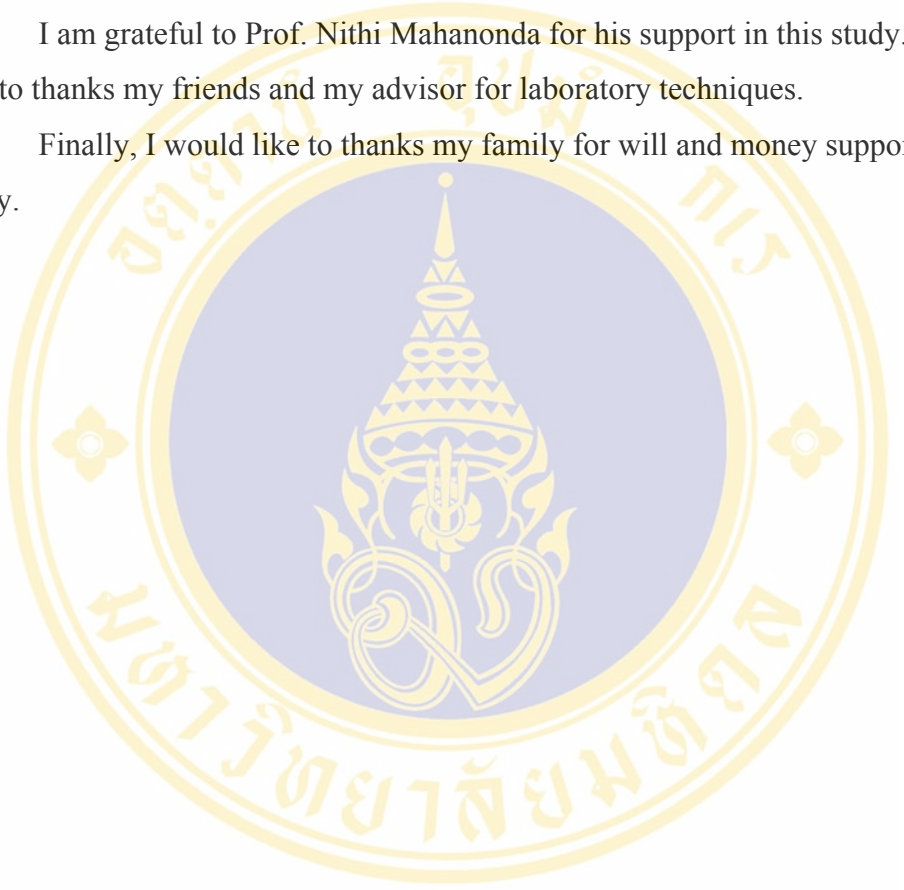

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ABSTRACT

Single nucleotide polymorphisms (SNPs) are DNA sequence variations that occur at least 1% in population but cause difference among individuals. SNPs generally do not directly cause disease but they may make a small contribution to disease. So, it has been expected that SNP maps of candidate genes will help identify genotypes involving in phenotypes of complex diseases. SNPs, however, are genetic variations that may be specific to each population. So, SNPs identification of disease candidate genes for each population will be necessary. This study presents SNPs of Cholesteryl Ester Transfer Protein (CETP) gene totally identified from 66 alleles of Thai subjects. CETP gene, located on chromosome 16q21, is one of candidate genes for Coronary Heart Disease (CHD). The product of CETP gene is a plasma glycoprotein that mediates the transfer of neutral lipids among blood lipoproteins. This capacity of CETP leads to the speculation that blood concentration of CETP may be associated with levels and sizes of blood lipoproteins. To understand the role of CETP gene in determining lipoprotein phenotypes in Thai population, all of common polymorphisms of this gene were identified by PCR-SSCP and direct DNA sequencing techniques. Thirteen SNPs were identified in this study. Eleven positions are known SNPs which publicized on online database: C-629A, G+279/in1A (TaqIB), C+8/in7T, T+24/in9G, G+29/in9A, I405V, R451Q, G-30/in15A, G+84A, G+184C and A+218G.. Two positions are novel SNPs that may be population specific. These SNPs are CETP_X1 that causing changing amino acid at highly conservative position in exon 3 and CETP_X2 that causing base substitution in intron 6. All of these SNPs will be validated for allele frequency and used for further studies: association analysis among affecting phenotypes, finding genetic markers for disease prediction and pharmacogenetic studies.

KEY WORDS: CHOLESTERYL ESTER TRANSFER PROTEIN (CETP)/ SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs)/ CORONARY HEART DISEASE (CHD)/ LIPOPROTEINS/ GENETICS

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(IDENTIFICATION OF SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs) OF CHOLESTERYL ESTER TRANSFER PROTEIN (CETP) GENE IN THAI POPULATION)

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บทคัดย่อ

Single Nucleotide Polymorphisms (SNPs) หรือ สนิป คือ ความหลากหลายทางพันธุกรรมระหว่างมนุษย์แต่ละคนที่เกิดจากการเปลี่ยนแปลงนิวคลีโอไทด์เพียงหนึ่งตำแหน่ง โดยความแตกต่างนี้ต้องมีความถี่ที่พบในประชากรมากกว่า 1% ซึ่งความแตกต่างนี้เองเป็นผลที่ทำให้มนุษย์แต่ละคนหรือคนแต่ละกลุ่มประชากรมีความแตกต่างกัน โดยปกติแล้ว SNP นั้นไม่ได้เป็นสาเหตุใหญ่ที่ทำให้เกิดโรคโดยตรง แต่ก็อาจส่งผลต่อการเกิดโรคได้โดยเฉพาะอย่างยิ่งถ้ามีความผิดปกติเกิดขึ้นในหลายๆตำแหน่ง ดังนั้นการจัดทำแผนที่สนิป (SNP maps) ของยีนที่คาดว่าเป็นสาเหตุของการเกิดโรค (candidate genes) จะสามารถนำมาใช้ในการพยากรณ์ความเสี่ยงของการเกิดโรคได้ นอกจากนี้ SNP ยังสามารถนำมาใช้ในงานทางด้านเภสัชพันธุศาสตร์ (pharmacogenomics) เพื่อใช้ค้นหายาตัวใหม่ และนำไปประยุกต์ใช้ในการศึกษาประสิทธิภาพและผลข้างเคียงของยาเฉพาะบุคคล (personnalized medicine) ได้ อย่างไรก็ตามความหลากหลายทางพันธุกรรมนี้จะแตกต่างกันไปในแต่ละกลุ่มประชากร ทั้งในด้านรูปแบบและความถี่ ไม่สามารถนำเอาข้อมูล SNP ของประชากรแต่ละกลุ่มมาแทนกันได้ ดังนั้นจึงมีความจำเป็นที่จะต้องจัดทำฐานข้อมูล SNP ในแต่ละกลุ่มประชากรขึ้นมา การวิจัยนี้เป็นการตรวจค้นหา SNP ของยีน Cholesteryl Ester Transfer Protein (CETP) ในประชากรไทย ยีน CETP นี้เป็นยีนที่อยู่บนโครโมโซมคู่ที่ 16 (16q21) ประกอบด้วย 16 exons, 15 introns และเป็นหนึ่งในยีนที่คาดว่าเกี่ยวข้องกับการเกิดโรคหลอดเลือดหัวใจอุดตัน

CETP คือโปรตีนที่ทำหน้าที่เป็นตัวขนส่งโคเลสเตอรอลเอสเทอร์และไตรกลีเซอไรด์ ระหว่างไลโปโปรตีนแต่ละชนิด ดังนั้น CETP จึงมีความสัมพันธ์กับปริมาณและขนาดของไลโปโปรตีน และเพื่อศึกษาความสัมพันธ์ของ CETP ต่อลักษณะที่แสดงออกของไลโปโปรตีนในประชากรไทย การวิจัยนี้จึงได้ทำการค้นหา SNP ของยีน CETP โดยทำการศึกษาจากกลุ่มประชากรไทยจำนวน 33 คน (66 alleles) โดยวิธี PCR-SSCP และ direct DNA sequencing ผลการศึกษาพบว่ามี SNP จำนวน11 ตำแหน่งที่เหมือนกับฐานข้อมูลสาธารณะ: C-629A, G+279/in1A (TaqIB), C+8/in7T, T+24/in9G, G+29/in9A, I405V, R451Q, G-30/in15A, G+84A, G+184C,A+218G และ 2 ตำแหน่งที่เป็น SNP ที่ยังไม่เคยค้นพบมาก่อน (CETP_X1: ทำให้เกิดการเปลี่ยนกรดอะมิโนที่ตำแหน่งอนุรักษ์ใน exon 3 และ CETP_X2: ใน intron 6) ซึ่ง SNP แต่ละตำแหน่งที่พบนี้ จะถูกนำมาวัดความถี่และศึกษาความสัมพันธ์ต่อการแสดงออกของลักษณะทางกายภาพในประชากรไทย ต่อไป

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CONTENTS

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CONTENTS (Cont.)

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CONTENTS (Cont.)

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LIST OF TABLES

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LIST OF FIGURES

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LIST OF FIGURES (Cont.)

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CHAPTER I
INTRODUCTION

Cholesteryl Ester Transfer Protein (CETP) is a 74 kDa plasma hydrophobic glycoprotein with 476 amino acids that enhances the transfer and exchange of cholesteryl ester (CE) and triglyceride (TG) between HDL and apoB-containing lipoproteins(1). This neutral lipid transfer is a central mechanism in Reverse Cholesterol Transport (RCT) pathway in the movement of excess cholesterol from the peripheral tissues back to the liver. CETP plays a critical role not only in the RCT pathway but also in the intravascular remodeling and recycling of HDL particles by combined action with lipoprotein lipase (LPL) or hepatic lipase (HL) to reduce HDL to small size species and ensure the reenter of the initial step of the RCT cycle(2, 3). By this mechanism, CETP acts as the antiatherogenic factor but it also acts as the proatherogenic fator by depleting plasma HDL-C levels and increasing CE content in all apoB-containing lipoproteins. In addition, CETP probably contributes to the formation of small dense LDL particles by it is biochemicaly involved in LDL metabolic pathway. It is well established that the presence of small, dense LDL particles is a recognized risk factor for Coronary Heart Disease (CHD). This is due to the evidential possiblility that small, dense LDL particles are more susceptible to oxidative modification and easily penetrate to the endothelial layer of the arterial wall. Furthermore, the apoB molecule on small, dense LDL undergoes a conformational change that leads to remain in the circulation longer and be more liable to oxidative modification and taken up into the vessel wall. Then, the atherosclerotic process can be developed(4).

In conclusion, CETP may be either proatherogenic or antiatherogenic depending on metabolic lipoprotein context and quantitative of CETP activity and concentration(5).

In some metabolic lipoprotein context that concentrations circulating of apoB-containing lipoprotein particles are elevated, as occurs in hyperlipidemic patients

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(hypercholesterolemia, mixed hyperlipidemia, and hypertriglyceridemia), both CETP activity and concentration are elevated significantly as compared with the normolipidemic stage(5). So, many studies try to reduce CETP activity and concentration in these patients.

In cholesterol-fed rabbits, the inhibition of CETP by injection of antisense oligodeoxynucleotides (ODNs)(3), a vaccine approach(6), and chemical inhibitor(7) to CETP have been developed. These inhibitors resulted in a reduction of CETP mRNA and mass in liver, a reduction in plasma total cholesterol, increasing in HDL-C concentration and reduction of aortic atherosclerotic lesion. In addition, CETP inhibitors may provide a useful therapeutic approach to raising HDL-C, lowering LDL-C and reducing the development of atherosclerosis in human(8).

Single nucleotide polymorphisms (SNPs) are DNA sequence variations that occur when single nucleotide in the genome sequence is altered. Data from Human Genome Project show that more than 99% of human DNA sequences are similar across the population (9). However, the only 1% variations in DNA sequence can have a major impact in how human differs among individuals such as the difference in abilities to absorb or clear certain drugs. 90% of these sequence variations are SNPs which occur in every 100 to 300 bases along the 3-billion bases in human genome. The variations considered as SNPs generally occur at least 1% of the population in both coding and non-coding region. Normally, SNPs do not directly cause disease or influence their response to drug but it may effect transcriptional or translational regulations, splicing, or RNA stability (9). Complex diseases, such as CHD, are believed to be influenced by complex interactions among multiple genes as well as environmental and lifestyle factors. It has been recently proposed that some SNPs on these genes may confer small disease phenotypes by themselves, or by interaction among them, or also by interaction with environmental factors or may be influenced by lifestyle. Each SNP in a single altered gene may make only a small contribution to the disease. Integration of these disease-related SNPs can eventually lead to the disease phenotype. So, it has been expected that SNP maps of candidate genes will help to identify the disease and development of suitable drug or personalized medicine

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(10). SNPs are genetic variation that may be specific to each population. So, SNPs identification especially for disease candidate genes, for our population will be urgently necessary.

Human CETP gene consists of 16 exons located on chromosome 16 (16q12-16q21) adjacent to LCAT gene(11). Many studies have demonstrated the effect of CETP polymorphisms and mutations on CETP activity and concentration, and HDL-C levels. They found that R451Q(12) and A373P(13) mutations are associated with high plasma CETP activity, low HDL-C. The other genetic alterations such as Int14A, D422G mutations(14-16) and I405V polymorphism(17) have been identified as cause of low or deficient CETP activity and elevated levels of HDL-C that cause hyperalphalipoproteinemia (HDL-C > 80 mg/dl) and increase risk of CHD(18).

CETP is involved in LDL catabolic pathway and thus should potentially effects the LDL particle size as described above. It can exert both proatherogenic and antiatherogenic. Therefore, CETP gene has been considered as one of candidate genes for CHD(2). The objective of this preliminary study was to identify SNPs in promoter, coding regions, intron-exon junctions, and 3' regions across the CETP locus in 66 alleles (n=33) of Thai normolipidemic subjects. Identification of the polymorphisms was performed by PCR-SSCP method following by automated DNA sequencing.

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OBJECTIVE

- To identify CETP SNPs in Thai Population.

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CHAPTER II
LITERATURE REVIEW

Coronary Artery Disease

Coronary Artery Disease (CAD) is the leading cause of cardiovascular mortality worldwide. It is project that CAD mortality rates will double from 1990-2020, with approximately 82% of the increase attributable to the developing world (19). Historically, abnormal levels of plasma lipids have helped define populations of patients at increased risk for development of coronary heart disease (CHD), however these values lose prognostic significance when viewed as predictors of disease at an individual patient level. LDL-C levels fail to differentiate populations of individuals with and without CHD. As seen in the Framingham Heart Study, 26 years follow-up data reveals that LDL-C levels were the same in 80% of patients who experienced a myocardial infraction (MI) versus those who did not experience an event (20). Therefore, not only quantitative but also qualitative of cholesterol abnormality (such as LDL particle size) should be considered as risk factor for CHD (20).

Lipoproteins

Cholesterol, cholesterol esters, triglycerides and phospholipids are essentially insoluble lipid. These lipid must, however, be moved from the tissue of origin to the tissues in which they will be stored or consumed. They are carried in blood plasma lipoproteins with specific carrier protein called apolipoprotein (21).

Lipoprotein particles are spherical which contain a central core of nonpolar lipids (primarily triglycerides and cholesteryl ester) and surface monolayer of polar lipids (primarily phospholipids) and apolipoproteins. At least nine different apolipoproteins are found in the lipoproteins of human plasma. These can be distinguished by their sizes, their reactions with specific antibodies, and their characteristic distribution in the lipoprotein classes. These protein components act as

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signal, targeting lipoproteins to specific tissues or activating enzymes that act on the lipoproteins (21). The lipoprotein structure was shown in Figure 2.1.

The density of lipoprotein particles is inversely related to their size, reflecting the relative contents of low-density, nonpolar core lipid, and high-density surface protein. Based on density and certain compositional and functional properties, the lipoproteins are usually separated into several classes as shown in Table1.1. In order of decreasing density, they are high density lipoprotein (HDL), low density lipoprotein (LDL), Intermediate density lipoprotein (IDL), very low density lipoprotein (VLDL) and chylomicron. The two largest classes contain mainly triglycerides in their cores. These are the chylomicrons, secreted from absorptive enterocytes, in which the apoB-48, and the VLDL, secreted by hepatocytes, which contain apoB-100. LDL and HDL contain mainly cholesteryl esters in their cores. The mature forms of these particles are not secreted directly from cell but rather are produced by metabolic process within the blood plasma. LDL is mainly produced as end products of the metabolism of VLDL. Components of HDL are secreted with chylomicrons and VLDL, as well as independently as HDL precursors. IDLs, which contain appreciable amounts of both triglycerides and cholesteryl esters in their core, are produced during the conversion of VLDL to LDL (22). The physical properties of each lipoprotein and lipoprotein metabolism are shown in Table2.1 and Figure 2.2.

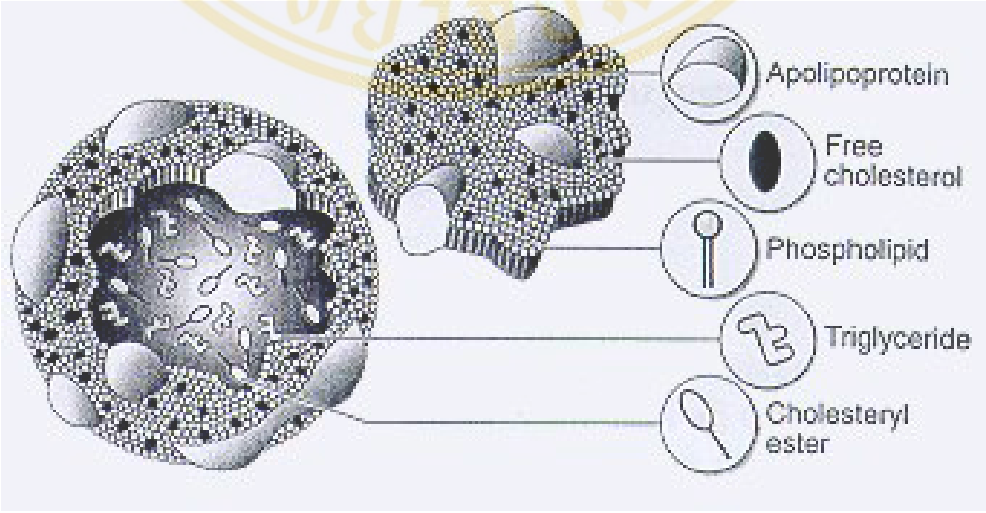

Figure 2.1 Lipoprotein structure (23).

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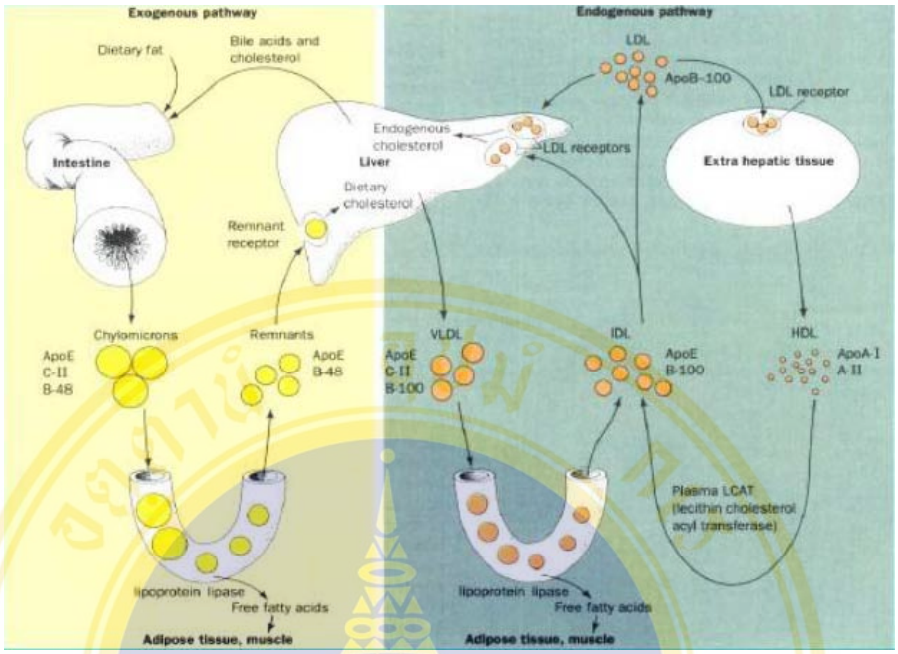

Figure 2.2 Lipoprotein pathway

Table 2.1 Physical properties of human plasma lipoprotein classes

Class	Density (g/ml)	Electrophoretic Mobility	Diameter (nm)	Molecular Weight
Chylomicrons	~ 0.93	Remains at origin	75-1200	50-1000 x 10^6
VLDL	0.93-1.006	Preβ-lipoproteins	30-80	10-80 x 10^6
IDL	1.006-1.019	Slow preβ-lipoproteins	25-35	5-10 x 10^6
LDL	1.019-1.063	β- lipoproteins	18-25	2,300,000
HDL_2	1.063-1.125	α- lipoproteins	9-12	360,000
HDL_3	1.125-1.210	α- lipoproteins	5-9	175,000
Preβ-HDL	~ 1.28	Preβ-lipoproteins	~ 5	67,000
LP(a)	1.040-1.090	Slow preβ-lipoproteins	25-30	~ 2,800,000

Reverse Cholesterol Transport (RCT) Pathway

Reverse cholesterol transport is the pathway in which cholesterol in peripheral tissues is transferred via plasma to the liver for either recycling or excretion from the body in bile. This pathway involves several identifiable steps as shown in Figure 2.3. First, small particles of discoidal shape, named preβ-HDL, are synthesized in liver and small intestine, or can be also resulting from hydrolysis of triglyceride-rich particles (22). These preβ-HDLs uptake cholesterol from peripheral cells, and their shape is changed to spherical particles, named HDL3 then HDL2, as they become enriched in esterified cholesterol (via an esterifying enzyme, lecithin:cholesterol acyltransferase (LCAT) associated with preβHDL particles) and phospholipids. This CE in form of

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HDL2 can then be delivered to the liver by two major routes. One is the uptake of HDL2 by the liver involves a selective receptor, named scavenger receptorB1 (SR-B1). The other is via CETP-mediated transfer to lipoprotein containing apo B and receptor-mediated uptake and catabolism in hepatocytes. While, triglycerides transfer reciprocally via CETP from apo B-containing lipoproteins to HDL, in which they are gradually hydrolyzed by hepatic lipase (HL) that reduces HDL size.

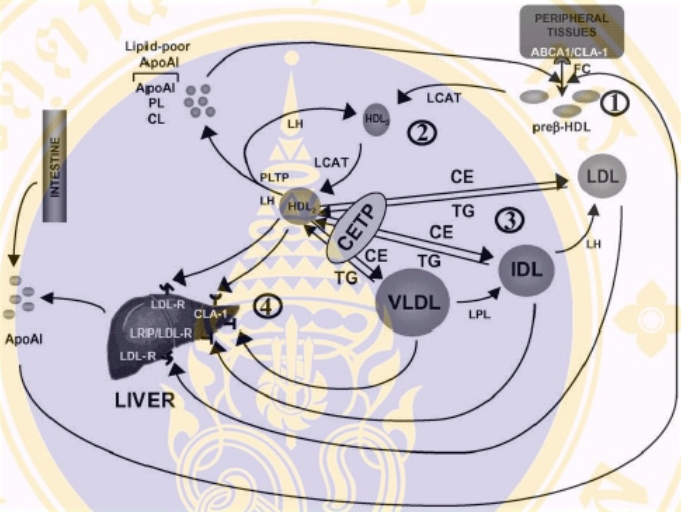

Figure 2.3. The key role of CETP in RCT (24) RCT can be divided into 4 major steps. 1) Cellular free cholesterol (FC) efflux, during which intracellular FC traverses the plasma membrane by passive diffusion or via the intermediary of a specific transporter, such as ABCA1 or a receptor such as SR-B1/CLA-1. FC is then taken up by a lipid-poor complex containing PL and apoAI to form preβ-HDL. 2) Preβ particles are transformed to HDL3 particles and subsequently to HDL2; such transformation involves esterification of FC by LCAT, thereby generating CE -enriched HDL2. 3) CETP redistributes CE from HDL particles to apoB-containing lipoproteins (VLDL, VLDL remnants, IDL, and LDL), enhancing their CE content but also favoring the formation of TG-enriched HDL2 particles. 4) The final step of RCT involves the return of CE to the liver, where HDL-CE may be taken up via a selective process involving SR-BI/CLA-1, while the intact particles can be taken up by HDL receptors and the LDL receptors (LDL-R). Finally, TG in CE-depleted HDL2 may be hydrolyzed by HL, thereby reducing HDL size and permitting the recycling of HDL particles and apoAI.

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Lipoprotein Heterogeneity

Lipoproteins represent a heterogeneous group of particles which span a spectrum of size and density, contain variable amounts of core cholesterol and triglycerides, and possess unique numbers and types of surface apolipoproteins which function to direct the processing and fate of individual lipoprotein particles. VLDL can be divided into large, light $VLDL_1$ (S_f 60-400) and smaller, denser $VLDL_2$ (S_f 20-60). When isolated by ultracentrifugation, HDLs separate into two major subfractions, which have been designated HDL_2 ($1.063 < d < 1.125$ g/ml) and HDL_3 ($1.125 < d < 1.21$ g/ml), LDL can be subdivided into large, light LDL I (d 1.025-1.034 g/ml) and LDL II (d 1.034-1.044 g/ml), and small dense LDL III (d 1.044-1.063 g/ml). At the University of California, Berkeley, works by Krauss and Blanche has results in a nomenclature that classifies individuals as LDL pattern A, B or I. LDL pattern A represents people with predominantly large buoyant particle with size $\geq$ 26.4 nm, LDL pattern B represents people with predominantly small dense LDL particle with size $\leq$ 25.7 nm , and LDL pattern I (intermediate) represents individuals with LDL particle midway between large and small, or individuals with multiple LDL peaks (from Lance K. 2000).

Normal populations require raised TG (in the form of triglyceride rich $VLDL_1$) and adequate activity of HL and CETP to form atherogenic levels of LDL III. Thus the combination of hypertriglyceridemia, increased small dense LDL and also low HDL are physiologically linked and known as the atherogenic lipoprotein phenotype (ALP) (25).

Atherogenic Property of Small Dense LDL

There is substantial evidence indicating that individuals with predominantly small dense LDL particles have an increased risk of developing CHD (26, 27). Small dense LDL particles are atherogenic probably because they are more susceptible to oxidative modification and can easily penetrate to the endothelial layer of the arterial wall (28). Furthermore, the apoB molecule on small, dense LDL undergoes a conformational change that leads to remain in the circulation longer and be more liable to oxidative modification and taken up into the vessel wall. Then, the atherosclerotic process can be developed (26) as shown in Figure 2.4.

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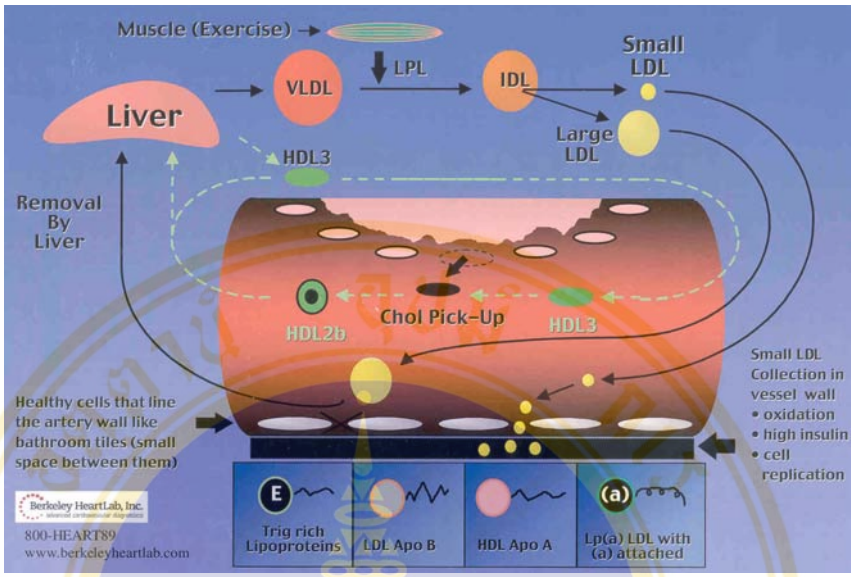

Figure 2.4 Atherogenicity of small dense LDL

Cholesteryl Ester Transfer Protein (CETP)

Human Cholesteryl Ester Transfer Protein, promoter of the hetero-exchange of neutral lipids, consists of 476 amino acids with a postulated hydrophobic peptide of 17 amino acid residues (29). The analysis of the CETP sequence indicates that the mature protein is composed of 45% of nonpolar residues, suggesting it to be highly hydrophobic in nature (30). However, the fact that CETP is readily soluble in water implies that such hydrophobic residues are mainly inaccessible to the aqueous phase. They form a hydrophobic pocket that permits the binding of neutral lipids (31). Study of structure-function relationships in CETP revealed that the C-terminal region plays a crucial role in neutral lipid binding (32-34). However, the binding of phospholipids seems to require a distinct site (35).

Structure of CETP

CETP is a member of a gene family of lipid-binding proteins that includes phospholipids-transfer protein (PLTP), lipopolysaccharide (LPS)-binding protein (LBP) and bactericidal/permeability-increasing protein (BPI) (36). LBP and BPI can both bind bacterial endotoxins and can modulate the host response to Gram negative bacterial infections. Whereas BPI is bactericidal and can prevent the neutrophil

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response to endotoxin (37). LBP promotes lipopolysaccharide-induced cell activation, probably through an interaction with cell surface CD14 (38). For humans, CETP amino acid homology is higher with LBP and BPI (26%) than with PLTP (20%) and is highest in the amino terminal half of the molecule (39). The crystal structure of human BPI has recently been solved (40). It is a boomerang-shaped molecule composed of two domains that have a very similar fold as shown in Figure 2.5. Each domain includes a hydrophobic pocket that opens onto the concave surface of the boomerang. In the BPI crystal structure, each hydrophobic pocket contained a phospholipid molecule that was bound through an interaction that involved primarily the acyl chains. Both immunochemistry and site-directed mutagenesis have been used to assess the structure–function relationships of CETP. A monoclonal antibody (mAb), TP2, that is specific for an epitope (that has been mapped to the carboxy-terminal 26 residues of CETP primary structure) can inhibit CETP-mediated cholesteryl ester, triglyceride, and phospholipid transfer (32, 41). This mAb does not prevent binding of CETP to the lipoprotein surface, but appears to neutralize cholesteryl ester and triglyceride transfer by preventing access to a neutral lipid-binding site on CETP (32). The mechanism responsible for TP2 inhibition of CETP-mediated phospholipid transfer has yet to be determined. A CETP variant that lacks residues 470–475 is unable to bind or to mediate the transfer of neutral lipids but does retain its ability to transfer phospholipid (34, 42) . As an increased negative surface charge of lipoproteins is associated with a higher binding affinity for CETP and an increased rate of CETP-mediated lipid efflux, electrostatic interactions are thought to be important in the association of CETP with the lipoprotein surface (Nishida et al., 1993). A model of CETP structure was present based on the binding characteristics of anti-CETP mAb. Four epitopes composed of CETP residues 215–219, 219–223, 223–227, and 444–450, respectively, are predicted to be situated on the external surface of the central β-sheet and a fifth epitope (residues 225–258) on an extended linker that connects the two domains of the molecule (43). In addition, human CETP contains four potential N-linked glycosylation sites in positions 88, 240, 341 and 396 (44). Glycosylation in position 88 appeared essential for normal lipid transfer activity (44). Whereas CETP without carbohydrate at asparagines 341 has about 40% higher activity than the fully

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glycosylated form, withdrawal of glycosylation in position 240 and 396 did not affect significantly CETP transfer activity (44).

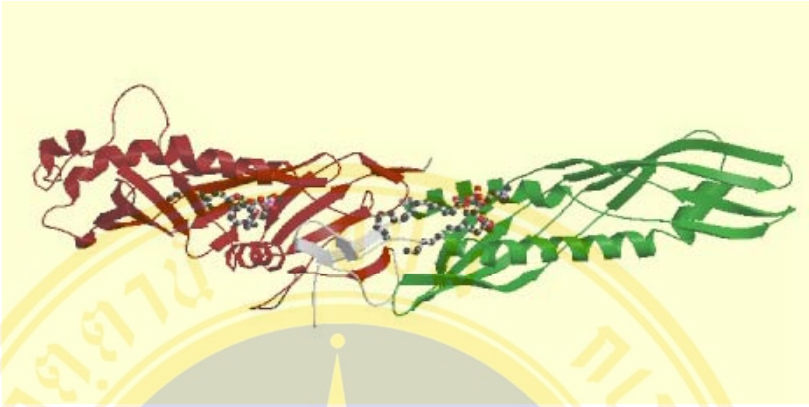

Figure 2.5 3D structure of LBP_BPI_CETP protein family

Source of CETP

In normolipidemic subjects, CETP concentration varies from 1 to 3 μg/ml plasma. It is noteworthy that plasma CETP levels have been shown to be about 25% higher in women than in men (45). However, plasma CETP levels are typically increased by 2 to 3 fold in subjects displaying hypercholesterolemia or mixed forms of hyperlipidemia involving elevated TG levels (46-48). In human, CETP is highly expressed in the spleen, liver and adipose tissue (30, 49). Although CETP is synthesized at low levels in a large number of tissues such as small intestine, adrenal glands, and macrophages, it is also detected in foam cells in human coronary and aortic atherosclerotic lesions (50).

Function of CETP

- **Lipid transfer activity**

CETP plays an important role in RCT pathway by mediating the transfer of CE and TG between HDL and apoB-containing lipoproteins (chylomicrons, VLDL, IDL, and LDL). In both rabbit (a species with naturally high CETP activity) and human plasma, CETP mediates all plasma CE and TG transfer activity but only about one third of phospholipids transfer activity. The remaining 70% of phospholipids transfer activity are mediated by another lipid transfer proteins, the phospholipids transfer protein (PLTP) (41). Actually all plasma lipoprotein particles may act as donor and

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acceptor in the CETP mediated lipid transfer process, the direction of net mass transfer depending mainly on both the proportion (Morton et al. 1983, Eisenberg et al. 1985) and the bidirectional transfer rate of each neutral lipid species in individual lipoprotein substrates (Barter et al. 1979). The observation both in vitro and in vivo results that CETP can promote the net mass transfer of CE from HDL and LDL towards VLDL with a reciprocal transfer of TG from VLDL towards LDL and HDL (51), no net mass transfers of either neutral lipids was demonstrated between HDL and LDL(52, 53).Therefore, pools of esterified cholesterol and triaclyglycerols are at equilibrium between LDL and HDL but not between VLDL and LDL or VLDL and HDL (54, 55).

- **Size redistribution of lipoprotein particles**

The capacity of CETP to redistribute neutral lipids between different lipoprotein particles can play a determinant role in the process of lipoprotein remodeling (56). In vitro studies revealed that CETP induces dramatic changes in the size distribution of both HDL and LDL (57-60). For HDL, CETP equally exerts major impact on the intravascular remodeling by allowing the enrichment of HDL particles in TG that will be further hydrolyzed by hepatic lipase, thus inducing reduction in the size of lipid core of the particle (61). Furthermore, CETP contributes to the formation of small, dense LDL particle, in subject with high TG levels by a preferential CE transfer from HDL to small, dense LDL, as well as through an indirect mechanism involving an enhanced CE transfer from HDL to large VLDL, the precursors of small, dense LDL particles in plasma (62). In addition, higher CETP levels are also associated with reduced HDL and LDL particle size and increased LDL particle number (46). All of these parameters are indicative of an increased risk for coronary artery disease.

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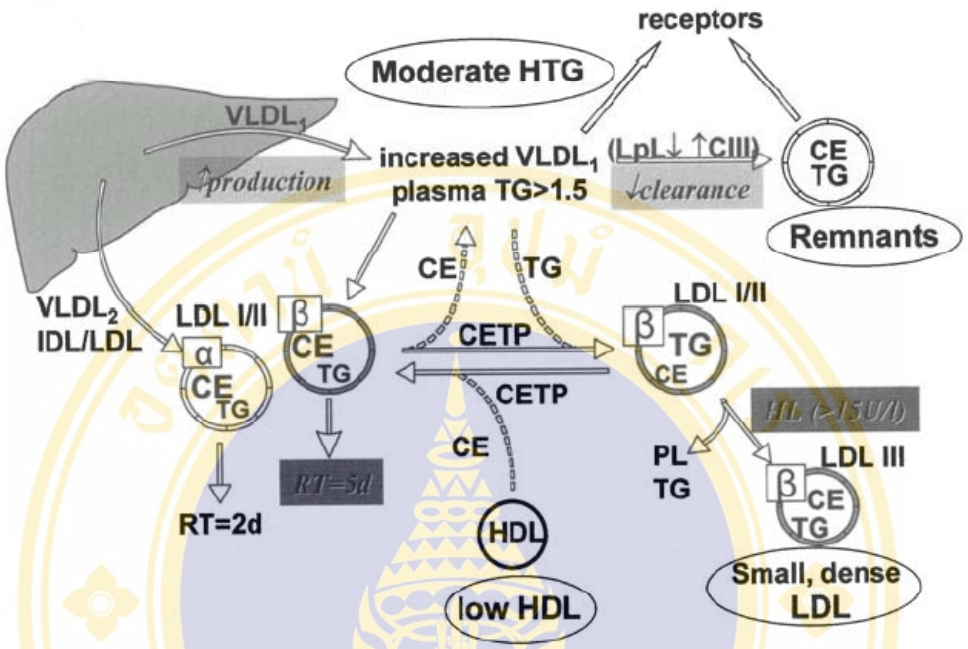

Figure 2.6. Metabolic model for the formation of small, dense LDL (63) In this postulated scheme, the key abnormality leading to the generation of small, dense LDL is the development of mild to moderate hypertriglyceridaemia (HTG), defined as a plasma triacylglycerol (TG) concentration of >1.5 mmol/l. Under these conditions, large triacylglycerol-rich VLDL (VLDL1) accumulates due to either overproduction in the liver or defective clearance from the circulation. Low lipoprotein lipase (LPL) activity or an excess of apoCIII (CIII; an inhibitor of LPL) can impede the ef.cient lipolysis of VLDL1. VLDL1, when lipolysed, gives rise to a population of LDL particles (denoted β) which have an altered apoB100 conformation. These particles fail to bind well to LDL receptors and so have a prolonged residence time (RT) in the circulation (d=days). Pool β LDL has therefore increased likelihood of undergoing remodelling. CETP removes cholesteryl ester (CE) and replaces it with triacylglycerol as the protein shuttles between VLDL, LDL and HDL particles. Triacylglycerol-enriched LDL is a good substrate for HL, an enzyme that removes triacylglycerol from smaller lipoprotein particles. Small, dense LDL is generated in this final lipolytic step.

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Role of CETP in the Development of Atherosclerosis

Expression of CETP in transgenic mice, a species that is naturally deficient in this protein, has yielded inconsistent results, with reports of both an increased susceptibility (64, 65) and protection (66) against atherosclerosis in different models. Whereas the effects in rabbits, an animal species that display high CETP activity and is highly susceptible to atherosclerosis, have been remarkably consistent and strongly support the therapeutic potential of CETP inhibition for blunting or even halting the development and progression of atherosclerosis (3, 6, 67, 68). In human, CETP inhibitors may provide a powerful therapeutic approach to raising HDL levels, lowering LDL levels, and reducing the development of atherosclerosis in human (8).

Inhibition of CETP

There is a strong inverse relationship between the plasma concentration of HDL-C and the development of CHD. Several studies demonstrated that the plasma concentration of HDL-C is a more powerful predictor of CHD than either total plasma cholesterol or the plasma concentration of LDL-C, indicating that the risk of CHD is increased by 2% to 3% for every 1% decrease in HDL-C (69). CETP is one modulator of HDL-C levels by its function. So, several approaches attempt to develop CETP inhibitors, including use of monoclonal antibodies (67, 70), antisense oligodeoxynucleotide; ODN (3, 71), vaccination (6), and a specific inhibitor against CETP (68, 72, 73).

The injection of a neutralizing monoclonal antibody, TP-2, to hamsters produced a dramatic reduction in CETP activity with a concomitant elevation of HDL-C (74). The intravenous injection of antisense ODN against rabbit CETP mRNA coupled to asialoglycoprotein carrier molecules significantly reduces both CETP mRNA and mass in the liver of cholesterol-fed rabbits (71) then reduction in plasma total cholesterol, increase of plasma HDL-C, increase of LDL receptor mRNA levels in liver, while no change in TG concentration was observed (3, 71). The immunization of cholesterol-fed New Zealand White rabbits with a peptide containing a region of CETP essential for neutral lipid transfer activity led to the production of antibodies against CETP and induced inhibition (~35%) of CETP activity. This vaccination was associated with significant increase (+42%) of HDL-C concentration, moderate

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decrease (-24%) in LDL-C and reduce in the surface area of aortic atherosclerotic lesion (~40%) as compared with nonvaccinated rabbits (6). A phase I human trial reports that the immunotherapeutic vaccine directed against CETP (CETi-1) is well tolerated and it does not generate laboratory abnormalities in healthy adults (75). CETi-1 is now under evaluation in a placebo-controlled phase II study in subjects with low levels of HDL-C. Furthermore, many chemical inhibitors of CETP such as JTT-705 have been tested in human. Treatment with the highest dose (900 mg/day) was associated with a highly significant 37% decrease in CETP activity, a 34% increase in HDL, and a 7% decrease in LDL (7). Nevertheless, this study did not evaluate the possibility as to whether such inhibition of CETP could either reduce or prevent cardiovascular disease. In addition, other CETP inhibitors are currently under development, such as Torcetrapib (Pfizer) and BAY-19-4789 (Bayer Pharma). However, no data on their actions are yet available (5).

In conclusion, several CETP inhibitors are currently in development and their action on lipid metabolism and on the treatment of atherosclerosis in human is now under investigation. Although there is not as yet a clear indication that CETP inhibitors can reduce or prevent atherosclerosis in humans, it is nonetheless evident that CETP inhibition is a powerful therapeutic tool for induction of elevation in plasma levels of antiatherogenic HDL.

CETP Gene

The human CETP gene spans approximately 25 Kbp consisting of 16 exons and 15 introns, located on chromosome 16 in 16q12-16q21 region. The size of the exon ranges from 32 to 250 bp (11). CETP mRNA encodes a preprotein of 493 amino acids, which contains a pre-peptide of 17 amino acids. Thus, the mature plasma CETP protein contains 476 amino acids (30).

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Single Nucleotide Polymorphisms

Single nucleotide polymorphisms or SNPs are DNA sequence variations that occur when a single nucleotide (A, T, G or C) in the genome sequence is altered. For a variation to be considered as a SNP, it must occur in at least 1% of the population. Data from Human Genome Project show that 99.9% of human DNA sequences are similar across the population. The only 0.1% variations in DNA sequence, however, can have a major impact on how human differs among individuals such as skin colors, difference in response to disease and ability to absorb or clear certain drugs. 90% of all human variations are SNPs which occur in every 100 to 300 bases along the 3-billion bases human genome. Two every three SNPs involve the replacement of cytosine (C) with thymine (T). SNPs can occur in both coding (gene) and non-coding regions of the genome. Many SNPs have no effect directly on cell functions, but scientist believes other could predispose people to diseases or influence their response to drugs. In deed, it may effect transcriptional or translational regulations, splicing, or RNA stability (9).

SNPs and Risk Factors in Disease Development

Complex diseases, such as coronary heart disease, are not caused by a genetic variation within a single gene but are influenced by complex interaction among multiple genes as well as environmental and lifestyle factors. Although both environment and lifestyle factors add tremendously to the uncertainty of developing a disease, it is currently difficult to measure and evaluate their overall effect on a disease process. Genetic factors may be more suitable to confer susceptibility or resistance to a disease and determine the severity or progression of disease. SNPs are genetic factors that most of them do not cause disease but it can help determine the likelihood that some one will develop a particular disease and can be used to search for and isolate the disease-causing gene. Moreover, SNPs are of great value to biomedical research and developing pharmaceutical products. Because SNPs do not change much from generation to generation, make it easier to follow them during population studies (9).

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CETP Polymorphisms and Susceptibility to Atherosclerosis in Humans

Several SNPs have been reported in human CETP gene. Many studies have demonstrated association between CETP SNPs and there contribution in plasma CETP and HDL levels. However, the relation between these polymorphisms and susceptibility to atherosclerosis is variable.

In the promoter region, a possible functional polymorphism has recently been reported, CETP/C-629A, in subjects from the ECTIM study (Etude Cas-Ttmoins de 1'Infarctus du Myocarde) that was set up to investigate the large differences in CHD incidence and mortality observed within European populations. The -629A allele was associated with lower CETP mass and higher HDL-C levels than the -629C allele (76). It was demonstrated that binding of the nuclear factor Sp1 and/or Sp3 to the -629A allele suppressed CETP promoter activity, whereas the binding of these nuclear factors to the -629C allele has no effect on promoter expression (76).

TaqIB (G+279/in1A), the extensive intronic polymorphism of CETP gene, was shown to be a silent base change affecting the 279th nucleotide in the first intron. Subjects with the B2 allele (absence of the *Taq*I restriction site) were more likely to have high HDL-C levels and low levels of CETP activity and mass (77, 78). This association has been implicated to be population specific (79, 80), and highly influenced by environmental factors such as alcohol consumption (81) and tobacco smoking (82). However, this polymorphism is unlikely to be functional by itself. In deed, the TaqIB polymorphism was found to be in nearly complete association with the CETP/C-629A polymorphism (83).

In an investigation of the common Isoleucine 405 to valine polymorphism (I405V) in exon 14 it was shown that the VV genotype was associated with lower CETP concentration than IV or II genotype. The levels of HDL-C were higher in hypertriglyceridemic men with the VV genotype than IV or II genotype. The prevalence of CHD was not significantly different among these three genotypes (84).

While other polymorphisms were shown to decrease plasma CETP activity, A373P and R451Q polymorphisms were shown to increase CETP activity. The study of Agerholm-Larsen B. and colleague show that all carriers of the 451Q allele also carried the 373P allele(13). Carriers of the 451Q/373P alleles had reduced levels of

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HDL-C and a paradoxically lower CHD risk when compared with the overall study population (12, 13).

SNPs and Drug Development

Today, pharmaceutical companies are limited to develop drugs that can response in every patient. In this way, companies attempt to find the drug targets that those individuals most likely to benefit, i.e., or personalized medicine. Individual has different response because SNPs may be associated with the absorbance and clearance of therapeutic agent. A high-density SNP map of the human genome will enable researchers to identify SNP variations that differ in patients who have a certain response when given a medicine. These SNPs could be used as part of a medicine response test to identify patients likely to benefit or experience a specific side effect. The B1B1 in TaqIB and CC allele in C-629A carriers of the CETP polymorphisms have a more atherogenic lipid profile, including low HDL, but they respond better to statin therapy. These results favor the hypothesis that CETP polymorphisms modify the effect of statin treatment and may help to identify patients who will benefit most from statin therapy (85, 86).

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CHAPTER III
MATERIALS AND METHODS

MATERIALS

1. Subjects

There were 33 unrelated subjects with Thai ethnic background used in this study. These subjects were classified into two groups, i.e., healthy subjects (n=27) and Coronary Artery Disease (CAD) (n=6) subjects from Department of Clinical Pathology, Navy hospital.

- Plasma lipid levels for healthy Thai subjects (control) under this study:

Total Cholesterol	<	200	mg/dl
LDL-Cholesterol	<	130	mg/dl
Triglycerides	<	200	mg/dl

- Plasma lipid levels for CAD patients under this study:
  (Before treatment with lipid lowering drugs)

Total Cholesterol	<	200	mg/dl
LDL-Cholesterol	<	130	mg/dl
Triglycerides	<	200	mg/dl

Subjects with some chronic disease, i.e., Diabetes Militus (DM), hypertension, hyperthyroidism and hypothyroidism were excluded from this study

2. Oligonucleotide Primers

Fifteen primer pairs were used for amplification of the entire CETP gene, partial part of promoter region and sixteen exon regions. These oligonucleotide primers were designed by Genefisher program. All of these primers were synthesized and purified by Operon, Germany. The sense and antisense oligonucleotide primers of each fragment are shown in Table 3.1

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Table 3.1 The oligonucleotide primers for amplification of promoter and exons 1-16 of the CETP gene.

Region	Primer	Sequence
Promoter	Sense (F2P_CETP)	5'-GCACTTGGCCATCTGGTCACA GTTGCTGCA-3'
	Antisense (RP_CETP)	5'-TATATGTATGTCCGCCCAGCCC CCACGGAA-3'
Exon1	Sense (FEx1_CETP)	5'-GCAAAAATGGTGCAGATGGTG GAGGGGAGA-3'
	Antisense (REx1_CETP)	5'-GGAGGCTCCTGGCATAGTGGGTGTCCA-3'
Exon2	Sense (FEx2_CETP)	5'-TGGGAGCCTCATCTCAGAGAG GCTGAGTCA-3'
	Antisense (REx2_CETP)	5'-CTTTCCTCCCTGTGGGCTGGGGGCAA-3'
Exon3&4	Sense (FEx34_CETP)	5'-CACCCTCGCCTAGACAAAATT GGAGGCTCA-3'
	Antisense (REx34_CETP)	5'-CCTGGAGTCCAGCTCCCACTT CCCCAGA-3'
Exon5	Sense (FEx5_CETP)	5'-CCTGGGCAGCATGTGGATACCA-3'
	Antisense (REx5_CETP)	5'-GGCCGTGGACACACTAACAGGA-3'
Exon6&7	Sense (FEx67_CETP)	5'-CACTAGGCGCTCCATGGATGCA-3'
	Antisense (REx67_CETP)	5'-CCCAAGGCCACATAGTGCAGCA-3'
Exon8	Sense (FEx8_CETP)	5'-GTGTGGATGCAGGGGACGGTGA-3'
	Antisense (REx8_CETP)	5'-GCTGGGGGATGGGGTTGTCAGA-3'
Exon9	Sense (FEx9_CETP)	5'-GGGCTCCTCCCAATCTCCCTGA-3'
	Antisense (REx9_CETP)	5'-CATGCGTGTGCACACACACACA-3'
Exon10	Sense (FEx10_CETP)	5'-AACTGCCCTTGGTCCCTGCGAA-3'
	Antisense (REx10_CETP)	5'-TGTGGGGGCTCTTGAAGCCAGA-3'
Exon11	Sense (FEx11_CETP)	5'-TTCCCATCTCCGAGGGCATGGA-3'
	Antisense (REx11_CETP)	5'-CCAGCAGAGGTGGTGAGAAGGA-3'
Exon12	Sense (FEx12_CETP)	5'-AATCAGGGGCCCTGAGCTAGGA-3'
	Antisense (REx12_CETP)	5'-GCCCCAGAAGAAAGGGGCCACA-3'
Exon13	Sense (FEx13_CETP)	5'-GAGACAAAAGCACTGGCTGCTA-3'
	Antisense (REx13_CETP)	5'-TTCTTTTGGTCTGGTTGCCTGA-3'
Exon14	Sense (FEx14_CETP)	5'-CATGAGGATGAATGCTTGTCCA-3'
	Antisense (REx14_CETP)	5'-GGTGAAATGGGAAGCTCTGTCA-3'
Exon15	Sense (FEx15_CETP)	5'-CTCCCACTACCCAGGGTGCAGA-3'
	Antisense (REx15_CETP)	5'-GCCCCTCTGTCTGTCTCCCCAA-3'
Exon16	Sense (FEx16_CETP)	5'-ACAGACAGAGGGGCCTCTACCA-3'
	Antisense (REx16_CETP)	5'-GGAAGGGCTGAAAAGAGGTGGA-3'
TaqIB	Sense (FTaqIB)	5'-GATGGGCTGAGTGGAGCTGTCA-3'
	Antisense (RTaqIB)	5'-GAGCAAGAGACTGAGGCCCAGA-3'

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3. Enzymes

All of the commercial enzymes used in this study were molecular biology grade. Taq DNA polymerase used for PCR-RFLP and PCR-SSCP was expressed from a recombinant plasmid according to the protocol of Pluthero et al.(87) as described in method 9. Commercial Taq DNA polymerase from ITS (Spain) was used to confirm the SSCP results and to perform DNA sequencing.

Table 3.2 List of enzymes and Restriction enzymes

Enzymes	Sources
Lysozyme	BioBasic Inc., Canada
Proteinase K	Invitrogen
Taq DNA polymerase	ITS (Spain)
NcoI	BioLabs (USA)
EcoRI	BioLabs (USA)
HpaII	BioLabs (USA)
HaeIII	BioLabs (USA)
BamHI	BioLabs (USA)
SphI	Fermentas
SmlI	Fermentas
StyI	Fermentas
ApoI	BioLabs (USA)
PstI	BioLabs (USA)

4. Chemicals

Table 3.3 List of 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
Agar-C	-	BioBasic Inc., Canada
Agarose	-	BMA, USA
7.5 M Ammonume acetate	-	Sigma, USA
Ammonium persulfate ($(NH_4)_2S_2O_8$)	288.19	USB, USA
Ampicillin ($C_{16}H_{18}N_3ONaO_4S$)	371.39	BioBasic Inc., Canada
Boric acid (H_3BO_3)	61.83	Merck, Germany
Bromophenol Blue ($C_{19}H_9Br_4O_5SNa$)		USB, USA
Bromophenol Blue-Xylene Cyanole Dye solution	-	Sigma, USA
Deionized Formamide	45.04	BioBasic Inc., Canada

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Table 3.3 List of chemical substances (Continued)

Chemical substances	Molecular weight	Source
2'-Deoxynucleotide 5'triphosphate (dNTP)	-	Pharmacia Biotech, USA
2',3'-Dideoxynucleotide 5'triphosphate (ddNTP)	-	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
Ethidium bromide ($C_{21}H_{20}N_3Br$)	394.30	Sigma, USA
Ethylenediamine tetraacetic acid (EDTA)	78.13	Merck, Germany
37% Formaldehyde (CH_2O)	30.00	Merck, Germany
Glycerrol ($HOCH_2CH(OH)CH_2OH$)	-	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	BioBasic Inc., Canada
Ladder Marker	-	Fermentas, 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)	116.21	BioBasic Inc., Canada
Polyethylene-20 Sorbitan minolaurate (Tween20)	-	Research Organics, USA
Potassium Chloride (KCl)	74.55	Sigma, USA
Phenylmethyl Sulfonyl Fluoride (PMSF, $C_6H_5CH_2SO_2F$)	174.19	BioBasic Inc., Canada
QIA quick Gel Extraction 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
Sodium hydroxide (NaOH)	40.00	Merck, Germany
Tris (Hydromymethy-amminomethane, $C_4H_{11}NO_3$)	121.1	Research Organics, USA
Xylene cyanol FF	554.6	Research Organics, USA

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5. Instruments

Table 3.4 List of instruments

Instruments	Source
Analytical balance	Scaltec
Autoclave	Hirayana, Japan
DNA Thermal cycler (Gene Amp PCR system 2400)	Perkin Elmer, USA
Hettich zentrifugen	Germany
Horizontal agarose gel electrophoresis set	Mupid II, Japan
Hoefer SE260	Pharmacia Biotech, USA
Hotplate stirrer SM22	Stuart Scientific, UK
Hot air oven	Memmert, Germany
Incubator	Heraeus, Germany
Microcentrifuge	Heraeus Sepatech, USA
Mini-PROTEIN®II Cell	BIO-RAD, USA
pH meter	Orion Research, USA
Pipetteman	Gilson, France
Power supply	Pharmacia Biotech, USA
Refrigerator	Sharp, Japan
UV-Transilluminator	Herolab, Germany
Vortex	IKA Work Inc., USA
Manual DNA sequencer	C.B.S. Scientific.co

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THE EXPERIMENTAL DESIGN

The aim of this study is to identify CETP SNPs in Thai subjects by PCR-SSCP following by DNA sequencing method. The different mobility shift patterns from SSCP analysis were characterized by DNA sequencing for both sense and antisense strands. The methods used in this study are described as followings.

- DNA extraction : all of DNA samples were extracted from peripheral blood cells by the Guanidine-HCl method (UCLA 1993)
- Polymerase Chain Reaction (PCR) : PCR is performed according to the method as described by Saiki et al.
- Restriction endonuclease digestion for SSCP analysis : Restriction endonuclease are enzymes that cleave DNA at specific sequence, yielding fragments of DNA molecules. This technique was required for generating the suitable fragment sizes for optimal sensitivity of SSCP analysis.
- Polymerase Chain Reaction-Single Strand Conformation Polymorphism (PCR-SSCP): The DNA fragment under this analysis is amplified by PCR. The PCR product is denatured to single-stranded DNA. In a non-denaturing condition, a DNA molecule containing changing base will have a different structure from wild type, resulting in a different mobility shift during electrophoresis. SSCP analysis was basically carried out according to the method described by Orita et al.(88)
- Silver staining: The silver staining of nucleic acids in polyacrylamide gels was originally described by Merril et al. (89)
- DNA sequencing: DNA sequencing was performed using the dideoxy chain termination method as describe by Sanger et al.(90)

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METHODS

1. Determination of lipid profiles

Lipid profiles were analyzed either by Department of Clinical Pathology, Faculty of Medicine, Siriraj Hospital, Mahidol University, or by the Department of Clinical Pathology, Navy Hospital, Bangkok. Venous blood (5-10 ml) was taken after 12-14 hours of fasting. Plasma total cholesterol and triglyceride levels were determined with automation by Hitachi 917 Autoanalyzer. The concentration of plasma HDL-C was measured after precipitation of LDL and VLDL fractions with dextran sulfate and $MgCl_2$ and plasma LDL-C level was calculated using the formula described by Friedwald et al.(91).

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 weighed and dissolved with approximately 800 ml distilled water. The pH of the solution was adjusted to 7.2 with HCl 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

1 mg of Proteinase K was weighed and dissolved with 1 ml of autoclaved ddH_2O and mixed. 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 until it was homogeneous and the volume was adjusted to 100 ml. The solution was incubated at 56°C for 1-2 hours until dissolved. This solution was used as such with no need to be autoclaved.

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d. 1 M Tris-HCl, pH 7.6

60.6 g of Tris-HCl was weighed and dissolved with 350 ml of ddH_2O. The pH of the solution was adjusted to 7.6 with approximately 13 ml of 6 M HCl. The volume of the solution was adjusted to 500 ml and sterilized by autoclaving.

e. 7.5 M Guanidine-HCl, pH 7.6

72.0 g of Guanidine-HCl was weighed and 1 M Tris-HCl was added. The volume of the solution was adjusted to 100 ml with autoclaved ddH_2O. This solution was filtered by 0.2 μm Nalgene filter.

f. 0.5 M EDTA, pH 8.0

93.0 g of EDTA disodium salt was weighed and dissolved in 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 1 M NaOH. The volume of the solution was adjusted to 500 ml. This 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, pH 7.6

10 ml of 1 M Tris-HCl pH 7.6 and 2 ml of 0.5 M EDTA pH 8.0 were diluted in ddH_2O and the volume was adjusted to 1 liter. The solution was sterilized by autoclaving.

2.2. Procedure

Ten ml of whole blood was collected into 50 ml capped tube which contained 400 μl of 5% EDTA (blood anti-coagulant). Two volumes of working solution A (20 ml) was added, mixed by vortex to prevent clumping and left stand for 10 minutes at room temperature. Then, the mixture was centrifuged at 3000 rpm for 10 minutes. After centrifugation, the supernatant was discarded and 2 volumes of working solution A was added again and the previous step was repeated until the supernatant was clear (not more than three times). For these step, the RBC were lysed while the WBC were packed in bottom of tube. For next step, the WBC were lysed by the following steps: First, 400 μl of autoclaved ddH_2O and 80 μl of 10 mg/ml of Proteinase K was added to the pellet and mixed with 1000 μl pipette-tip in order to thoroughly mix the content.

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Second, another 400 ml of autoclaved ddH_2O was added and resuspended further. Third, 300 µl of 10% SDS was added and the sample was mixed gently by rocking the tube back and forth, air-bubble was avoided. After mixing, the solution was incubated at 37°C for overnight. Next step, all of proteins were precipitated by Guanidine-HCl. 300 µl of 7.5 M Guanidine-HCl was added and gently mixed. The whole solution was mixed vigorously and incubated at 68-70°C for 3 hours. The mixture was centrifuged at 3000 rpm for 10 minutes at 4°C. Only the above clear solution was reincubated at 68-70°C for additional 30 minutes or until the pellet looks like dark green jelly. The mixture was recentrifuged as above. The supernatant was transferred to a new 50 ml capped tube with 5 ml of cold absolute ethanol. The tube was gently rocked back and forth until cotton-like strand of DNA appeared and then stored at -20 for 1-2 hours. The cotton-like strand of DNA was transferd to a new 1.5 eppendroff tube with 800 µl of cold absolute ethanol by 1000 µl micropipette. The mixture was centrifuged at 12000 rpm for 15 minutes then the supernatant was discarded. 700 µl of 70% ethanol was added to the sample, invert to loosen the pellet and left the sample strand for 1 minute. The mixture was centrifuged at 12000 rpm for 15 minutes and the supernatant was discarded. The DNA sample was dried with the cap open at 37°C or room temperature. 100-250 µl of TE 10-1 buffer was added to the sample and incubated at 37°C for overnight. The sample was stored at -20°C until use. The genomic DNA quality was verified by agarose gel electrophoresis.

3. Polymerase Chain Reaction (PCR)

3.1. Principle

Polymerase Chain Reaction (PCR) is a technique for amplification of DNA via reaction carried out entirely in vitro. The reaction starts with separation of DNA sample into single strands. The desired target DNA is selected using oligonucleotide primers which will bind specifically to this sequence. Once the sequence-specific primers are bound to the target, a heat stable DNA polymerase can generate additional copies of the target sequence. The new DNA copies of the target in turn serve as templates to make more copies still in a chain reaction.(92)

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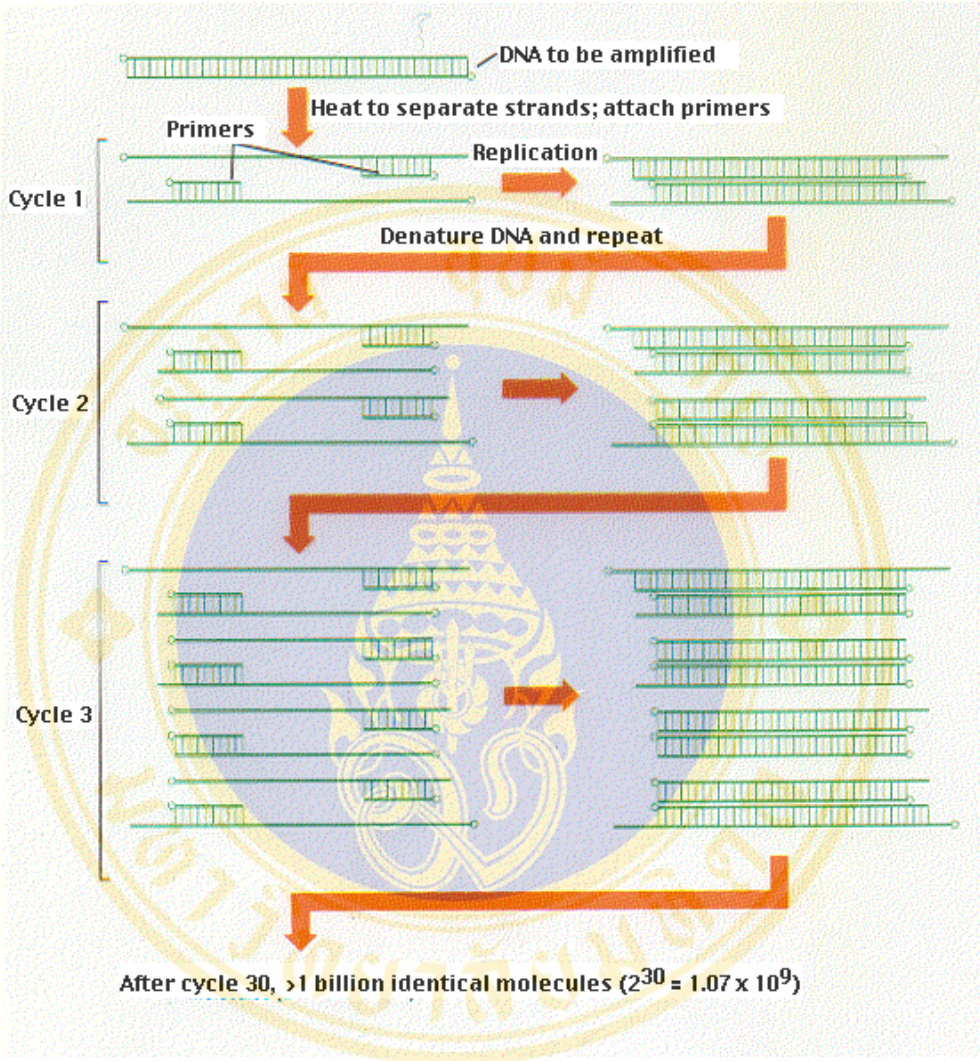

Figure 3.1 The schematic diagram of polymerase chain reaction (PCR)

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3.2. Reagents preparation

a. 10X PCR buffer (15 mM $MgCl_2$)

200 µl of 1 M Tris-HCl pH 8.4, 15 µl of 1 M $MgCl_2$, 500µl of 1M KCl and 10µl of Tween 20 were mixed and the volume was adjusted to 1000 µl with sterilized ddH_2O.

b. 10 mM dNTPs

10 µl of each 100 mM dNTPs (dATP, dTTP, dGTP, dCTP) were mixed in 60 µ l of sterilized ddH_2O.

c. 1 mM dNTPs

10 µl of 10 mM dNTPs was diluted to 100 µl with sterilized ddH_2O.

d. Taq DNA polymerase

As described in method 10 (P. 44)

e. DNA template (1:50)

2 µl of genomic DNA extracted by Guanidine-HCl method was diluted to 100 µl with autoclaved ddH_2O.

f. Pooled DNA template

10 µl of each 1:50 diluted DNA template were mixed together.

g. 10 pmol Oligonucleotide primers

An oligonucleotide primer was diluted to 10 pmol with sterilized ddH_2O.

3.3. Procedure

The target sequence was amplified in 1X PCR reaction buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 8.4), 0.2 pmol of each primer, 0.1 mM of each dNTPs (dATP, dTTP, dGTP, dCTP) and 0.5 unit Taq DNA polymerase in final volume of 25 µl. The reaction mixture of each exon is shown in table 3.5. The reactions were placed into a Perkin Elmer 2400 Thermal cycle. The cycling condition was as follow: The denaturation step at 94°C for 5 minutes in the initial cycle. The amplification profile for each reaction was as presented in Table 3.6 and extension at 72 °C were prolonged for the final cycle. These amplification reactions were run for 35 cycles.

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Table 3.5 The PCR reaction mixtures of promoter and exon 1-16 of CETP gene

Region	2.5 µl of 10 X PCR Buffer with $MgCl_2$ (mM)	1 mM dNTPs (µl)	10 pmol of each primer (µl)	Template (µl)	Taq DNA polymerase (unit)	Final volume (µl)
Promoter	2.5	2.5	0.5	3.0	0.5	25
Exon1	2.5	2.5	0.5	3.0	0.5	25
Exon2	2.5	2.0	0.5	3.0	0.5	25
Exon3&4	2.5	2.5	0.5	3.0	0.5	25
Exon5	2.5	1.5	0.5	3.0	0.5	25
Exon6&7	2.5	2.5	0.5	3.0	0.5	25
Exon8	2.5	1.5	0.5	3.0	0.5	25
Exon9	1.5	2.0	0.5	3.0	0.5	25
Exon10	2.5	1.5	0.5	3.0	0.5	25
Exon11	2.5	2.0	0.5	3.0	0.5	25
Exon12	2.5	1.5	0.5	3.0	0.5	25
Exon13	1.5	1.5	0.5	3.0	0.5	25
Exon14	2.5	1.5	0.5	3.0	0.5	25
Exon15	2.5	1.5	0.5	3.0	0.5	25
Exon16	2.5	2.5	0.5	3.0	0.5	25
TaqIB	2.5	2.5	0.5	3.0	0.5	25

Table 3.6 The cycling conditions of the PCR reaction of promoter and exons 1-16 of CETP gene

Region	Amplification profile	Fragment length (bp)
Promoter	94°C, 60 s : 64°C, 60 s : 72°C, 60 s	810
Exon1	94°C, 30 s : 66°C, 30 s : 72°C, 60 s	427
Exon2	94°C, 60 s : 66°C, 60 s : 72°C, 45 s	304
Exon3&4	94°C, 60 s : 66°C, 60 s : 72°C, 60 s	425
Exon5	94°C, 60 s : 60°C, 60 s : 72°C, 30 s	218
Exon6&7	94°C, 60 s : 62°C, 60 s : 72°C, 60 s	492
Exon8	94°C, 60 s : 64°C, 60 s : 72°C, 30 s	253
Exon9	94°C, 30 s : 68°C, 30 s : 72°C, 45 s	332
Exon10	94°C, 60 s : 62°C, 60 s : 72°C, 30 s	208
Exon11	94°C, 60 s : 62°C, 60 s : 72°C, 45 s	355
Exon12	94°C, 60 s : 66°C, 60 s : 72°C, 45 s	307
Exon13	94°C, 60 s : 56°C, 60 s : 72°C, 30 s	243
Exon14	94°C, 60 s : 56°C, 60 s : 72°C, 30 s	226
Exon15	94°C, 60 s : 66°C, 60 s : 72°C, 30 s	269
Exon16	94°C, 60 s : 64°C, 60 s : 72°C, 60 s	435
TaqIB	94°C, 60 s : 64°C, 60 s : 72°C, 60 s	304

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4. Agarose Gel Electrophoresis

4.1. Principle

The agarose gel is immersed in buffer and the DNA fragments are loaded into a well at one end of the gel and made to move through the gel by the application of an electric current. DNA is highly negatively charged and so will move towards the positive anode. However, the polysaccharide matrix of the gel retards the DNA by a process of sieving, so the small fragment move through faster and thus fragments separate according to size. The DNA fragments were visualized by staining with ethidium bromide. This dye binds to DNA and fluoresces under ultraviolet radiation (93).

4.2. Reagent preparations

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 in distilled water. The volume of the solution was adjusted to 1 liter.

b. 1X TBE buffer

100 ml of 10X TBE buffer was diluted to 1 liter with distilled water.

c. Loading dye

0.125 g each of bromophenol blue and xylene cyanol FF were weighed and dissolved in 25 ml ddH_2O. Then, 15 ml of glycerol was added and mixed. The volume of the mixture was adjusted to 50 ml with ddH_2O.

d. 250 ng/µl of 100 bp ladder marker

5 µl of 1 µg/µl stock 100 bp ladder marker was taken and then 15 µl of steriled water was added.

e. Ethidium bromide solution

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

f. 2% agarose gel

2 g of agarose was weighed and dissolved in 100 ml of 1X TBE buffer. The mixture was heated until boil and no granule of agarose remained visible. The melting gel was poured into an electrophoresis chamber set with comb which was inserted. The agarose gel was left stand to solidify at room temperature for 30 minutes.

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4.3. Procedure

Agarose gel was immersed in 1X TBE buffer at a depth of 1-2 mm. 5 µl of PCR products were pipetted and mixed with 2 µl of loading dye. The mixture was loaded in each well on 2% agarose gel and electrophoresed for 30 minutes at 100 volts. After electrophoresis, the PCR products were stained by ethidium bromide solution for 1-2 minutes and destained for 10 minutes then visualized under ultraviolet light. The sized of fragment were estimated by comparison with 100 bp DNA ladder marker under the same condition.

5. Restriction endonuclease digestion of PCR fragment for SSCP analysis

5.1. Principle

Restriction endonucleases are enzymes that cleave DNA at specific sequences to generate a set of smaller fragments(21). For SSCP, this technique was required the optimal DNA fragment size. A large fragment of an amplified product (>250 bp) is difficult to identify by SSCP technique. But for some short fragment (<250 bp), this technique was also required depending on secondary structure formation of that fragment. The restriction enzyme of each exon is shown in Table 3.7.

Table 3.7 Restriction endonuclease digestion for SSCP analysis

Region	Restriction enzyme	Restriction sequence	Length of PCR product (bp)	Fragment length (bp)
Promoter	EcoRI and NcoI	G↓AATT_C C↓CATG_C	810	109, 181,234,286
Exon1	HpaII	C↓CG_G	427	142, 285
Exon2	HphI	GGTGA(N)$_7$_N↓	304	136, 168
Exon3&4	HaeIII	GG↓CC	425	167, 285
Exon5	StyI	C↓CWWG_G	218	76, 142
Exon6&7	BamHI	G↓GATC_C	492	185, 307
Exon9	SphI	G_CATG↓C	332	163, 169
Exon11	SmlI	C↓TYRA_G	355	122, 233
Exon14	PstI	C_TGCA↓G	226	100, 126
Exon16	StyI	C↓CWWG_G	435	174, 261

When,

R: A or G; W: A or T; Y: C or T; N: G or A or T or C.

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5.2. Reagents

Reagents used for digesting PCR products were restriction enzyme, 10 X buffer which was supplied together with its enzyme. All of these restriction enzymes were incubated at 37°C.

5.3. Procedure

0.8 u of restriction enzyme, 1 µl of 10 X buffer and 1 X of supplement (for some fragment) were mixed and adjusted the volume to 10 µl with sterilized ddH_2O. The mixture was incubated at recommended temperature approximately 12-14 hours. After digestion, the mixture was heated to inactivate enzymatic activity at 80°C for 20 minutes and kept at 4°C.

6. Restriction endonuclease digestion of TaqIB polymorphism

6.1. Principle

TaqIB position is a well known polymorphism of CETP gene. This polymorphism is G to A substitution in 279^{th} nucleotide in intron 1 that caused of absence TaqI restriction site (TC**G**A) that were more likely to have high HDL-C levels and low levels of CETP activity and mass (77, 78). For this study, we characterized this common SNP by RFLP method.

6.2. Procedure

Five µl of PCR product, 0.8 u of restriction enzyme and 1 µl of 10 X buffer were mixed and adjusted the volume to 10 µl with sterilized ddH_2O. The mixture was incubated at 65°C approximately 12-14 hours. After digestion, the mixtures were run in 2% agarose gel electrophoresis.

7. Single strand conformation polymorphism (SSCP)

7.1. Principle

The SSCP analysis was firstly described by Orita et al.(88) SSCP analysis is wildly used for mutation detection because of its simplicity and versatility. PCR-SSCP is based on two steps. First, the DNA sequence of interest is PCR amplified, and second, the amplified DNA is analysed by SSCP method. For SSCP, the double strand DNA was heat denatured to single-strand DNA, in a non-denaturing condition, and

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folded structure of the single-strand DNA is determined by intramolecular interaction. This sequence based secondary structures (conformer) affect mobility of the DNA during electrophoresis on a non-denaturing polyacrylamide gel. A DNA molecule containing changing base will have a different secondary structure from wild type, resulting in a different mobility shift during electrophoresis. Subsequently, silver staining will be used for visualization. The schematic diagram of SSCP is shown in Figure 3.2.

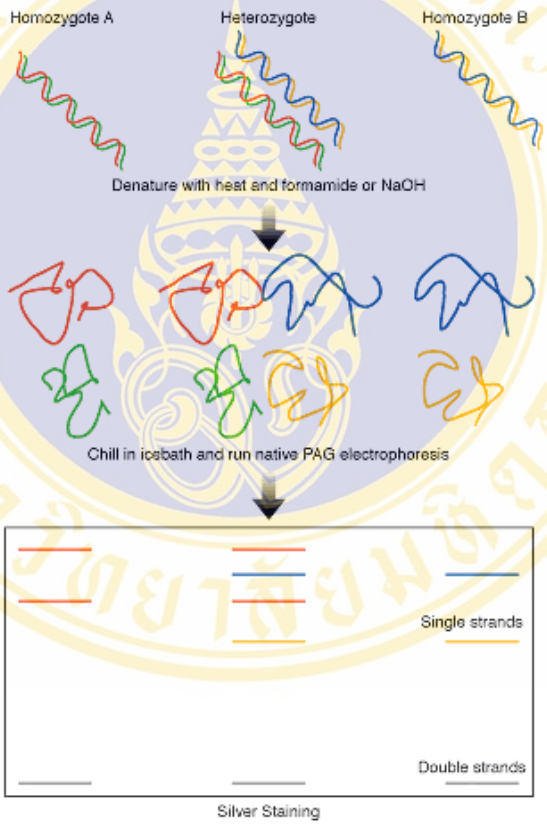

Figure 3.2 The schematic diagram of single strand conformation polymorphism (SSCP).

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7.2. Reagent preparation

a. 50%T Acrylamide stock solution with 2% cross link

49 g of acrylamide and 1 g of N'N'-methylene-bis-acrylamide were weighed and dissolved in 100 ml sterilized miliQ water. The solution was filtered with Whatman paper no.1 and stored at 4°C in dark.

b. 30.8%T Acrylamide stock solution with 2.7% cross link

30 g of acrylamide and 0.8 g of N'N'-methylene-bis-acrylamide were weighed and dissolved in 100 ml sterilized miliQ water. The solution was filtered with Whatman paper no.1 and stored at 4°C in dark.

c. Formamide dye (Bromophenol blue – Xylene cyanol stock solution)

200 µl of Bromophenol blue-Xylene cyanol FF solution, 800 deionized formamide, 200 µl of 0.5 M EDTA were aliquoted. The solution was homogeneously mixed and kept at -20°C in dark.

d. 20% Ammonium persulfate (20% APS)

0.2 g of ammonium persulfate was weighed and dissolved in 1 ml of ddH_2O. The solution was freshly prepared, kept away from light and stored at 4°C.

7.3. Preparation of the gel plates.

The glass plates were cleaned with water and detergent and then thoroughly rinsed with dH_2O to remove detergent residues. The plates were cleaned again with 70% ethanol. Detergent microfilms left on the glass plates may result in a high background upon staining the gel.

7.4. Preparation of Non-denaturing polyacrylamide gel

The polyacrylamide gel solution was the mixture of the reagents as shown in Table 3.9. Then the total volume of the mixture was adjusted to 10 ml with sterilized miliQ water. 70 µl of 20% ammonium persulfate and 7 µl of TEMED were added and mixed gently. Immediately, the solution was carefully poured into the sandwiched glass plates without any air bubble using a 1000 µl pipette until the glass plate sandwich was full. Then, the comb was inserted into the top of the gel and left stand for at least 30 minutes at room temperature.

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Table 3.8 The Mixtures of Non-denaturing polyacrylamide gels.

Percentage of gel	Acrylamide stock solution (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
8%T , 2.7%C	2.6	0.5	6.9
10%T, 2.7%C	3.25	0.5	6.25

7.5. Procedure

One µl of PCR product or 2 µl of restriction enzyme digested PCR product was mixed with 10 µl of formamide dye mix. The mixture was denatured by heat for 5-10 minutes. After denaturation, the mixture was immediately chilled on ice and loaded onto non-denaturing acrylamide gel in 0.5X TBE buffer. Electrophoresis was carried out with the Hoefer apparatus (Amersham Ltd.) and mini-PROTEAN® II cell (BIO-RAD) at 100 volts at 4°C. The percentage of gel for each fragment and times for running electrophoresis were shown in table 3.9. After electrophoresis, the DNA fragments were visualized by the silver staining method.

Table 3.9 The percentages of polyacrylamide gel and times for running electrophoresis for each fragment.

Fragment	Percentage of polyacrylamide gel	Times for running electrophoresis	Digestion
Promoter	8%T , 2%C	3:30 hours	✓
Exon1	10%T. 2.7%C	5:30 hours	✓
Exon2	8%T , 2%C	3:00 hours	✓
Exon3&4	8%T, 2.7%C	4:00 hours	✓
Exon5	8%T , 2%C	2:30 hours	✓
Exon6&7	8%T, 2.7%C	4:00 hours	✓
Exon8	10%T , 2%C	4:00 hours	×
Exon9	8%T , 2%C	2:30 hours	✓
Exon10	10%T, 2.7%C	3:30 hours	×
Exon11	8%T, 2%C	2:00 hours	✓
Exon12	8%T , 2.7%C	5:00 hours	×
Exon13	8%T , 2.7%C	5:00 hours	×
Exon14	8%T, 2.7%C 10%T, 2.7%C	4:30 hours 3:30 hours	× ✓
Exon15	10%T, 2%C	5:00 hours	×
Exon16	8%T , 2%C	3:30 hours	✓

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8. Silver staining method (Non-diamine chemical reduction stains)

8.1. Principle

Silver stain is used for the visualization of proteins and nucleic acids on polyacrylamide gels and blotting matrices. It has achieved widespread application because of its great sensitivity and easily to record without any special equipment. There are three basic silver staining protocols 1) the diamine or ammoniacal stains, 2) the non-diamine chemical reduction stains and 3) the silver stains based on photodevelopment. For this study, Non-diamine chemical reduction stain was used because it is relatively rapid and simple to perform. This method silver nitrate is used to provide silver ions for reaction with protein and nucleic acid sits under acidic conditions. Image development is initiated by placing the gel in an alkaline solution, utilizing sodium carbonate to maintain the alkaline pH, while the silver ions are selectively reduced by the oxidation of formaldehyde and buffered by the sodium carbonate. This development is stopped by the addition of acetic acid(94).

8.2. Reagent preparation

Reagents for silver staining comprise three solutions 1) fix and stop solution, 2) staining solution and 3) developing solution.

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

100 ml of gracial acetic acid was diluted to 1000 ml with distilled water.

b. Staining solution

2 g of silver nitrate was weighed and dissolved in 1000 ml of deionized miliQ water.

c. Developing solution

3 g of sodium carbonate was weighed and dissolved in 100 ml of deionized miliQ water. The solution was chilled to 10°C in the refrigerator. Immediately before use, 150 µl of 37% formaldehyde was added.

8.3. Procedure

After electrophoresis, the cover glass plates were taken out. The gel was placed in plastic tray and fixed with 100 ml of fix solution to remove electrophoresis buffer from the gel and to prevent diffusion of small extension products. Then, the tray was either agitated well for 30 minutes or until the tracking dyes were no longer visible or left strand for overnight at room temperature. The gel was removed from the staining

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solution and rinsed with deionized miliQ water for no longer than 5-10 seconds to remove excess silver nitrate. 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, and the mixture was agitated for 3 minutes. The gel was rinsed twice in distilled water. The gel was collected by wrapping with cellophane membrane, then air dried for overnight at room temperature.

9. DNA sequencing

9.1 Principle

The Sanger's DNA sequencing method is used for characterizing DNA sequence variations after abnormal patterns are observed by SSCP analysis. The Sanger's method is also known as the dideoxy method because this procedure uses dideoxynucleoside triphosphate (ddNTP) analogs to interrupt DNA synthesis. Normally, the 3'-hydroxyl group of the primer reacts with an incoming deoxynucleoside triphosphate (dNTP) to form a new phosphodiester bond. When dNTP is replaced by ddNTP, strand elongation is stopped after the analog is added because the analog lacks the 3'-hydroxyl group needed for the next step. The structures of dideoxynucleoside triphosphate are shown in Figure 3.3. In the Sanger sequencing, DNA synthesis is initiated from a primer that has been labeled at one end with a radioisotope. Four separate reactions are run, each including one dideoxynucleotide (either A, C, G or T) in addition to its normal counterpart. Incorporation of a dideoxynucleotide stops further DNA synthesis as described above. Thus, a series of labeled DNA molecules are generated, each termination of the base is represented by the dideoxynucleotide in each reaction. These fragments of DNA are then separated according to size by gel electrophoresis and detected by exposure of the gel to x-ray film (autoradiography) or silver staining when the sequencing primer is not radioactively labeled. The DNA sequence corresponds to the order of fragments read from the gel. Figure 3.4 show the dideoxy DNA sequencing method.

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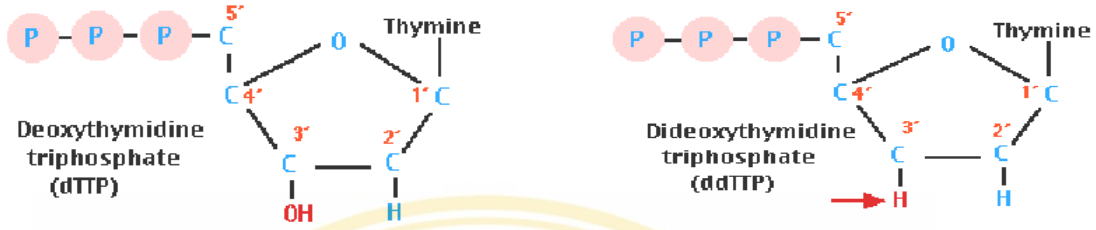

Figure 3.3 The structure of dideoxynucleoside triphosphate

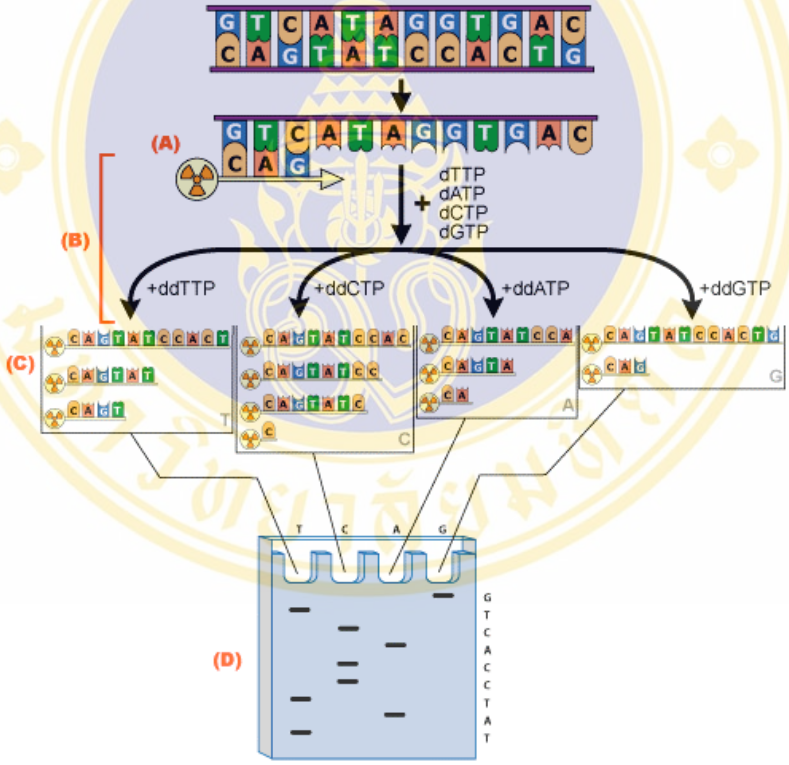

Figure 3.4 The Sanger DNA sequencing method.

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Automated DNA sequencing

Large-scale DNA sequencing is frequently performed using automated system. Each dideoxynucleotide used in this system was linked to a fluorescent molecule that gives all the fragments terminating in that nucleotide a particular color. All four labeled ddNTPs are added to a single tube. The resulting colored DNA fragments are then separated by size in a single electrophoresis gel contained in a capillary gel in a single peak, and the color associated with each peak is detected using a laser beam. The automated fluorescent sequencing detection was shown in Figure 3.5.

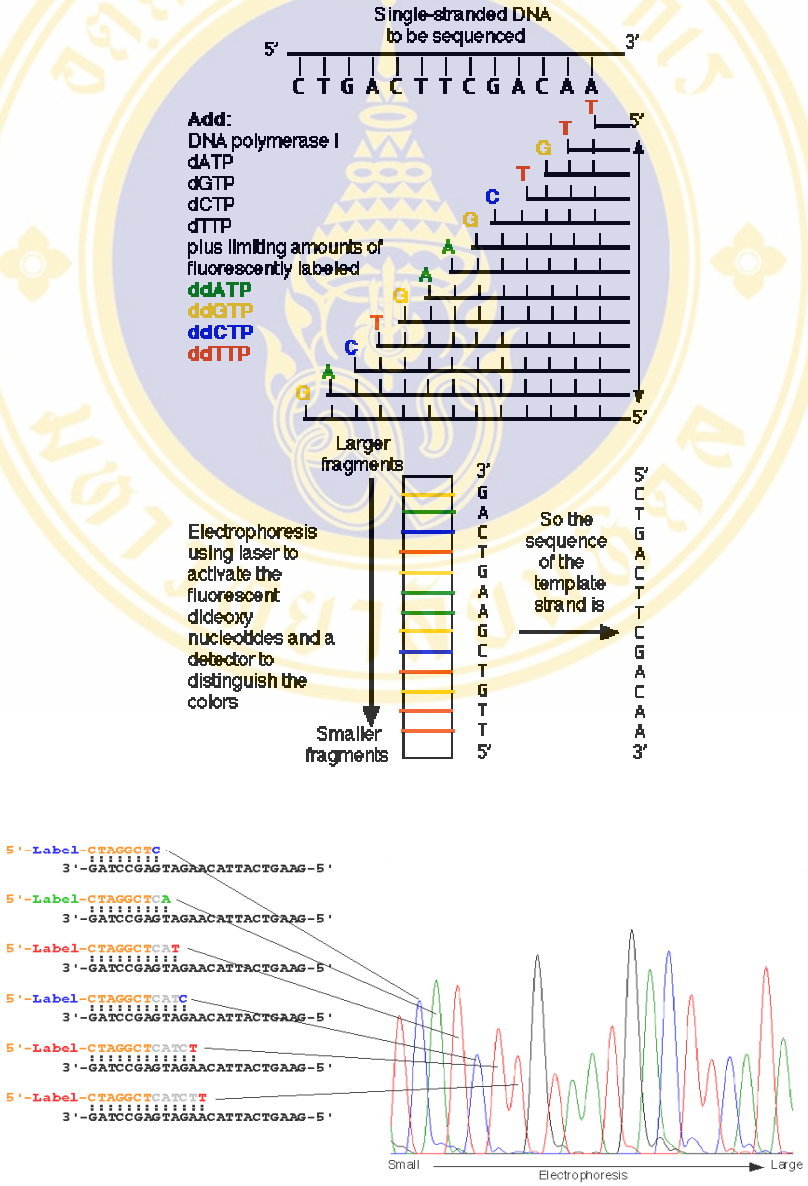

Figure 3.5 The automated DNA sequencing method (http://medlib.med.utah.edu/block2/biochem/Formosa/menu.html)

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9.2 DNA template preparation

The DNA template for sequencing reaction was obtain for purified PCR product. The PCR products have to be cleaned to remove primers, nucleotides, polymerase, and salts which affect the sequencing reactions. 120 µl of PCR products that have fragments ranging from 70 bp to 10 kb were run on gel electrophoresis. The DNA band was excised with a clean, sharp scalpel and purified by using QIA quick Gel Extraction Kits (QIAGEN). In elution step, however, the purified PCR product was eluted by 30 µl of sterilized ddH_2O to decrease salt and increase DNA concentration.

9.3 Procedure for automated DNA sequencing

Purified PCR product of a DNA sample with mobility shift was used as template for automated DNA sequence analysis at Ramatibodi hospital.

9.4 Manual DNA sequencing

9.4.1 DNA sequencing reaction

Purified PCR product of a DNA sample was used as template for manual DNA sequencing reaction. This reaction was performed by using a commercial kit, SILVER SEQUENCE™ DNA sequencing system (Promega, German).

10.5 µl of template DNA are prepared as describe above (section 9.2), 5 µl of DNA sequencing 5X buffer and 0.45 µl of 10 pmol primer were mixed and the volume was adjusted to 16 µl with nuclease-free water. Then, 1 µl of sequencing grade *Taq*DNA polymerase (5u/µl) supplied by the manufacture (Promega) was added to the mixture and mixed briefly by pipetting. After that, 4 µl of the mixture was aliquot to four tubes each labeled with G, A, T and C containing 2 µl of the appropriate d/ddNTP mix in each tube. The mixture was mixed briefly and then 1 drop of mineral oil was added. Next, the reaction tubes were placed to the thermal cycler machine. The cycling condition was as following. The denaturation step was set at 95°C for 2 minutes in the initial cycle. The amplification profile was 95°C for 30 seconds, 66°C for 30 seconds and 72°C for 1 minute. This amplification reaction was run for 60 cycles and then soaked at 4°C. After the thermal cycling program was completed, 3 µl of DNA sequencing stop solution was added and kept at -20°C until used.

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9.4.2 Reagents preparation

a. Bind Silane solution

1 ml of 95% ethanol was added with 5 µl of glacial acetic acid and 3 µl of Bind Silane solution.

b. 40% (19:1) Acrylamide stock solution with 5% cross link

19 g of acrylamide and 1 g of N'N'-methylene-bis-acrylamide were weighed and dissolved in 50 ml sterilized miliQ water. The solution was filtered with Whatman paper no.1 and stored at 4°C in dark.

9.4.3 Preparation of the gel plate

The plates were cleaned with water and detergent and then thoroughly rinsed with deionized water to remove detergent residues. The plates were cleaned again with 70% ethanol and prepared as following and then these two plates were joined face to face with 0.5 mm spacers.

a. Short glass plate preparation

The short glass plate was treated with binding solution each time a gel is prepared. Binding solution was dropped and wiped a scrupulously using a tissue on all four sides of the glass (about 1 inch wide). The area in the middle of the plate was treated with glass-loose solution (i.g., Rain-away) to prevent the gel from sticking to the plate. After 4-5 minutes, 70% ethanol was applied to the plate and wiped a paper tissue using gentle pressure to remove excess treated solution. After electrophoresis, middle area of the gel with sequence data can be cut and pealed off from the plate by Whatman paper. The gel is then dried and kept as such permanently. After performing the DNA sequencing, the plate was cleaned by soaking in 10% NaOH for at least 1 hour or preferable overnight.

b. Long glass plate preparation

Treatment of the long glass plate with glass-loose solution each time was unnecessary if water beaded in the surface of the plate. The cleaned long glass plate was wiped using a tissue saturated with glass-loose solution. After 4-5 minutes, the excess glass-loose solution was removed by wiped a paper tissue with 70% ethanol.

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9.4.4 8%(19:1) denaturing polyacrylamide gel preparation with 7M urea

Twenty-one grams of urea was weighed and dissolved in the mixture of 10 ml of 40% acrylamide stock solution with 5% cross link (19:1 w/w) and 5 ml of 10X TBE buffer by stirring machine. The total volume of the mixture was adjusted to 50 ml with sterilized miliQ water then the solution was filtered with Whatman paper no.1. 250 µl of 20% ammonium persulfate and 20 µl of TEMED were added and mixed gently. Immediately, the solution was carefully poured into the sandwiched glass plates without any air bubble until the glass plate sandwich was full. Next, the smooth side of comb was inserted into the top of the gel and left stand for at least 4 hours at room temperature. After that, the comb was drawn from the gel.

9.4.5 Polycrylamide gel electrophoresis for sequencing reactions

All of the products of sequencing reactions were denatured by heat for 3-5 minutes. After denaturation, the mixtures were immediately chilled on ice and 5 µl of each set of sequencing products was loaded onto the denaturing acrylamind gel in 1X TBE buffer. Electrophoresis was carried out with microprocessor controller electrophoresis at 1200-15000 volts at 45-60°C for 3-4 hours. After electrophoresis, the DNA fragments were visualized by the silver staining method as described in method 7.

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

10.1 Reagent preparation

a. LB broth

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

b. LB agar

2.5 g of Bacto-tryptone, 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.

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c. 10 mg/ml Ampicillin stock

0.1 g of ampicillin 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 μm Nalgene filter and kept at 4°C.

e. 100 mM PMSF (Phenylmethyl Sulfonyl Fluoride)

0.0174 of PMSF was weighed and dissolved in 1 ml 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 of autoclaved dH_2O.

g. 10 mM DTT

10 μl of 1 M DTT stock was diluted to 1000 μl 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 μl of 0.5 M EDTA were aliquoted. The volume was adjusted to 40 ml with autoclaved dH_2O.

j. Prelysis buffer

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

k. Lysis buffer

50 μl of 1 M Tris-HCl pH 8.0, 250 μl of 1 M KCl, 10 μl of 0.5 M EDTA, 50 μl of 10 mM PMSF, 25 μl of Tween20, and 25 μl 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 μl of 1 M $CaCl_2$ and 100 μl of 1 M Tris-HCl, pH 8.0 were aliquoted. The volume was adjusted to 10 ml with autoclaved dH_2O.

10.2 Preparation of fresh competent *E. coli*. cells

Twenty μl of frozen stock *E.coli*. ($DH_5\alpha$) was inoculated into 2 ml LB broth and then incubated overnight at 37°C with shaking. 500 μl of the culture was inoculated to 50 ml of new LB broth and shaked at 37°C to an OD_{550} of approximately

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0.5. Then, 3 ml of inoculum was aliquoted into focal 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 µl of cold 50 mM $CaCl_2$, 10 mM Tris-HCl pH 8.0 solution. The mixture was transferred to new 1.5 ml Eppendorf tube.

10.3 Transformation

Five µl of recombinant p*Taq* plasmid was mixed with 200 µl of the competent *E.coli*. cells prepared as described above. 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 ampicillin 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 µl of supernatant was discarded. The cell pellet was gently resuspended and spreaded on LB agar with 100 mg/l ampicillin.

10.4 Gene Expression

A single colony of transformed *E.coli*. was inoculated to 2 ml LB groth with ampicillin (100 mg/l) and incubated at 37°C overnight with shaking. 400 µl of inoculum was subcultured into 40 ml LB broth with ampicillin (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 focal 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 discarded. 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 Eppendorf tube and placed on ice bath for 5 minutes. The mixture was centrifuged at

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1,200 rpm for 15 minutes. The supernatant was transferred to new 1.5 Eppendorf tube and then added with 100 µl of 10 mM DTT into 1 ml of lysate. The *Taq* DNA polymerase activity was assayed by PCR reaction.

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CHAPTER IV
RESULTS

1. Promoter of CETP Gene

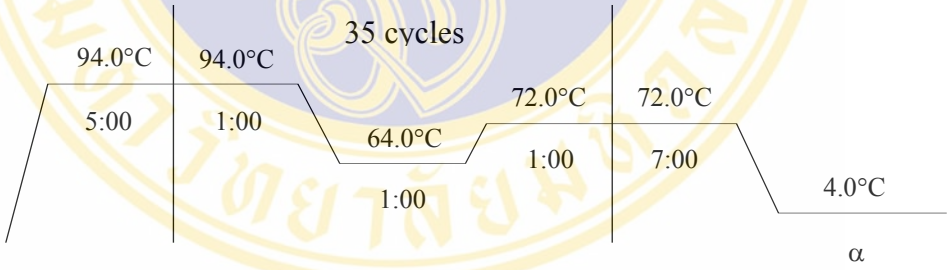
1.1 DNA amplification of promoter

The promoter of CETP gene is consisted of approximately 5700 bp. In this study, a region of this promoter was partially amplified (-26 to -836) with the primers F2P_CETP and RP_CETP. The nucleotide sequences of these primers are as shown below. The amplification condition and the amplified products are presented in Figure4.1.

F2P_CETP 5'-GCA CTT GGC CAT CTG GTC ACA GTT GCT GCA-3' = 30 bp

RP_CETP 5'-TAT ATG TAT GTC CGC CCA GCC CCC ACG GAA-3' = 30 bp

A)



B)

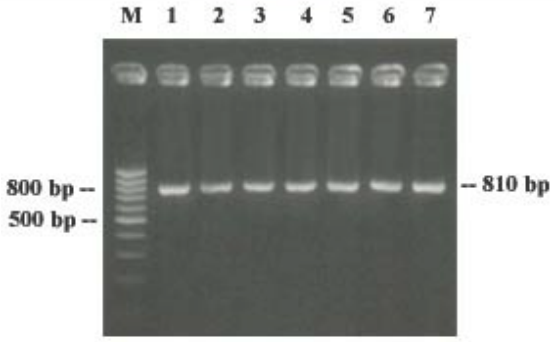


Figure 4.1 Amplification condition of promoter region (Panel A) and ethidium bromide staining of 2% agarose gel containing partially amplified fragments of promoter (810 bp) of CETP gene from different individuals (Panel B). Lane M was 100 bp ladder marker. Lanes 1 to 7 were PCR products of each test sample.

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1.2 Restriction endonuclease digestion of PCR fragment of promoter

810 bp of PCR product was digested with two restriction enzymes, *EcoR*I and *Nco*I. The digestion produced four fragments of 109, 181, 234 and 286 bp as shown in Figure4.2. This restriction endonuclease digestion was performed to improve the sensitivity of the SSCP analysis.

A) Restriction site for *Nco*I: 5' C↓CATG_G 3'

*EcoR*I: 5' G↓AATT_C 3'

GCACTTGGCC ATCTGGTCAC AGTTGCTGCA GGGCAGTTCT TGGCCCCAGC
TGTAGGTAAA GTACTGTATG TTGTAATTTT TTGAAAGATA ACACGTTCAC
ACAACTCAGA ATTGAAATGC CACAGACATT CCCCCTGCTC CGCCCCTTTC
CCCCGGATAC CCAGTTTCTC CCGGAGGCAG CCAATGATCT CAGAGGCTGT
ATACACACCC AGAGTTATTT TATGCATATC AAGGAAAGTC TACATAGAGG
ACTGTTTCTG GGGTACCCAG ATGCAGCGTC AAATG**C↓CATG_**G AATACTACA
GTGAGGACAT TATCCTTTCA AGCTTTCAAA TCAGAGCAAG GGAAAGGTCG
ATGCTAGAGT TTCTCTAGCA CCCATGAAGC CCTCTCCCTT TTTCTACTGA
GTTTTACTTT ACAGGCAACA GCAGGCTTCA AGCTTGGGGT CATTGTCGGG
CAACAGTATC TGGCAA**G↓AAT** **T_C**AATGTCTT TTTCTCATAG TCATTGTATT
TTGGCCTCTT TCTATTTATG GCAACTGAGA GAGAAAGCTT ATTCCTAGAT
ATATGTATTT AAGTAAAAAA TAAAT**G↓AATT_** **C**ATGGAAACA TATTAAGCAA
TTATCCAGAT AACATAAGGG ATGGCAAAAA TGGTGCAGAT GGTGGAGGGG
AGACAAGTAG AAGTTGGGGT GCTCTTGTTG AATGTCTGGC TCTGAACTCT
AGAGGAGGCC GCAGGGGCTG GGCAGGAAGG AGGTGAATCT CTGGGGCCAG
GAAGACCCTG CTGCCCGGAA GAGCCTCATG TTCCGTGGGG GCTGGGCGGA
CATACATATA

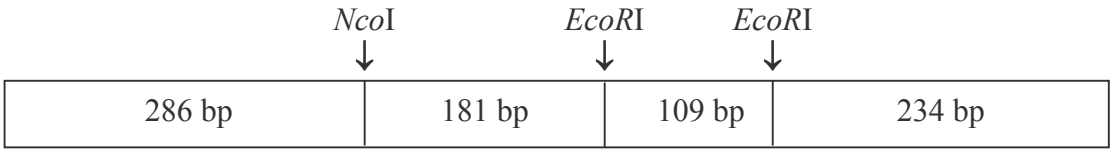

Figure 4.2 *EcoR*I and *Nco*I digestions of an amplified fragment of promoter region of CETP gene. The nucleotide sequences of PCR fragment of promoter region showing the *Nco*I and *EcoR*I sites (Panel A) and the map of *Nco*I and *EcoR*I digestions of this fragment (Panel B).

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1.3 SSCP analysis of promoter

The amplified products of the promoter, after *NcoR*I and *EcoR*I digestions, were analyzed by SSCP on 8%T, 2%C non-denaturing polyacylamide gel. Three different SSCP patterns were observed in different samples.

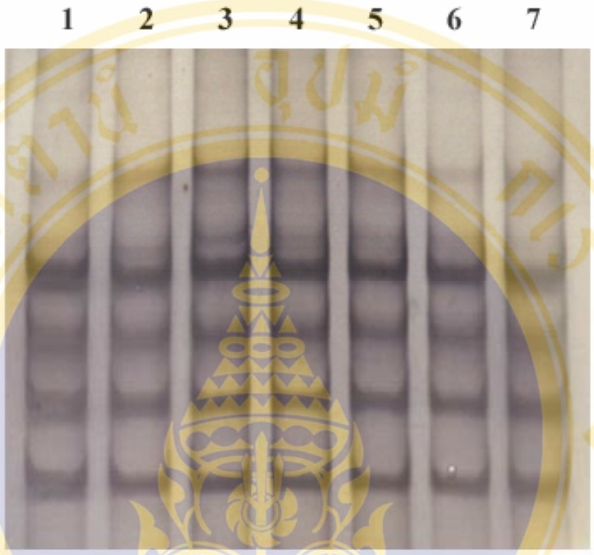

Figure 4.3 SSCP analysis of the amplified fragments (with *Nco*I and *EcoR*I restriction) of promoter sequences of CETP gene. Lanes 1 to 7 were single-stranded DNA pattern of test samples. Lanes 2, 3 and 4 were DNA samples with different SSCP patterns.

1.4 DNA sequence analysis of promoter

All three amplified fragments with different SSCP patterns were subjected to automated DNA sequencing. Heterozygous A to C substitution at -629 was found as shown in Figure 4.4.

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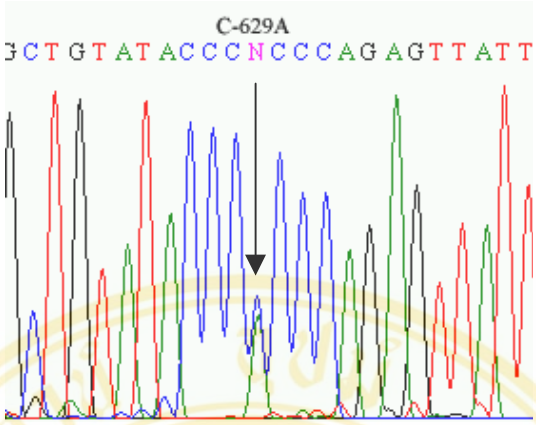


Figure 4.4 Partial DNA sequence of promoter region with mobility shift in SSCP pattern. The sequencing reactions were performed with both sense (F2P_CETP) and anitsense (RP_CETP) primers. Heterozygousity (A/C) at nucleotide -629 is indicated by an N (marked with an arrow).

2. Exon 1 of CETP Gene

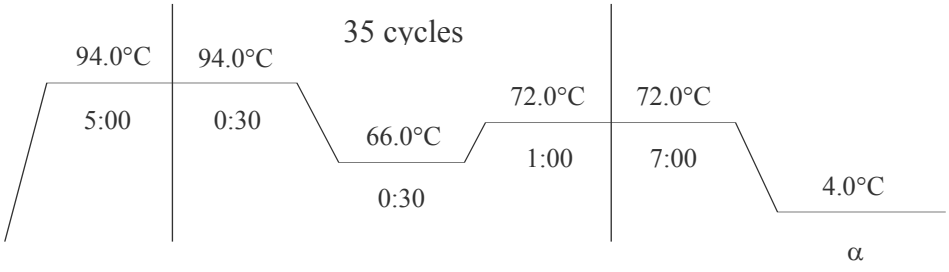
2.1 DNA amplification of exon 1

Exon 1 of CETP gene is consisted of 248 bp. In this study, exon1 with exon-intron junctions (427 bp) was amplified with the primers FEX1_CETP and REX1_CETP. The nucleotide sequences of these primers are as shown below. The amplification condition and the amplified products are presented in Figure 4.5.

FEX1_CETP 5'-GCA AAA ATG GTG CAG ATG GTG GAG GGG AGA-3'=30 bp

REX1_CETP 5'-GGA GGC TCC TGG CAT AGT GGG TGT CCA-3' =27 bp

A)



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B)

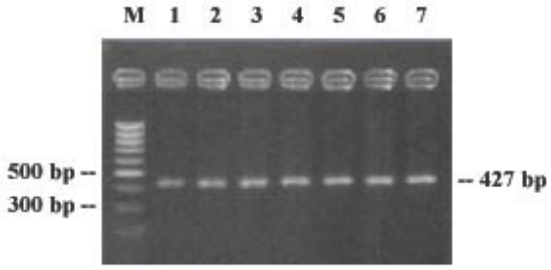

Figure 4.5 Amplification condition of exon 1 (Panel A) and ethidium bromide staining of 2% agarose gel containing 427 bp of exon 1 from different individuals (Panel B). Lane M was 100 bp ladder marker. Lanes 1 to 7 are normal PCR products of each test sample.

During DNA amplification, an abnormal double-banded PCR product of exon 1 was found in one subject with high HDL-C level (Figure 4.6). The lower band was normal 427 bp PCR product as confirmed by automated DNA sequencing (data not shown). The upper band was an unknown PCR product.

Figure 4.6 Ethidium bromide staining of 3.5% agarose gel of exon1 containing normal 427 bp PCR product (Lanes A and B) and an abnormal double-banded PCR product from high HDL-C subject (Lane C). Lane M was 100 bp ladder marker.

2.2 Restriction endonuclease digestion of exon 1

427 bp of exon 1 PCR product was digested with a restriction enzyme, *Hpa*II. The digestion produced two fragments of 142 and 285 bp as shown in Figure 4.7.

A) Restriction site for *Hpa*II: 5’ C↓CG_G 3’

GCAAAAATGG TGCAGATGGT GGAGGGGAGA CAAGTAGAAG TTGGGGTGCT
CTTGTTGAAT GTCTGGCTCT GAACTCTAGA GGAGGCCGCA GGGGCTGGGC
AGGAAGGAGG TGAATCTCTG GGGCCAGGAA GACCCTGCTG **CC↓CG_G**AAGAG
CCTCATGTTC CGTGGGGGCT GGGCGGACAT ACATATACGG GCTCCAGGCT
GAACGGCTCG GGCCACTTAC ACACCACTGC CTGATAACCA TGCTGGCTGC

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CACAGTCCTG ACCCTGGCCC TGCTGGGCAA TGCCCATGCC TGCTCCAAAG
GCACCTCGCA CGAGGCAGGC ATCGTGTGCC GCATCACCAA GCCTGCCCTC
CTGGTGTGTA AGTATCAGTG CATCTGTCTG CCCTGCCAGG GGTCTTTTCA
TGGACACCCA CTATGCCAGG AGCCTCC

B)

Figure 4.7 *Hpa*II digestion of an amplified exon 1 of CETP gene. The nucleotide sequences of exon 1 showing the *Hpa*II site (Panel A) and the map of *Hpa*II digestion of exon 1 (Panel B).

2.3 SSCP analysis of exon 1

The amplified products of exon 1 were analyzed by SSCP on 10%T, 2.7%C non-denaturing polyacylamide gel. There was no different SSCP pattern detected in all DNA samples under this study. A number of representative SSCP patterns are shown in Figure 4.8.

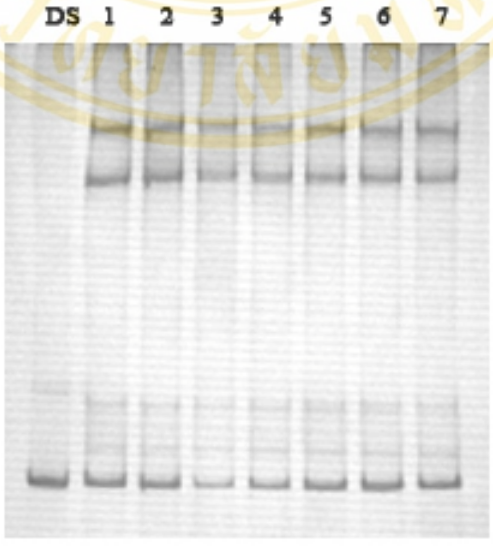

Figure 4.8 SSCP analysis of exon 1 of CETP gene. Lane DS is double-stranded DNA pattern. Lanes 1 to 7 were single-stranded DNA pattern of test samples. There was no different SSCP pattern in all DNA samples under this analysis.

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2.4 Automated DNA sequencing of exon 1

Purified abnormal double-banded PCR product was subjected to automated DNA sequencing. The scrambled peaks were found in both sense and antisense sequencing reactions as shown in Figure 4.9. Comparison of the results of sense and antisense sequencing reactions showed that abnormal allele was presumably due to a deletion of 18 nucleotides (as shown in Figure 4.10.). This result was later confirmed with manual DNA sequencing (Figure 4.11).

A)

B)

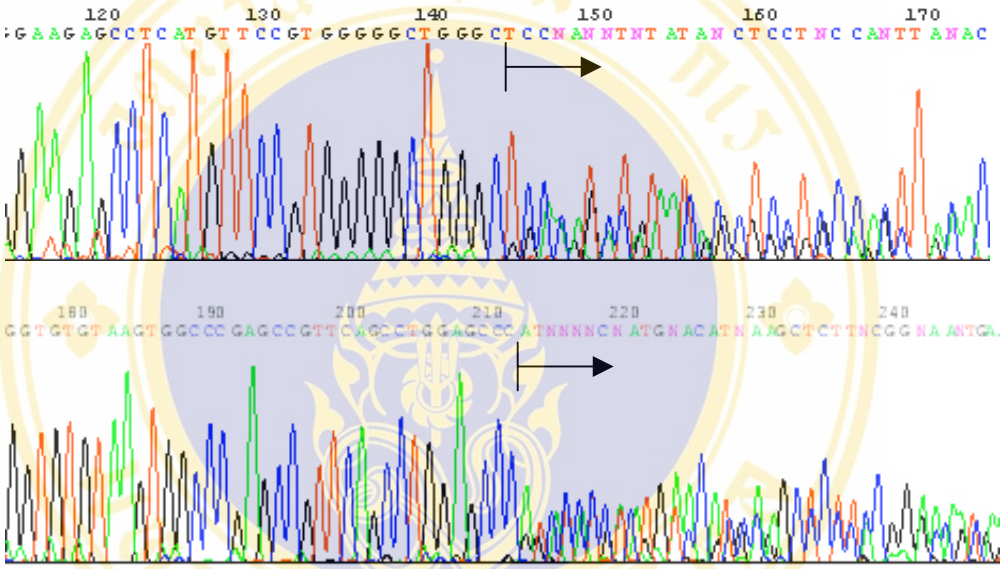

Figure 4.9 Partial DNA sequences with scrambled peaks of abnormal band of exon1. The sequencing reactions were performed with both sense (FEX1_CETP; Panel A) and antisense (REX1_CETP; Panel B). The scrambled peaks were apparent in both reactions.

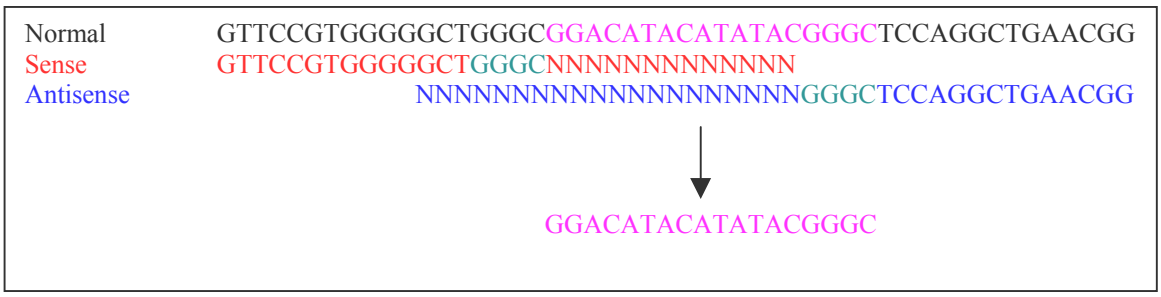

Figure 4.10 The sequence comparison between the normal and abnormal PCR band (upper band). Both sense and antisense sequences of the abnormal band were presented here with the wild-type sequence. The deletion of 18 nucleotides was indicated under an arrow.

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2.5 Manual DNA sequencing

The scrambled data obtained from automated DNA sequencing was clarified by manual DNA sequencing method. The abnormal PCR product was purified and used as template for manual DNA sequencing reaction using SILVER SEQUENCE^{TM} DNA sequencing system (Promega). Each set of sequencing product (C, T, A, G) was loaded on 8%(19:1) denaturing polyacrylamind gel. The result was shown in Figure 4.11. Two bands at same positions along the sequencing gel were observed. The nucleotide sequences from this gel is shown in Figure 4.12.

Figure 4.11 Silver staining of manual DNA sequencing of abnormal double-banded PCR product of exon1. For this representative DNA sequence data, the sequencing

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reaction was performed by sense primer (FEX1_CETP). The two bands at same position began from the red-colored line.

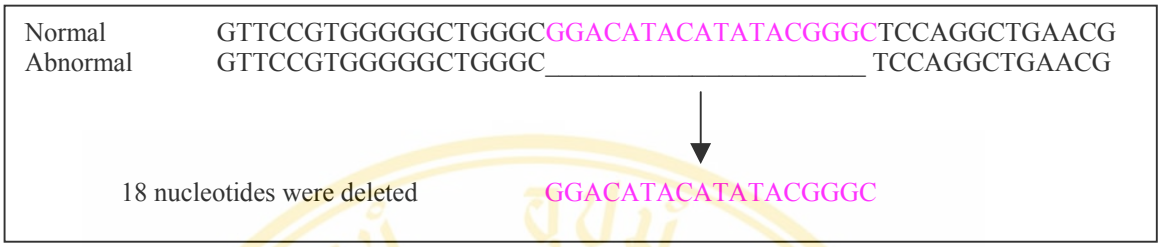

Figure 4.12 Partial nucleotide sequences of normal and abnormal allele from manual DNA sequencing method. Deletion of 18 nucleotides was observed.

3. TaqIB position in intron 1

3.1 Partial DNA amplification of intron 1

TaqIB is a common polymorphism in CETP gene. The intron1 of CETP gene is consisted of 902 bp. In this study, 343 bp of this intron was partially amplified with the primers FTaqIB and RTaqIB. The nucleotides sequences of these primers are as shown below.

FTaqIB 5'-GAT GGG CTG AGT GGA GCT GTC A-3' = 22 bp

RTaqIB 5'-GAG CAA GAG ACT GAG GCC CAG A-3' = 22 bp

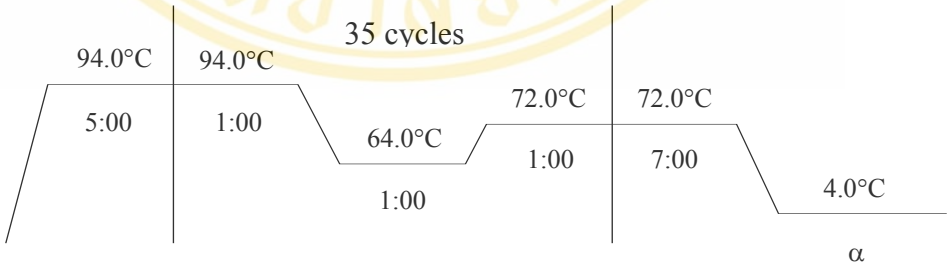

Figure 4.13 Amplification condition of TaqIB fragment.

3.2 Restriction endonuclease digestion of TaqIB polymorphism

TaqIB polymorphism is the G to A substitution at the 297th nucleotide in intron1. B1 allele is digestible with *Taq*I restriction enzyme because the restriction site (TC**G**A) is present while B2 is undigestible with *Taq*I because the restriction site (i.e.,

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TCAA) is absent. In this experiment, 343 bp of PCR product from a heterozygote would reveal cut (B1) and uncut (B2) fragments after *Taq*I digestion.

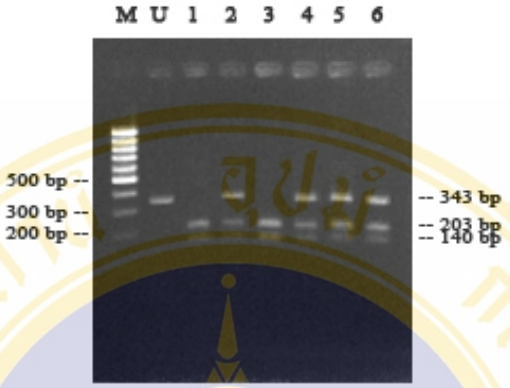


Figure 4.14 Ethidium bromide staining of 2% agarose gel containing *Taq*I- restricted fragments from different individuals. Lane M was 100 bp ladder marker and lane U was uncut PCR product. Lanes 1 to 6 were digested PCR products of each test sample. Lanes 1 and 3 are homozygous B1B1 while lanes 2, 4, 5 and 6 are heterozygous B1B2.

4. Exon 2 of CETP Gene

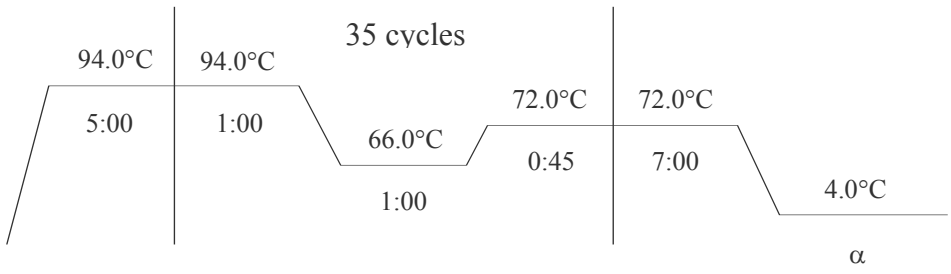
4.1 DNA amplification

Exon 2 of CETP gene is consisted of 115 bp. In this study, exon2 included with exon-intron junctions (304 bp) was amplified with the primers FEX2_CETP and REX2_CETP. The nucleotides sequences of these primers are as shown below. The amplification condition and the amplified products are presented in Figure 4.15.

FEX2_CETP 5'-TGG GAG CCT CAT CTC AGA GAG GCT GAG TCA-3' = 30 bp

REX2_CETP 5'-CTT TCC TCC CTG TGG GCT GGG GGC AA-3' = 26 bp

A)



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B)

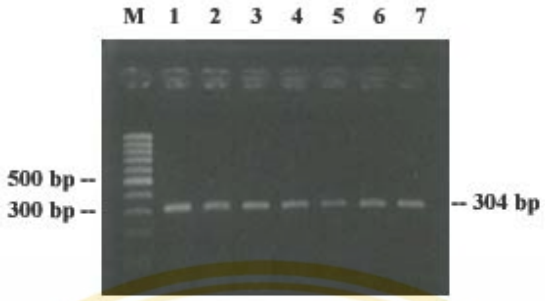

Figure 4.15 Amplification condition of exon 2 (Panel A) and ethidium bromide staining of 2% agarose gel containing 304 bp of exon 2 from different individuals (Panel B). Lane M was 100 bp ladder marker. Lanes 1 to 7 were PCR products of each test sample.

4.2 Restriction endonuclease digestion of exon 2

304 bp of PCR product was digested with a restriction enzyme, *Hph*I. The digestion produced two fragments of 136 and 168 bp as shown in Figure 4.16.

A) Restriction site for *Hph*I: 5' GGTGAnnnnnnn_n↓ 3'

TGGGAGCCTC ATCTCAGAGA GGCTGAGTCA TGGCCAAGGC AGTTGGGGTG
GGAGCAGGGG GCTTGGTGTG GGCCTGCAGC CCTCATCCAC TGCCCTCCCT
CTAGTGAACC ACGAGACTGC CAA**GGTGA**TC CAGACC↓GCCT TCCAGCGAGC
CAGCTACCCA GATATCACGG GCGAGAAGGC CATGATGCTC CTTGGCCAAG
TCAAGTATGG GTTGCACAAG TGAGTCGGGC CTCGGGTGTG ACCTGGCTGG
GGGTAGGGTG GCGGGAGGAA CAGCCTGGGC TTCCCCCAGC CACAGGGAGG
AAAG

B)

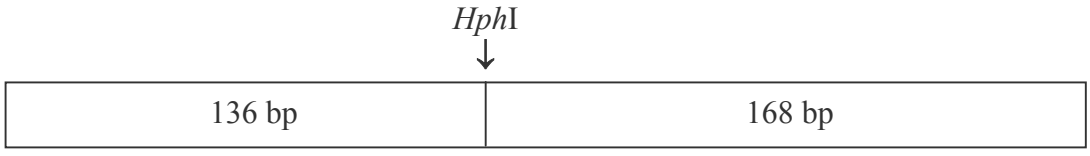

Figure 4.16 *Hph*I digestion of an amplified exon 2 of CETP gene. The nucleotide sequences of exon 2 showing the *Hph*I site (Panel A) and the map of *Hph*I digestion of exon 2 (Panel B).

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4.3 SSCP analysis of exon 2

The amplified products of exon 2 were analyzed by SSCP on 8%T, 2%C non-denaturing polyacylamide gel. There was no different SSCP pattern detected in all DNA samples under this study. A number of representative SSCP patterns are shown in Figure 4.17.

Figure 4.17 SSCP analysis of exon 2 of CETP gene. Lane DS is double-stranded DNA pattern. Lanes 1 to 7 were single-stranded DNA pattern of test samples. There was no different SSCP pattern in all DNA samples under this analysis.

5. Exons 3 and 4 of CETP Gene

5.1 DNA amplification of exons 3 and 4

Exons 3 and 4 of CETP gene is consisted of 135 and 71 bp, respectively. In this study, exons 3 and 4 included with intron 3 and exon-intron junctions (425 bp) was amplified with the primers FEX34_CETP and REX34_CETP. The nucleotides sequences of these primers are as shown below. The amplification condition and the amplified products are presented in Figure 4.18.

FEX34_CETP 5’-CAC CCT CGC CTA GAC AAA ATT GGA GGC TCA-3’ = 30 bp

REX34_CETP5’-CCT GGA GTC CAG CTC CCA CTT CCC CAG A-3’ = 28 bp

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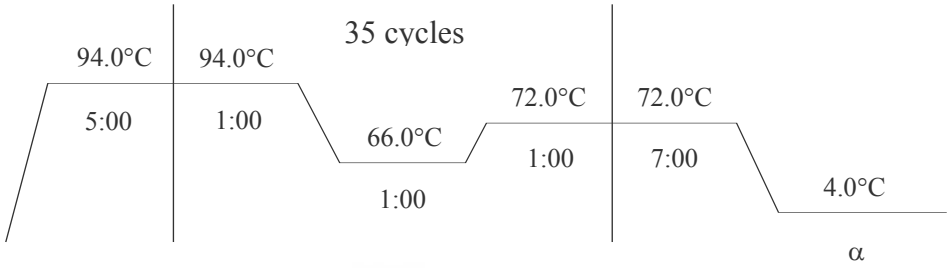
A)



B)

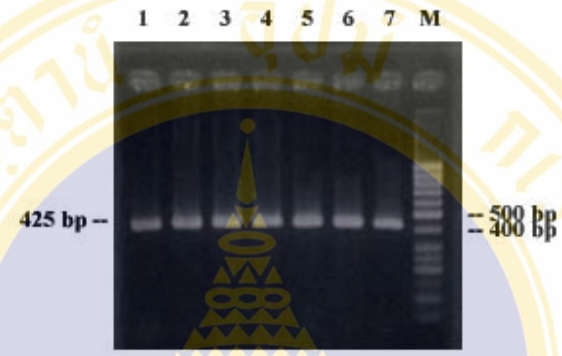


Figure 4.18 Amplification condition of exons 3 and 4 (Panel A) and ethidium bromide staining of 2% agarose gel containing exons 3 and 4 of CETP gene from different individuals (Panel B). Lene M was 50 bp ladder marker. Lanes 1 to 7 were PCR products of each test sample.

5.2 Restriction endonuclease digestion of exons 3 and 4

425 bp of PCR product was digested with a restriction enzyme, *Hae*III, producing two fragments of 167 and 258 bp as shown in Figure 4.19.

A) Restriction site for *Hae*III: 5’ GG↓CC 3’

CACCCTCGCC	TAGACAAAAT	TGGAGGCTCA	CTCCTTGGGC	TCCCTGGATG
ACCCCCAACA	TCCTTCCTCA	CTTCCATTCC	TTCCCAGCAT	CCAGATCAGC
CACTTGTCCA	TCGCCAGCAG	CCAGGTGGAG	CTGGTGGAAG	CCAAGTCCAT
TGATGTCTCC	ATTCAGAACG	TGTCTGTGGT	CTTCAAGGGG	ACCCTGAAGT
ATGGCTACAC	CACTGCCTGG	TGGTAAGCAT	TCCTGTCAGC	TGATGCCCCA
TGCCCT**GG↓CC**	CTCTCTGGGT	GGAGGGCTGA	ATGAGGTCTG	GGTCCTTGGC
TCTTTCCAGG	CTGGGTATTG	ATCAGTCCAT	TGACTTCGAG	ATCGACTCTG
CCATTGACCT	CCAGATCAAC	ACACAGCTGA	GTATGTGTCA	AGCGTCCTCT
GGGGAAGTGG	GAGCTGGACT	CCAGG		

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B)

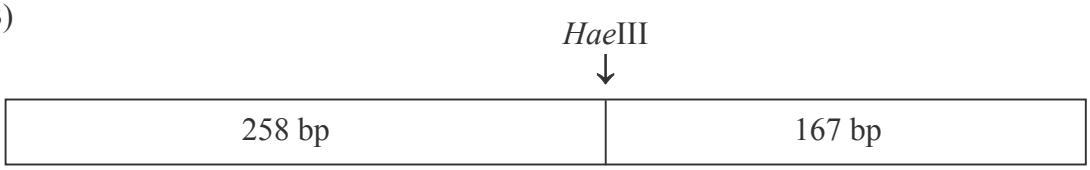

Figure 4.19 *Hae*III digestion of an amplified exons 3 and 4 of CETP gene. The nucleotide sequences of exons 3 and 4 showing the *Hae*III site (Panel A) and the map of *Hae*III digestion of exons 3 and 4 (Panel B)

5.3 SSCP analysis of exons 3 and 4

The amplified products of exons 3 and 4 were analyzed by SSCP on 8%T, 2.7%C non-denaturing polyacylamide gel. A different SSCP pattern was observed in one subject of this region.

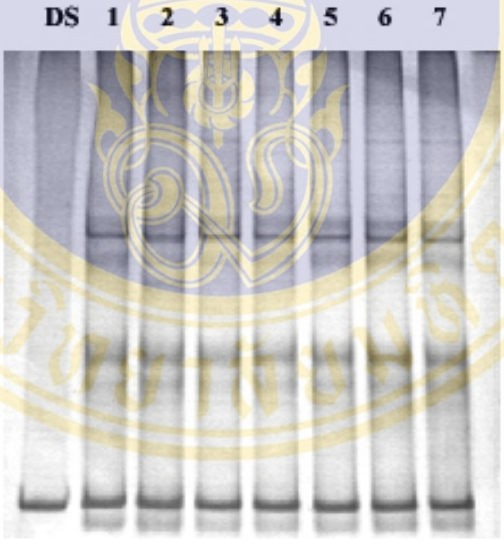

Figure 4.20 SSCP analysis of exons 3 and 4 of CETP gene. Lane DS is double stranded DNA pattern. Lanes 1 to 7 were single-stranded DNA pattern of test samples. Lane 2 was DNA sample with different SSCP pattern.

5.4 DNA sequencing of exons 3 and 4 of CETP gene

A different SSCP pattern was subjected to automated DNA sequencing. Novel heterozygous substitution in exon 3 (code: CETP_X1) at highly conservative position was founded as shown in Figure 4.21

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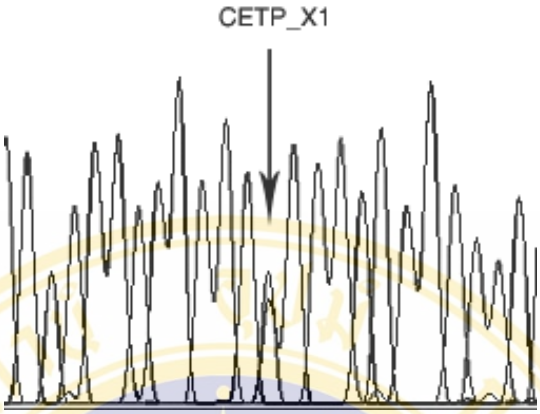


Figure 4.21 Partial DNA sequence of SSCP pattern of exons 3 and 4. The sequencing reactions were performed with both sense (FEX34_CETP) and antisense (REX34_CETP) primers. Novel heterozygous subsitiution is indicated by an arrow.

6. Exon 5 of CETP Gene

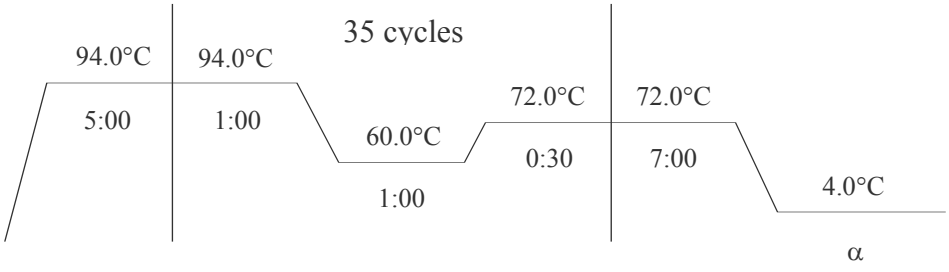
6.1 DNA amplification of exon 5

Exon 5 of CETP gene is consisted of 88 bp. In this study, exon5 included with exon-intron junctions (218 bp) was amplified with the primers FEX5_CETP and REX5_CETP. The nucleotide sequences of these primers are as shown below. The amplification condition and the amplified products are presented in Figure 4.22.

FEX5_CETP 5'-CCT GGG CAG CAT GTG GAT ACC A-3' = 22 bp

REX5_CETP 5'-GGC CGT GGA CAC ACT AAC AGG A-3' = 22 bp

A)



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B)

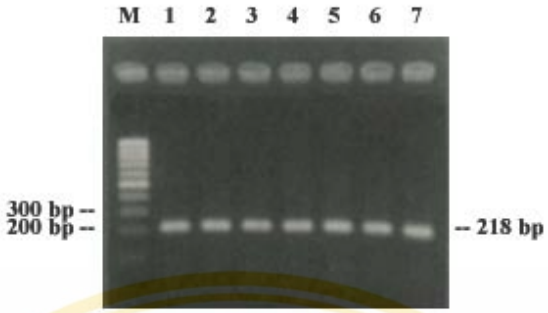

Figure 4.22 Amplification condition of exon 5 (Panel A) and ethidium bromide staining of 2% agarose gel containing 218 bp of exon 5 from different individuals (Panel B). Lane M was 100 bp ladder marker. Lanes 1 to 7 were PCR products of each test sample.

6.2 Restriction endonuclease digestion of exon 5

218 bp of PCR product was digested with a restriction enzyme, *Sty*I, producing two fragments of 76 and 142 bp as shown in Figure 4.23.

A) Restriction site for *Sty*I: 5' C↓CWWG_G 3'

CCTGGGCAGC ATGTGGATAC CATCTGATAG CGGAGGCTGC CCTGAGGTCA
TGTCGGGTCT CCCTGCAGCC TGTGACTCTG GTAGAGTGCG GACCGATGCC
CCTGACTGCT ACCTGTCTTT CCATAAGCTG CTCCTGCATC **TC↓CAAG_G**GGA
GCGAGAGTAA GTACACCACC CTGTGCCCCC ATTCCTGTCG TGCCCATCCT
GTTAGTGTGT CCACGGCC

B)

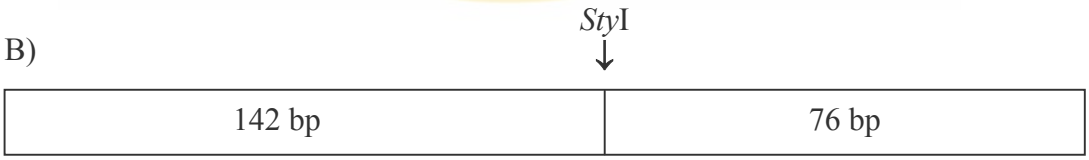

Figure 4.23 *Sty*I digestion of an amplified exon 5 of CETP gene. The nucleotide sequences of exon 5 showing the *Sty*I site (Panel A) and the map of *Sty*I digestion of exon 5 (Panel B).

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6.3 SSCP analysis of exon 5

The amplified products of exon 5 were analyzed by SSCP on 8%T, 2%C non-denaturing polyacylamide gel. There was no different SSCP pattern detected in all DNA samples under this study. A number of representative SSCP patterns are shown in Figure 4.24.

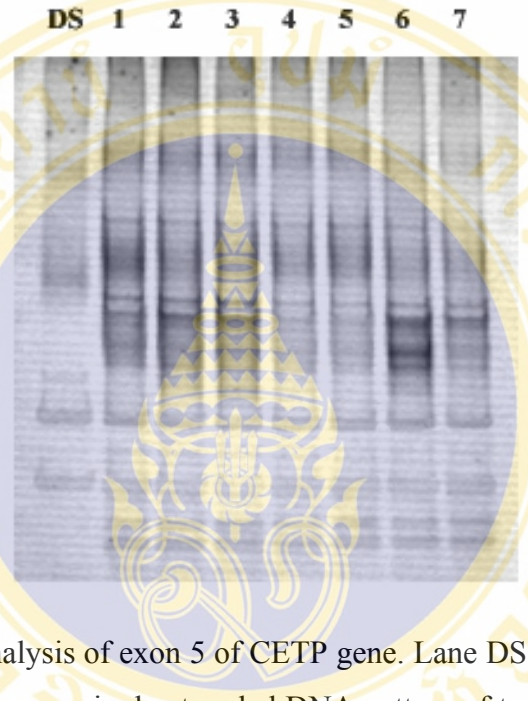

Figure 4.24 SSCP analysis of exon 5 of CETP gene. Lane DS is double stranded DNA pattern. Lanes 1 to 7 were single-stranded DNA pattern of test samples. There was no different SSCP pattern.

7. Exons 6 and 7 of CETP Gene

7.1 DNA amplification of exons 6 and 7

Exons 6 and 7 of CETP gene is consisted of 70 and 61 bp, respectively. In this study, exons 6 and 7 included with intron 6 and exon-intron junctions (492 bp) was amplified with the primers FEX67_CETP and REX67_CETP. The nucleotides sequences of these primers are as shown below. The amplification condition and the amplified products are presented in Figure 4.25.

FEX67_CETP 5’-CAC TAG GCG CTC CAT GGA TGC A-3’ = 22 bp

REX67_CETP5’-CCC AAG GCC ACA TAG TGC AGC A-3’ = 22 bp

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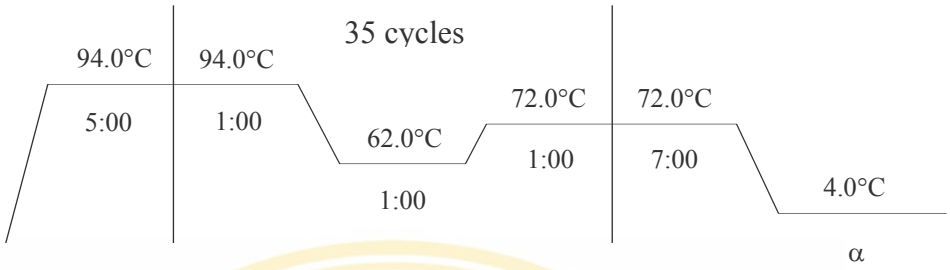
A)



B)

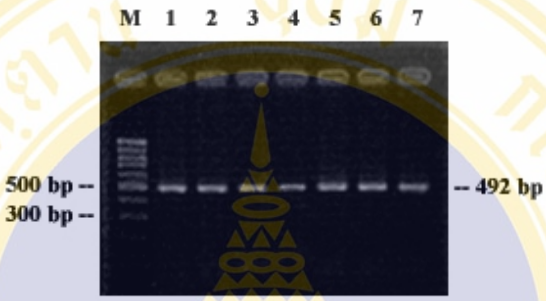


Figure 4.25 Amplification condition of exons 6 and 7 (Panel A) and ethidium bromide staining of 2% agarose gel containing exons 6 and 7 of CETP gene from different individuals (Panel B). Lane M was 100 bp ladder marker. Lanes 1 to 7 were PCR products of each test sample.

7.2 Restriction endonuclease digestion of exons 6 and 7

492 bp of PCR product was digested with a restriction enzyme, *BamH*I, revealing two fragments of 185 and 307 bp as shown in Figure 4.26.

A) Restriction site for *BamH*I: 5' G↓GATC_C 3'

CACTAGGCGC TCCATGGATG CACAGGACTG GTCAGGGGCT CATTGTGGTG
CTTGCTGCCT TCAGGCCTGG GTGGATCAAG CAGCTGTTCA CAAATTTCAT
CTCCTTCACC CTGAAGCTGG TCCTGAAGGG ACAGGTGAGT GAGGCTGGCT
GACTCCCTGT GGTCCAGGCC ATGCCCAGGA GGCT**G↓GATC_C** CTTTCCTCCC
TGCCTTTCCC TGAGAAGGTG CCACTCCCAC CTTCTCCATG TGGCCAGTCC
CCTGTGCCGG TCCCCAGCAC TGCCACCACC ACGCAGCTGG AAGGAGGCAC
TCCGTCTGGC CTCCTTTCCT GCCTGGAAAG CACCTGCTCT GTCTGCCCCA
GATCTGCAAA GAGATCAACG TCATCTCTAA CATCATGGCC GATTTTGTCC
AGACAAGGGC TGGTGAGTGC GTTTCTGTCT GCATGCCTCA GAAGACAGCA
GTGGGAGCCA GAAAGCCACC TGCTGCACTA TGTGGCCTTG GG

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B)

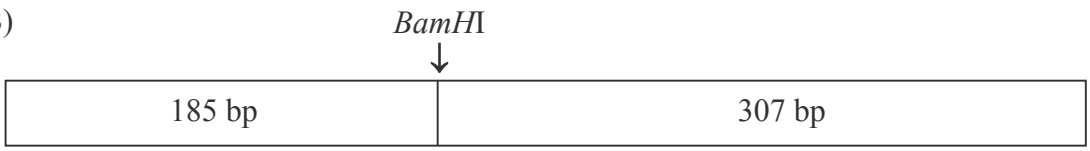

Figure 4.26 *BamH*I digestions of an amplified exons 6 and 7 of CETP gene. The nucleotide sequences of exons 6 and 7 showing the *BamH*I site (Panel A) and the map of *BamH*I digestion of exons 6 and 7 (Panel B).

7.3 SSCP analysis of exons 6 and 7

The amplified products of exons 6 and 7, after *BamH*I digestion, were analyzed by SSCP on 8%T, 2.7%C non-denaturing polyacylamide gel. Three different SSCP patterns were observed from different subjects in this region.

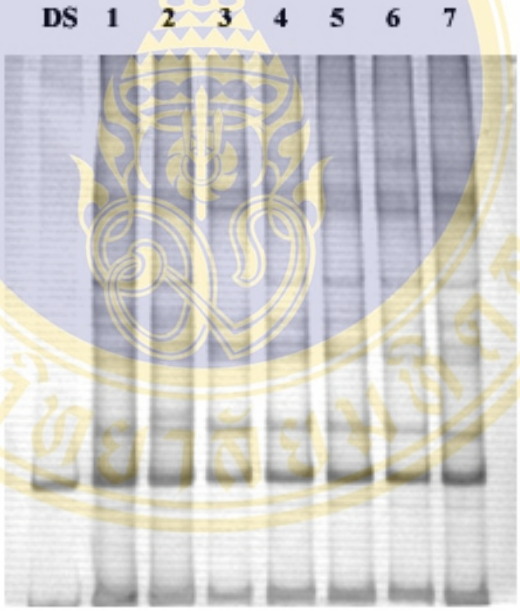

Figure 4.27 SSCP analysis of exons 6 and 7 of CETP gene. Lane DS is double stranded DNA pattern. Lanes 1 to 7 were single-stranded DNA pattern of test samples. Lanes 1, 2 and 3 were DNA samples with different SSCP patterns.

7.4 DNA sequencing of exons 6 and 7 of CETP gene

PCR fragments with different SSCP patterns were subjected to automated DNA sequencing. Two polymorphisms were founded. There are a novel heterozygous substitution in intron 6 (code: CETP_X2) and a known heterozygous T to C transition at +8/intron 7 at the position as shown in Figure 4.28.

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A) B)

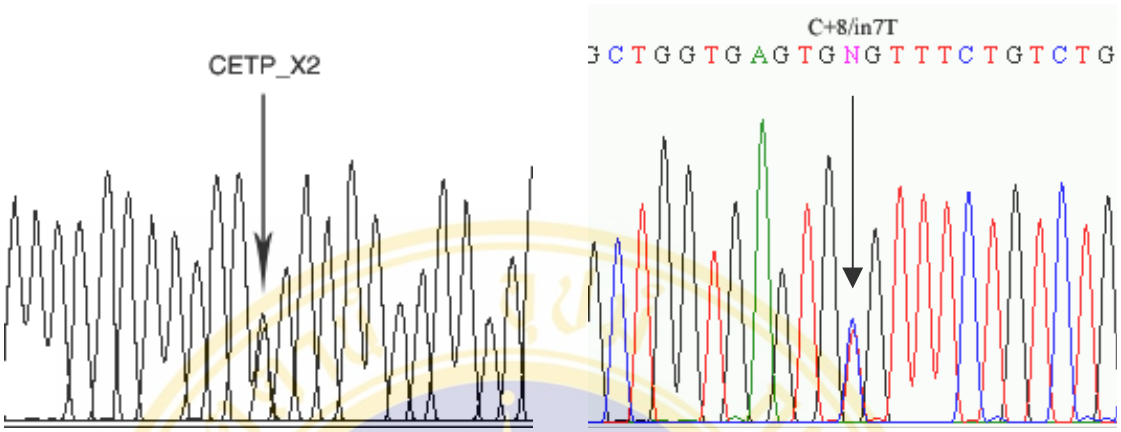


Figure 4.28 Partial DNA sequences of SSCP patterns of exons 6 and 7. The sequencing reactions were performed with both sense (FEX67_CETP) and antisense (REX67_CETP) primers. Novel heterozygous substitution in intron6 (Panel A) and heterozygous (C/T) at +8 intron7 position (Panel B) are indicated by arrows.

8. Exon 8 of CETP Gene

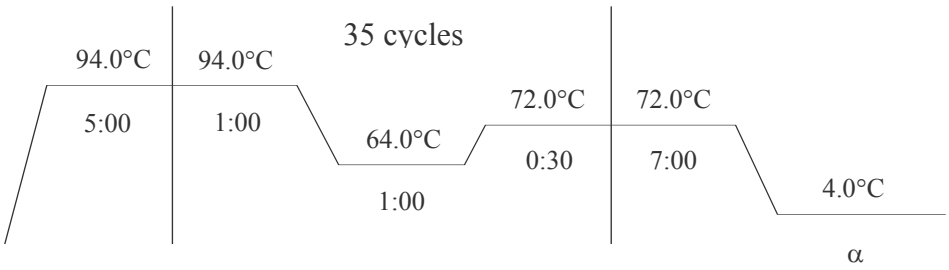
8.1 DNA amplification of exon 8

Exon 8 of CETP gene is consisted of 92 bp. In this study, exon 8 included with exon-intron junctions (253 bp) was amplified with the primers FEX8_CETP and REX8_CETP. The nucleotides sequences of these primers are as shown below. The amplification condition and the amplified products are presented in Figure 4.29.

FEX8_CETP 5'-GTG TGG ATG CAG GGG ACG GTG A-3' = 22 bp

REX8_CETP 5'-GCT GGG GGA TGG GGT TGT CAG A-3' = 22 bp

A)



Copyright by Mahidol University

B)

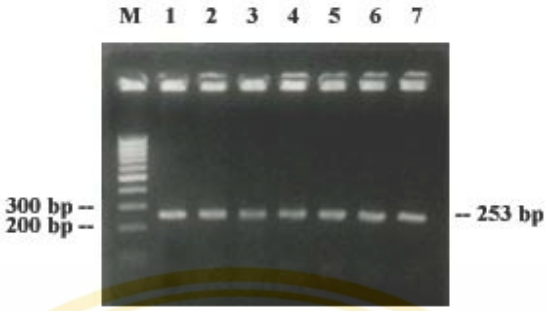


Figure 4.29 Amplification condition of exon 8 (Panel A) and ethidium bromide staining of 2% agarose gel containing 253 bp of exon 8 from different individuals (Panel B). Lane M was 100 bp ladder marker. Lanes 1 to 7 were PCR products of each test sample.

8.2 SSCP analysis of exon 8

The amplified products of exon 8 were analyzed by SSCP on 10%T, 2%C non-denaturing polyacylamide gel. There was no different SSCP pattern detected in all DNA samples under this study. A number of representative SSCP patterns are shown in Figure 4.30.

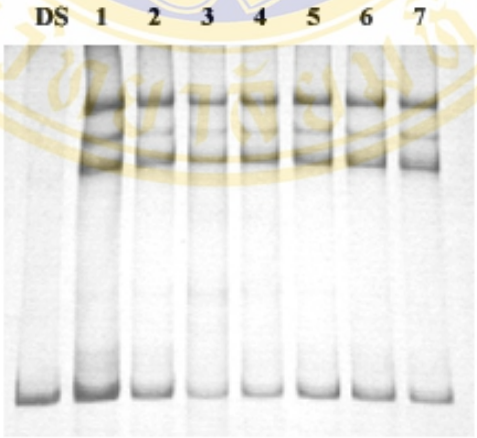


Figure 4.30 SSCP analysis of exon 8 of CETP gene. Lane DS is double-stranded DNA pattern. Lanes 1 to 7 were single-stranded DNA pattern of test samples. There was no different SSCP pattern in all DNA samples under this analysis.

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9. Exon 9 of CETP Gene

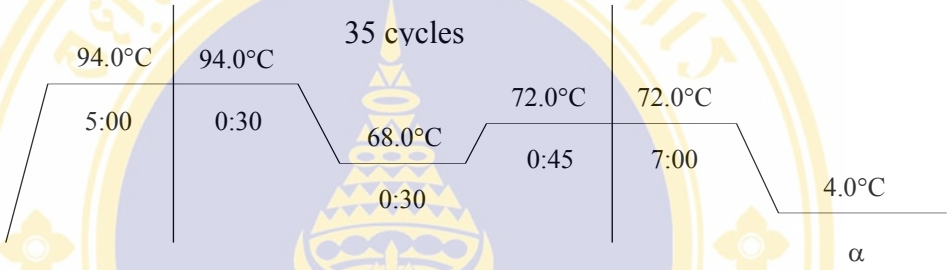
9.1 DNA amplification of exon 9

Exon 9 of CETP gene is consisted of 180 bp. In this study, exon 9 included with exon-intron junctions (332 bp) was amplified with the primers FEX9_CETP and REX9_CETP. The nucleotides sequences of these primers are as shown below. The amplification condition and the amplified products are presented in Figure 4.31.

FEX9_CETP 5'-GGG CTC CTC CCA ATC TCC CTG A-3' = 22 bp

REX9_CETP 5'-CAT GCG TGT GCA CAC ACA CAC A-3' = 22 bp

A)



B)

Figure 4.31 Amplification condition of exon 9 (Panel A) and ethidium bromide staining of 2% agarose gel containing 332 bp of exon 9 from different individuals (Panel B). Lane M was 100 bp ladder marker. Lanes 1 to 7 were PCR products of each test sample.

9.2 Restriction endonuclease digestion of exon 9

332 bp of PCR product was digested with a restriction enzyme, *Sph*I, producing two fragments of 163 and 169 bp as shown in Figure 4.32.

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A) Restriction site for *Sph*I: 5' G_CATG↓C 3'

GGGCTCCTCC	CAATCTCCCT	GAAGCTGGAC	CTGAGCCCAG	TAGGGACACA
CAGGGTCCAG	CCAGCGTCCT	GGCTTCCTCC	AGGGTCATTT	CATCTACAAG
AATGTCTCAG	AGGACCTCCC	CCTCCCCACC	TTCTCGCCCA	CACTGCTGGG
GGACTCCC**G_C**	**ATG↓C**TGTACT	TCTGGTTCTC	TGAGCGAGTC	TTCCACTCGC
TGGCCAAGGT	AGCTTTCCAG	GATGGCCGCC	TCATGCTCAG	CCTGATGGGA
GACGAGTTCA	AGGTGAGTGG	GTGGGGCTGG	GCTGCTAGGG	GATCCAGATG
GCATGTGGTA	TGTGTGTGTG	TGCACACGCA	TG	

B)

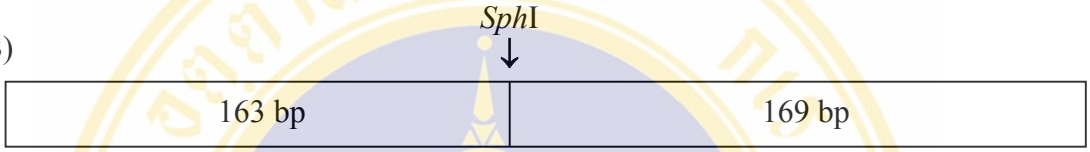

Figure 4.32 *Sph*I digestion of an amplified exon 9 of CETP gene. The nucleotide sequences of exon 9 showing the *Sph*I site (Panel A) and the map of *Sph*I digestion of exon 9 (Panel B).

9.3 SSCP analysis of exon 9

The amplified products of exon 9 were analyzed by SSCP on 8%T, 2.7%C non-denaturing polyacylamide gel. Two different SSCP patterns were observed in different subjects in this region.

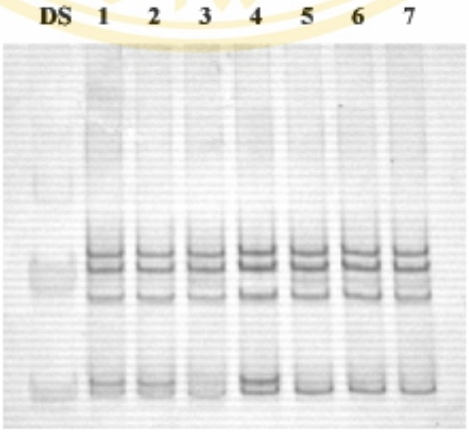

Figure 4.33 SSCP analysis of exon 9 of CETP gene. Lane DS is double-stranded DNA pattern. Lanes 1 to 7 were single-stranded DNA patterns of test samples. Lanes 2 and 3 were DNA samples with different SSCP patterns.

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9.4 DNA sequencing of exon 9 of CETP gene

Amplified exon 9 with different SSCP patterns were subjected to automated DNA sequencing. Two polymorphisms were observed in one DNA sample. There are heterozygous G to T transversion at +24/intron 9 position and heterozygous A to G transition at +29/intron 9 position as shown in Figure 4.34.

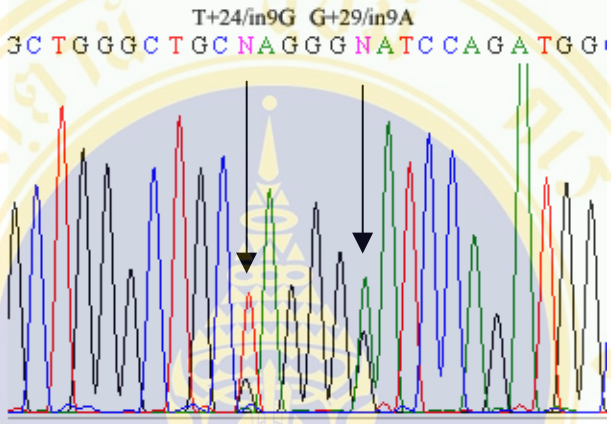

Figure 4.34 Partial DNA sequence of SSCP pattern of exon 9. The sequencing reactions were performed with both sense (FEX9_CETP) and antisense (REX9_CETP) primers. Heterozygous (T/G) at +24 intron9 position and heterozygous (G/A) at +29 intron 9 position are indicated by arrows with N.

10. Exon 10 of CETP Gene

10.1 DNA amplification of exon 10

Exon 10 of CETP gene is consisted of 51 bp. In this study, exon 10 included with exon-intron junctions (208 bp) was amplified with the primers FEX10_CETP and REX10_CETP. The nucleotide sequences of these primers are as shown below. The amplification condition and the amplified products are presented in Figure 4.35.

FEX10_CETP 5'-AAC TGC CCT TGG TCC CTG CGA A-3' = 22 bp

REX10_CETP5'-TGT GGG GGC TCT TGA AGC CAG A-3' = 22 bp

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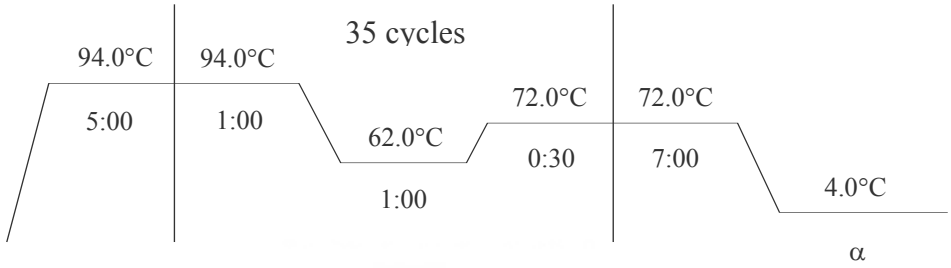
A)



B)

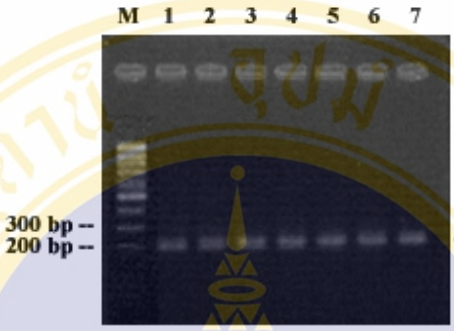


Figure 4.35 Amplification condition of exon 10 (Panel A) and ethidium bromide staining of 2% agarose gel containing 208 bp of exon 10 from different individuals (Panel B). Lane M was 100 bp ladder marker. Lanes 1 to 7 were PCR products of each test sample.

10.2 SSCP analysis of exon 10

The amplified products of exon 10 were analyzed by SSCP on 10%T, 2.7%C non-denaturing polyacylamide gel. There was no different SSCP pattern detected in all DNA samples under this study. A number of representative SSCP patterns are shown in Figure 4.36.

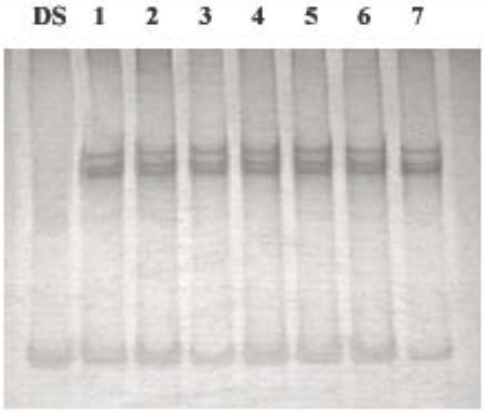


Figure 4.36 SSCP analysis of exon 10 of CETP gene. Lane DS is double-stranded DNA pattern. Lanes 1 to 7 were single-stranded DNA patterns of test samples. There was no different SSCP pattern in all DNA samples under this analysis.

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11. Exon 11 of CETP Gene

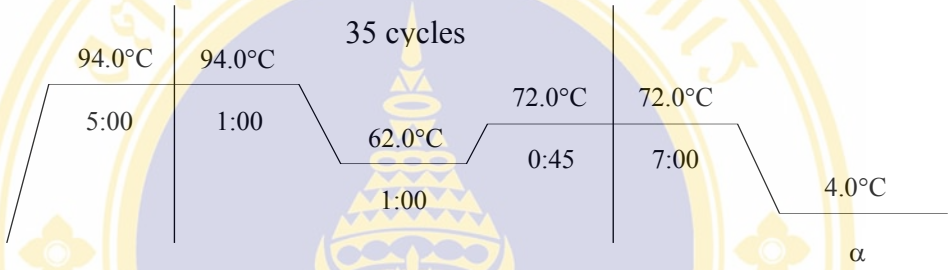
11.1 DNA amplification of exon 11

Exon 11 of CETP gene is consisted of 165 bp. In this study, exon 11 included with exon-intron junctions (355 bp) was amplified with the primers FEX11_CETP and REX11_CETP. The nucleotides sequences of these primers are as shown below. The amplification condition and the amplified products are presented in Figure 4.37.

FEX11_CETP 5'-TTC CCA TCT CCG AGG GCA TGG A-3' = 22 bp

REX11_CETP5'-CCA GCA GAG GTG GTG AGA AGG A-3' = 22 bp

A)



B)

Figure 4.37 Amplification condition of exon11 (Panel A) and ethidium bromide staining of 2% agarose gel containing 355 bp of exon 11 from different individuals (Panel B). Lane M was 100 bp ladder marker. Lanes 1 to 7 were PCR products of each test sample.

11.2 Restriction endonuclease digestion of exon 11

The 355 bp of PCR product of exon 11 was digested with a restriction enzyme, *Sml*I. The digestion produced 122 and 233 bp fragments as shown in Figure 4.38.

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A) Restriction site for *Sml*I: 5' C↓TYRA_G 3'

TTCCCATCTC CGAGGGCATG GACTGCTGCT CCGTGATGGG CCCCTGTCCT
GGCCATGGGA CCCCTGTCTT CCACAGGTTG TCGGCGGCTT CCCCAGCCAG
GCCCAAGTCA CCGTCCACTG **CC↓TCAA_G**ATG CCCAAGATCT CCTGCCAAAA
CAAGGGAGTC GTGGTCAATT CTTCAGTGAT GGTGAAATTC CTCTTTCCAC
GCCCAGACCA GCAACATTCT GTAGCTTACA CATTTGAAGA GGTGAGGCGG
GTGCAGGGAG AGGTGGTGGT GGGGGAACTG ACTCACATAT GGGCCGCAGA
GGGCAGGGGC TGGGGGTCTC TGAAGCCTCC AGATCCTTCT CACCACCTCT
GCTGG

B)

Figure 4.38 *Sml*I digestion of an amplified exon 11 of CETP gene. The nucleotide sequences of exon 11 showing the *Sml*I site (Panel A) and the map of *Sml*I digestion of exon 11 (Panel B).

11.3 SSCP analysis of exon 11

The amplified products of exon 11 were analyzed by SSCP on 8%T, 2%C non-denaturing polyacylamide gel. There was no different pattern detected in all DNA samples under this study. A number of representative SSCP patterns are shown in Figure 4.39.

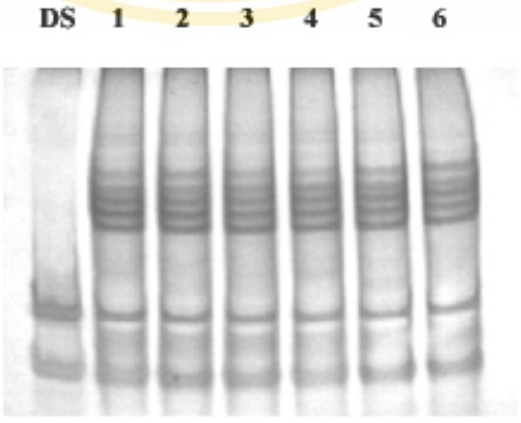

Figure 4.39 SSCP analysis of exon 11 of CETP gene. Lane DS is double-stranded DNA pattern. Lanes 1 to 7 were single-stranded DNA pattern of test samples. There was no different SSCP pattern in all DNA samples under this analysis.

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12. Exon 12 of CETP Gene

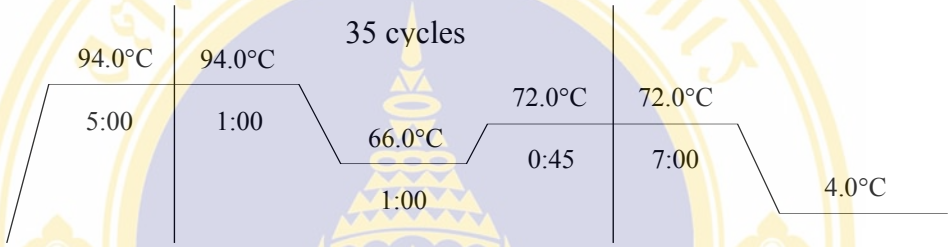
12.1 DNA amplification of exon 12

Exon 12 of CETP gene is consisted of 68 bp. In this study, exon 12 included with exon-intron junctions (307 bp) was amplified with the primers FEX12_CETP and REX12_CETP. The nucleotides sequences of these primers are as shown below. The amplification condition and the amplified products are presented in Figure 4.40.

FEX12_CETP 5'-AAT CAG GGG CCC TGA GCT AGG A-3' = 22 bp

REX12_CETP5'-GCC CCA GAA GAA AGG GGC CAC A-3' = 22 bp

A)



B)

Figure 4.40 Amplification condition of exon 12 (Panel A) and ethidium bromide staining of 2% agarose gel containing 307 bp of exon 12 from different individuals (Panel B). Lane M was 100 bp ladder marker. Lanes 1 to 7 were PCR products of each test sample.

12.2 SSCP analysis of exon 12

The amplified products of exon 12 were analyzed by SSCP on 8%T, 2.7%C non-denaturing polyacylamide gel. There was no different pattern detected in all DNA samples under this study. A number of representative SSCP patterns are shown in Figure 4.41.

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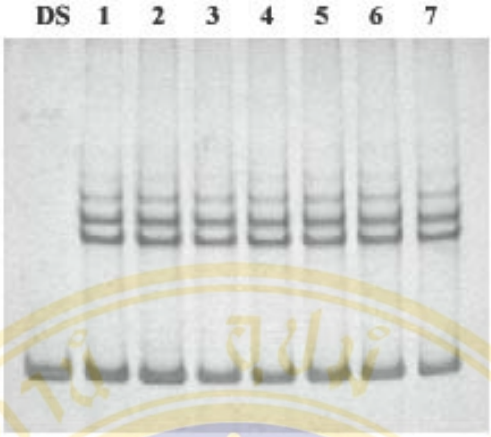


Figure 4.41 SSCP analysis of exon 12 of CETP gene. Lane DS is double-stranded DNA pattern. Lanes 1 to 7 were single-stranded DNA pattern of test samples. There was no different SSCP pattern in all DNA samples under this study.

13. Exon 13 of CETP Gene

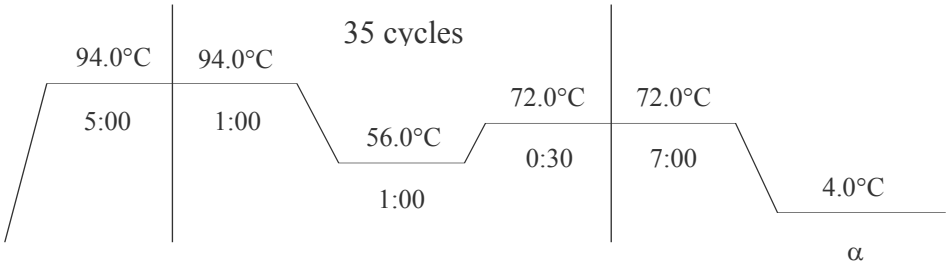
13.1 DNA amplification of exon 13

Exon 13 of CETP gene is consisted of 34 bp. In this study, exon 13 included with exon-intron junctions (243 bp) was amplified with the primers FEX13_CETP and REX13_CETP. The nucleotide sequences of these primers are as shown below. The amplification condition and the amplified products are presented in Figure 4.42.

FEX13_CETP 5'-GAG ACA AAA GCA CTG GCT GCT A-3' = 22 bp

REX13_CETP5'-TTC TTT TGG TCT GGT TGC CTG A-3' = 22 bp

A)



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B)

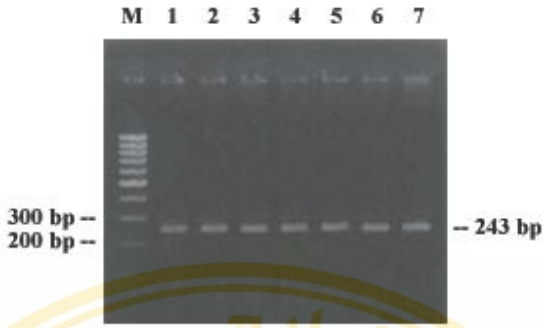

Figure 4.42 Amplification condition of exon 13 (Panel A) and ethidium bromide staining of 2% agarose gel containing 243 bp of exon 13 from different individuals (Panel B). Lane M was 100 bp ladder marker. Lanes 1 to 7 were PCR products of each test sample.

13.2 SSCP analysis of exon 13

The amplified products of exon 13 were analyzed by SSCP on 8%T, 2.7%C non-denaturing polyacylamide gel. There was no different pattern detected in all DNA samples under this study. A number of representative SSCP patterns are shown in Figure 4.43.

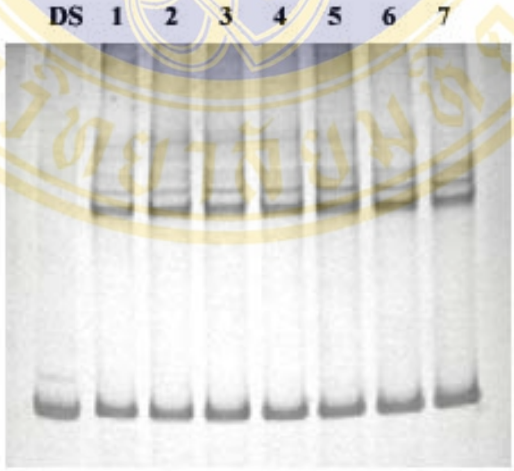

Figure 4.43 SSCP analysis of exon 13 of CETP gene. Lane DS is double-stranded DNA pattern. Lanes 1 to 7 were single-stranded DNA patterns of test samples. There was no different pattern in all DNA sample under this study.

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14. Exon 14 of CETP Gene

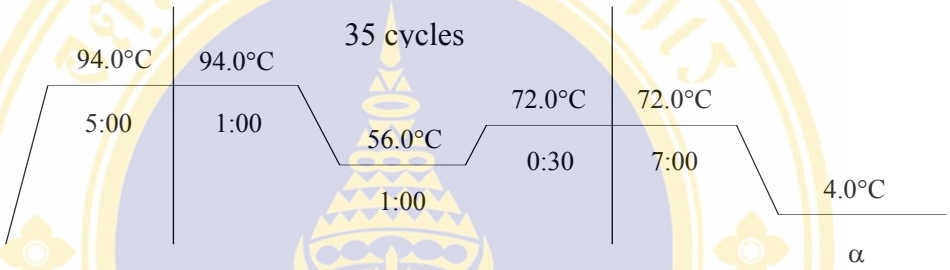
14.1 DNA amplification exon 14

Exon 14 of CETP gene is consisted of 73 bp. In this study, exon 14 included with exon-intron junctions (226 bp) was amplified with the primers FEX14_CETP and REX14_CETP. The nucleotide sequences of these primers are as shown below. The amplification condition and the amplified products are presented in Figure 4.44.

FEX14_CETP 5'-CAT GAG GAT GAA TGC TTG TCC A-3' = 22 bp

REX14_CETP5'-GGT GAA ATG GGA AGC TCT GTC A-3' = 22 bp

A)



B)

Figure 4.44 Amplification condition of exon 14 (Panel A) and ethidium bromide staining of 2% agarose gel containing 226 bp of exon 14 from different individuals (Panel B). Lane M was 100 bp ladder marker. Lanes 1 to 7 were PCR products of each test sample.

14.2 Restriction endonuclease digestion of exon 14

The 226 bp of PCR product of exon 14 was digested with a restriction enzyme, *Pst*I, producing two fragments of 100 and 126 bp as shown in Figure 4.45.

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A) Restriction site for *Pst*I: 5' C_TGCA↓G 3'

CATGAGGATG	AATGCTTGTC	CAGGCCGTGC	AGCATCTGCC	TTGTGGGTCA
CTTCTGTGCT	CCAGGGAGGA	CTCACCATGG	GCATTTGATT	GCAGAGCAGC
TCCGAGTCCG	TCCAGAGCTT	C**C_TGCA↓G**TCA	ATGATCACCG	CTGTGGGCAT
CCCTGAGGTC	ATGTCTCGTA	AGTGTGGGCT	GGAGGGGAAA	CTGGGTGCCG
AGGCTGACAG	AGCTTCCCAT	TTCACC		

B)

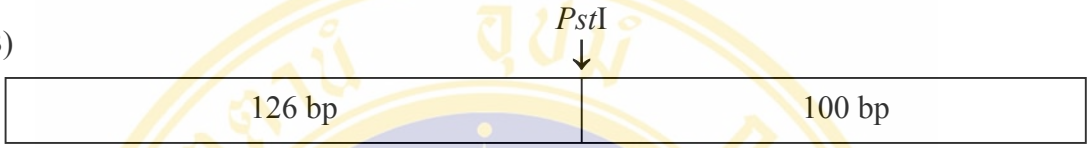

Figure 4.45 *Pst*I digestion of an amplified exon 14 of CETP gene. The nucleotide sequences of exon 14 showing the *Pst*I site (Panel A) and the map of *Pst*I digestion of exon 14 (Panel B).

14.3 SSCP analysis of exon 14

The amplified products of exon 14 with and without restriction enzyme digestion were analyzed by SSCP on 10%T, 2.7%C and 8%T, 2.7%C non-denaturing polyacylamide gel, respectively. There was no different SSCP pattern detected in all DNA samples under this study. A number of representative SSCP patterns are shown in Figure 4.46.

A) B)

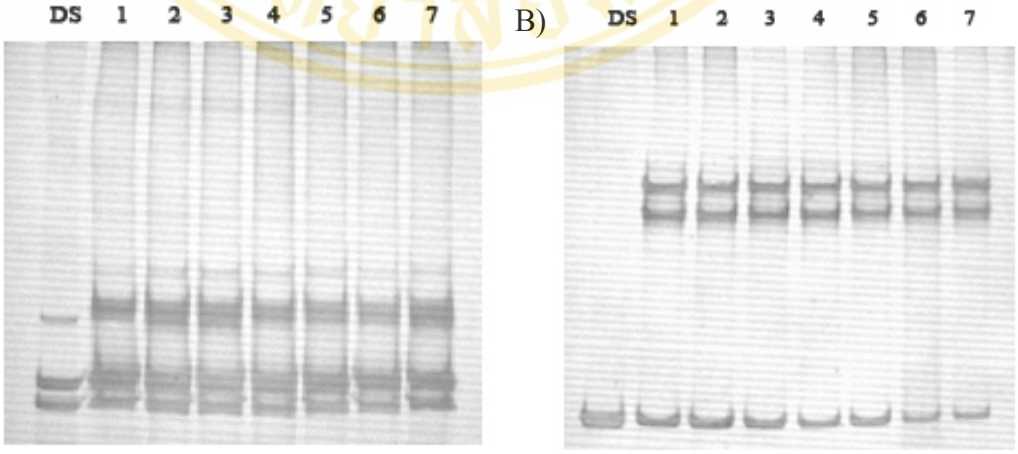

Figure 4.46 SSCP analysis of exon 14 of CETP gene with (Panel A) and without (Panel B) restriction enzyme, PstI, digestion. Lane DS is double-stranded DNA pattern. Lanes 1 to 7 were single-stranded DNA patterns of test samples. There was no different SSCP pattern in all DNA samples under this analysis.

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14.4 Pooled DNA sequencing of exon 14 of CETP gene

This experiment was an attempt to identify SNPs by direct DNA sequencing as an alternative method of PCR-SSCP and DNA sequencing. This method was tried to accelerate the identification of SNPs in this gene. Pooled DNA of twenty samples was used as template for automated DNA sequencing. Heterozygous A to G transition at first base of codon 405 was founded as shown in Figure 4.47. This A to G transition causes the substitution of valine (GTC) with isoleucine(ATC) at codon 405 of CETP.

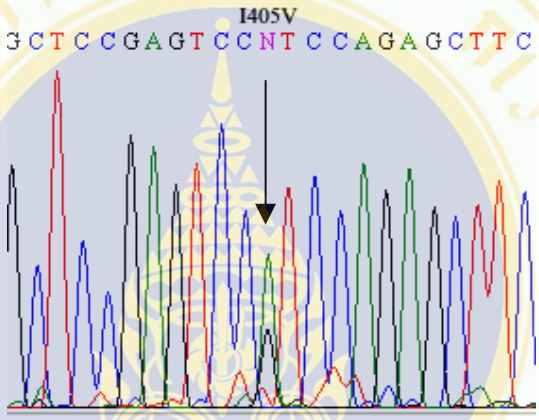

Figure 4.47 Partial pooled DNA sequence of exon 14. The sequencing reactions were performed with both sense (FEX14_CETP) and antisense (REX14_CETP) primers. Heterozygous (A/G) at first nucleotide of condon 405 is indicated by an arrow with N.

15. Exon 15 of CETP Gene

15.1 DNA amplification of exon 15

Exon 15 of CETP gene is consisted of 86 bp. In this study, exon 15 included with exon-intron junctions (269 bp) was amplified with the primers FEX15_CETP and REX15_CETP. The nucleotide sequences of these primers are as shown below. The amplification condition and the amplified products are presented in Figure 4.48.

FEX15_CETP 5’-CTC CCA CTA CCC AGG GTG CAG A-3’ = 22 bp

REX15_CETP5’-GCC CCT CTG TCT GTC TCC CCA A-3’ = 22 bp

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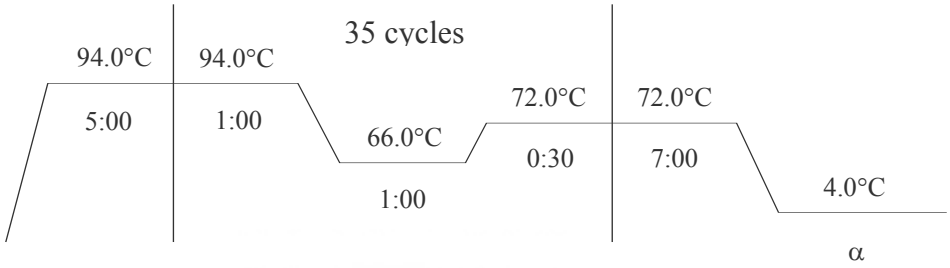
A)



B)

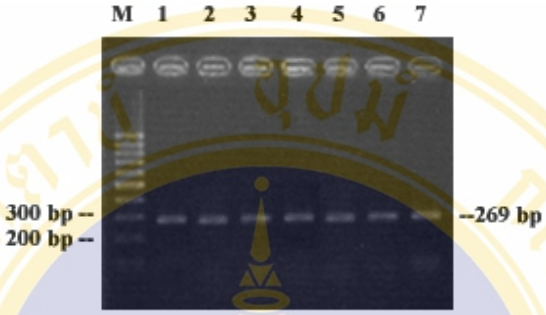


Figure4.48 Amplification condition of exon 15 (Panel A) and ethidium bromide staining of 2% agarose gel containing 269 bp of exon 15 from different individuals (Panel B). Lane M was 100 bp ladder marker. Lanes 1 to 7 were PCR products of each test sample.

15.2 SSCP analysis of exon 15

The amplified products of exon 15 were analyzed by SSCP on 10%T, 2%C non-denaturing polyacylamide gel. A different SSCP pattern was observed in this region as shown in Figure 4.49.

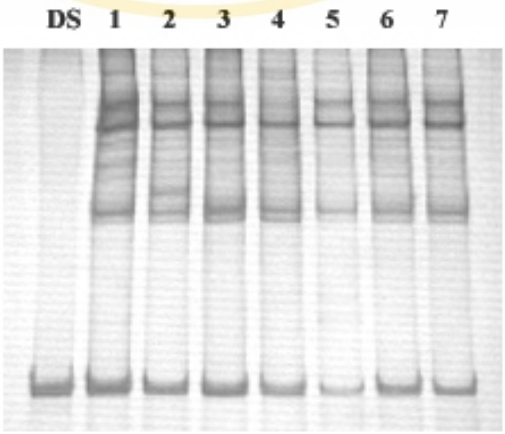


Figure 4.49 SSCP analysis of exon 15 of CETP gene. Lane DS is double-stranded DNA pattern. Lanes 1 to 7 were single-stranded DNA patterns of test samples. Lane 2 was DNA sample with different SSCP pattern.

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15.3 DNA sequencing of exon 15 of CETP gene

The amplified fragment with different pattern was subjected to automated DNA sequencing. Heterozygous A to G transition at second base of codon 451 was founded as shown in Figure 4.50. This A to G transition causes the substitution of glutamine (CAA) with arginine (CGA) at codon 451 of CETP.

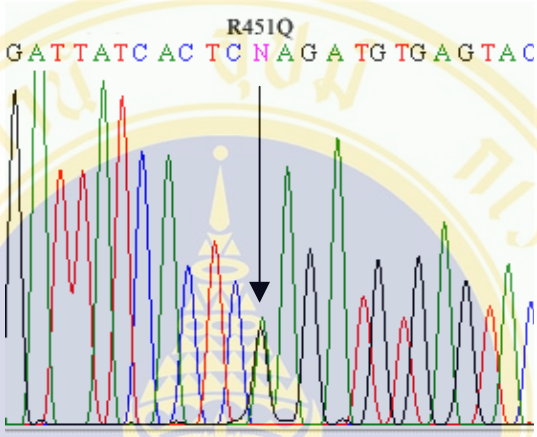

Figure 4.50 Partial DNA sequence of SSCP pattern of exon 15. The sequencing reactions were performed with both sense (FEX15_CETP) and antisense (REX15_CETP) primers. Heterozygous (A/G) at second nucleotide of condon 451 is indicated by an arrow with N.

16. Exon 16 of CETP Gene

16.1 DNA amplification of exon 16

Exon 16 of CETP gene is consisted of 253 bp. In this study, exon 16 included with exon-intron junctions (435 bp) was amplified with the primers FEX16_CETP and REX16_CETP. The nucleotide sequences of these primers are as shown below. The amplification condition and the amplified products are presented in Figure 4.51.

FEX16_CETP 5’-ACA GAC AGA GGG GCC TCT ACC A-3’ = 22 bp

REX16_CETP5’-GGA AGG GCT GAA AAG AGG TGG A-3’ = 22 bp

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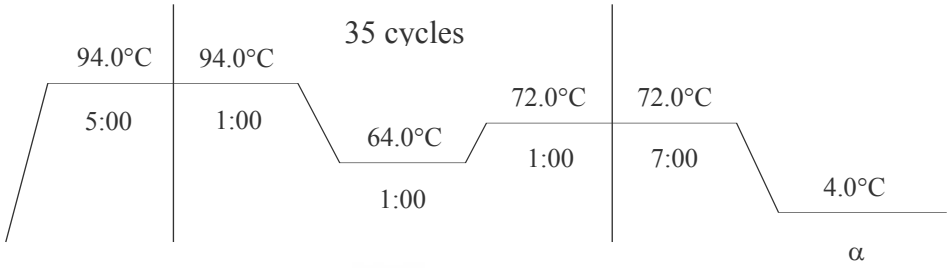
A)



B)

Figure 4.51 Amplification condition of exon 16 (Panel A) and ethidium bromide staining of 2% agarose gel containing 435 bp of exon 16 from different individuals (Panel B). Lane M was 100 bp ladder marker. Lanes 1 to 7 were PCR products of each test sample.

16.2 Restriction endonuclease digestion of exon 16

The 435 bp of PCR product of exon 16 was digested with a restriction enzyme, *Sty*I, producing two fragments of 174 and 261 bp as shown in Figure 4.52.

A) Restriction site for *Sty*I: 5' C↓CWWG_G 3'

ACAGACAGAG GGGCCTCTAC CAGCTTGGCT CCCTCCTGGT GGCCTGGGAG
TCAGCCCAGC TCGCCCCTCT CTCCTACTGC CCCTCCCTTC AGGGCTTCCT
GCTGCTGCAG ATGGACTTTG GCTTCCCTGA GCACCTGCTG GTGGATTTCC
TCCAGAGCTT GAGCTAGAAG TCT**C↓CAAG_G**A GGTCGGGATG GGGCTTGTAG
CAGAAGGCAA GCACCAGGCT CACAGCTGGA ACCCTGGTGT CTCCTCCAGC
GTGGTGGAAG TTGGGTTAGG AGTACGGAGA TGGAGATTGG CTCCCAACTC
CTCCCTATCC TAAAGGCCCA CTGGCATTAA AGTGCTGTAT CCAAGAGCTG
CGGAGTCCTT CTTCTGTGGC TGGCGGGTAG AGGGGGGGGG AAGGGATTGT
CTCACCAGTG CCGTCCACCT CTTTTCAGCC CTTCC

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B)

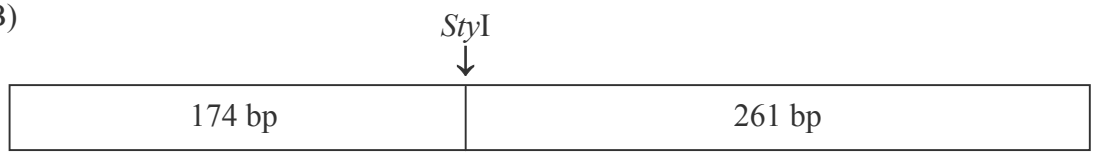

Figure 4.52 *Sty*I digestion of an amplified exon 16 of CETP gene. The nucleotide sequences of exon 16 showing the *Sty*I site (Panel A) and the map of *Sty*I digestion of exon 16 (Panel B).

16.3 SSCP analysis of exon 16

The amplified products of exon 16 were analyzed by SSCP on 8%T, 2%C non-denaturing polyacylamide gel. Three different SSCP patterns were observed in different subjects in this region.

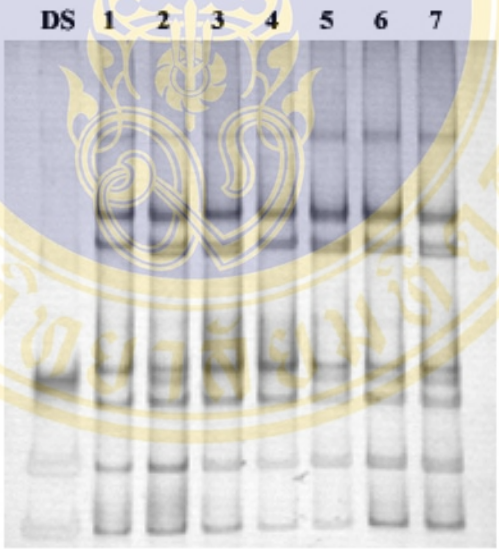

Figure 4.53 SSCP analysis of exon 16 of CETP gene. Lane DS is double-stranded DNA pattern. Lanes 1 to 7 were single-stranded DNA patterns of test samples. Lanes 1, 2 and 3 were DNA samples with different SSCP patterns.

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16.4 DNA sequencing of exon 16 of CETP gene

PCR fragment with different SSCP patterns were subjected to automated DNA sequencing. Four polymorphism positions were founded. There are heterozygous G to A transition at -30/intron 15 position, heterozygous A to G transition at +84 position, heterozygous C to G transversion at +184 position and heterozygous G to A transition at +218 position in 3' region as shown in Figure 4.54.

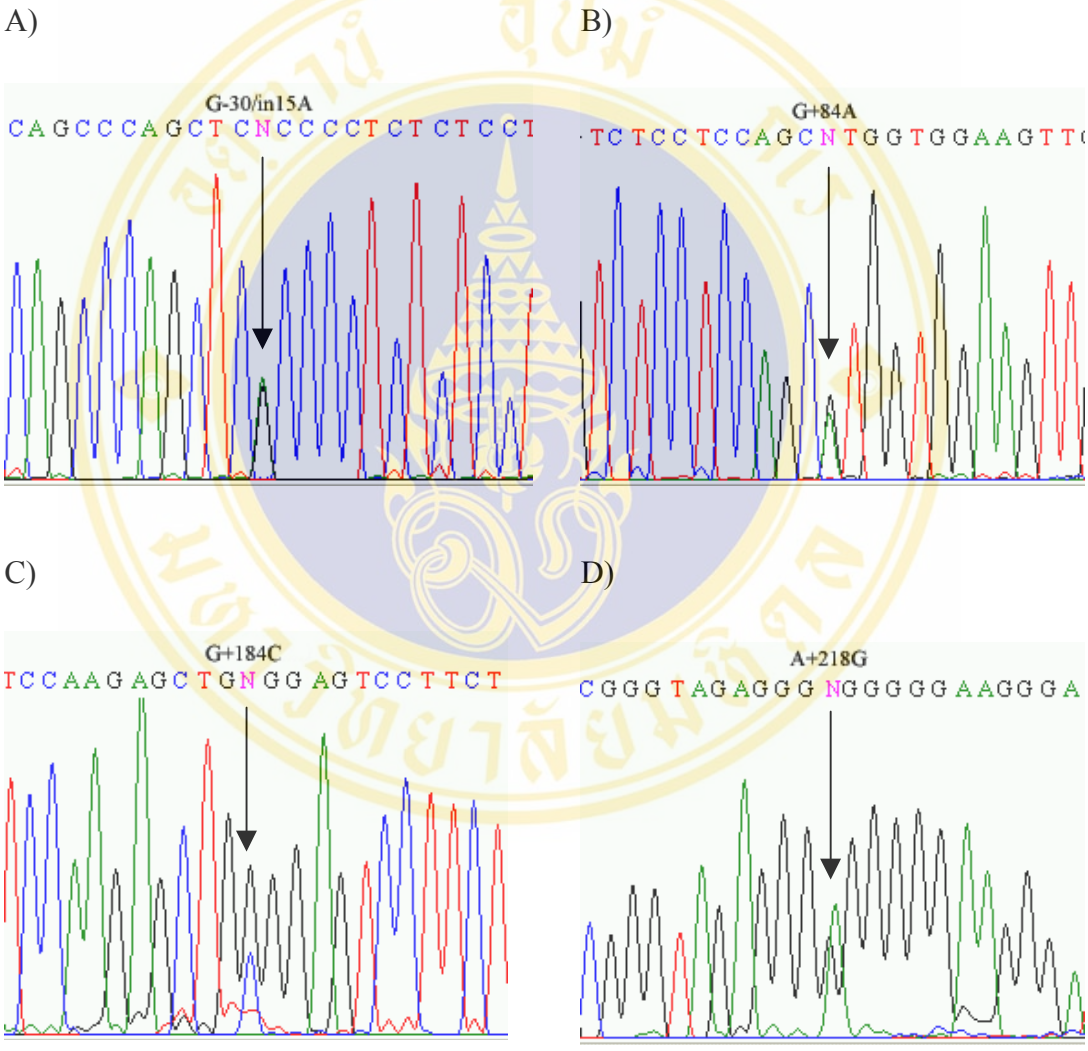

Figure 4.54 Partial DNA sequences of SSCP patterns of exon 16. The sequencing reactions were performed with both sense (FEX16_CETP) and antisense (REX16_CETP) primers. Heterozygous (G/A) at -30 intron15 position
B) Heterozygous (A/G) at nucleotide +84 of 3' region C) Heterozygous (C/G) at +184 position of 3' region D) Heterozygous (G/A) at +218 position of 3' region. All of the heterozygous positions are indicated by arrows with N.

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CHAPTER V
DISCUSSION

CETP is a plasma glycoprotein that plays an important role in reverse cholesterol transport (RCT) pathway. It enhances the transfer and exchange of CE and TG between lipoproteins. In RCT pathway, CETP acts as an antiatherogenic factor. However, it also acts as a proatherogenic factor by depleting plasma HDL-C levels and increasing CE content in all apoB-containing lipoproteins. So, it is speculated that un-equilibration of CETP activity and concentration can induce cardiovascular phenotype.

Several studies have demonstrated the effect of some polymorphisms and mutations in CETP gene on CETP activity and concentration, size of HDL and LDL particles, and HDL-C levels. These associations, however, are population specific. This thesis research was assigned to identify SNPs in this CETP gene in our population.

In the future, the effect of these identified SNPs on various cardiovascular phenotypes will be investigated using association analysis.

Methods

Single nucleotide polymorphisms (SNPs) are the most frequent form of DNA variation. Due to their abundance and slow mutation rate within generations, they are thought to be the next generation of genetic markers that can be used in a myriad of important biological, genetic, pharmacological, and medical applications(95). There are several strategies for SNP discovery and mapping. These strategies include restriction fragment length polymorphism (RFLP) analysis, single strand conformation polymorphism (SSCP) analysis, and DNA sequencing method (96). RFLP is a conventional method in which changing bases are searched at random. SSCP is a favoring method for detection of unknown nucleotide sequence polymorphism by phenomenon of mobility shift. The mobility shift is probably due to a conformational change of single-stranded DNAs caused by a single nucleotide substitution on

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polyacrylamide gel electrophoresis (97). Automated DNA sequencing is the most sensitive method for detection of altering base but it is an expensive process. The method currently being used to discover SNPs involves the large-scale repetitive sequencing of cloned DNA segments from pools of 10-50 subjects, followed by computer alignment of multiple sequences to detect positions where deviation in the sequence between subjects occurs(98). In this study, we tried to identify SNPs by sequencing of pooled DNA from 20 samples. The data show that only SNPs with high-allele frequency were identified while SNPs with low-allele frequency may be lost (Data not shown). As described above, DNA sequencing of each sample, however, consumes considerably high cost. Thus, under limited cost, PCR-SSCP was used for SNPs discovery in this study. Nevertheless, SSCP sensitivity is approximately 80%. So, some SNPs are possibly missed in SSCP analysis. As exemplified in exon 14, a SNP was yet discovered by DNA sequencing albeit no abnormal SSCP pattern was observed in this exon. Furthermore, this study did attempt to increase our SSCP sensitivity by using more than one SSCP conditions for each amplified DNA fragments under analysis. The high-allele frequency SNPs such as I405V was detected by pooled DNA sequencing method but it was not detected by SSCP analysis. This missing may be due to the un-sensitive condition of SSCP analysis. Nevertheless, no single genotyping method is ideally suited for all application (99). So, SSCP is still the best method for this kind of study.

Results

Sixty-six alleles of Thai subjects (n=33) were used to identify SNPs of CETP gene by PCR-SSCP analysis and automated DNA sequencing method. Thirteen SNPs were found in this study as shown in Table 5.1 and Figure 5.1.

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Table5.1 SNPs of CETP gene

Polymorphism	Position	Type of variation
C-629A	5’	C/A
G+279/in1A (TaqIB)	Intron1	G/A
CETP_X1	Exon3	X1
CETP_X2	Intron6	X2
C+8/in7T	Intron7	C/T
T+24/in9G	Intron9	T/G
G+29/in9A	Intron9	G/A
I405V	Exon14	G/A
R451Q	Exon15	G/A
G-30/in15A	Intron15	G/A
G+84A	3’	G/A
G+184C	3’	G/C
A+218G	3’	A/G

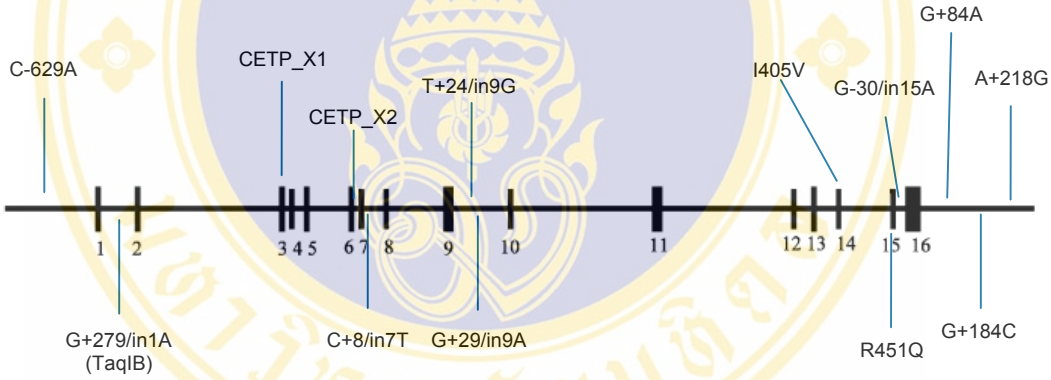

Figure 5.1 SNPs of CETP gene and gene structure.

Because of the important role of CETP in lipid metabolisms and modulations, the CETP gene has gained considerable interest ever since its discovery. Thus, it should not be surprising that we found only one novel exonic SNP, CETP_X1 in exon 3, and one novel intronic SNP, CETP_X2, while others SNPs are known polymorphisms.

CETP_X1 polymorphism is predicted to cause a nonconservative substitution in highly conservative position. CETP_X2 is located in intron 6. This position is probably a functional SNP because it is close to exon-intron junction region. The effect of these two polymorphisms will be studied in the future.

For known polymorphisms generally available in public domain, both functional SNPs and those with unknown function were discovered in CETP gene.

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Functional SNPs in CETP locus may be divided into two groups: those which are associated with low and high CETP activity and/ or mass. Some of these SNPs may effect HDL-C levels. First group such as C-629A, TaqIB and I405V are associated with lower CETP mass and higher HDL-C levels. Second group such as R451Q and A373P are shown to be associated with increase CETP activity. In this study, R451Q was also observed in our subjects. Many SNPs in introns and 3' region: C+8/in7T, T+24/in9G, G+29/in9A, G-30/in15A, G+84A, G+184C and A+218G discovered in this study are of unknown function at present.

Nearly all SNPs discovered in this study are known SNPs. The allele frequency in Thai population will be validated later for future association analysis.

Moreover, not only SNPs were identified but the other genetic variation, i.e., deletion, was also detected in this study. The heterozygous 18 nucleotides deletion in untranslated region of exon 1 was found in one subject with high HDL-C level (119 mg/dl). The result was revealed as scrambled DNA sequence data by automated DNA sequencing analysis and finally by manual DNA sequencing specifically for the deleted allele. This deletion may be possibly implicated in high HDL-C level in this subject.

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CHAPTER VI
CONCLUSION

Function of CETP is to transfer and exchange CE and TG among lipoproteins, therefore, CETP gene has been considered as one candidate gene of CHD. SNPs map of this candidate gene has been expected to be used as genetic markers involving in disease phenotype of CHD. In this study, polymorphisms of this gene were identified by PCR-SSCP and direct DNA sequencing techniques. Thirteen SNPs of CETP gene were identified from 33 Thai subjects as shown in Table 5.1. Eleven positions are known SNPs which have been publicized on databases (NCBI and GeneCanvas). Two positions are novel SNPs that may be population specific. One of them is predicted to change an amino acid at highly conservative position in exon 3. Another one causes a base substitution in intron 6 next to the intron-exon junction. The functional consequences of these novel SNPs will be proved in the future.

All of these SNPs will be validated for allele frequency and then tested directly for association to clinical phenotypes. The data will be used as part of a potential genetic panel to evaluate genetic predisposition to develop CHD in our population. Moreover, variations on CETP gene may be likely useful in pharmacogenetics study in the future.

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