A STUDY ON THE GENETIC POLYMORPHISM OF PARAOXONASE 1 IN THAI POPULATION

ARTHIT SRIPIROM

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Entitled

A STUDY ON THE GENETIC POLYMORPHISM OF PARAOXONASE 1 IN THAI POPULATION

Arthit Sripirom
.....
Mr.Arthit Sripirom
Candidate

.....
Assoc.Prof.Winai Wananukul,
M.D.,F.R.C.P.
Major-Advisor

.....
Assoc.Prof.Thanyachai Sura,
M.D.,F.R.C.P.
Co-Advisor

Krongtong Yoovathaworn
.....
Assoc.Prof.Krongtong Yoovathaworn,
Ph.D.
Co-Advisor

.....
Prof. M.R. Jisnuson Svasti,
Ph.D.
Dean
Faculty of Graduate Studies

.....
Assoc.Prof.Jutamaad Satayavivad,
Ph.D.
Chair
Master of Science Programme in
Toxicology
Faculty of Science

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was submitted to the faculty of Graduate Studies, Mahidol University for the degree of Master of Science (Toxicology)
on
4 April 2006

Arthit Sripirom
.....
Mr.Arthit Sripirom
Candidate

Krongtong Yoovathaworn
.....
Assoc.Prof.Krongtong Yoovathaworn,
Ph.D.
Member

.....
Assoc.Prof.Winai Wananukul,
M.D.,F.R.C.P.
Chair

Dhasanai Suriyachan
.....
MG.Assoc.Prof.Dhasanai Suriyachan,
Ph.D.
Member

.....
Assoc.Prof.Thanyachai Sura,
M.D.,F.R.C.P.
Member

.....
Prof.M.R.Jisnuson Svasti,
Ph.D.
Dean
Faculty of Graduate Studies
Mahidol University

.....
Prof.Amaret Bhumiratana,
Ph.D.
Dean
Faculty of Science
Mahidol University

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Arthit Sripirom

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A STUDY ON THE GENETIC POLYMORPHISM OF PARAOXONASE 1 IN THAI POPULATION.

ARTHIT SRIPIROM 4636548 SCTX/M

M.Sc. (TOXICOLOGY)

THESIS ADVISORS: WINAI WANANUKUL, M.D., THANYACHAI SURA, M.D., KRONGTONG YOOVATHAWORN, Ph.D.

ABSTRACT

Human paraoxonase1 (PON1) plays a role in the detoxification of organophosphorus (OP) compounds and the protection of low-density lipoproteins (LDL) from oxidative modification. This lipoprotein is responsible for causing atherosclerosis. Some PON1 polymorphisms have been found to be responsible for variations in its catalytic activity and the level of expression. Consequently, these polymorphisms may be the causes of susceptibility to OP poisoning and vascular diseases.

The objectives of this study were to determine the allele frequency of PON1 polymorphisms at position -108 and position 192 in Thai population and to investigate the effect of PON1 genotype on lipid profile in these subjects. The studied group consisted of unrelated individuals (n =160). The PON1 genotypes were determined by PCR-RFLP and found that C and T alleles were 0.54 and 0.46, respectively, at position -108. For position 192, Q and R alleles were 0.57 and 0.43, respectively. The allele frequencies of PON1 gene in Thai population differ from that of European, Americans and Africans. In contrast, the similarity was observed among Asian population. Both PON1 gene polymorphisms show linkage disequilibrium (LD). In this study, the difference of lipid profile among the genotype was not observed.

KEY WORDS: PARAOXONASE 1/ GENE / POLYMORPHISM

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การศึกษาความหลากหลายของยีน PARAOXONASE 1 (PON1) ในกลุ่มประชากรไทย
(A STUDY ON THE GENETIC POLYMORPHISM OF PARAOXONASE1 N THAI POPULATION)

อาทิตย์ ศรีภิรมย์ 4636548 SCTX/M

คณะกรรมการควบคุมวิทยานิพนธ์: วินัย วานานุกูล, M.D., ธันยชัย สุระ, M.D., กรองทอง ยุวถาวร, Ph.D.

บทคัดย่อ

ยีน Paraoxonase1 (PON1) มีบทบาทสำคัญในการทำลายความเป็นพิษของสารกลุ่มออแกนโนฟอสฟอรัส และช่วยป้องกันการเกิด oxidation ของไลโปโปรตีนชนิด LDL ซึ่งมีส่วนทำให้เกิดหลอดเลือดแข็งได้ จากการศึกษาที่ผ่านมาพบว่ายีน PON1 มีความหลากหลาย และความหลากหลายนี้ทำให้การทำงานและปริมาณของเอ็นไซม์ของแต่ละคนแตกต่างกันด้วย ซึ่งจะส่งผลให้แต่ละคนมีความไวต่อการเกิดพิษหรือโรคหลอดเลือดแข็งแตกต่างกัน

วัตถุประสงค์ของงานวิจัยครั้งนี้เพื่อศึกษาความถี่ของยีน PON1 ที่ตำแหน่ง -108 และ 192 ในกลุ่มประชากรไทย และเพื่อที่จะศึกษาผลของ PON1 จีโนไทป์ที่มีต่อระดับของไขมันในกลุ่มที่ศึกษานี้ โดยศึกษาความหลากหลายของจีโนไทป์ด้วยวิธี PCR-RFLP ในกลุ่มประชากร 160 คน จากการศึกษาพบว่ามีความหลากหลายของยีน PON1 ความถี่ของอัลลีลล์ C และ T เป็น 0.54 และ 0.46 ตามลำดับ ในตำแหน่ง -108 และสำหรับตำแหน่ง 192 ความถี่ของอัลลีลล์ Q และ R เป็น 0.57 และ 0.43 ตามลำดับ เมื่อเปรียบเทียบความถี่ของอัลลีลล์ที่ได้กับกลุ่มประชากรอื่น พบว่าประชากรไทยแตกต่างจากกลุ่มประชากรในแถบยุโรป อเมริกา และแอฟริกา แต่มีค่าใกล้เคียงกับประเทศในแถบเอเชียด้วยกัน จากการศึกษาพบว่า อัลลีลล์ C และ T ของตำแหน่ง -108 กับ อัลลีลล์ Q และ R ของ ตำแหน่ง 192 ของยีน PON1 มีการถ่ายทอดไปด้วยกัน (linkage disequilibrium) และจากการศึกษาผลของ PON1 จีโนไทป์ต่อระดับของไขมันในเลือด พบว่าระดับของไขมันในเลือดไม่แตกต่างกันในแต่ละจีโนไทป์ของกลุ่มประชากรนี้

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LIST OF ABBREVIATIONS

˚C	= degree Celsius
µg/dL	= microgram per milliliter
A	= adenine
apo	= apolipoprotein
bp	= base pair
C	= cytosine
cDNA	= complementary deoxyribonucleic acid
CHD	= coronary heart disease
DBP	= diastolic blood pressure
dCTP	= deoxycytidine 5'-triphosphate
ddNTP	= dideoxynucleotide triphosphate
dL	= deciliter
DNA	= deoxyribonucleic acid
dNTP	= deoxynucleotide triphosphate
et al.	= et alii
G	= guanine
g	= gramme
g/dL	= gram per deciliter
HDL	= high density lipoprotein
KDa	= kilo Dalton
l	= liter
LDL	= low density lipoprotein
min	= minute
mL	= milliliter
mM	= millimolar
mmHg	= milliliter of mercury
mRNA	= messenger ribonucleic acid
ng	= nanogram

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LIST OF ABBREVIATIONS (CONTS.)

nm	= nanometer
PCR	= polymerase chain reaction
RFLP	= restriction fragment length polymorphism
rpm	= revolutions per minute
SBP	= systolic blood pressure
T	= thymine
TC	= total cholesterol
TG	= triglyceride
U/L	= unit per liter
UV	= ultraviolet
Vs	= versus

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CHAPTER I
INTRODUCTION

Atherosclerosis is one of the leading causes of death worldwide. It is characterized by the thickening of arterial wall from deposits of plaque, resulting in reduced blood flow. In the coronary circulation, plaque rupture and the consequence of thrombosis may lead to sudden arterial blockade causing heart attack. The traditional risk factors of atherosclerosis include age, hypercholesterolemia, hypertension, smoking, gender, diabetes mellitus, and genetic factors; Moreover, factors including dietary fat, cholesterol, triglyceride, obesity, and lack of exercise are also risk factors. An initiating event of plaque formation is oxidative modification of low-density lipoproteins (LDL). LDL is the major lipoprotein in blood circulation. A possible mechanism of LDL oxidation is induced by reactive oxygen species (ROS) and reactive nitrogen species. Circulating oxidized LDL (ox-LDL) originates from mild oxidation in the arterial wall by cell-associated lipoxygenase and myeloperoxidase. The ox-LDL induces atherosclerosis by stimulating monocyte infiltration, smooth muscle cell migration and proliferation in the subendothelial layer of the vessel. Macrophages engulf ox-LDL and become foam cells. Several growth factors secreted from foam cells stimulate smooth muscle cell (SMC) proliferation and migration to subendothelial layer, resulting in the protrusion of endothelial wall into the lumen together with fatty streak formation and calcification. Ox-LDL contributes to atherothrombosis by inducing endothelial cell apoptosis, and thus plaque erosion, by impairing the anticoagulant balance in the endothelium, stimulating tissue factor production by smooth muscle cells, and inducing apoptosis in macrophages (Merten & Holvoet, 2001). Several studies reported that high-density lipoprotein (HDL) plays role in the inhibition of LDL oxidation. The antioxidant hypothesis has been considered to be, the presence in the HDL, enzymes that possess antioxidant property against oxidative stress.

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Paraoxonase1 (PON1) is an HDL-associated enzyme that contributes the protective effect of HDL against oxidative damage of the plasma lipoproteins and cell membranes. PON1 is a calcium-dependent esterase enzyme, whose physiological role is to protect LDL from oxidative modification by preventing the accumulation of lipoperoxide in LDL (Mackness, Sharon & Durrington, 1991). PON1 plays role in detoxification of organophosphate pesticides by its paraoxonase activity; the metabolism of drugs that contain lactone and carbonate-ring moiety (drugs and pro-drugs) by lactonase activity (Draganov & La Du, 2004). PON1 is synthesized in the liver and secreted into circulation by anchoring with HDL via the hydrophobic N-terminal region. At present, PON1 is widely studied in patients with various diseases, and found to associate with dementia, diabetes, chronic renal disease, and coronary heart disease. Numerous studies suggested that these diseases may arise from the effect of oxidative stress as a result of the inability of our body to regulate the balance between antioxidant and oxidant. PON1 is associated with these diseases due to its antioxidant property. The activity of PON1 varies among individuals. The interethnic difference in PON1 gene was found to be responsible for PON1 activity variation among populations.

PON1 gene is located on the long arm of chromosome 7 between q22-q23. The expression of PON1 is developmentally regulated, having only one-fourth to one-third of their adult PON1 levels at birth. Additionally, PON1 levels plateau between 6 to 15 months of age, and that the variation in PON1 levels with age is common among individuals (Cole et al., 2003). The variability of PON1 is associated with polymorphisms of PON1 gene. The complete sequencing of PON1 gene has illustrated that PON1 contains about 200 polymorphic sites in both exon and intron regions. However, only promoter and coding regions contribute to the enzyme concentration and activity. Thus, the polymorphisms in these two regions would affect the concentration and activity of the enzyme.

At the promoter region, PON1 contains at least five polymorphisms with varying degrees of influence over gene expression. These polymorphisms are located at positions -909 (C or G), -832(A or G), -162(A or G), -126(C or G), and -108(C or T).

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Haplotype analysis of the promoter region showed that the -108 (C or T) polymorphisms are the main contributor to serum PON1 variation, accounting for 23-24 % of the total variations and also a slight contribution (1.1 % total variations) from the -162 (A or G) site. The sites at -909 and -832 cause little or no difference in serum PON1 levels (Brophy, Jampsa, Clencenning, Mckinstry & Jarvik, 2001). In addition, within the position C-1O8 T, there are two-fold difference in PON1 concentration. The presence of C allele leads to a higher enzyme concentration than T allele, resulting from the polymorphic site locating in the centre of consensus binding site for the ubiquitous transcription factors Sp1 and Sp3. This consensus site is abolished by the presence of the -108T (Leviev & James, 2000).

Polymorphisms of PON1 in coding region at the position 192 (Q/R), leading to amino acid changes from glutamine (Q) to arginine (R), and methionine (M) to leucine (L) at the position 55 (M/L). The position Q192R has been widely studied because the two alloenzymes have different affinities and catalytic activities towards a number of substrates such as paraoxon (toxic metabolite of organophosphate). An individual, who was carrying low activity PON1 (QQ genotype), exposed to organophosphate may be susceptible to toxicity of organophosphate. On the other hand, the polymorphism at the position 55(L/M) does not affect the interaction of PON1 with substrates, but it is associated with lower serum concentration and activity of the enzyme (Blatter-Garin, 1997). Subsequent analysis showed strong linkage disequilibrium with the position -108 (C or T) polymorphisms in the promoter region of the gene. Most studies have been trying to explain the association of PON1 polymorphisms with several pathological conditions. The polymorphic sites which were widely studied included the positions Q192R, L55M, and C-108 T. An individual who carries low activity of PON1, such as 55MM genotype and -108TT genotype, has disease risk. At the position 192, the risks of disease are dependent on the substrates for each genotype. The 192QQ genotype is efficient for hydrolyzing the nerve agents such as soman and sarin. In contrast, the 192RR genotype is efficient for the hydrolysis of organophosphate pesticide. In addition, the 192QQ genotype is more efficient in protecting LDL from oxidation than 192RR genotype.

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The recent numerous studies demonstrated the association of PON1 genotype with the risk of CHD in various populations such as French (Ruiz et al., 1995), Japanese (Imai et al., 2000), and Indian (Sanghera, Saha, Aston, & Kamboh, 1997). However, some studies reported no association such as Spanish (Aubo et al., 2000), British (Lawlor et al., 2004), and Japanese (Suehiro et al., 1996). It is likely that other factors such as the large inter-individual variation in serum PON1 activity or concentration have a significant role in the potentially protective effect of PON1 against CHD. Unfortunately, the enzyme activity of PON1 can be inhibited by environmental factors such as smoking, alcohol, diet, etc. The dietary polyphenols can affect gene expression of PON1 via nuclear receptor such as aryl hydrocarbon receptor (AhR) and xenobiotic responsive element (XRE) (Gouedard, Baruki, Morel, 2004). Moreover, HDL plays role in PON1 activity as it acts as carrier of PON1 in the circulation and several lines of evidence suggested that PON1 may have a role in lipoprotein metabolism. First, PON1 is an important structural component of the apolipoprotein (apo) A-I containing lipoprotein subpopulation of HDL particles (Blatter, James, Messmer, Barja, Pometta, 1993). Second, purified PON from human HDL decreases the oxidative modification of LDL in vitro (Mackness, Arrol, Durrington, 1991). Third, plasma PON1 activity is significantly related to variation in plasma concentrations of triglyceride, and LDL cholesterol (Saha, Roy, Teo, Tay, Ratnam, 1991). The differences in the distribution of risk factors and disease among racial and ethnic groups are a function of the frequency of specific genotypes and interaction with environmental factors (Kuller, 2004)

Variant PON1 genotype frequency is in accordance with geography and race. The prevalence of PON1 gene at position 192R allele ranges from 2 to 63 percent depending on the population. Caucasians have the lowest frequency of PON1 192R allele while Africans and Asians have high frequency of the allele. At the promoter region, Caucasians have the lowest frequency of PON1 -108C allele while Africans and Asians have high frequency of the allele. PON1 status in Thai subjects had previously been studied by Phuntuwat et al in a selected population. (2005).

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Risk of CHD tends to increase in Thai population due to the change of life-style, the drinking and eating manner. Obesity may be one of the problems in the future (Kantachuvessiri, 2005). Several studies have found the association between obesity and CHD caused by hyperlipidemia, especially LDL, leading to atherosclerosis. Thus, the study of PON1 polymorphism in Thai population may be useful for the evaluation of the risk of atherosclerosis, as it is genetic dependent. Moreover, PON1 genotype can be used for the prediction the risk of organophosphate toxicity and response to some medications. The data obtained from this study may be useful for further studies on the association of PON1 genotype and risk of many diseases in Thai population.

This study aims to estimate the incidence of polymorphisms of the PON1 gene in Thai population and the effect of these polymorphisms on lipid profile.

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CHAPTER II
OBJECTIVES

The objectives of this study are as follows:

1. To determine the allele frequencies of PON1 gene in Thai population.
2. To study the association between PON1 genotype and plasma lipoproteins.

Significance of this study

The overall study will provide data regarding PON1 allele frequency distribution in Thai population, and the association between PON1 genotype and plasma lipoprotein levels. Moreover, the data of genotype may be used in susceptibility prediction to environmental chemicals.

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CHAPTER III
LITERATURE REVIEWS

1. Lipoproteins

Plasma lipoproteins are soluble macromolecules (pseudomicellar particle) representing complexes of lipids (triglycerides, cholesterol, and phospholipids) and one or more specific proteins, referred to as apolipoproteins. There are several types of lipoproteins with different chemical compositions, physical properties, and metabolic functions. Their common roles are to transport lipids from one tissue to others.

1.1 Lipoprotein structures (Baynes & Dominiczak, 2005)

A lipoprotein particle contains a hydrophobic core of cholesterol esters, triacylglycerols, amphipathic phospholipids, and free cholesterol, together with apopoteins from an outer layer. The binding of core lipids with the phospholipids and protein coat are non-covalent, occurring primary through hydrogen bonding and van der Waals forces. This binding of lipid to protein is loose enough to allow the ready exchange of lipids among the plasma lipoproteins and cell membranes. However, the binding is strong enough to allow the various classes and subclasses of lipoproteins to be isolated by a variety of analytical techniques (Figure 1).

1.2 Classification of lipoprotein (Burtis & Ashwood, 2001)

Lipoprotein particles present in plasma form a continuum of size and density (Table1). They are chylomicrons, very-low density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). HDL can be further divided by density into two

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subpopulations (HDL_2 and HDL_3). VLDL and IDL are triacylglycerol-rich, whereas LDL is cholesterol-rich. The density of these particles increases and the size decreases with decreasing triacylglycerol contents, ranging from chylomicrons (the lightest) to VLDL, IDL, LDL, and HDL (the heaviest). HDL contains several proteins, some cholesterol and phospholipids, but relatively little triacylglycerol.

In addition to their density, lipoproteins may be separated according to their electrophoretic property. This technique is called electrophoresis. On electrophoresis, α- lipoproteins (HDL) migrate furthest towards the anode (+ electrode), followed by pre-ß lipoproteins (VLDL), ß-lipoproteins (LDL), and IDL is located between ß- and pre-ß lipoproteins. Chylomicron remains at the cathode end (- electrode), at the origin of electrophoretic strip (Figure 2).

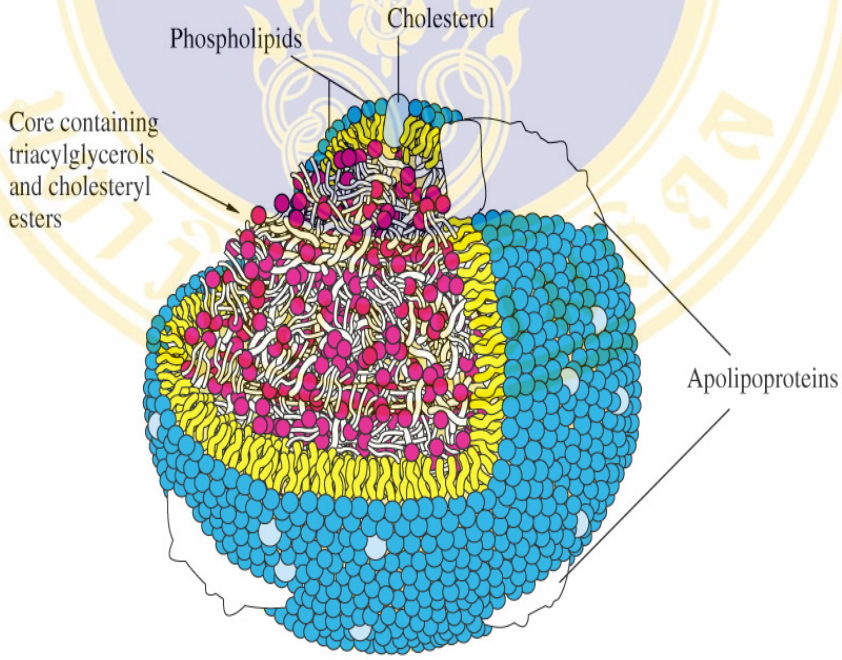

Figure 1. Structure of lipoprotein. A core of neutral lipids, including triglycerides and cholesteryl ester, is coated with phospholipids in which apolipoproteins and cholesterol are embedded (Horton, Moram, Ocly, Rawn, and Serimgeous, 2002)

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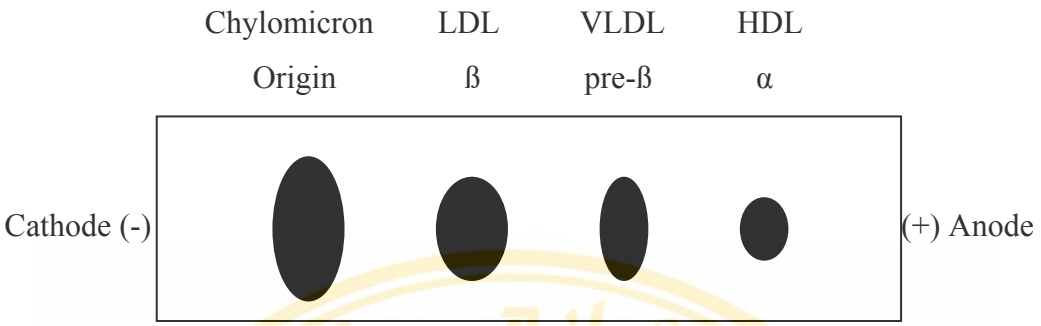

Figure 2. Migration of plasma lipoprotein on electrophoresis

Table 1. Characteristic of human plasma lipoproteins (Burtis & Ashwood, 2001)

Variable	chylomicron	VLDL	IDL	LDL	HDL	Lp(a)
Density (g/mL)	<0.95	0.95-1.006	1.006-1.019	1.019-1.063	1.063-1.210	1.040-1.130
Electrophoretic mobility	Origin	Pre-ß	Between ß and pre-ß	ß	α	Pre-ß
Molecular weight (dalton)	0.4-30 x 10^9	5-10 x 10^6	3.9-4.8 x 10^6	2.75 x 10^6	1.8-3.6 x 10^5	2.9-3.7 x 10^6
Diameter (nm)	>70	25-70	22-24	19-23	4-10	25-30
Lipid-protein ratio	99:1	90:10	85:15	80:20	50:50	75:25 to 64:36
Major lipids	Exogenous triglyceride	Endogenous triglyceride	Endogenous triglyceride, cholesterol esters	Cholesterol esters	phospholipids	Cholesterol esters, phospholipids
Major proteins	A-I B-48 C-I C-II C-III	B-100 C-I C-II C-III E	B-100 E	B-100	A-I A-II	(a) B-100

VLDL, very-low density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; Lp(a), lipoprotein (a).

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1.3 Lipoprotein metabolism (Burtis & Ashwood, 2001)

The main pathways of lipoprotein metabolism are divided into exogenous and endogenous pathways.

1.3.1 Exogenous pathway

Lipoproteins in this pathway are of dietary origin. The nascent chylomicrons are assembled from dietary triglyceride and cholesterol in secretory vesicles of the Golgi apparatus and introduced into circulation through the intestinal villi. The lipid content of nascent chylomicrons consists mainly of triglyceride (90% by mass), whereas the protein components include apoprotein (apo) B-48 and A (2% by mass). Shortly after entering the circulation, these particles acquire apo C and apo E from circulating HDL (Figure 3). Apo C-II now presents on the surface of chylomicrons and activates the lipoprotein lipase (LPL) attached to the luminal surface of endothelial cells. The enzyme rapidly hydrolyzes the triglycerides to free fatty acids. The fatty acids are associated with albumin and can either be taken up by muscle cells as an energy source or into adipose cells for storage. Simultaneously, some of the phospholipids and apo A are transferred from chylomicron particle onto HDL. The newly formed particle, the chylomicron remnant, contains 80% to 90% of the triglyceride content of the original chylomicron. Because of the presence of apo B-48 and apo E on its surface, the chylomicron remnant can be recognized by hepatic remnant receptors and internalized by endocytosis. Then, the components of the particle are hydrolyzed in the lysosomes. The cholesterol remnant can be incorporated into newly synthesized lipoprotein or stored as cholesteryl ester. Furthermore, the cholesterol from these remnants can down-regulate HMG-CoA (3-hydroxy-3-metylglutaryl-CoA) reductase.

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1.3.2 Endogenous pathway

Lipoproteins in this pathway are of hepatic origin because hepatocytes have the ability to synthesize triglycerides from carbohydrates and fatty acids. In addition, when dietary cholesterol is insufficient, the hepatocytes also synthesize their own cholesterol by increasing the activity of HMG-CoA reductase. The endogenous triglycerides and cholesterol are packed in secretory vesicles in the Golgi apparatus, transported by exocytosis into extracellular space, and introduced into circulation in the form of nascent VLDL (Figure 3). This triglyceride-rich particle (55% by mass) contains apo B-100, apo E, and small amount of apo C on its surface. Additional apo C is transferred after secretion from circulating HDL. As with chylomicron metabolism, apo C-II presents on the surface of VLDL and activates LPL on endothelial cells, leading to the hydrolysis of VLDL triglyceride and the release of free fatty acids. During the hydrolysis of VLDL triglyceride, the apo C is transferred back to HDL. VLDL particles are converted to VLDL remnants, some of which are taken up by the liver. The rest are converted to small, dens particles called IDL. Large IDL particles, which also have several molecules of apo E, bind the hepatic remnant receptors and are removed from circulation about 50% of the IDL. Surface material from IDL, including some phospholipids, free cholesterol, and apolipoproteins, are transferred to HDL, or form HDL de novo in the circulation. Cholesterol esters are transferred from HDL to LDL. The net result of lipolysis and cholesteryl esters exchange reaction is the replacement of much of the triglyceride core of the original VLDL with chlolesteryl esters. IDL undergoes further hydrolysis, in which most of the remaining triglycerides are removed and all apolipoproteins except B-100 are transferred to other lipoproteins. This process ends with ultimate formation of LDL.

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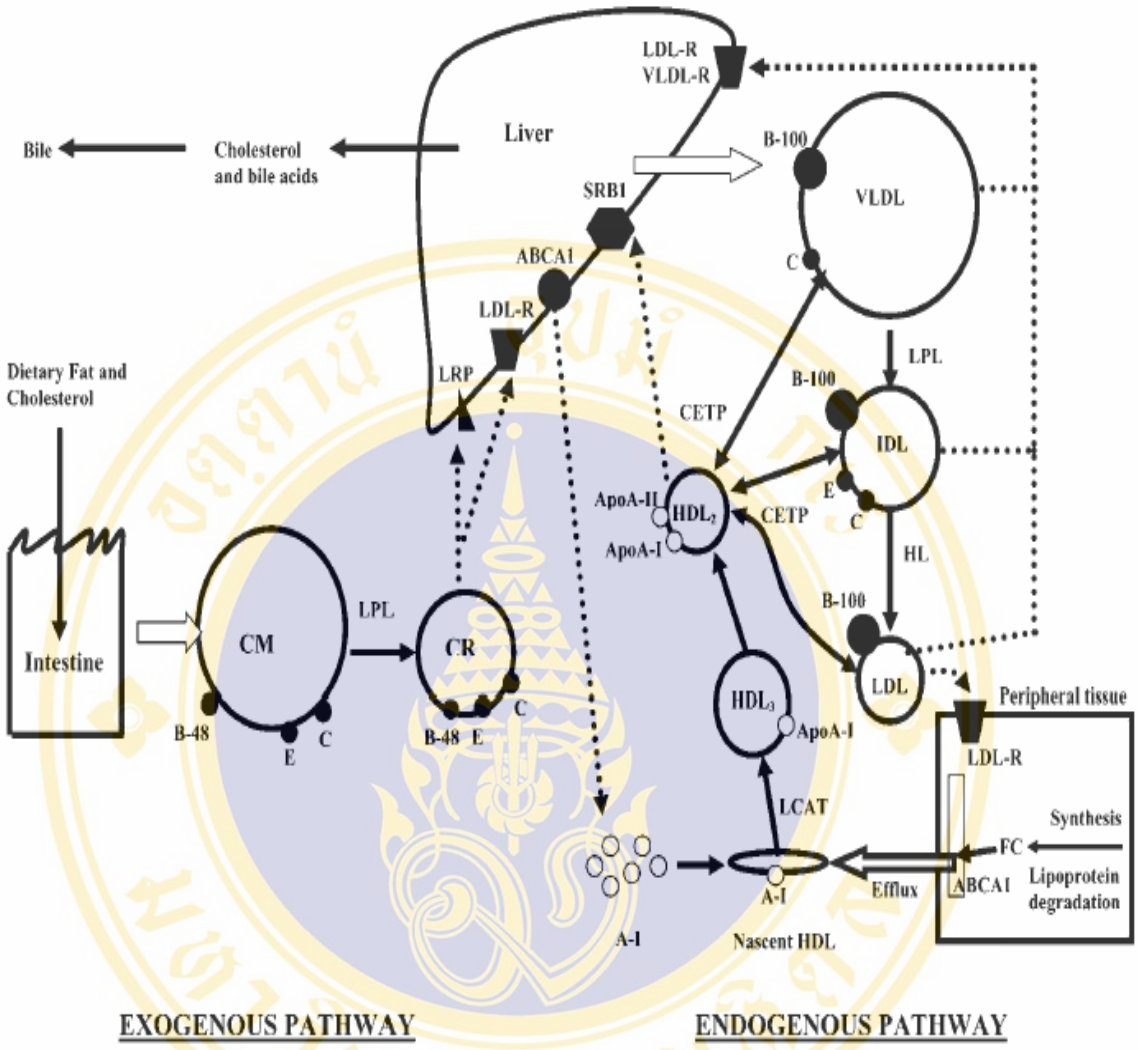

Figure 3. Schematic diagram of lipoprotein metabolism. CM, Chylomicron; CR, Chylomicron remnant; FC, Free cholesterol; HL, hepatic lipase; LDL-R, LDL receptor; VLDL-R, VLDL receptor (Chan, Barrett, & Watts, 2004).

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2. Atherosclerosis (Madammanchi, Vendrov, & Runge, 2005)

Atherosclerosis is a progressive disease characterized by the accumulation of lipids and fibrous elements in the large arteries. Normally, the lumen of healthy artery is lined by a confluent layer of endothelial cells. Normal endothelium forms a surface, which repels cells floating in the plasma and exerts strong antithrombolytic effect. The arterial wall consists of three well-defined layers. The subendothelial layer is called the intima, the middle layer is called the media [this layer contains vascular smooth muscle cells (VSMC)], and the outer layer is advantitia that is composed of looser connective tissue and containing relevant nerve. The earliest lesion of atherosclerosis consists of subendothelial accumulation of cholesterol-engorged macrophages called foam cells. Such "fatty streak" lesions are not clinically significant, but they are the precursors of more advanced lesions characterized by the accumulation of lipid-rich necrotic debris and smooth muscle cells (SMC). Early atheromas typically have "fibrous cap" consisting of SMC and extracellular matrix that encloses a lipid-rich necrotic core. Atheromatous plaque can become increasing complex with calcification, ulceration at the luminal surface, and hemorrhages from small vessels (Figure.4).

An important initiating event for atherosclerosis is the transport of oxidized low-density lipoprotein (ox-LDL) across the endothelium into the arterial wall. This is likely to occur at the site of endothelial damage which is caused by ox-LDL itself as well as physical or chemical agents and infections. Endothelial cells, smooth muscles, and macrophages are sources of oxidants for oxidative modification of phospholipid. Ox-LDL can damage endothelial cells and induces the expression of adhesion molecules such as P-selectin and chemotactic factor such as monocyte chemoattractant protein1 (MCP-1) and macrophage stimulating factor (mCSF). These processes lead to leathering, activation, and attachment of monocytes and lymphocytes to the endothelial cells. Endothelial cells, leukocytes, and SMC then secrete growth factors and chemoattractants that affect the migration of monocytes and leukocytes into the subendothelial space. After activation of growth factor, Monocytes transformed into macrophages and generate reactive oxygen species (ROS), which convert ox-LDL into highly oxidized LDL and taken up by macrophage to form foam cells. Foam cells combine with leukocytes to become fatty streak. Foam cells secrete a growth factor

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that induces SMC migration into the intima layer. SMC proliferation, coupled with continuous influx, propagation of monocytes and macrophages, converts fatty streak to more advanced lesions and ultimately to fibrous plaque that will protrude into the arterial lumen. Later, calcification can occur and fibrosis continues, yielding fibrous caps that surround a lipid-rich core. This formation may also contain dead or dying SMC.

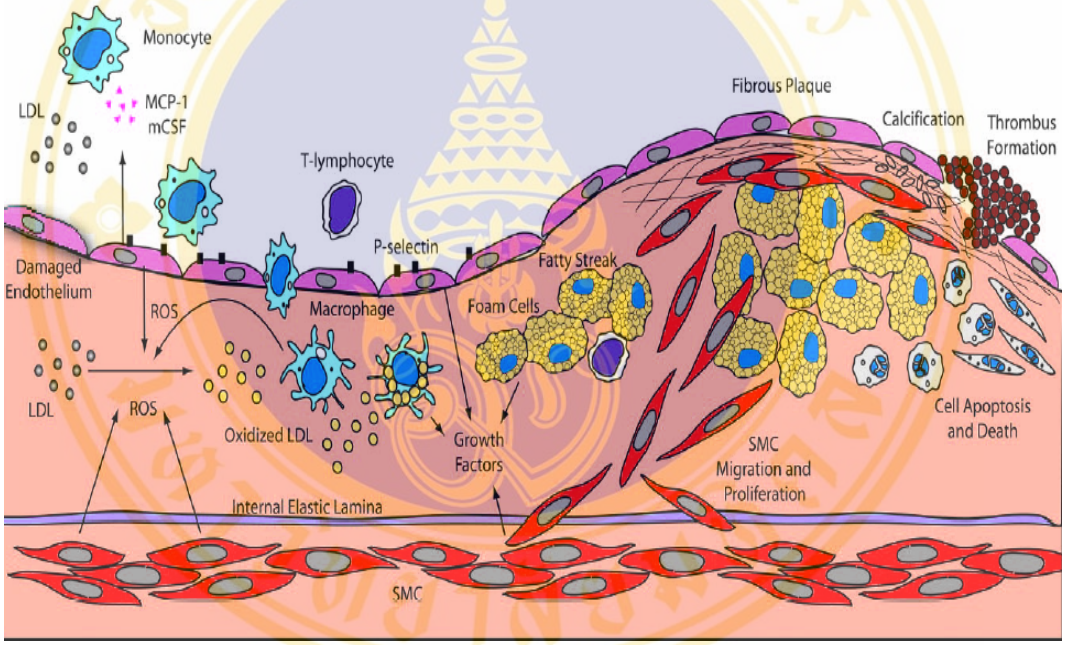

Figure 4. Development of Atherosclerosis (Madammanchi et al., 2005)

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2.1 Oxidative stress and atherosclerosis (Yokoyama, 2004)

Oxidative stress is an imbalance between oxidants and antioxidants in favor of the oxidants. The link between oxidative stress and atherosclerosis is originally derived from oxidative modification hypothesis as a key step in generation of macrophage-derived foam cells and initiation of atherosclerosis. All mammalian cells produce reactive oxygen species (ROS). ROS encompass a variety of diverse chemical species including superoxide anions (O^{2-}), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($HO^{\cdot}$). Accumulating evidence implicate ROS as specific signal molecule under both physiological and pathological conditions. Thus, ROS can mediate phenotype modulations in vascular endothelial and smooth muscle cells (ie. growth, apoptosis, and survival). Overproduction of ROS or increased oxidative stress is considered as a major mechanism involved in the pathogenesis of endothelial dysfunction, and might serve as a common pathogenic mechanism in conditions such as hypercholesterolemia, hypertension, and diabetes. O_2 promotes cellular damage by reacting with nitric oxide (NO) and reducing vascular NO bioavailability, and by exerting harmful effects including direct injury of endothelial and smooth muscle cells, oxidation of LDL, and induction of redox sensitization gene. Atherosclerosis represents a series of highly specific cellular and molecular responses that can best described the same as inflammatory processes. One possible mediator of inflammation is myeloperoxidase (MPO), a heme protein secreted from activated neutrophils, monocytes, and macrophages. MPO generates several reactive species, including hypochlorous acid (HOCl), chloramines, tyrosyl radicals, and nitrogen dioxide. It also functions as a major enzymatic catalyst for initiating of LDL oxidation and conversion of the lipoprotein into a high-uptake form for macrophage, leading to foam cell formation.

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2.2 Antioxidant property of HDL and Atherosclerosis

High-density lipoprotein (HDL) is regarded as one of the most important protective factors against atherosclerosis. Traditionally, HDL's protective function has been attributed to its active participation in the reverse cholesterol transport of cholesterol. Within the last few years, another mechanism has been proposed that HDL may function as antioxidant (Merten & Hobvoet, 2002). The capacity of HDL to inhibit the oxidative modification of LDL is potentially antiatherogenic. Numerous studies have revealed that HDL inhibits the transmigration of monocytes and cytotoxicity induced by oxidized LDL, and the oxidized LDL-induced adhesion of monocytes to endothelial cells. Lipophilic antioxidant carried by HDL can scavenge oxygen-derived free radicals and thereby interrupts the cascade of LDL oxidation, which is induced by transition ion. HDL depletes hydroperoxyeicosatetraenoic acid and hydroperoxydecadieonic acid of LDL, which are formed by lipoxygenase, and then induces the non-enzyme oxidation of phospholipids. HDL then transports these molecules to the liver for elimination (Eckardstein, Hersberger, & Rohere, 2005). HDL protects oxidative modification of LDL by using the enzyme that lies on it. Paraoxonase1 (PON1) is the most important enzyme on HDL that plays role in the inhibition of LDL oxidation by catalyzing the breakdown of oxidized phospholipids in LDL. The hypothetical models for mechanism of HDL destruction of biologically active lipids in Mildly- Oxidized LDL (MM-LDL) are shows in Figure 5. Reactive oxygen species may be formed in the arterial wall (A) in areas sequestered from plasma antioxidants by a variety of possible mechanisms, some of which may involve cyclooxygenase, lipoxygenase, inducible nitric oxide synthase, cytochrome P450, mitochondrial respiration, or free iron (B). These oxygen radicals may then "seed" LDL within the subendothelial space by oxidation of phospholipids in LDL (C). This process involves abstraction of hydrogen atoms from bis-allylic methylenes (D). An addition of molecular oxygen at multiple sites may generate multi-oxygenated phospholipids (E) which may be substrates for PON in HDL (F). In cases where PON concentrations are low or depleted or where lipid peroxide levels are excessive, oxidized phospholipids may undergo oxidative fragmentation (G) to form molecules (H) which evoke the characteristic inflammatory responses in endothelial cells induced by MM-LDL. These oxidative phospholipids may then be substrates for the second

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line of defense, platelet activating factor acetylhydrolase (PAF-AH) (I). PAF-AH hydrolyzes these biologically active lipids into molecules which do not evoke the characteristics inflammatory responses in endothelial cells induced by MM-LDL. Further oxidative decomposition of lipids in LDL, including cholesteryl esters, leads to the deposition of highly oxidized LDL (J) present in the necrotic core of advanced atherosclerotic lesion.

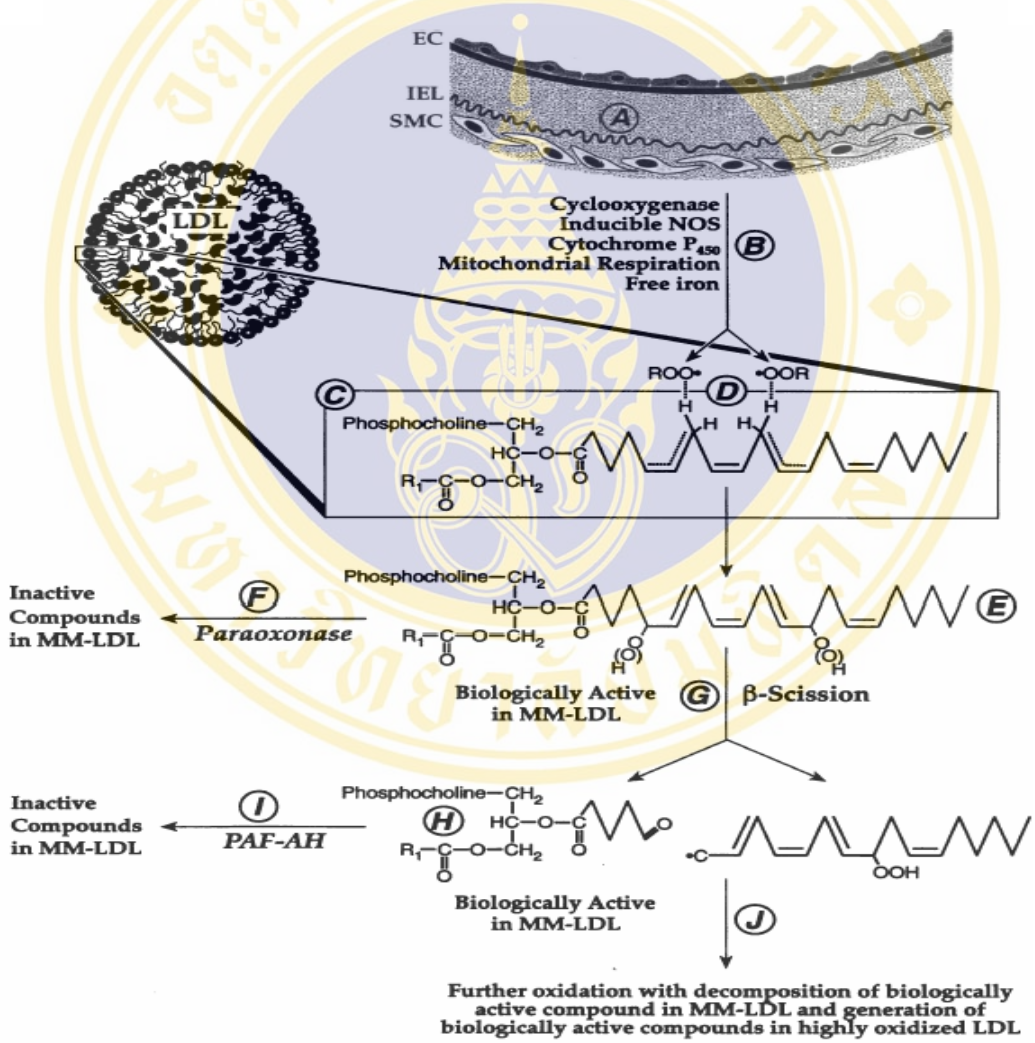

Figure 5. Hypothetical models for mechanism by which HDL destroys biologically active lipids in Mildly- Oxidized LDL (Watson et al., 1995)

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3. PARAOXONASE1 GENE (PON1)

3.1 Paraoxonase gene family

The paraoxonase (PON) gene family consists of three members, PON1, PON2, and PON3, located adjacent to each other on the long arm of chromosome 7 between q22.1 and 22.3 in humans. The three human PON genes share approximately 65% similarity at the nucleotide level (Table 2). Among mammalian species, however, each of the three gene shares 60-90% identity at amino acid level and 61-89 % identity on the nucleotide level. Codon 106 (lysine) presents in PON1 is missing in all PON2 and PON3 (Primo-Pramo, Sorenson, Teiber & La Du, 1996). From an evolutionary, PON2 appears to be the oldest member followed by PON3 and then PON1. In human, PON1 mRNA expression is limited to the liver. Similarly, PON3 is expressed primarily in the liver, with expression also seen in the kidney (Reddy et al., 2001). In contrast, PON2 is more widely expressed and is found in variety of tissues including the heart, kidney, liver, lung, placenta, small intestine, spleen, stomach, and testis (Ng et al, 2001). In addition, while PON1 and PON3 associate with HDL in circulation, PON2 protein is undetectable in HDL.

3.2 Paraoxonase 1 (PON1)

Paraoxonase1 gene has 9 exons, which encode for 355 amino acids. Serum paraoxonase1 has a molecular mass of 45 KDa. Paraoxonase-1 (PON1) is a high-density lipoprotein (HDL)-associated serum enzyme whose primary physiological role is to protect low-density lipoproteins (LDL) from oxidative modifications. Early research focused on the observation that PON1 could hydrolyze organophosphorus (OP) compounds, including commonly used insecticides. Indeed, the enzyme (EC 3.1.8.1) was initially characterized as an organophosphate hydrolase, and its name was derived from one of its most commonly used in vitro substrate, paraoxon. More recently, in addition to its role in lipid metabolism and, hence, in cardiovascular disease and atherosclerosis, PON1 has been shown to play a role in the metabolism of drugs.

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Table 2 Percentage of identity in PON1-like cDNAs (below the diagonal) and respective deduced proteins (above the diagonal).

	H *PON1*	M *PON1*	R *PON1*	H *PON2*	M *PON2*	D *PON2*	T *PON2*	C *PON2*	H *PON3*	M *PON3*
H *PON1*	–	83	86	64	62	64	67	68	60	56
M *Pon1*	84	–	79	65	62	63	64	66	57	58
R *PON1*	89	81	–	64	63	63	66	66	57	60
H *PON2*	72	70	71	–	84	90	72	71	64	60
M *Pon2*	69	65	70	85	–	85	68	68	62	59
D *PON2*	68	69	72	91	85	–	72	72	63	60
T *PON2*	71	71	72	72	70	72	–	97	63	60
C *PON2*	71	67	72	70	69	74	97	–	64	60
H *PON3*	67	65	66	70	67	70	69	69	–	80
M *Pon3*	65	61	63	65	65	66	65	65	84	–

Note. H = Human, M = Mouse, R = Rabbit, D = Dog, T = Turkey, C = Chicken (Primo-Parmo et al., 1996)

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PON1 is synthesized by the liver. Regarding the expression of human paraoxonase1 during development are showed that PON1 levels plateau between 6 to 15 months of age, and that variability in age at which PON1 levels plateau is quite variable among individuals (Cole et al., 2003). The gene expression is regulated by the transcription factor Sp1 which the mutations at this site have impact on PON1 synthesis, such as the polymorphism of -108 promoter region. In addition, the sterol-sensitive transcription factor SREBP-2 regulates and has impact on the PON1 synthesis (Deakin, Leviev, Guernier, & James, 2003). Direct sequencing of PON1 N-terminal sequence from human has demonstrated an unusual property: the mature form retains its N-terminal hydrophobic signal sequence. Retention of this signal peptide has been postulated to be the consequence of an evolutionary substitution of large, polar residues at the signal-peptide cleavage site where smaller residues are typically present, thereby eluding cleavage by signal peptidases. The unusual property of signal sequence arises from the changing of the histidine (H) and glutamine (Q) amino acid in positions 20 and 21, respectively, to alanine by mutagenesis (Sorensen et al., 1999).

Signal peptide sequences play a crucial role in directing peptide to secretory pathway, as well as determining their insertion to the membrane. It explains one of the major differences between PON gene family which PON2 is not secreted, in contrast to PON1 and PON3 (Deakin et al., 2002). The release of PON1 from cells must accommodate the need to prevent or minimize exposure of hydrophobic tail to the aqueous environment of the plasma. In addition, the release of PON1 to circulation needs the suitable acceptor; the most suitable one is a lipid complex. HDL is a lipid complex molecule which acts as acceptor and promotes PON1 release, but other lipid complex molecules are not. PON1 is inserted into the external membrane of cells from which it may be transferred to HDL during transient association of the lipoprotein with cell membrane. HDL is associated with cell membrane via the scavenger receptor B1 (SR-B1), previously identified as a receptor for HDL in the reverse cholesterol transport process (Eckardstein, Hersberger & Rohere, 2005). The study on the association of PON1 with HDL reveals that the enzyme doses not distribute across the entire HDL spectrum, because HDL exhibits extensive heterogeneity in term of both size and composition. Size heterogeneity has clinical relevance: large diameter HDL

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particles (HDL_2) are more closely linked to protective cardiovascular effects than the smaller diameter HDL_3. The research finding has proposed that PON1 may firstly associate with smaller HDL_3 type particles, which subsequently are transformed to larger HDL_2 particles (Bergmeter, Siekmeter, & Gross, 2004). In this context, PON1 would form the normal metabolic course of HDL, in which remodeling leading to an increase in particle size, notably due to accumulation of lipid components. The other parameter, which defines HDL heterogeneity, is the peptide composition. The structural peptides of HDL particle are apoA1 and A2, which this heterogeneity may reflect specific function of HDL subfractions. Recent reports showed that the PON1 exclusively binds with apoA1-associated HDL subfraction (Noto et al., 2001), but the apoA2 displaces PON1 from HDL and impairs its antioxidant properties (Ribas et al., 2004).

In plasma, PON1 does not bind to LDL, which is a quantitatively major plasma lipoprotein. Minor quantities of the enzyme (< 5%) are found within VLDL. The phenomenon would seem to be the impact of signal sequence on PON1 biology. The signal sequence can form transmembrane structure, and this is suggested to anchor PON1 to HDL (Sorensen et al, 1999). The hydrophobic core of signal sequence is usually flanked on the N-terminal side by polar amino acid, consistent with its eventual location in a more hydrophilic environment. Such an N-terminal sequence would be difficult to accommodate within VLDL and LDL as they contains more hydrophobic cores of esterified cholesterol and triglycerides. The hydrophobic core of HDL is less voluminous, and with the aid of amphipathic peptides such as apo A1 and A2, so HDL may better accommodate the structural requirements of PON1 (Harel et al., 2004)

The stability of PON1 requires apoA1, since lipid-poor apoA1 does not promote release of PON1 from cells. Although phospholipid vesicle can effectively promote PON1 release from cells, but enzyme activity is more rapidly lost from PON1 phospholipid complexes. In addition, the important role of apoA1 is to stabilize the enzyme, and to increase its activity leading to a more active molecule (Oda, Bielicki, Berger, & Forte, 2001). Thus, apoA1 can stabilize and promote PON1 release.

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3.3 The architecture of PON1 (Harel et al., 2004)

PON1 is a six-bladed ß-propeller, and each blade contains four strands (Figure.6). The ‘velcro’ closure characteristic of this fold is complemented by disulfide bridge between Cys42 (strand 6D) and Cys353 (strand 6C). This covalent closure of the N and C termini is rarely seen in ß-propellers with more than four blades, but conserved throughout the PON family. Two calcium ions are seen in the central tunnel of the propellers, one at the top (Ca1) and one in the central section (Ca2). Ca2 is most probably structural calcium whose dissociation leads to irreversible denaturation. Ca1 is assigned as the catalytic calcium. It seems to interact with protein residues (the side chain oxygen of Asn224, Asn270, Asn168, Asp269 and Glu53), 2.2-2.5 A° away. Two other potential ligands are water molecule and one of the oxygen phosphate ions. The two calcium ions exhibit markedly different affinities. Ca1’s ligation is stronger than Ca2’s. However, two of Ca’s ligating residues (Asn224 and Asp269) exhibit distorted dihedral angles. PON1 expression in animal cells is glycosylated. Glycosylation is not essential for hydrolytic activity of PON1, but may be important in increasing their solubility and stability or in preventing nonspecific binding to cell membranes (as proposed for other HDL-associated enzymes).

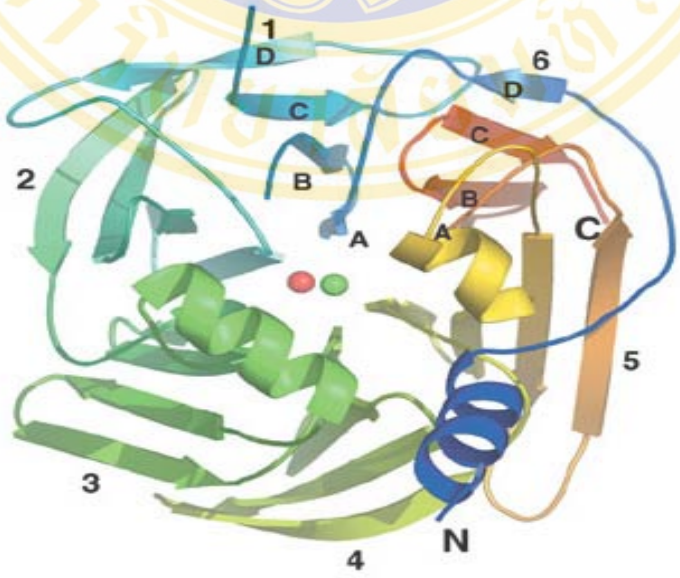

Figure 6. The structure of PON1 (Harel et al., 2004)

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3.4 The catalytic mechanism of PON1

At the very bottom of active site cavity lies upper calcium (Ca1), and phosphate ion that was present in the mother liquor (Figure.7). One of the phosphate's oxygen is only 2.2 A° from Ca1. The phosphate ion may bind in a mode which is similar to that of intermediates in the hydrolytic reactions catalyzed by PON. One of its negatively charged oxygen, the one nearest to Ca1, may mimic the oxyanionic moiety of these intermediates, which is stabilized by positively charged calcium. Two other phosphates oxygen may be mimic the attacking hydroxyl ion and oxygen of the alkoxy or phenoxy leaving groups of ester and lactone substrates.

The catalytic mechanism can be elucidated in Figure 8. His115 (the closer nitrogen of which only 4.1 A° from Ca1) acts as a general base to deprotonate a single water molecule and generate the attacking hydroxide, whereas His134 acts in proton shuttle mechanism to increase His115's basicity. Notably, His115 adopts distorted dihedral angles, a phenomenon observed in catalytic residues of many enzymes. In support of postulated mechanism are the H115Q mutation, which causes a marked decrease (2 x 10^4 fold) in activity, and H134Q mutation, which produces a milder decrease (6-150 fold). Notably, Cys284, which is conserved in all PON family, has been proposed to serve as alternative functions of PON1 related to atherosclerosis (Aviram et al., 1998). Cys284 is part of a highly conserved stretch that includes active site His285, and is packed against four highly conserved residues from the adjacent strands (Leu267, Val268, Glu303 and Leu305). It is unlikely to have a functional role, but its mutation is likely to destabilize the core structure, thereby affecting function indirectly. Indeed, the some study found that Cys284 mutants are poorly expressed and relatively unstable (Harel et al., 2004).

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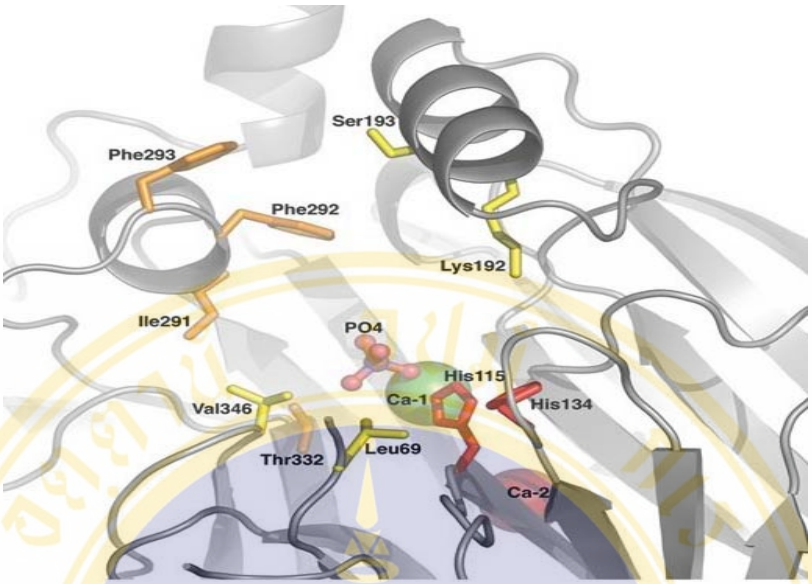

Figure 7. Active site of PON1 enzyme (Harel et al., 2004)

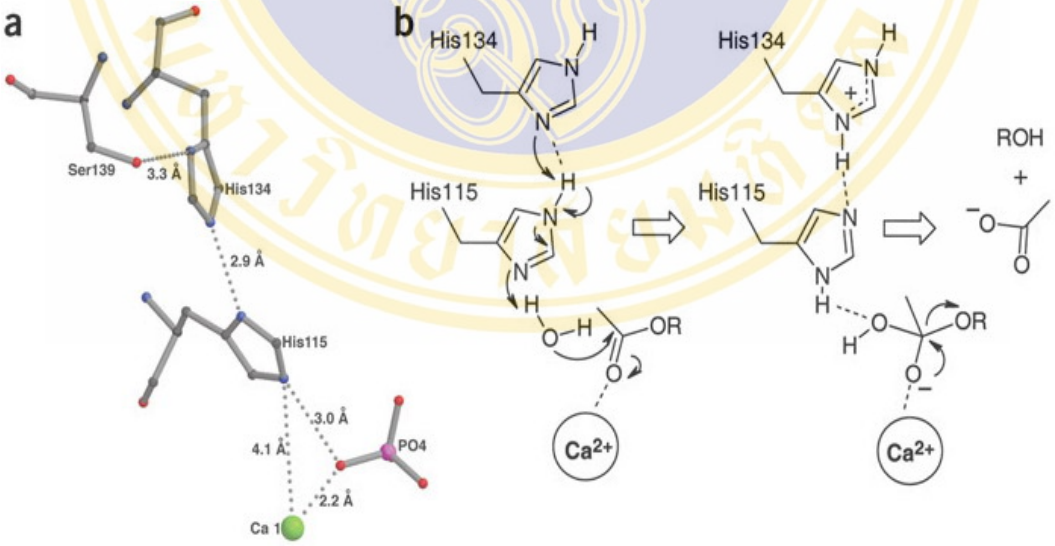

Figure 8. The postulated catalytic site and mechanism of PON1 (Harel et al., 2004)

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3.5 Human PON1 polymorphisms and point mutations

The current number of polymorphisms identified in human PON1 gene from complete gene sequencing reveals that there are 7 in the 5′ -untranslated regions, 171 are in the intronic regions, and 15 are in the 3′- untranslated regions. In addition to the 2 common exonic polymorphic sites (Leu55Met) in exon 3 and (Gln192Arg) in exon 6, there are at least 3 structural variants within the coding regions. The latter mutations are: Trp194stop in exon 6 (Jarvik et al., 2003), Arg160Gly in exon 5 found in Chinese Hun population (Wang et al., 2003), and the Finnish mutant Ile102Val in exon 4 (Marchesani et al., 2003). The important polymorphic sites of 5′-promoter regions are located at positions -909 (C or G), -832 (A or G), -162 (C or G), -108 (C or T) (Brophy et al, 2001). At present, the functional polymorphic sites were divided into two regions (coding regions and promoter regions). At the coding regions, there are two polymorphisms in the PON1 coding region at positions $Gln^{192}\rightarrow$Arg (Q192R) and $Leu^{55}\rightarrow$Met (L55M). The position Q192R has been more widely studied, because the two alloenzymes have different affinities and catalytic activities towards a number of substrates. The R allele hydrolyses paraoxon faster than the Q allele, whereas the Q allele is more active toward sarin, soman, and diazoxon. For some substrates, there is no difference in hydrolytic rate, e.g. phenylacetate (Clement E, 2004). The alteration in enzyme activity caused by this single amino acid change is explained by the structure of the enzyme. Amino acid 192R is an important active-site residue (Harel et al, 2004). The difference in activity towards certain OPs is important as it affects susceptibility to some toxic substrates. The Q192R polymorphism also alters the enzyme's ability to protect LDL from oxidation in vitro with the Q form being the most protective (Aviram et al., 1998).

The PON 55M polymorphism does not affect the interaction of PON1 with its substrates, but is associated with lower serum activity and concentration of the enzyme (Blatter Garin et al., 1997). Numerous studies found that individuals with the M allele have lower PON1 mRNA levels (Leiev, Deakin & James, 2001). Subsequent analysis showed strong linkage disequilibrium with the C -108 T polymorphism in the promoter of the gene. The L55 isoform was more stable and resistant to proteolys

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which may explain part of its association with higher serum PON1 levels [these data were supported by the recent publication of the PON1 crystal structure which showed a key role of L55 in the correct packing of the protein].

For the promoter region, sequencing of the promoter of the PON1 gene led to the discovery of at least five polymorphisms with varying degrees of influence over gene expression. These polymorphisms are located at positions -909 (C or G), -832 (A or G), -162 (A or G), -126 (C or G), and -108 (C or T). There have been reported that promoters containing the polymorphisms GAAC, as opposed to CGGT, at positions -909, -832, -162 and -108 respectively, are up to two times more active. These variations in promoter activity were shown to be physiologically relevant as they correlated with significant differences in serum PON1 concentration and activity (Brophy et al., 2001). The polymorphisms have also been detected in the 3′ untranslated region of the PON1 gene, but their significance is still unknown. Identification of clinically significant polymorphisms has been hindered by the fact that there is significant linkage disequilibrium between all the promoter polymorphisms. Haplotype analysis showed that the C (-108) T polymorphism was the main contributor to serum PON1 variation, accounting for 23–24% of the total variations, and also reported a slight contribution (1.1% total variations) from the A (-162) G site. The positions -909 and -832 made little or no contribution to serum PON1 levels. Reported gene assays using promoter regions of varying lengths have shown that the approximately 200 bp region covering the -108 and -162 polymorphisms is sufficient for transcription of the PON1 gene [deleting this region completely abolishes promoter activity, and it is an essential regulatory region of the PON1 promoter]. The -108 site is the most significant contributor to PON1 serum variation. The polymorphism is located in the centre of a consensus binding site for the ubiquitous transcription factors Sp1 and Sp3. This consensus site is abolished by the presence of the -108T variant. Binding of Sp1 to the -108 site is weaker in the presence of T than C, suggesting an effect of the polymorphism on Sp1 binding (Deakin et al., 2003). There are multiple Sp1 sites in this region of the PON1 promoter, so the effect of the polymorphism is likely to be positional. The -162 polymorphism lies over a potential NF-1 (nuclear factor-1) binding site, and the

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activity is low for G variant. This may explain the effect of the change at -162 on gene expression. There is significant linkage disequilibrium between the promoter polymorphisms and the coding region polymorphisms. Several studies revealed linkage disequilibrium between -108C and the 192R coding region polymorphism, which is associated with a lower level of protection against CHD. Although Q192R is independently associated with variance in enzyme activity, it is possible that the -108C may partly compensate for the lower protection of PON1 192R complicating the relationship between PON1 genotype and disease.

3.6 Determining an individual's PON1 status

Most studies investigating the association of PON1 polymorphisms with sensitivity to OP toxicity or with diseases (particularly cardiovascular diseases) have examined only the nucleotide polymorphisms (Q192R, L55M, C-108T) using polymerase chain reaction (PCR)-based assays. A functional genomic analysis, however, provides a much more informative approach, as direct measurement of an individual's PON1 function (plasma activity) takes into account all polymorphisms that might affect activity. This is accomplished through the use of a high-throughput enzyme assay involving two PON1 substrates, paraoxon and diazoxon. In addition to providing a functional assessment of the plasma PON1-192 alloforms, this approach also provides the plasma level of PON1 for each individual, thus encompassing the two factors that affect PON1 levels or activity. The catalytic efficiency of PON1 to degrade OP determines the degree of protection provided by PON1, accounting for higher concentrations of PON1 and better protection. Thus, it is essential to know PON1 levels and activity. Plasma PON1 activity varies up to 40-fold, and up to 13-fold differences in PON1 protein levels (Costa, Cole, Jarvik, & Furlong, 2003). Recent studies investigating the role of PON1 in cardiovascular diseases have provided evidence that PON1 status (encompassing genotype and activity levels) is a much better predictor of disease than PON1 genotype alone (Mackness et al, 2001). Although it might be possible to determine all of the PON1 SNPs (single nucleotide polymorphisms) for a given individual, doing so would not provide an accurate assessment or prediction of the individual's PON1 status. On the other hand, a two-

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substrate enzymatic analysis provides an accurate assessment of an individual's PON1 level and position 192 functional statuses (i.e. the individual's PON1 functional genomics). This assay can classify individuals into three phenotypes of PON1 192 activitty (Figure 9.); QQ, QR, RR, thus the functional position of the 192 genotype is accurately inferred. In addition, PON1 activities within a genotype provide information on the levels of PON1 in the plasma of a given individual within that genotype group, which is most relevant to assess an individual's sensitivity to a specific OP. The PON1 phenotype at position 192 can be verified by genotyping the individual's DNA. However, with very few exceptions, this functional genomic analysis is very accurate, and does not require verification (Jarvik, 2003). An example of possible discordance is an individual who is genotyped as a PON1Q/R heterozygote but whose PON1 status functional analysis indicates a PON1192Q or PON1192R homozygote; this would indicate that one of the alleles is nonfunctional, and underlines the advantage of the PON1 status analysis over straight genotyping. Though most of the variation in serum PON1 activity can be explained by its polymorphisms in the coding region (Q192R) and the 5'-regulatory region (T-108C), modulation of PON1 by a variety of other factors should also be considered. Alcohol, smoking, drugs, diet and certain physiological and pathological conditions can increase or decrease PON1 activity, and their contribution to measured levels is important (Costa, Vitalove, Cole , & Furlong, 2005)

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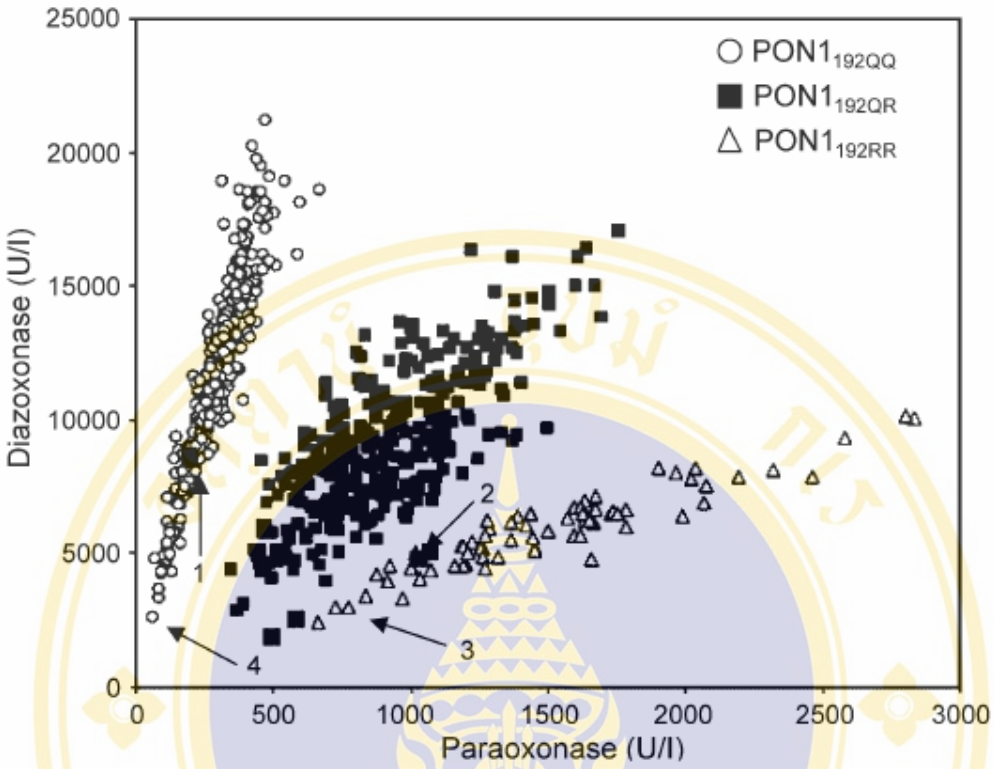

Figure 9. Determination of PON1 status. Plot of diazoxonase vs. paraoxonase activities. Note that the two-substrate assay provides an accurate inference of PON1Q192 genotype (QQ, QR or RR, as confirmed by PCR), as well as the level of plasma PON1 activities (PON1 status). (Costa et al, 2005)

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3.7 The frequencies of PON1 polymorphism in various populations

There are many studies on the distribution of PON1 gene. Most of PON1 polymorphisms were studied at the positions 192 (Q/R), 55 (L/M), and -108 (C/T). The allele frequencies of PON1 were different in each country. Table 3 provides a list of allele frequencies from populations around the world.

Table 3. The distribution of allele frequency of PON1 in various populations

POPULATION	-108C	55M	192R	Reference
African-American	0.85	0.21	0.63	Chen et al., 2003
African	0.83	0.17	0.66	Sklan et al.,2004
Afro-Brazilian	ND	0.29	0.53	Allebrandt et al., 2002
American	0.5	0.36	0.33	Brophy et al, 2001
Caribbean	0.65c	0.29	0.46	Chen et al., 2003
Caucasian-American	0.38\	0.46	0.27	Chen et al., 2003
European	0.46	0.35	0.31	Leviev and James, 2000
Israeli	0.40	0.39	0.33	Bryk et al, 2004
Japanese	0.48	0.06	0.60	Suehiro et al., 2000
Mexican	0.45	0.84	0.49	Rojas-Garcia et al., 2005
Spanish	0.38	0.41	0.30	Hernandez et al., 2003
Thai	0.25	0.05	0.29	Phuntuwate et al., 2005

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4. Paraoxonase1 and sensitivity to organophosphorus compounds' toxicity

Many organophosphorus (OP) compounds are triesters of phosphoric acid. The majority are insecticides. Acute exposure to OP causes neurotoxicity, in the form of a cholinergic syndrome. Only OP with a P=O moiety can interact with acetylcholinesterase. Commonly used insecticides, such as diazinon and chlorpyrifos, have a P = S bond that are needed to be converted to their oxygen analogs. Bioactivation occurs by a process of oxidative desulfuration catalyzed by cytochrome P450 enzymes (Figure 10). PON1 can hydrolyze and hence detoxify a number of insecticidal organophosphates which include some nerve agents (Draganove & La Du., 2004). The existence of polymorphisms in PON1 that confers different hydrolyzing activities toward OP, as well as different levels of expression, has long inspired the hypothesis that certain individuals may be more sensitive to OP toxicity (La Du, Billecke, Shu, Haley, & Broomfield, 2001).

The ability of PON1 to hydrolyze several OP in vitro has long been taken as an indication that it may modulate OP toxicity in vivo. Furthermore, the presence of polymorphisms in PON1, which confers different enzyme levels and catalytic efficiency, has suggested that certain individuals may be more susceptible to the toxic effect of OP exposure. In earlier studies, PON1 purified from rabbit serum was injected into rats or mice to increase plasma hydrolytic activity toward OP substrates. When challenged with an OP (chlorpyrifos oxon is used in most instances), animals that have received exogenous PON1 were more resistant than the controls to the acute cholinergic toxicity. Interestingly, PON1 provided some protection against chlorpyrifos, the parent compound that is used as insecticide, when given before or even after this OP (Li, Furlong, & Costa, 1995). More recently, PON1 knockout mice, which have no detectable plasma and liver hydrolytic activity toward paraoxon and diazoxon and very limited chlorpyrifos-oxonase activity, have dramatically increased sensitivity to the toxicity of chlorpyrifos oxon and diazoxon, and a small increased sensitivity to their respective parent compounds, but they did not show an increased sensitivity to paraoxon. Administration of pure human PON1 192Q or PON1 192R to PON1 knockout revealed that PON1 192R provided better protection than PON1 192Q toward chlorpyrifos oxon, while both alloforms were equally effective in

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protecting against the toxicity of diazoxon (Li et al., 2000). A kinetic analysis of substrate hydrolysis by purified human alloforms carried out under physiological conditions (low NaCl concentration), provided an explanation for the in vivo findings. In case of chlorpyrifos oxon, both PON1 alloforms have high catalytic efficiency and that of PON1 192R is significantly higher. Catalytic efficiency is still high in case of diazoxon, although lower than with chlorpyrifos oxon, but no alloform specific difference is evident. For paraoxon, the PON1 192R alloform is much more efficient than the PON1 192Q alloform in vitro assays, but its overall catalytic efficiency is very low and unable to confer protection in vivo (Li et al., 2000). This confirms the hypothesis that PON1 is not efficient in hydrolyzing paraoxon at low concentrations, suggesting that PON1 may not degrade paraoxon efficiently in vivo. Additional experiments carried out in PON1 transgenic mice (TG) (mice expressing either human PON1 192Q or PON1 192R on a knockout background) provided further evidence for such conclusions. For example, human PON1 (hPON1) 192R-TG mice were significantly less sensitive to the toxicity of chlorpyrifos oxon than hPON1Q192- TG mice, despite having the same level of PON1 protein in liver and plasma. Altogether, these animal experiments indicated that the role of PON1 in modulating the toxicity of OP varies depending on the substrates. In the case of paraoxon, no apparent in vivo effect of PON1 is seen; with diazoxon, protection or susceptibility is dictated primarily by the level of expression of PON1, but independent from the Q192R genotype. In the case of chlorpyrifos oxon, both the level of expression and the Q192R genotype are important determinants of susceptibility.

There were evidences of PON1 associate with sensitivity to OP toxicity in vivo. The farmer exposure to diazinon cause chronic central and peripheral nervous system abnormalities, and have a higher proportion of PON1-192R polymorphism (Mackness et al, 2003). A few studies have also investigated PON1 genotypes and plasma activity in cohorts of veterans from the 1990–91 Persian Gulf War in the US and the UK (Hotopf et al, 2003). In two studies, lower paraoxonase activity was found in veterans who experienced symptoms associated with the Gulf War Syndrome compared to controls. The finding of low PON1 activity in neonates (Cole et al, 2003) had suggested that PON1 levels may be lower before birth. In addition, an mother with

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low PON1 status would be predicted not to be able to provide protection for her fetus against exposure to some OP. Recently, offspring of mothers with low PON1 status exposed to chlorpyrifos were reported to have significant smaller head circumference compared to those born to mothers with high PON1 activity or those not exposed to chlorpyrifos (Berkowitz et al, 2004).

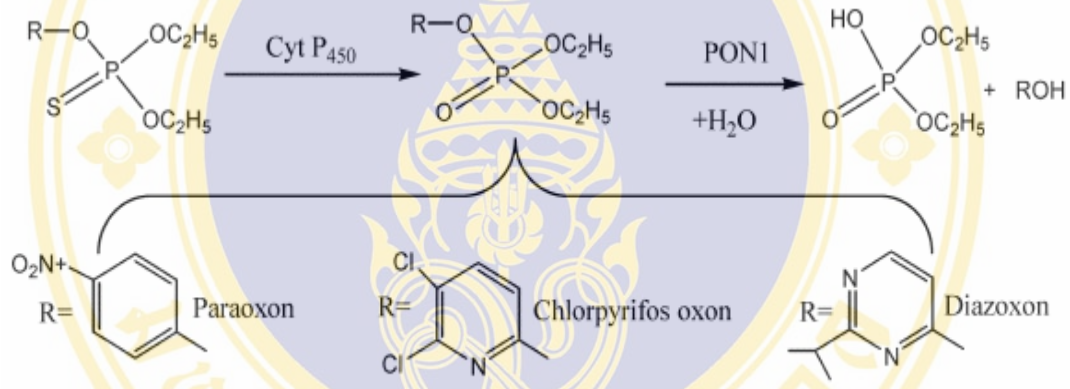

Figure 10. Hydrolysis of oxon metabolites of commonly used insecticide (Draganove & La Du, 2004)

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5. PON1 and cardiovascular disease

The influence of PON1 and its polymorphisms on the risk of cardiovascular disease appears related to its ability to metabolize oxidized lipids and to prevent their formation. Oxidized LDL is believed to plays a central role in monocyte chemotaxis and macrophage differentiation, which are early events in the progression of atherosclerosis, whereas HDL destroys these biologically active oxidized lipids. PON1, the major enzyme responsible for this protective effect, is associated with HDL particle. HDL particle appears to accept PON1 directly by high-affinity desorption as it is released from the liver, in a process that requires both apolipoprotein and phospholipids. PON1 can prevent the formation of oxidized LDL and inactivate LDL-derived oxidized phospholipids once they are formed, and also protect phospholipids in HDL from oxidation (Aviram et al., 1998). PON1 hydrolyzes phospholipid and cholesteryl ester hydroperoxides and hydroxides derived from arachidonic acid or linoleic acid, and also reduce linoleic acid hydroperoxides to linoleic acid hydroxides (Avrium et al., 2000). The hydrolysis of phospholipid hydroperoxides by PON1 produces aldehyde or ketone derivatives which serve as substrates for platelet-activating factor acetylhydrolase (PAF-AH) and generates lysophospholipids such as lysophosphatidyl choline (Watson et al., 1995). An oxidized HDL lipids are converted by apolipoprotein A-1 to phosphatidylcholine aldehydes that are subsequently hydrolyzed to lysophosphatidylcholine by PON1, without detectable release of linoleate, arachidonate, or their hydroperoxy or hydroxy derivatives. Thus, PON1 appears to have esterase-like, peroxidase-like, and phospholipase-like activities that inhibit the formation of proinflammatory oxidized phospholipids and degrade them once they are formed.

It is likely that variability among individuals in the PON1-catalyzed hydrolysis of oxidized lipids arises from two sources. The first source of variability is the polymorphism at the position Q192R, which results in different substrate-specific catalytic efficiencies of hydrolysis for 192R and 192Q alleles. The strong association of PON1 192R with vascular disease predicts that this isoform would have a lower catalytic efficiency of hydrolysis of oxidized lipids than PON1 192Q (Lawlor et al, 2004). Previous studies reported that both isoforms reduced total lipid-peroxide

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content of human atherosclerotic lesion homogenates, with Q allele being twice as efficient as R allele. The second source of variability in PON1 activity is PON1 abundance in the plasma, which has been largely ignored in studies examining the association of PON1 with atherosclerosis or other diseases. Studies of PON1 genotype as a predictor of human vascular disease have reported mixed results, but overall they suggest that genotype is one of many predictors of vascular disease (Heijsmans et al., 2000). The 192R and PON1 55L alleles are in strong linkage disequilibrium in multiple ethnic groups. Recent data suggest that the PON1 L55M polymorphism is correlated with the plasma PON1 concentration both through linkage disequilibrium with promoter polymorphisms and possibly through increased protein turnover of the PON 55M isoform (Brophy et al., 2001).

6. PON1 and drug metabolism

The ability of PON1 to hydrolyze some novel substrates, most notably lactones and carbonate esters (Figure. 11), has been reported (Billecke et al, 2000). This finding may have several important significances. First, PON1 may be involved in the metabolism of drugs, in which case the PON1 status of an individual would be relevant to a drug's effectiveness and/or side effects. Several studies revealed that the diuretic spironolactone and the hypocholesterolemic drugs mevastatin, lovastatin, and simvastatin are hydrolyzed by PON1, and that the Q and R isoforms hydrolyze these drugs with approximately the same efficiency. Pravastatin was found to increase serum apolipoprotein A1 and HDL cholesterol to a greater extent in men with the RR genotype of PON1 than in those with the QQ genotype (Malin R et al, 2001). Simvastatin therapy also increases plasma paraoxonase activity, possibly by increasing the transcriptional activity of the PON1 gene (Tomas et al, 2000). The therapeutic response related to PON1 activity is independent of position 192 and position 55 polymorphisms. Second, novel classes of the so-called prodrugs may be developed by incorporating a lactone or cyclic carbonate moiety into other molecules, which can thus be inactivated or bioactivated in vivo by PON1. Inactivation has been shown to occur with glucocorticoid γ-lactones, while activation was found in the antibacterial

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prodrug, prulifloxacin. The latter is hydrolyzed at a higher rate by type R than by type Q. Interestingly, the free cysteine in position 284 of PON1, which is not required for the hydrolysis of paraoxon or phenylacetate, is essential for the lactonase activity of the enzyme, as it is essential to protect LDL against oxidation. Third, PON1 may exert other effects on endogenous substrates, in addition to protecting LDL from oxidation. It has been proposed that PON1 protects against homocysteinylation by hydrolyzing homocysteine thiolactone, but the catalytic efficiency for this reaction is low (Jakubowski, 2000). Although the Q isozyme is more efficient in protecting against LDL oxidation, the R type hydrolyzes thiolactones more readily.

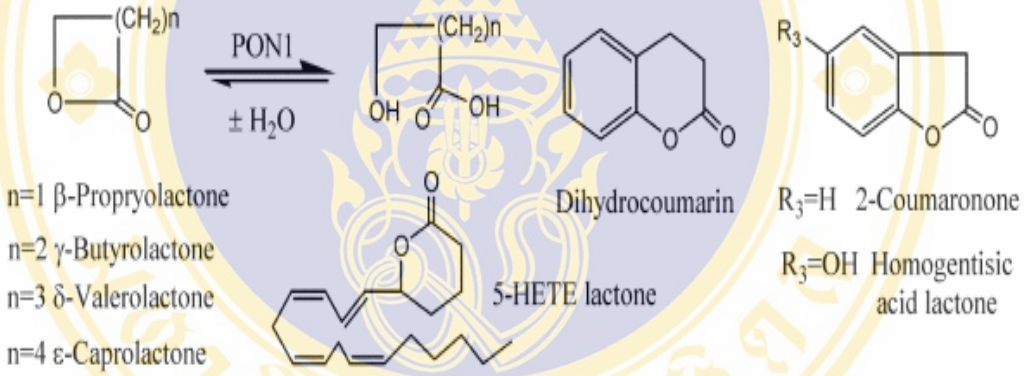

Figure11. Lactone hydrolysis and lactonization of hydroxy acids (Draganove & La Du, 2004)

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7. Modulation of paraoxonase1 activity

7.1 Modulation of PON1 by exogenous compounds

Environmental chemicals

PON1 activity is completely dependent upon calcium, and EDTA irreversibly abolishes its activity. Other cations, however, have been shown to have an inhibitory effect on PON1 activity. Barium, lanthanum, copper, zinc, and mercury were found to inhibit PON1 activity from human liver (Gonzavo, Gill, Hernandaze, Villanueva, & Pla, 1997). Similar results were obtained in a subsequent study in human serum from PON1 192Q individuals, where an inhibitory effect of manganese, cobalt, cadmium and nickel was also identified (Debord, Bollinger, Merle and Dantoine, 2003). In case of mercury and copper, further biochemical studies suggested that a free thiol group on the Cys 285 residue may be the molecular target. More recent experiments have shown that cadmium, iron, zinc, and mercury are the most potent in vitro inhibitors of PON1 192R activity, greater than 80% inhibition. Overall, PON1 192Q appears to be somewhat less sensitive to inhibition by metals, with the exception of lead (Cole, Li, Richter, Furlong, & Costa, 2002).

Drugs

An increase in serum-PON1 activity was found in patients treated with simvastatin and other statins (Gouedard et al., 2003). In a cohort study of aspirin users, a significant increase of plasma PON1 activity and concentration was reported. Such effect may be due to the anti-inflammatory effect of aspirin, as serum PON1 levels are reduced during the inflammatory response in animal models; alternatively, aspirin may act as an antioxidant (Blatter-Garin, Kalix, De Pre, James, 2003). The anti-inflammatory glucocorticoid, dexamethasone, caused an eight fold increase in PON1 mRNA (Ali et al., 2003). Finally, the cholinergic muscarinic antagonist, atropine, was shown to inhibit human plasma PON1 in vitro (Radic and Reiner, 1986).

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7.2 Modulation of PON1 by life-style factors

Smoking

Cigarette smoke extract was found to inhibit human plasma PON1 activity (Nishio & Watanabe, 1997). This effect was not reversed by antioxidants such as vitamin E, vitamin C, superoxide dismutase, and catalase, but it was antagonized by GSH, *N*-acetylcysteine, 2-mercaptoethanol, DTT, and L-cysteine. The finding support that free thiols are the center of the inhibitory effect. Compounds suggested to be responsible for inhibition of PON1 activity are various reactive aldehydes (acetaldehyde, formaldehyde and α,β-unsaturated aldehydes, such as acrolein and crotonaldehyde), as well as aromatic hydrocarbons (Nishio & Watanabe, 1997).

Alcohol

The finding that moderate doses of ethanol exert a protective role in cardiovascular disease has prompted studies on the possible modulation of PON1 by alcohol. The observations in animals and humans have shown that low levels of ethanol increase PON1 activity and levels. The studies in healthy middle-aged men and postmenopausal women showed an increase of plasma PON1 activity following moderate alcohol consumption of wine, beer or spirits (Van der Gaag et al., 1999). In another study, light drinkers had a 39.5% higher, whereas heavy drinkers had a 45% lower serum plasma PON1 activity compared to non-drinkers. PON1 polymorphisms do not appear to be a factor in PON1 modulation by light and heavy drinking, and the regulation of PON1 expression may be related to effects of alcohol on protein kinase C, which may be involved in the phosphorylation of an Sp1 binding site in the promoter region of PON1 (Rao et al, 2003).

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7.3 Modulation of PON1 activity by diet

Fat-rich diet

The proatherogenic diet caused a significant fall in PON1 activity or mass, which correlated with a reduction in HDL-cholesterol. Diet with high *trans*-unsaturated fat contents can reduce PON1 activity (De Roos, Schouten, Scheek, van Tol, & Katan, 2002). Meals rich in used cooking fat, which contains high contents of oxidized lipid, induced a significant fall in PON1 activity.

Antioxidants

PON1 activity is protected by the antioxidant polyphenols including quercetin and glabridin. The consumption of pomegranate juice, high amount of polyphenol, and other antioxidants, can raise PON1 activity up to 20% (Kaplan et al, 2002). Quercetin and naringenin act as ligand for the AhR (aryl hydrocarbon receptor). AhR is ligand activated transcription factor which binds to XRE (xenobiotic responsive element). Over expression of AhR increased the induction of the PON1 gene by polyphenols. AhR specifically bound to an XRE-like sequence in the PON1 promoter (Gouedard at al., 2004). The effects of vitamins C and E on PON1 activity were not clear. Some studies have reported that PON1 activity correlated positively with the quantity of vitamins C and E in diet (Jarvik et al., 2002). However, another study did not show the positive result (Rantala et al, 2002).

7.4 Effects of age on PON1 activity

PON1 during development

Age is a major determinant of PON1 activity. The studies in humans have also shown that serum PON1 activity is very low at birth and increases over time, reaching a plateau between 6 and 15 months of age. PON1 activity in the fetus may be even lower, as indicated by a 24% lower activity in premature babies (33–36 weeks of gestation) compared to term babies (Cole al., 2003). Low PON1 activity during development could imply a relevant risk of developing the toxicity of organophosphate insecticides.

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PON1 and aging

PON1 activity is quite constant over time, once it reaches adult values. However, the recent investigations have reported a progressive decrease in PON1 activity in the elderly (Seres, Paragh, Deschen, Fulop, & Khaili, 2004). This decline in PON1 activity may be related to the development of oxidative stress conditions with aging, and would have an impact on the increased incidence of atherosclerosis with age.

7.5 PON1 in certain physiological and pathological conditions

Plasma PON1 activity has been found to decrease in a number of pathological conditions and diseases. Both insulin-dependent (type 1) and non-insulin-dependent (type 2) diabetes have been consistently associated with lower serum PON1 activity (Mackness, Durrington, Boulton, Hind, & Mackness, 2002). This low PON1 activity is independent of genotype, and may be due to altered glycation of HDL and/or PON1, lower rate of synthesis or higher rate of catabolism, or increased oxidative stress. Decreased serum PON1 activity has also been found in patients with chronic renal failure undergoing hemodialysis (Dantoine et al., 1998), and patients with rheumatoid arthritis (Tanimoto et al, 2003). Patients with Alzheimer's disease and vascular dementia also displayed lower (30–40%) serum PON1 activity (Paragh et al, 2002). Pregnancy has been found associated with lower PON1 activity in humans (Weitman, Vodienik, & Lech, 2003). Moderate exercise was found to increase serum PON1 activity by 14% (Senti et al, 2003).

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CHAPTER IV
MATERIALS AND METHODS

1. Subjects

The subjects were recruited from the participants in the Electricity Generating Authority of Thailand (EGAT) study project of Ramathibodi Hospital since 1998. They were 160 unrelated males and females from four locations of EGAT (Bang Krui, Bhumibol Dam, Khao Leam Dam, and Srinakarin Dam). All subjects gave their written informed consent. The project was approved by Ethical Clearance Committee on Human Rights Related to Researches Involving Human Subjects, Faculty of Medicine, Ramathibodi Hospital, Mahidol University. All volunteers were requested to fill out their information in a questionnaire including smoking, drinking status, age, medical history. Physical examinations were also recorded.

Sample size calculation

Sample size was calculated by the formula shown below;

$$N = \frac{Z^2 pq}{D^2}$$

Where N = number of subject, Z = z-score of 95% confidence interval, D = the percentage of error, p = the allele frequency of wild type, and q = the allele frequency of mutant allele.

In this study, the allele frequencies of Chinese population was used, p = 0.4 and q = 0.6, (Wang et al., 2003) and 10% of error to calculated number of subject. After calculation, it was assumed that the number of subject should be at least 92 subjects.

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MATERIALS

1. Enzymes

Alw I (5,000 U/mL)	Biolabs
BsrB I (10,000U/mL)	Biolabs
Taq DNA polymerase (5000 U/mL)	Invitrogen

2. Oligonucleotide primers

Oligonucleotide primers used in this study were as described by Campo et al. (2004) for the position –108 of promoter region and were designed according to an available published PON1 sequence by Primer 3 software for the position 192 of coding regions. All oligonucleotide primers were synthesized and purified by the Operon Biotechnologies.

The sequence of oligonucleotide primers for DNA amplification in the promoter region of PON1 gene at position -108 were;

Sense strand: 5’ AGCTAGCTGCGGACCCGGCGGGGAGGAG 3’

Antisense strand: 5’ GGCTGCAGCCCTCACCACAACCC 3’

The sequence of oligonucleotide primers for DNA amplification in the coding region of PON1 gene at position 192 were;

Sense strand: 5’ TATTGTTGCTGTGGGACCTG3’

Antisense strand: 5’ CAAATCCTTTCTGCCACCACT3’

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3. Chemical substances

Absolute Ethanol	Merck
Boric acid	Sigma
Bromphenol Blue	Sigma
100 mM dNTP	Beorinhger
100 mM dCTP	Beorinhger
100 mM dGTP	Beorinhger
100 mM dTTP	Beorinhger
DNA blood mini kit	QIAGEN
Ethidiumbromide ($C_{21}H_{20}N_3Br$, MW=394.3)	GIBCOBRL
Ethyldiamine tetraacetic acid disodium salt dehydrate ($C_{10}H_{14}N_2Na_2O_8.H_2O$, MW= 394.24)	GIBCOBRL
Ficoll	Sigma
Ladder marker	Biolabs
Magnesium Chloride	Invitrogen
Nusieve 3:1 agarose	Cambrex
10X PCR buffer	Invitrogen
Seakem LE Agarose	FMC
Trisma base	Sigma

4. Instruments

Autoclave	Hirayama, Japan
Autopipette	Pipetteman, France
Px2 thermo cycler	Thermo electron corporation, USA
Horizontal agarose gel electrophoresis set	Mupid, Japan
Biological safety cabinet class II	Heraeus Sepatech, USA
Microcentrifuge	Scientific Inc
Power supply electrophoresis	EC apparatus Co., USA
UV-transluminator (Dolphin-Doc)	Wealtech Crop., USA

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5. Miscellaneous

Microcentrifuge tube	Scientific plastic, USA
Pipette tip	Biohit, USA
Thin wall PCR reaction tube	Scientific plastic, USA

METHODS

1. Determination of lipid profiles

Lipid profiles were analyzed by Department of Pathology, Ramathibodi Hospital. Blood samples were obtained after a 12-hour fasting. Serum total cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides were measured by using enzymatic calorimetric assays (Bochiringer Mannhein). Serum low-density lipoproteins (LDL) cholesterol level was calculated using the Friedewald's formula (Friedewald, Levy, and Fredrickson, 1972).

2. DNA extraction

The genomic DNA was extracted from whole blood by QIAamp DNA blood mini kit (QIAGEN). The principle is based on the ability of DNA to bind specifically to silica gel membrane while contaminants passed through. The procedures followed the manufacture's instruction. Briefly, the optimized buffers were added to lyse blood sample to give the optimal condition for stabilization of nucleic acid, and to enhance selective DNA absorption to membrane. Next, alcohol was added to precipitate DNA, and lysates were loaded into the spin column. Washing buffer was used to remove impurities. The purified DNA was eluted in water or low-salt buffer. DNA was collected into a 1.5 ml centrifuge tube. The concentration and purity of DNA were determined by measuring absorbance (spectrophotometer) at wavelengths 260 nm, 280 nm, and 320 nm. DNA samples were kept at –20°C until use.

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3. DNA amplification by polymerase chain reaction (PCR)

Principles

The PCR is an in vitro DNA amplification method that involves a repeated cycling process of a number of defined stages. The technique enables large amounts of DNA to be produced from very small amounts of starting material. The processes of DNA amplification mimic the basic mechanism of DNA replication. The reagents required for the PCR include a DNA polymerase, each of four nucleotide dNTP, and source of template DNA. The technique also demands the availability of two oligonucleotide primers designed to complement DNA sequence flanking the region of interest. DNA amplification takes place in repeated cycle made up of these defined stages; denaturation, annealing and extension. In the first stage, denaturation, the template DNA is heated at the higher than 90°C for more than 60 seconds to separate the double-stranded DNA and produces two single strands. The second stage temperature, known as annealing stage, is reduced to 40-60°C for time interval of between 30 and 60 seconds to allow the oligonucletide primer to bind to their complementary DNA sequence on the single strand. In the final stage of the cycle, an enzymatic primer extension reaction is carried out to produce complementary copies of the initial single strands from the primers bound to the DNA. This step usually takes place at 72°C for 5-10 minute by a DNA polymerase that is able to withstand the high denaturating temperature. The three separated stages are usually repeated between 25 and 40 times. In this way, the double stranded products of the previous cycle become new templates for the next cycle. Therefore, the amount of the specific target DNA is flanked by the primers essentially doubled in each round (Figure.12). This result shows in the near exponential accumulation of specific target DNA sequence of up to million-fold in 3-4 hours.

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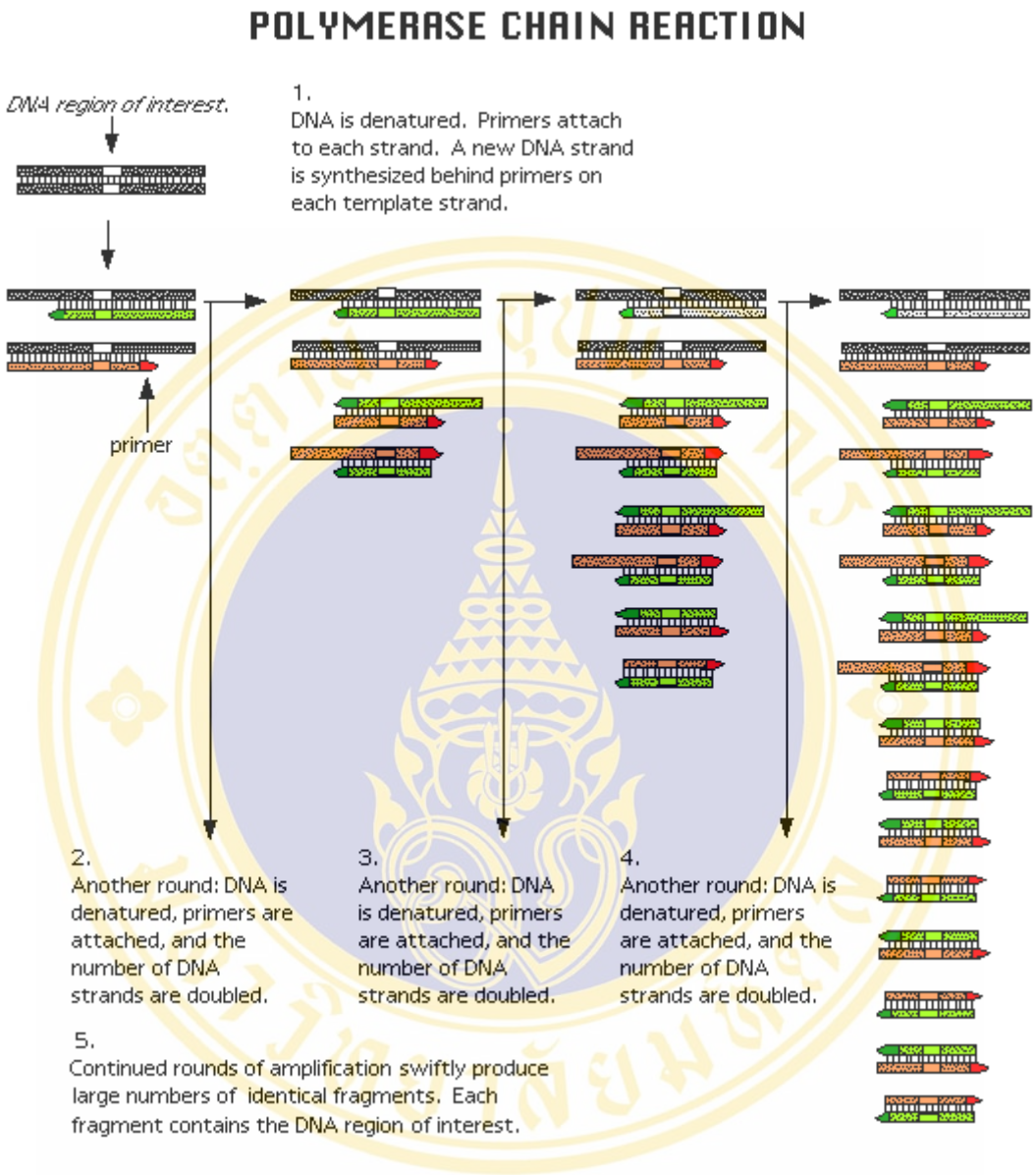

Figure 12 Principles of PCR amplification

(http://www.accessexcellence.org/RC/VL/GG/polymerase.html)

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Procedure

The template for PCR was genomic DNA, which was extracted by DNA blood mini kit. The oligonucleotide primers were synthesized for target sequence of promoter region at position –108 and coding region at position 192.

At position-108 of promoter region, target sequences were amplified in 1X reaction buffer provided by manufacture. A 25 μl reaction volume contained 10 μl of diluted genomic DNA, 1.5 μl of 2 mM dNTP, 0.5 μl of 20 μM sense oligonucleotide primers, 0.5 μl of 20 μM antisense oligonucleotide primers, 1.25 μl of 50 mM $MgCl_2$, 2.5 μl of 10X reaction buffer, 0.1 μl 5000 U/ml of taq DNA polymerase, and 8.65 μl ddH_2O. Reaction was performed in 0.2 ml thin wall micro-tube with the thermal cycler. The thermal profiles were started at 95°C for 5 min and then involved 35 cycles of denaturation at 95°C for 1 min, primer annealing at 68 °C for 1min and extension at 72 °C for 1 min. The time of final cycle DNA extension up to 10 min in attempt to ensure that all new DNA templates were fully extend. The amplified products were determined by electrophoresis through 2% agarose gel in 1X TBE with 100 base pair (bp) of DNA marker. The size of PCR product is 240 bp.

At the position 192 of coding region, the target DNA sequence was amplified in the same thermal cycle and reagent mixture as the position –108, except for primer annealing and oligonucleotide primers sequence. The annealing temperature in this position was 61°C. The size of PCR products was 155 bp.

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4. Analysis of Restriction Fragment Length Polymorphism (RFLP)

Principles

Restriction endonucleases

Restriction endonucleases are bacterial enzymes that cut double-strand DNA molecules in a precise and reproducible manner. Three different classes of restriction endonucleases have been recognized. Each class has a slightly different mode of action. The type II restriction endonucleases are the most important and frequently used as cutting enzymes. Restriction enzymes recognize specific sequence in double-strand DNA and cleave the DNA, usually within the recognition size, to yield fragments of defined length. The specific recognition sequence of double-strand DNA for the vast majority of type II restriction enzyme is usually between 4 and 6 base pairs in length and generally palindromes (a sequence of bases is the same both strand when read in 5' to 3' direction). Restriction enzymes cut the DNA at every point at which the target sequence occurred. Many restriction enzymes cut at the middle of the recognition sequence to produce a "blunt end" whereas others cut at different nucleotide usually 2 or 4 bases apart resulting in DNA fragments with short single strand overhang at each end which are know as "sticky or cohesive ends" by using restriction endonuclease to digest DNA physical map of molecule can be obtained. (Figure.13)

RFLP is restriction fragment length polymorphism. This technique is based on sequence polymorphisms that occur in the human genome and the genome of every other organism. Sequence polymorphisms are slight sequence difference (usually single base pair change) that occurs from the individual-to-individual once every few hundred base pairs. Restriction fragment produced when a DNA molecule is treated with a restriction endonuclease enzyme that binds to a DNA molecule at specific sequence and markers for detecting a number of diseases and has provided a new horizontal for human genetic. Each difference from the consensus human genome sequence that occurs in some fraction of human population, every individual has some of them. Some of sequence affect recognition site for restriction enzyme, resulting in

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the variation from individual to individual in the size of DNA fragment produced by digestion with particular restriction enzyme.

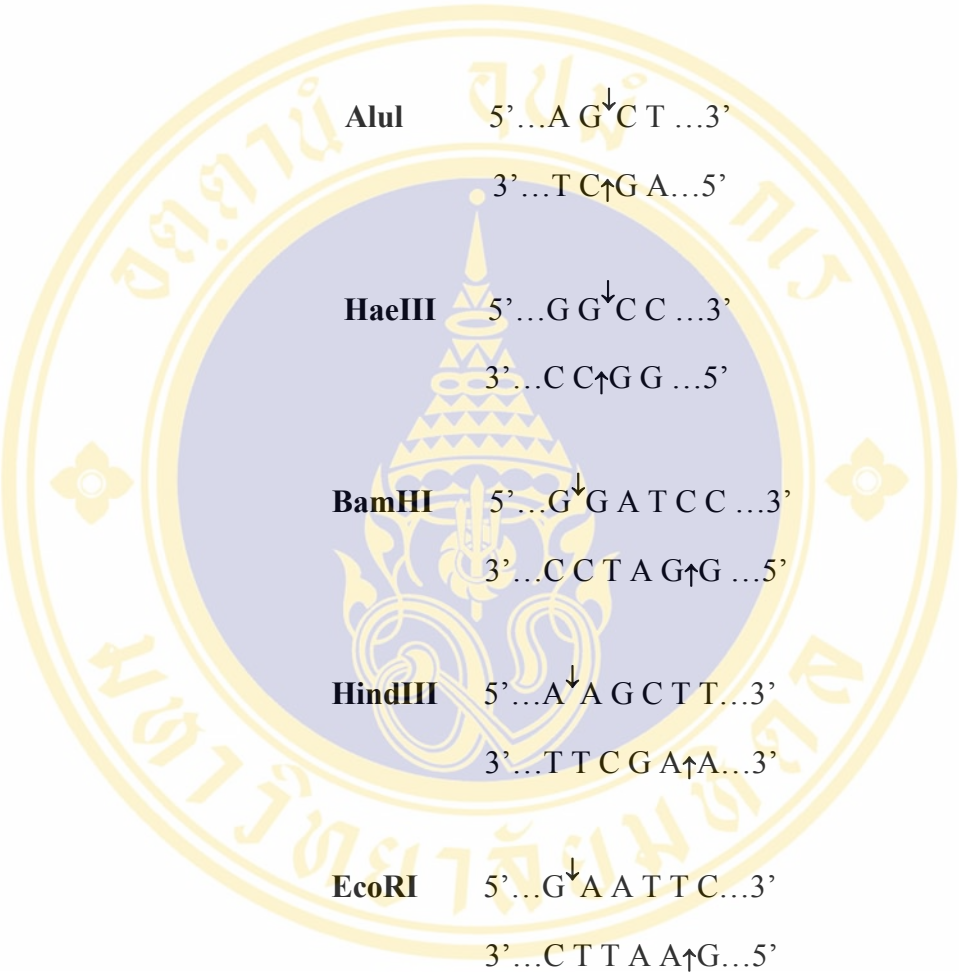

Figure 13. Characteristics of restriction enzymes. AluI and HaeIII produce bunt ends. BamHI, HindIII, and EcoRI produce "sticky" ends.

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Procedures

BsrB I digestion of position C-108 T

The PCR-amplified 240 bp DNA was subjected to digestion. The 10 µL reaction mixture contains 5 µL of PCR products, 0.5 µL of BsrB I (10000 U/mL), 1 µL of 10X NEBuffer 2, and 0.35 µL of ddH_2O. The digestion reaction was incubated at 37°C followed by electrophoresis on 3% nusieve agarose gel. The DNA fragments were observed by ethidium bromide staining and were visualized under ultraviolet light. The DNA patterns were photographed by UV-transluminator (Dolphin-DOC).

Alw I digestion of position Q192R

The PCR-amplified 155 bp was subjected to digestion. The 10 µL reaction mixture contains 5 µL of PCR products, 0.5 µL of Alw I (5000 U/mL), 1 µL of 10X NEBuffer 4, and 0.35 µL of ddH_2O. The digestion reaction was incubated at 37 °C followed by electrophoresis on 3% nusieve agarose gel. The DNA fragments were observed by ethidium bromide staining and were visualized under ultraviolet light. The DNA patterns were photographed by UV-transluminator (Dolphin-DOC).

5. Agarose gel electrophoresis for analysis of DNA fragments

Principels

Electrophoresis is a separation method based on the difference in mobility that analytes exhibit in an applied electric field. Electrophoretic method can be applied for the separation of a wide variety of samples, including proteins, nucleic acids, amino acids, and carbohydrates. Electrophoresis is the movement of electric charged particles or molecules in conductive medium, which is usually an aqueous buffer, and also referred to as an electrolyte or run buffer. The mixture of analytes is introduced into the medium containing the run buffer, and electric field is applied. Upon application of electric field, the anions start moving toward the positive electrode (anode). Difference in charges and sizes leads to different mobility and thus separation of different sample components. Similarly, positive charged ions move towards the cathode in an applied electric field.

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Reagent preparation

a. 10X TBE buffer pH 8.0

Weigh 108 g Trisma base, 55 g boric acid and 9.3 g EDTA disodium salt. Add ddH_2O up to 1 L and sterilize by autoclave.

b. Ethidium bromide solution

Weight 1 g of ethidium bromide and dilute in 100 mL of water.

c. 2% Agarose gel

Weigh 1 g of agarose gel, dissolve in 50 mL of 1X TBE buffer and 20 mL of distilled H_2O, boil, and pour the melting gel in the gel cassette. Let the gel stand at room temperature until it completely polymerizes.

d. 3% Nusieve agarose gel (FMC seakem LE agarose:nusieve =1:1)

Weigh 0.75 g seakem LE agarose and 0.75 g nuseive agarose, dissolve in 50 mL of 1X TBE buffer and 20 mL of distilled H_2O, boil, and pore the melting gel in the gel cassette. Let the gel strand at room temperature until it completely polymerizes.

e. Loading buffer

Weigh 4 g Ficoll and 0.06 g bromophenol blue. Add 25 µL of 10X TBE buffer and aliquot into 1.5 mL tube. Store at -20°C.

f. 250 ng/µL Ladder marker

Take 10 µL of 1 µg/mL stock Ladder Maker. Add 50 µL TE buffer and 20 µL Ficoll.

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Procedures

PCR products were separated by 2% agarose gel in 1X TBE buffer. DNA sample was loaded on the analytical gel with appropriate size marker (100 bp ladder marker) at 100 volts for approximately 60 min. DNA patterns were visualized under ultraviolet light and photographed by UV-transluminator (Dolphin-DOC).

Restriction enzyme digestion products were separated by 3% nusieve agarose gel (FMC seakem LE: nusieve agarose; 1:1) in 1X TBE buffer, the DNA samples were loaded on the analytical gel with appropriate size marker (100 ladder marker) at 100 volts for approximately 60 min. DNA patterns were visualized under ultraviolet light and photographed by UV-transluminator (Dolphin-DOC).

6. DNA Sequencing

Principles

The dideoxy chain termination method was developed by Sanger et al (1981). In this method, PCR-amplified DNA was used as a template. A single strand-DNA fragment whose sequence is to be determined is used as template for the synthesis of the complementary strand. The new strand grows from the 5' to 3' end. In this technique, direction of growth is true for all nucleic acid synthesis. The synthesis can be interrupted at every possible site in a population of molecules. The interruption of synthesis depends on the presence of 2'3'-dideoxyribonucleoside triphosphate (ddNTPs). The 3'-hydroxyl group of deoxyribonucleoside triphosphates has been replaced by hydrogen. This ddNTP can be incorporated in a growing DNA chain, but they lack 3'-hydroxyl group to form a bond to another nucleoside triphosphate. The incorporation of a ddNTP into the growing chain causes termination at that point (Figure.14). The presence of small amounts of ddNTPs in a replicating mixture causes random termination of chain growth.

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The principle of the method is summarized in Figure 15. Primers and templates are denatured to separate the two strands of DNA template, and then annealed to form the primer-template complex. The mixture is then divided into four samples. One (the T sample) is incubated with DNA polymerase in the presence of a mixture of ddTTP and a low concentration of TTP, together with the other three deoxynucleoside triphosphates (one of which is labelled with ^{32}P) at normal concentration. As the DNA chains are built up on the 3' end of the primer, and the position of the T's will be filled. At the end of incubation, there are remain a mixture of chains terminating with T at their 3' end but all having the same 5' end (the 5' end of the primer). Similarly, incubations are carried out in the presence of each of the other three dideoxy derivatives, giving mixtures terminating at the positions of C, A, and G, respectively. The four mixtures are fractionated in parallel by electrophoresis on acrylamide gel under denaturing conditions. This system separates the chains according to size, the small ones moving quickly and the large ones slowly. As all the chains in the T mixture end at T, the relative positions of the T's in the chain will define the relative sizes of the chains, and therefore their relative positions on the gel after fractionation. The actual sequence can then simply be read off from an autoradiograph of the gel. The method is comparatively rapid and accurate, and sequences of up to about 300 nucleotides from the 3' end of the primer can usually be determined.

Procedures

DNA templates were cleaned to remove primer, nucleotide, polymerase, and salt that affect the sequencing reactions by using QIAquick PCR amplification kit (QIAGEN). Next step was labeling of the DNA by PCR, the reaction was performed in 10 µl reaction mixtures; 2 µl of big dye solution (fluorescent dideoxynucleotide), 3 µl of reaction buffer, 0.5 µl of 3.2 pmol primer (sense or anti-sense), 0.3 µl of ddH_2O, 4 µl of purified DNA. The reaction mixtures were placed into the PCR thermocycler and the cycles of temperature were as follows; hot start at 96 °C for 1 min, and then involve 35 cycles of denaturation at 96 °C for 10 seconds, annealing at 50 °C for 5 seconds, extension at 60 °C for 4 min, respectively. After PCR reaction was completed, 10 µl of labeled DNA was precipitated by adding 1 µl of 3M sodium acetate, 1µl of 125 mM EDTA, and 25 µl of absolute ethanol. Then, the reaction

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mixture was let stand at room temperature for 30 min. The reaction mixture was centrifuged at 13,000 rpm for 30 min and then the supernatants were discarded. Seventy microliter of 70% ethanol was added and then centrifuged at 13,000 rpm for 10 min. After centrifugation, the reaction mixture was dried to collect the pellet of labeled DNA. Then, the DNA sequence was read by automated sequencer [ABI PRISM on model 3100 version 3.7 at bioservice unit (BSU) of National Science and Technology Development Agency (NSTDA)].

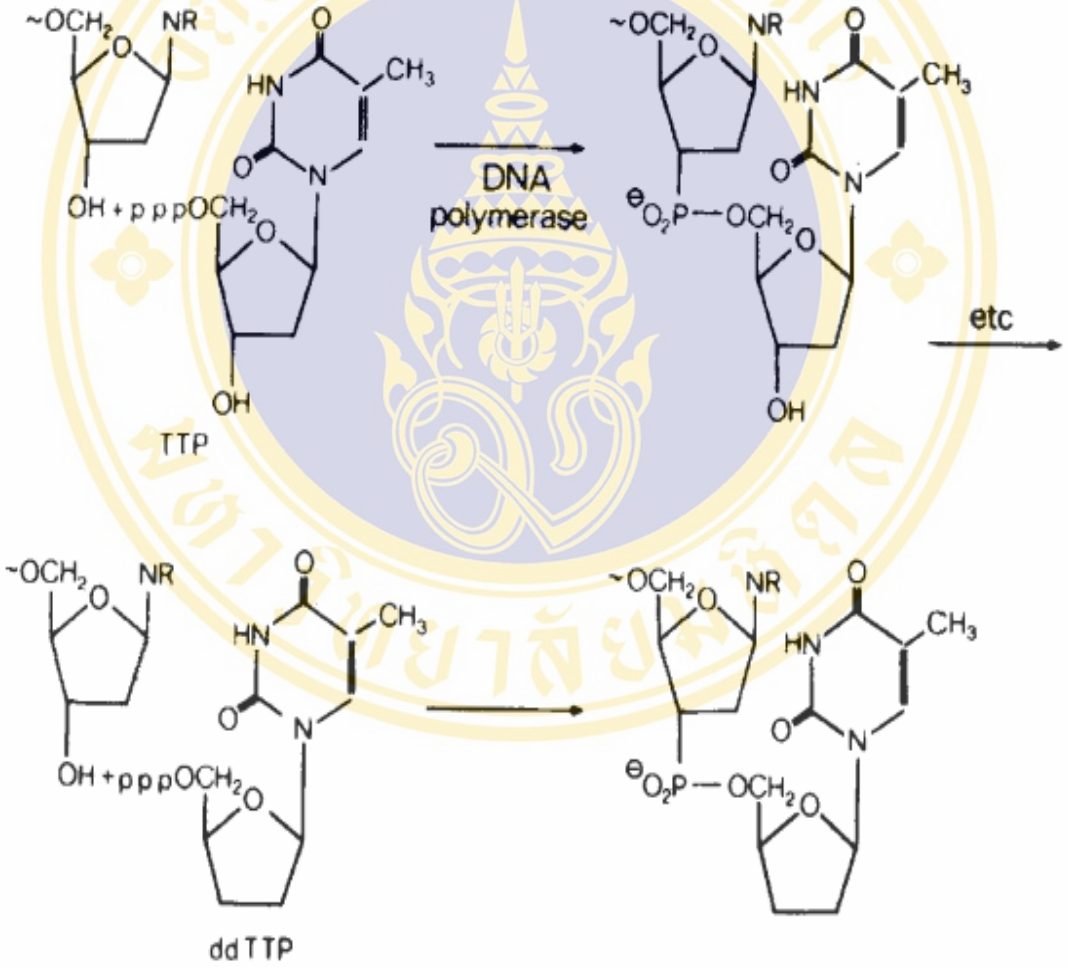

Figure 14. Diagram showing chain termination with dideoxythymidine triphosphate (ddTTP). The top line shows the DNA polymerase-catalysed reaction of the normal deoxynucleoside triphosphate (TTP) with the 3'terminal nucleotide of the primer; the bottom line, the corresponding reaction with ddTTP (Sanger, 2004).

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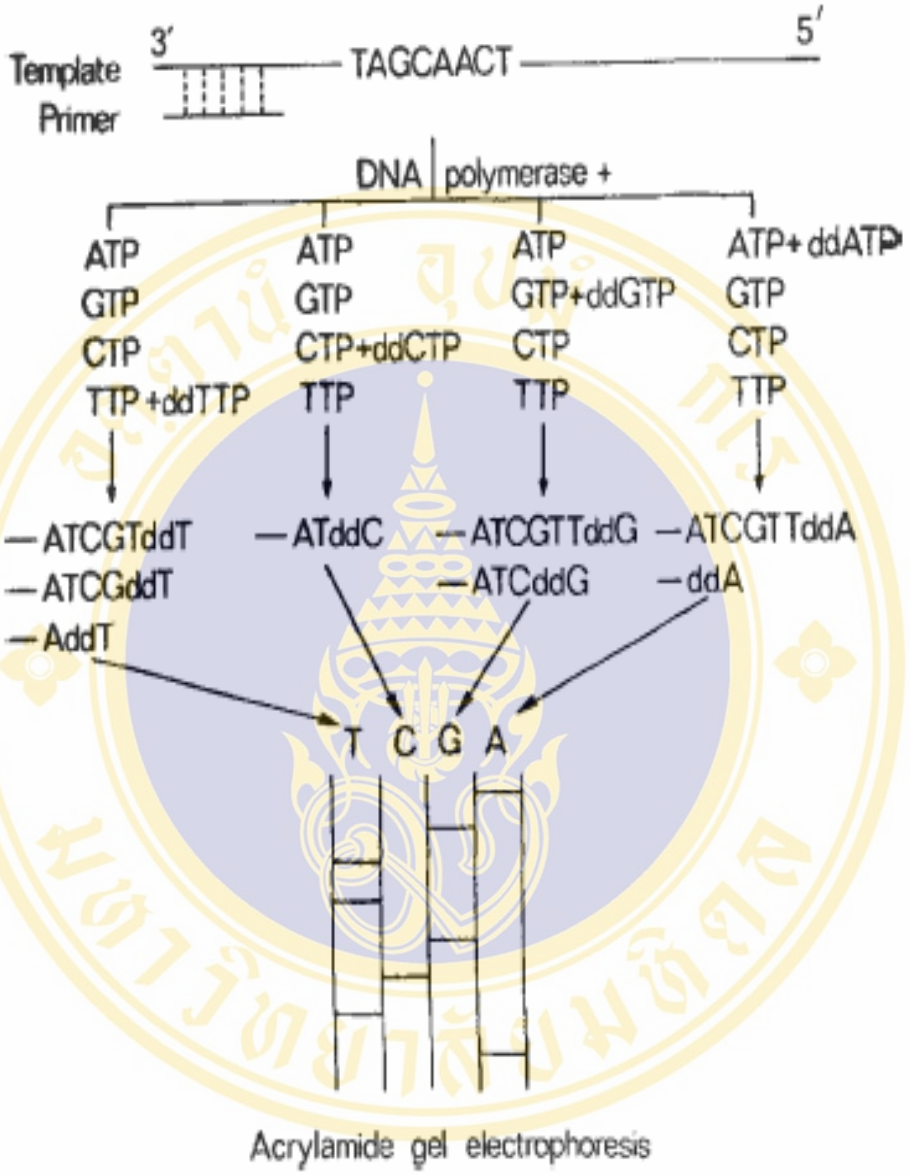

Figure 15. Principle of the chain-terminating method (Sanger, 2004)

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7. Statistical analysis

Statistical analysis employed the Statistical Package for Social Science 10 (SPSS) for Windows. The normality of sample distribution of each continuous parameter was tested with Kolmogorov-Smirnov test. Because data of HDL were not in a normal distribution, they were transformed to normal distribution by reciprocal technique. In contrast, triglyceride and total cholesterol were transformed to normal distribution by using logarithmic technique. The genotype frequency was calculated by gene-counting method. The Chi-Square goodness of fit was used for determination to Hardy-Wienberg proportion of PON1 genotype and test for linkage disequilibrium (LD). The allele frequency of this study was compared with the published frequencies for the PON1 gene in other countries and tested for significant variation by chi-square analysis. Analysis of variance (ANOVA) was used for testing the mean values of lipoproteins and PON1 genotype. Stepwise regression analysis was performed, and partial regression coefficients were used to estimate the variation of lipid profile that was due to genotype and other influent factors.

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CHAPTER V
RESULTS

1. Characteristics of the subjects

The characteristics of the subjects are summarized in Table 4. The subjects of this study consisted of 160 individuals (123 males and 37 females). The mean age of the subjects was 42.0 years. Body mass index (BMI) and waist-hip ratio (WHR) were 23.86 Kg/m^2 and 0.89, respectively. Systolic blood pressure (SBP) and diastolic blood pressure (DPB) was 123.26 mmHg and 75.93 mmHg, respectively. The mean fasting blood glucose (FBG) was 94.13 mg/dl. Compare with females, males had significantly higher WHR (0.90 vs 0.84: $p < 0.05$), SBP (125.88 vs 114.54; $p< 0.05$), and DPB (77.42 vs 70.96; $p< 0.05$). There were no significant differences in mean age (41.9 vs 42.6), BMI (23.99 vs 23. 45 Kg/m^2), and FBG (95.30 vs 90.18 mg/dl) between males and females.

As shown in Table 4, the average values of total cholesterol (TC), triglyceride (TG), high density lipoprotein (HDL-C), low density lipoprotein (LDL-C), and very low density lipoprotein (VLDL-C) were 231.25, 159.67, 53.96, 141.39, and 35.89 mg/dL, respectively. There was no significant difference in the levels of TC and LDL-C between males and females. However, the levels of TG, HDL-C, and VLDL-C were significantly different accounting for TG levels of males were higher than females (175.67 vs 106.46 mg/dL; $p < 0.05$), and the VLDL-C levels of males were higher than females (40.28 vs 21.29 mg/dL; $p< 0.05$), but the HDL levels of females were higher than males (63.1 vs 51.9 mg/dL; $p<0.05$). The percentage of subjects with diabetes, smoking, hypertension, hypercholesterolemia, alcohol drinking, and exercise were 5.1%, 32%, 9%, 15.4%, 62.2%, and 46.2%, respectively. The percentage of smoking and alcohol drinking was significantly higher in males than in females (41.7% vs 2.9% and 73.6% vs 22.9%, respectively).

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Table 4. The characteristics of subjects

Characteristics	Males	Females	Total	Reference Value **
Number	123	37	160	-
Age(year)[1]	41 ± 5	43 ± 6	42 ± 5	-
BMI(Kg/m^2)[1]	24 ± 3	23 ± 4	24 ± 3	19-25 Kg/M^2
WHR*	0.9 ± 0.05	0.84 ± 0.06	0.89 ± 0.06	< 1.0
SBP (mmHg)*	126 ± 18	115 ± 16	123 ±18	< 120 mmHg
DBP (mmHg)*	77 ± 13	71 ± 10	76 ± 12	< 80 mmHg
FBG(mg/dl)[1]	95 ± 39	90 ± 24	94 ± 36	< 110 mg/dL
TC (mg/dl)[1]	234 ± 42	234 ± 33	231 ± 40	< 200 mg/dL
TG (mg/dl)[1]*	176 ± 91	106 ± 50	160 ± 88	< 150 mg/dL
HDL-C (mg/dl)[1]*	51 ± 13	64 ± 15	54 ± 15	40-59 mg/dL
LDL-C (mg/dl)	142 ± 48	139 ± 34	141 ± 45	< 100 mg/dL
VLDL-C (mg/dl)[1*]	40 ± 41	21 ± 10	36 ± 37	20- 40 mg/dL
Diabetes; n (%)	7 (5.8%)	1 (2.9%)	8 (5.1%)	-
Smoker; n (%)*	50 (41.7%)	1 (2.9%)	51 (32.9%)	-
History of Hypertension; n (%)	13 (10.7%)	1 (2.9%)	14 (9%)	-
History of hypercholesterolemia; n (%)	20 (16.5%)	4 (11.4%)	24 (15.4%)	-
Alcohol; n (%) *	89 (73.6%)	8 (22.9%)	97 (62.2%)	-
Exercise; n (%)	58 (47.9%)	14 (40.0%)	72 (46.2%)	-

Values are mean ± SD ([1]Mann-Whitney U). Value are shown as percentage (Chi-square analysis)

Significant differences between results for males and females:

* *P-value* < 0.05

** National Cholesterol Education Program (NCEP)

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2. Analysis of PON1 polymorphism

2.1 The position -108 (C >T) of promoter region analysis

Isolated DNA of PON1 gene was amplified by using PCR technique with two specific oligonucleotide primers, sense and antisense strands. The amplified products of this position are 240 bp as shown in Figure 16.

Figure16. Agarose gel electrophoresis of amplified fragment of position-108 (240 bp) in lanes 1-7. Lane C is the negative control and lane M contains 100 bp ladder molecular weight markers.

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The previous report showed that polymorphism of PON1 gene at position -108 of promoter region was the nucleotide substitution (C > T) (Brophy et al., 2001). Genotype of the position -108 was determined by restriction endonuclease BsrB I, which recognizes sites with 5'…CCGCTC…3' and cuts the DNA between the 5'GC3'. Three hundred and twenty alleles (320) from 160 unrelated DNA samples were amplified by PCR technique. The amplified products were incubated with 2 U of BsrB I in 10X NEBuffer 2 overnight at 37 °C. The DNA was fractionated by electrophoresis on 3% nusieve agarose gel (FMC SeaKem LE agarose: Nusieve = 1:1) in 1X TBE buffer, staining with ethidium bromide, and visualized under UV illuminator. The position -108 of the promoter region after cutting with BsrB I gave a band indicating the site where enzyme is able to cut. All of amplified fragments contained a restriction site for BsrB I. The BsrB I restriction map is shown in Figure17. After digestion, the -108CC homozygote is identified by 28 and 212 bp fragments (lane 12), -108CT heterozygote is characterized by 28, 212, and 240 bp (lanes 1, 3, 4, 5, 6, 7, 8, 9, 10, 11) and -108TT homozygote contain a non-digested 240 bp fragment (lane 2.) as shown in Figure 18.

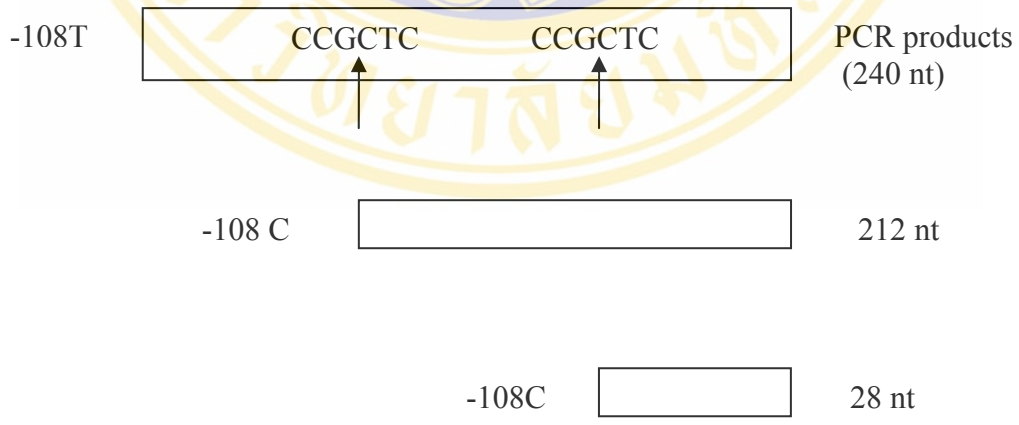

Figure17. The BsrB I restriction map of C-108 T PON1 gene

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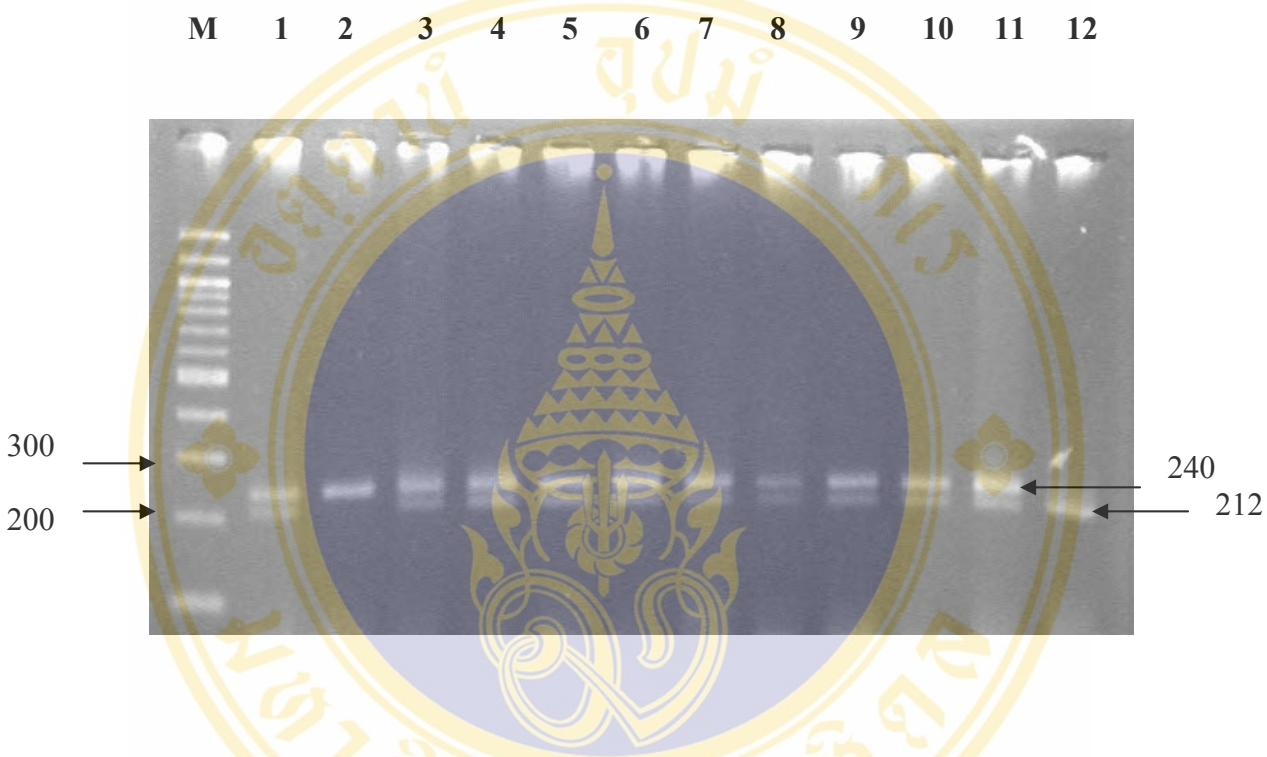

Figure18. DNA band pattern after digested with BsrB I restriction enzyme. -108CC is identified by 212 bp and 28 bp (not shown) (lane 12), -108CT heterozygote by 240 bp, 212 bp, 28 bp (lanes 1,3,4,5,6,7,8,9,10,11), and -108TT homozygote contains a single band of 240 bp (lane 2). Lane M contains molecular weight standard markers.

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2.2 The position 192 (Q >R) of coding region analysis

For this position, isolated DNA of PON1 gene was amplified by PCR technique with two specific oligonucleotide primers, sense and antisense strands. The amplified product of this region was 155 bp as shown in Figure 19.

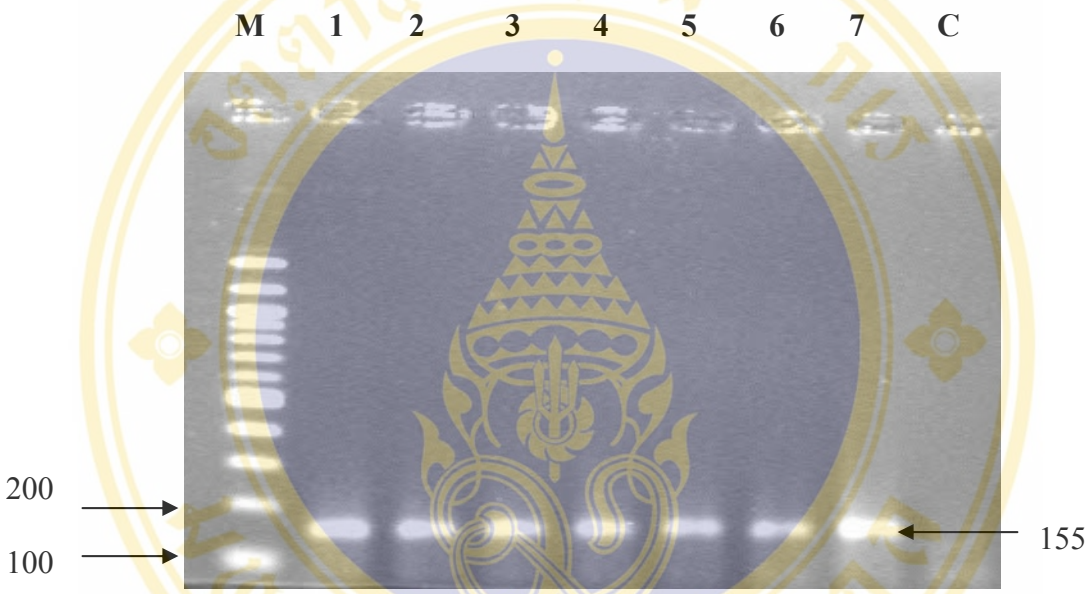

Figure 19. Agarose gel Electrophoresis of Amplified Fragment of Position 192 (155 bp) in lanes 1-7. Lane C is the control and lane M contains 100 bp ladder molecular weight markers.

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The previous report showed that polymorphism of coding region of PON1 at the position 192 was the A to G substitution, which results in amino acid substitution of arginine (CGA) for glutamine (CAA) at codon 192 (Humbert et al, 1993). Genotype for PON1 polymorphism in this position was determined by restriction endonuclease AlwI, which recognizes site with 5'…GGATC(N_4)…3' and cuts the DNA at the fourth base further from the recognition site. Three hundred and twenty alleles (320) from 160 unrelated DNA samples were amplified by PCR technique. The amplified product was incubated with 2 U of AlwI in NEBuffer 4 overnight at 37 °C. The DNA fragments were fractionated by electrophoresis on 3% nusieve agarose gel (FMC SeaKem LE Agarose: Nusieve = 1:1) in 1X TBE buffer, stained with ethidium bromide, and visualized under UV illuminator. When PON1 gene was cut with AlwI, it gave a band indicating the site where enzyme was able to cut. All of these amplified DNA fragments contain a restriction site for AlwI. The AlwI restriction map is shown in Figure 20. QQ homozygote was characterized by a single 155 base pair (lanes 1 and 9). QR heterozygote contains 155 bp, 93 bp, and 63 bp. RR homozygote was illustrated by 93 bp and 63 bp (Figure 21.)

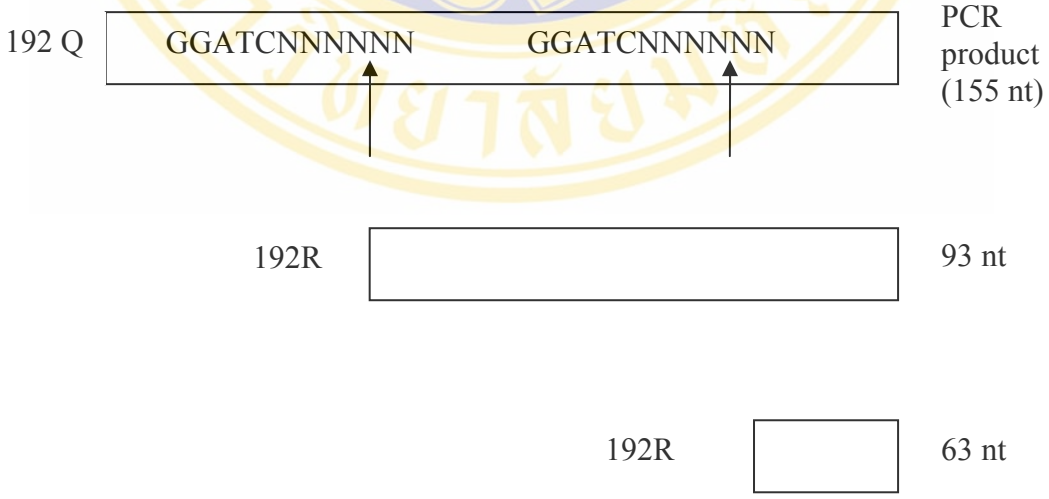

Figure 20. The AlwI restriction map of PON1 gene at position 192

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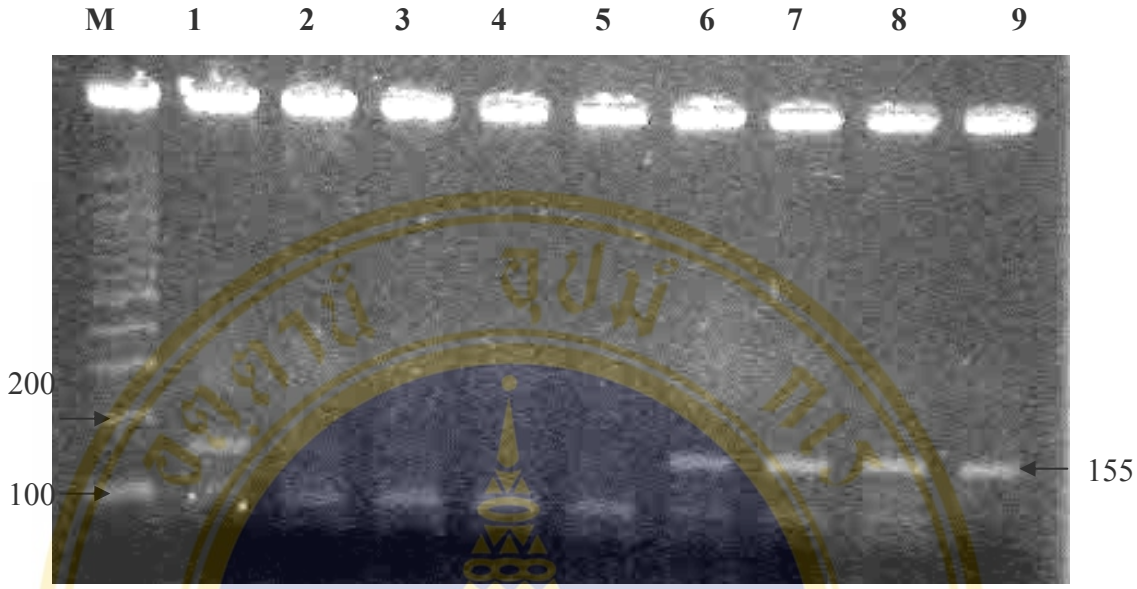

Figure 21. DNA band pattern after digested with AlwI restriction enzyme. QQ homozygote is characterized by a single 155 base pair AlwI fragment (lanes 1,9), QR heterozygote by 155 bp, 92 bp, 63*bp fragment (lanes 6,7,8) and RR homozygote contains 92 bp and 63 bp fragment (lanes 2,3,4,5). Lane M contains ladder markers.
* 63 bp is not visualized by this experiment

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3. PON1 gene sequencing

The polymorphism of PON1 was characterized and reported such as the position -108 of promoter region and the nucleotide change in codon 192 of coding region. In this study, we confirmed the polymorphic sites of PON1 at positions 192 and -108 by using DNA sequencing method. Figure 22 and 23 show the reverse strand nucleotide sequence of PON1 gene at position -108 of the promoter region and the forward strand nucleotide sequence at position 192 of the coding region, which was derived from amplified target DNA with specific primers, respectively. Both nucleotide sequences were similar to the published data.

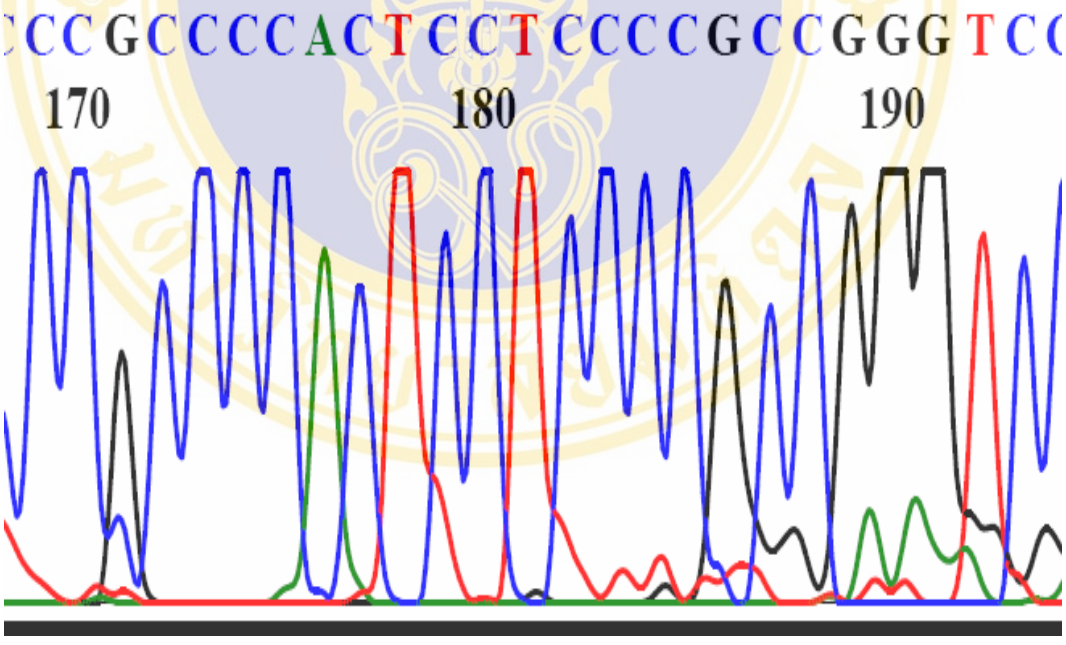

Figure 22. PON1 gene sequence at position -108, the 176th bp (A) in this DNA sequence is equivalent to position -108 of the promoter region of PON1 gene.

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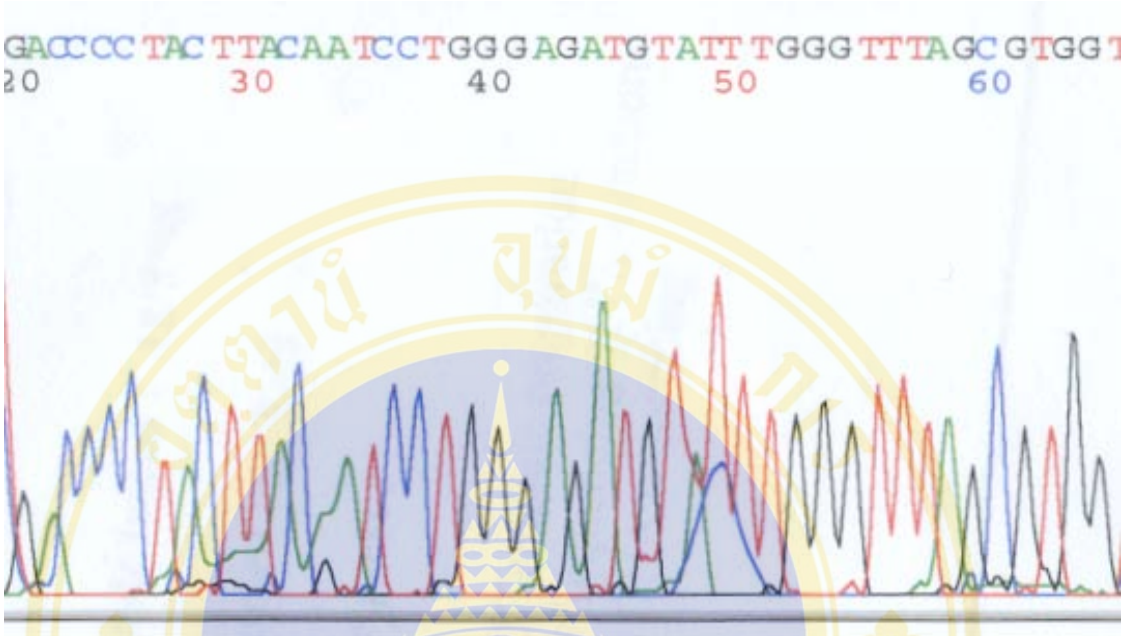

Figure 23. PON1 gene sequence at Position 192, the 32nd-34th (CAA) bp in this DNA sequence is equivalent to codon 192 of PON1 gene.

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4. PON1 allele frequency analysis

The allelic frequency can be calculated by gene counting method, which the frequency of the wild type allele is equal to number of wild type alleles divided by the total number of alleles. The frequency of mutant alleles is also equal to number of mutant alleles divided by the total number of alleles [since the homozygotes have two of given alleles, and heterozygotes have only one, the total number of alleles is twice the number of individuals (each person carries two alleles)]. Table 5 shows allelic frequency of PON1 polymorphisms at positions -108 and 192. The 320 alleles were analyzed in this study. At position C-108 T, the allele frequencies of -108C and -108T were 0.54 and 0.46, respectively. The allele frequencies of position Q192R were 0.57 and 0.43 for Q allele and R allele, respectively.

Table 5. Allele frequency of PON1

		Allele Frequencies			
		C-108 T		Q192R	
	Number	C [a]	T [b]	Q [a]	R [b]
Subjects	160	0.54	0.46	0.57	0.43

[a] Frequency of PON wild type alleles = $\frac{\text{number of wild type alleles}}{\text{number of total alleles}}$

[b] Frequency of PON mutant alleles = $\frac{\text{number of mutant alleles}}{\text{number of total alleles}}$

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5. PON1 genotype frequency

5.1. Position -108 of the promoter region

The genotype of each individual was determined by site-directed endonuclease, BsrB I restriction enzyme. Each subject could be assigned a genotype consisting of two alleles. Table 6 shows the distribution of three genotypes of this region, a predominant homozygote (CC), heterozygote (CT), and homozygote (TT). The genotype distribution of PON1 polymorphism in this region was 27.5%, 53.8%, and 18.8% for CC, CT, and TT genotype, respectively.

5.2 Position 192 of the coding region

The genotype of each individual was determined by site-directed endonuclease, AlwI restriction enzyme. Each subject could be assigned a genotype consisting of two alleles. Table 6 shows the distribution of three genotypes of PON1 gene in this position, a predominant homozygote (QQ), heterozygote (QR), and homozygote (RR). The genotype distribution of PON1 polymorphisms was 34.4%, 44.4 %, and 21.3% for QQ, QR, and RR, respectively.

The PON1 genotypes in both positions were tested for the assumption of Hardy-Weinberg equilibrium (HWE). HWE shows that allele and genotype frequencies remain unchanged (or in equilibrium) over time if the population is very large and random mating, as well as, no mutation, migration, and selection are taking place. In this study, chi-square test of goodness of fit was used to compare an observed number with expected numbers (Hardy-Weigberg law). The observed values are the actual numbers of three genotypes in the sample population, and the expected values are derived from the prediction of genotypes occurring as p^2, 2pq, and q^2. An analysis for PON1 gene is present in Table 7 and 8 for the positions -108 and 192, respectively. Since the critical Chi-Square for one degree of freedom at 0.05 levels was 3.841. Therefore, distribution of PON1 gene at positions -108 and 192 were not deviated from Hardy-Weinberg Law.

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Table 6. Distribution of PON1 genotypes in 160 subjects

	C-108 T			Q192R		
	CC	**CT**	**TT**	**QQ**	**QR**	**RR**
Frequency	44	86	30	55	71	34
Percent (%)	27.5	53.8	18.8	34.4	44.4	21.3

Table 7. Chi-Square test for Hardy-Weinberg proportion of the position -108

	C -108 T			Total
	CC	**CT**	**TT**	
Observed Numbers (O)	44	86	30	160
Observed proportions	0.275	0.5375	0.1875	1.0
Expected proportions	p^2	2pq	q^2	1
	0.2916	0.4968	0.2116	1.0
Expected Number (E)	46.66	79.48	33.86	160
$\chi^2 = (O-E)^2/E$	0.1516	0.5349	0.4400	1.1265*

p = frequency of C allele (0.54), q = frequency of T allele (0.46).

* The critical chi-square for one degree of freedom at 0.05 levels is 3.841.

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Table 8. Chi-Square test for Hardy-Weinberg proportion of the position 192

	Q192R			Total
	QQ	QR	RR	
Observed Numbers (O)	55	71	34	160
Observed proportions	0.3438	0.4438	0.2125	1.0
Expected proportions	p^2	2pq	q^2	1
	0.3249	0.4902	0.1849	1.0
Expected Number (E)	51.98	78.43	29.58	160
$\chi^2 = (O-E)^2/E$	0.1755	0.7039	0.6604	1.5398*

p = frequency of Q allele (0.57), q = frequency of C allele (0.46).

* The critical Chi-Square for one degree of freedom at 0.05 levels is 3.841.

6. Haplotype frequency estimation of PON1 gene

From genotype data of PON1 at positions C-108 T and Q192R, the individual genotype was combined as shown in Table 9. The random combination of Q and R alleles at position 192 and C and T alleles at position -108 was expected to give rise to nine possible combinations. The most common genotypes were 192QR and -108CT (26.3%). The random combinations of genotype were also expected to give four haplotypes (QC, QT, RC, and RT). The haplotype frequency was calculated from the combined genotype data. Although these results were manually calculated as described below, one could also use the expectation-maximization algorithm. The haplotype frequencies were calculated as in Appendix I, when $n_{11/11} = 23$, $n_{11/12} = 26$, $n_{12/12} = 6$, $n_{11/21} = 18$, $n_{12/22} = 11$, $n_{21/21} = 3$, $n_{21/22} = 18$, $n_{22/22} = 13$ and $n_{1122} = 42$. The parameters of the haplotype frequencies estimation equation were $\alpha = 0.6430$, $\beta = 0.9941$, $\gamma = 0.7801$, $\xi = -1.6004$, $\eta = -0.9231$. Because $D = -39.4031 < 0$, the likelihood surface is unimodal. The maximum estimate of the phase was $\varphi = 0.7849$,

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thus the haplotype frequencies were h_{11} (QC) = 0.3843, h_{12} (QT) = 0.1813, h_{21} (RC) = 0.1595, h_{22} (RT) = 0.2749 (Table 10)

Table 9. Combined genotype frequency of PON1 for positions –108 and 192

Genotype	Haplotype	Symbol	Number	%
QQ / CC	QC / QC	11/11	23	14.4
QQ / CT	QC /QT	11/12	26	16.3
QQ / TT	QT / QT	12/12	6	3.8
QR / CC	QC / RC	11/21	18	11.3
QR / CT	QC / RT	11/22	42	26.3
QR / TT	QT / RT	12/22	11	6.9
RR / CC	RC / RC	21/21	3	1.9
RR / CT	RC / RT	21/22	18	11.3
RR / TT	RT / RT	22/22	13	8.1
	Total		160	100

Table 10. The haplotype frequency of PON1

Haplotype	QC	QT	RC	RT
Frequency	0.3843	0.1813	0.1595	0.2749
Percent (%)	38.43	18.13	15.95	27.49

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7. Linkage disequilibrium analysis (LD)

The analysis of linkage disequilibrium among PON1 polymorphisms is presented in Table 11. Chi-Square test was used to determine the linkage disequilibrium, since the critical chi-square for one degree of freedom at 0.05 levels was 3.841. Therefore, we would observe linkage disequilibrium between position -108 of the promoter region and position 192 of coding. The coefficients of LD (D) between two positions were estimated as shown in Table 12. We observed 33.03% genetic linkage disequilibrium between PON1 polymorphisms at the positions -108 and 192.

Table 11. Chi-Square test for linkage disequilibrium among PON1 polymorphism

	Gamete				
	QC	QT	RC	RT	Total
Observed Number (O)	123	58	51	88	320
Observed frequencies	0.3843	0.1813	0.1595	0.2749	1
Expected frequencies	P_QP_C	P_QP_T	P_RP_C	P_RP_T	
	0.3078	0.2622	0.2322	0.1978	1
Expected Number (E)	99	84	74	63	320
$\chi^2 = (O-E)^2/E$	5.8182	8.0476	7.1486	9.9206	30.935*

P_Q, P_R, P_C, P_T are the allele frequencies of Q, R, C, T, respectively.

* The critical Chi-Square for one degree of freedom at 0.05 levels is 3.841.

Table 12. The coefficient of LD estimation

Parameter	Equation		Value
D	$P_{QC}P_{RT} - P_{QT}P_{RC}$	(0.3843)(0.2749) – (0.1813)(0.1595)	0.0767
D_{max}	P_CP_R	(0.54)(0.43)	0.2322
$D' = \frac{D}{D_{max}}$		$\frac{0.0767}{0.2322}$	0.3303

D_{max} is the maximum that D can have, which is $D_{max} = \min(p_1q_2, q_1p_2)$ when $D > 0$

or $= \min(p_1p_2, q_1q_2)$ when $D < 0$

PQC, PRT, PQT, PRC are haplotype frequencies of QC, RT, QT, RC, respectively.

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8. The comparison of PON1 allele frequency in various populations

There were several studies reporting the allele frequency of PON1 polymorphism in various populations. When the allele frequency of PON1 in each study was compared, each population significantly differed from the other populations, and it was concluded that PON1 polymorphisms were different among ethnic or racial groups. Two-sample χ^2 analysis showed that the allele frequencies of PON1 at position -108 (Table13.) in our study differed significantly from that previously reported in Thai, African-American, Caucasian-American, Caribbean, Israeli, Mexican and European. The similarities were observed for Japanese and Chinese Hun population. For position 192 (Table 14), the differences of allele frequencies in our study could be observed as previously reported in Thai (previous study), Chinese, German, Netherlands, Costa-Rica, British, French, Turkish, Indian and Finish. However, no significant differences were found when compared to Japanese and Mexican.

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Table 13. Comparison of allele frequencies in position -108

Population	N	Allele Frequencies		Different from Thai population		Reference
		C	T	χ^2	*p*-value	
Thai (Present study)	160	0.54	0.46	-	-	-
Chinese	474	0.57	0.43	0.8173	NS	Wang et al. 2003
Japanese	132	0.48	0.52	2.3236	NS	Suehiro et al. 2000
European	374	0.46	0.54	5.8498	< 0.05	Leviev and James. 2000
Mexican	214	0.45	0.55	6.206	< 0.05	Rojas-Garcia et al. 2004
Caribbean	203	0.65	0.35	8.983	<0.01	Chen et al.2003
Caucasian-American	82	0.38	0.62	10.634	< 0.01	Chen et al. 2003
Israeli	157	0.40	0.60	12.926	< 0.01	Bryk,B et al. 2005
African-American	117	0.85	0.15	43.363	< 0.001	Chen et al. 2003
Thai	202	0.25	0.71	57.746	<0.001	Phuntuwate, W et al, 2005

χ^2 values (df =1): p-vales of 0.05 = 3.84
0.01 = 6.63
0.001 = 13.95

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Table 14. Comparison of allele frequencies in position 192

Population	N	Allele frequency		Different from Thai population		Reference
		Q	R	χ^2	p	
Thai (Present study)	160	0.57	0.43	-	-	-
Japanese	132	0.60	0.40	0.6690	NS	Suehiro et al. 2000
Mexican	214	0.51	0.49	2.3974	NS	Rojas-Garcia et al 2004
Finnish	135	0.69	0.31	5.2707	< 0.05	Clarimon et al. 2004
Indian	175	0.69	0.31	8.734	< 0.01	Sanghera et al. 1998
Dutch	187	0.69	0.31	9.0432	< 0.01	Leus et al. 2000
Turkish	381	0.69	0.31	14.383	< 0.001	Ayancioglu et al. 1999
Thai	202	0.71	0.29	15.723	< 0.001	Phuntawate, W et al, 2005
English	282	0.71	0.29	17.911	< 0.001	Mackness et al. 2001
African-American	117	0.37	0.63	20.989	< 0.001	Chen et al. 2003
French	1170	0.71	0.29	26.980	< 0.001	Helbecque et al.1999
Chinese	474	0.40	0.60	27.330	< 0.001	Wang et al. 2003
Costa Rica	518	0.76	0.24	44.195	< 0.001	Sen-Banerjee et al. 2000
German	535	0.78	0.22	49.243	< 0.001	Gardemann et al. 2000

χ^2 values (df =1): p-vales of 0.05 = 3.84
0.01 = 6.63
0.001 = 13.95

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9. Association between PON 1 polymorphism and lipid profile

To evaluate whether the allelic variation at PON1 gene significantly affects the serum lipid levels, plasma total cholesterol (TC), triglycerides (TG), HDL-C, LDL-C and VLDL-C were determined. The subjects who had the history of medications that affect lipoprotein level were excluded for analysis. Tables 15 and 16 showed the geometric mean of TC, TG, HDL-C, VLDL-C and mean of LDL-C levels in the different PON1 genotype groups at the positions C–108 T and Q192R, respectively. The analysis of variance (ANOVA) was used to examine the equality of the mean values among the phenotypes. The statistical analyses showed that no significant difference in the geometric mean cholesterol levels among genotypes was observed in both the positions -108 and 192. In addition, the genotype combination of PON1 in this study was not significantly different for the cholesterol levels (Table 17).

Table 15. Comparison of Lipid Levels among genotype of position -108

Parameter variable	Position -108			*p*-value
	CC (30)	CT (78)	TT (28)	
TG (mg/dL)	144 (121-172)	123 (110-138)	164 (164-202)	0.063
TC (mg/dL)	227 (212-242)	225 (217-234)	229 (218-241)	0.903
HDL-C (mg/dL)	50 (47-55)	52 (49-56)	50 (47-55)	0.726
VLDL-C (mg/dL)	31 (24-38)	25 (22-28)	34 (27-44)	0.067
LDL-C (mg/dL)∗	136 (118-154)	143 (134-152)	134 (119-150)	0.580

Values are showed as geometric mean (95% Confidence interval) and ∗mean (95% Confidence interval)

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Table 16. Comparison of Lipid Levels among genotype of position 192

Parameter Variable	Position 192			*p*-value
	QQ (53)	QR (63)	RR (30)	
TG (mg/dL)	150 (128-174)	126 (110-144)	135 (113-161)	0.216
TC (mg/dL)	228 (217-240)	224 (214-234)	228 (216-242)	0.801
HDL-C (mg/dL)	49 (46-53)	54 (48-55)	40 (45-53)	0.092
VLDL-C (mg/dl)	32 (29-38)	25 (22-30)	27 (25-32)	0.186
LDL-C (mg/dL) ∗	137 (123-150)	137 (125-147)	149 (133-164)	0.440

Values are shown as geometric mean (95% Confidence interval) and ∗mean (95% Confidence interval)

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Table 17. Comparison of lipid levels among haplotype

Haplotype	N	TG	TC	HDL-C	VLDL-C	LDL-C
QQ/CC	22	167 (134-209)	226 (206-248)	48 (42-53)	38 (28-50)	135 (112-159)
QQ/CT	25	132 (105-165)	226 (211-241)	50 (46-56)	27 (21-36)	140 (122-159)
QQ/TT	6	169 (78-366)	246 (215-282)	50 (40-64)	40 (13-125)	124 (56-192)
QR/CC	15	132 (98-177)	224 (204-246)	53 (46-58)	31 (21-46)	129 (98-160)
QR/CT	38	118 (100-140)	224 (211-239)	55 (46-57)	25 (21-30)	142 (130-153)
QR/TT	10	148 (98-223)	221 (200-245)	52 (44-65)	29 (17-50)	132 (106-160)
RR/CC	3	78 (27-231)	243 (107-553)	61 (36-199)	15 (5-46)	172 (58-402)
RR/CT	15	122 (96-154)	226 (208-246)	40 (44-56)	26 (21-33)	149 (127-172)
RR/TT	12	176 (137-227)	227 (212-244)	48 (40-51)	36 (26-49)	141 (122-161)
p-value		0.073	0.977	0.480	0.103	0.818

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10. Multivariate regression analysis

Factor influencing lipoprotein levels were analyzed by stepwise multiple regression analysis. The factors include age, log BMI, gender, PON1 genotype, smoking, alcohol, exercise, SPB, DPB, DM, hypertension, and hypercholesterolemia. In this study, PON1 genotype did not contribute to variation of the plasma lipoprotein levels. Gender and WHR had significant contribution up to 14.7% of HDL levels variation. For the variation of TG levels, WHR, alcohol, and exercise had significant contribution to 26.1%. For the variation of TC, alcohol had significantly contributed to 3.3% of the variation in TC levels. These factors did not contribute to the variation of LDL levels. The detail of results from multiple regression analysis is shown below:

Transform HDL

Transform HDL = 0.006 – 0.003 (gender) + 0.02 (WHR)

* $r^2 = 0.147$, p = 0.007

* Transform HDL = 1/ HDL

* Gender was categorized as 1 (male) and 2 (female)

Log TG

Log TG = 0.699 + 1.538(WHR) + 0.105 (Alcohol) - 0.067 (Exercise)

* $r^2 = 0.261$, p = 0.036

* Alcohol was categorized as 1 (non-drinker) and 2 (current drinker)
  Exercise was categorized as 1 (non-exercise) and 2 (exercise)

Log TC

Log TC = 2.304 + 0.03 (Alcohol)

* $r^2 = 0.033$, p = 0.024.

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CHAPTER VI
DISCUSSION

Human serum paraoxonase1 (PON1, EC 3.1.8.1) is a calcium-dependent glycoprotein with hydrolase activity. PON1 enzyme appears to be under the control of an autosomal gene. There are many polymorphic sites of PON1 gene which may affect enzyme function. Previous studies reported that the polymorphism of PON1 gene at position -108 of the promoter region and position 192 of the coding region plays important role in the enzyme function. The nucleotide substitution of C to T was observed at position -108 (C > T), and this polymorphism causes the difference in enzyme concentration. The position C-108 T is located in the centre of a consensus binding site for the ubiquitous transcription factors Sp1 and Sp3. The binding of Sp1 to the -108 site is weaker in the presence of T than C because T disrupts the GGCGGG recognition sequence for Sp1 and results in decreased affinity for haplotype nuclear extracts and Sp1 (Deakin et al, 2003). The polymorphisms of Sp1 may affect mRNA level, resulting in low plasma enzyme concentration and low enzyme activity. The nucleotide A to G substitution at codon 192 involves an exchange of amino acid glutamine (Q) to arginine (R) leading to changing of enzyme activity, and the variation of enzyme activity is also dependent on substrates. The determination of PON1 polymorphism can be detected by either phenotyping or genotyping. The genotyping method is used to determine the polymorphism of PON1 gene due to its accuracy and reproducibility. Polymerase chain reaction (PCR) is used in the genotyping technique to amplify DNA at a desired position. After that PCR product is digested with specific restriction enzyme.

The distribution of PON1 allele frequencies is different among ethnic or racial groups. For position -108, the prevalence of -108C allele ranges from 38 to 85 percent depending on the population studied; Asians and Africans have high frequencies of -108C allele. For position 192, the prevalence of 192R allele ranges from 20 to 70

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percent depending on the population studied; Asians and Africans have high frequencies of 192R allele (Garcia-Rojas et al., 2004).

The assumption of Hardy-Weinberg law shows that in large randomly mating population of diploid, without the influence of mutation, migration, or selection, equilibrium will be achieved for an autosomal with two alleles. This law predicts that allele frequencies and genotype frequencies will not change from generation to generation and the genotype will occur according to the binomial distribution $p^2+2pq+q^2 = 1$. To determine whether a population is at equilibrium, the observed and expected genotypes can be compared by the Chi-square test. In this study, the allele frequencies were not deviated from those expected assuming Hardy-Weinberg equilibrium for position -108 (χ^2 = 1.1265, df =1; p = 0.05) and position 192 (χ^2 = 1.5398, df = 1; p = 0.05), respectively. The Hardy-Weinberg equilibrium has been used to determine relationship between genotype frequencies and allele frequencies in population.

Phuntuwate et al, (2005) previously studied PON1 status in 202 Thai subjects who attended routine medical check at the hospital by using genotyping method. The results showed that the frequencies of PON1 C-108 T were 25% and 71% for C and T allele, respectively. For Q192R, allele frequencies were 71% and 29% for Q and R allele, respectively. The present study using the genotyping technique, which was PCR based protocol and restriction fragment length polymorphism was investigated in 160 Thai subjects. The prevalence of allele frequencies were 54% and 46% for C and T alleles at position -108, respectively. For position 192, 57 % of Q allele and 43% of R allele were observed. Comparing with the previous study reported by Phuntuwat, allele frequencies were markedly different. This discrepancy might be explained by sample selection method. Sample selection of the previous study excluded subjects who had hypertension, diabetes, and hypercholesterolemia. These diseases are risk factors for cardiovascular disease (CVD). If the 192R allele was associated with CVD, the presence of R allele may be decreased in this group of subject. In contrast, the present study did not have any exclusion criteria and the samples were randomly selected from workers in many dams located in various parts of the country. Therefore,

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the result of the present study would more likely to represent the general Thai population.

Comparison of PON1 allele frequencies estimated in this study with those reported for other populations showed that there are significant differences among the Thai population, African-American, Caucasian-American, Caribbean, Israeli, and European for position -108. For the position 192, the differences between Thai population, Chinese, German, Dutch, Costa Rica, English, French, Turkish, Finnish, Indian, and African-Americans were observed. These differences are mainly due to differences in frequencies of the -108 C allele (low in European countries and high in African countries) and R192 allele (low in European countries and high in African and Asian countries). The similarity of these PON1 allele frequencies were observed among Thai, Japanese, and Chinese populations for position -108, and the similarity could be observed among Thai, Japanese, and Mexican populations for position 192.

The differences in PON1 allele frequencies among the Thai, European, African, and American populations might be due to the resemblance in ethnic background and geographical isolation. The similarities of these PON1 allele frequencies in Asian populations may be due to the similarities in ethnic and geographical distribution. The similarities of PON1 allele frequencies at position 192 between Thai and Mexican populations may be due to a combination of population admixture (Native American mixed with Caucasian) leading to genetic drift (Lisker, Ramírez, González-Villalpando, Stern, 2005)

Several studies reported that PON1 activity and localization are intimately linked with HDL. Nevertheless, it has been hypothesized that PON1 gene may have a role in lipid metabolism and levels. Several lines of evidence suggest that PON1 may have a significant role in lipoprotein metabolism. First, PON is an important structural component of the apolipoprotein AI-containing lipoprotein subpopulation of HDL particles (Blatter et al., 1993). Second, PON purified from human HDL decreases the oxidative modification of LDL in vitro (Mackness et al., 1991). Third, plasma PON activity is significantly related to variation in plasma concentrations of triglycerides,

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LDL cholesterol (LDL-C), apo AII and apoB (Saha et al., 1991). For these reasons, we were interested in identifying associations between genotype of PON1 and fasting plasma lipoprotein variable in Thai population. In order to define the role of PON1 polymorphism in the lipid levels in Thai population, we determined the lipid profile in a studied group. In the analysis, lack of association between PON1 genotypes and lipid profile was found at the positions -108, 192 and combined genotype of both positions. These results are consistent with the previous data reported in other studies (Arca et al., 2002; Roberson et al., 2003). Several studies have been showed that the influence of the polymorphism of PON1 on distributions of plasma lipid is complex and related to the other influential or non-genetic factors. In this study, the correlation between PON1 genotypes and lipid levels was not observed. This may be due to the small sample size or non-genetic risk factors that are particularly strong in the studied group. Moreover, there was at least a 13-fold variation in PON1 activity among individuals with the same genotype (Humbert et al, 1993; Davies et al 1996; Richter and Furlong 1999). There were at least 20 genes participated in lipoprotein metabolism and influent the lipoprotein levels (Hegele, RA., 1995). Some of these genes might be strongly associated with lipoprotein levels than PON1 gene. Thus, the association of PON1 gene and lipoprotein levels was not observed.

To determine the parameters which influence the lipoprotein level, multivariate regression analysis was performed. Our finding indicated that the polymorphisms of PON1 were not correlated with the lipoprotein levels, but gender, WHR (waist-to-hip ratio), alcohol consumption, and exercise were contributors to the variation of lipoprotein levels. Previous studies reported that the enzyme activity of PON1 varied in individuals with the same genotype (Humbert et al, 1993; Davies et al 1996; Richter and Furlong 1999). The enzyme activity can be modulated by many factors. A study examining serum PON1 activity in rats reported that female rats had higher activity than the males. WHR, that is used to classify obesity in adult, showed an inverse relationship with PON1 activity (Wang et al, 2004). A study conducted to examine the association of PON1 and alcohol consumption showed that light drinkers had a 39.5% higher, whereas heavy drinkers had a 45% decrease in PON1 activity compared with non-drinkers. These events were confirmed by an animal study, which reported that

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light ethanol feeding caused a 20-50% increase PON1 activity and 59% increase in the levels of liver PON1 mRNA. In contrast, heavy alcohol consumption caused a 25% decrease in serum and liver PON1 activity with a 51% decrease in the liver PON1 mRNA level (Rao et al., 2003).

Numerous studies have been conducted to investigate the role of PON1 polymorphism in the genetic susceptibity to coronary heart disease (CHD). However, these studies revealed discrepancy. Some studies have shown association between PON1 polymorphisms and atherosclerosis, although others did not observe such evidence (Imai et al., 2000; Arca et al., 2002). This variability could be due to ethnic differences in the studied populations, since the allele frequencies of PON1 polymorphism differed among ethnic groups. Although the present work did not aim to study the association of PON1 polymorphism and the cardiovascular disease because the numbers of subject are too small to detect the CHD, the allele frequencies may be used to predict the susceptibility to such diseases. In Thai population, the presence of high frequency of 192R allele was observed. The R isoform was found to be less effective for hydrolyzing lipid peroxides than the 192Q isoform. If the PON1-192R allele was positively associated with coronary heart disease, Q allele would be protective against atherosclerosis (Mackness et al., 1999). However, the incidence of cardiovascular disease in Thai population was lower than Caucasian population (Lin Khor, 2001). There would be other factors that are more potent than PON1 for development of CHD. These factors may include life-style and type of food consumption. As the incidence of cardiovascular disease is increasing in Thai population, we may assume that Thai population will be more susceptible to CHD than Caucasian population without the contribution of these factors.

It has long been known that PON1 can hydrolyze several organophosphates (OP) in vitro, and has been taken as an indication that it may modulate OP toxicity in vivo. The polymorphisms of PON1, which cause different enzyme levels and catalytic efficiency, would indicate that certain individuals may be more susceptible to the toxic effect of OP exposure. As mentioned previously, the -108C allele gave rise to a high concentration of enzyme and 192R allele had high activity for paraoxon. The population having this phenotype would be more resistant to organophosphate

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pesticide intoxication due to high allele frequencies of 192R and -108C of the PON1 gene

A limitation of our study is that PON1 activity has not been measured. Thus, the influence of PON1 activity on the lipid profile is still unclear. Comparing with PON1 genotype, PON1 status is a better predictor to evaluate the effect of PON1 enzyme on lipoprotein as PON1 status was controlled by a number of functional polymorphisms in both the coding and the promoter regions, which may affect either the level or the substrate specificity. Thus, the further study should aim for studying the association between PON1 status and plasma lipoprotein level. In addition, the association of PON1 gene and other diseases should also be evaluated in Thai population.

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CHAPTER VII
CONCLUSION

1. The polymorphisms of PON1 gene at position -108 of the promoter region and at the position 192 of the coding region were found in Thai population.

2. The allele frequencies of PON1 gene at position C-108 T are 54% and 46% for C and T allele, respectively. The prevalence of genotype is 27.5% for CC genotype, 53.8% for CT genotype, and 18.8% for TT genotype.

3. The allele frequencies of PON1 gene at position Q192R are 57% and 43% for Q and R allele, respectively. The prevalence of genotype is 34.4% for QQ homozygote, 44.4% for QR heterozygote, and 21.3% for RR homozygote.

4. The allele frequencies distribution showed that Thai populations are related to Asians, but differed from Caucasian and Africa populations.

5. The association of PON1 polymorphism and lipoprotein level was not observed.

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APPENDIX

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Appendix A

The haplotype frequencies estimation (Mano et al., 2004)

The step for haplotype frequencies estimation was showed below;

1. The combined genotype of two loci was observed and count.
2. The α, β, γ, ξ, η were calculated for estimates D value

$$\alpha = \frac{1}{f}(q_{11} + q_{22} - q_{12} - q_{21}),$$
$$\beta = \frac{1}{f^2}(q_{11}q_{22} + q_{12}q_{21}),$$
$$\gamma = \frac{1}{f^2}q_{11}q_{22}.$$

Where $f = n_{1122}/ N$, $q_{11} = n_{11}/ 2N$

3. The D value can be estimated by using the equation below;

$$D = 4\zeta^3 - 27\eta^2 > 0,$$

Where

$$\zeta = \frac{1}{3}\left(\alpha^2 + \frac{3}{4} - 6\beta\right),$$
$$\eta = \frac{1}{27}\left(\alpha - \frac{3}{2}\right)(2\alpha^2 + 3\alpha - 18\beta) - 2\gamma,$$

4. If D value is less than zero (D<0), the likelihood surface is unimodal and φ value is estimated from the algorithm;

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$$\varphi_{\times} = \frac{1}{2} - \frac{\alpha}{3} + \sqrt[3]{-\frac{\eta}{2} + \frac{1}{6}\sqrt{-\frac{D}{3}}} + \sqrt[3]{-\frac{\eta}{2} - \frac{1}{6}\sqrt{-\frac{D}{3}}},$$

If $D \geq 0$, the likelihood surface is bimodal and φ value is estimated from the Algorithm;

$$\varphi_{+} = \frac{1}{2} - \frac{\alpha}{3} + 2\sqrt{\frac{\zeta}{3}}\cos\frac{\theta}{3},$$

$$\varphi_{-} = \frac{1}{2} - \frac{\alpha}{3} - 2\sqrt{\frac{\zeta}{3}}\cos\frac{\theta - \pi}{3},$$

When

$$\cos\theta = -\frac{\eta}{2}\left(\frac{3}{\zeta}\right)^{3/2}, (0 < \theta < \pi),$$

5. φ value can be used to estimate the haplotype frequencies follow algorithm below;

$$\hat{\varphi} = \frac{\hat{h}_{11}\hat{h}_{22}}{\hat{h}_{11}\hat{h}_{22} + \hat{h}_{12}\hat{h}_{21}},$$

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Then, replace in equation;

$$\hat{h}_{11} = \frac{1}{2N}\left(n_{11} + n_{1122}\frac{\hat{h}_{11}\hat{h}_{22}}{\hat{h}_{11}\hat{h}_{22} + \hat{h}_{12}\hat{h}_{21}}\right),$$

$$\hat{h}_{12} = \frac{1}{2N}\left(n_{12} + n_{1122}\frac{\hat{h}_{12}\hat{h}_{21}}{\hat{h}_{11}\hat{h}_{22} + \hat{h}_{12}\hat{h}_{21}}\right),$$

$$\hat{h}_{21} = \frac{1}{2N}\left(n_{21} + n_{1122}\frac{\hat{h}_{12}\hat{h}_{21}}{\hat{h}_{11}\hat{h}_{22} + \hat{h}_{12}\hat{h}_{21}}\right),$$

$$\hat{h}_{22} = \frac{1}{2N}\left(n_{22} + n_{1122}\frac{\hat{h}_{11}\hat{h}_{22}}{\hat{h}_{11}\hat{h}_{22} + \hat{h}_{12}\hat{h}_{21}}\right).$$

When h = haplotype frequencies estimation

N = total number of sample

n = number of sample in each haplotye

11 = wild type allele of locus 1 and wild type allele of locus 2

12 = wild type allele of locus 1 and mutant allele of locus 2

21 = mutant allele of locus 1 and wild type allele of locus 2

22 = mutant allele of locus 1 and mutant allele of locus 2

n_{1122} = number of sample in heterozygote genotype combination of both loci (it cannot inferred to haplotype)

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Appendix B.1

Position C-108 T; U55877 Human paraoxonase [gi: 1575033]

1 AAGCTTGACT GTCCTTTCCC ATGGCAATTT ACTTCAGCTT GTTTGATTTC CCCTCCCCGA

61 CTGGACTAGG CACCTATTCT CTGTCTTCTC TCTTTACAGT TGGAAGGAGC AAAATGGGAC

121 TTTTGGCTGA AAGTGCTGAG CTCCTGCGGT GGGGGCTGAC CGCAAGCCGC GCCTTCTGTG

181 CACCTGGTCG GCCCAGCTAG CTGCGGACCC GGCGGGGAGG GGTGGGGCGG GCCAATCGGC

241 GCTGCCCCAG CAGGGCTGCG GCTGCAGGCA GCAGAGCCTC CTAGCCCGTC GGTGTCTGCG

301 CCCATCGATC CCTTTGTCTA TCCCCGACCA TGGCGAAGCT GATTGCGCTC ACCCTCTTGG

361 GGATGGGACT GGCACTCTTC AGGAACCACC AGTCTTCTTA CCAGTAAGTT GGGTTGTGGT

421 GAGGGCTGCA GCCTTCGTCC TCCTCACTCC CTGCCAGCT CACGAGCCCC CAGAAATTG

481 GGTCCAGGCT GTTAACAGAA CATTTACAAG CAGGTGCTAG ATGCACACTC TGCGGGGGTT

541 TCATAAATGT TTGTCCAGCC GTCATGTGTC ACTTGAAAAC AAACTCTTGC TCATGGCCTG

601 AGACATGGGA TAAGGAGGCA ATTGCCAGGG GTGTGAGTGC CCAGGTTGTC AGCATACACG

661 AATTAATTTT TCATGAAGAT TTGTGCCC TGGCTACAGA TTCACTTCTC AACCTGGAAA

721 GAACAAACTA ACCTGGCTGG GGGTTCTCCT GATATTTTGT GTATTAGAGA AAGGAGCCCC

781 TGTCGTTCTG CGCCTTGACT GCCTGGCCAT GGCAAACTTC CAGGCTTGAA GATTCTCAAA

841 TTGAGGCATC TGAGGGCCAC AGCAAAACTG CGTGTGGACC CGAGCAGGTG GAGCTAACGT

901 GTGCTGTGAT GGTTCGCTTC AGTGGGGGCA GGCGGGGAAG AGGGAACCCT GGAGCCAGCA

961 TCTGATGTCC TGGGGTTTAG CTCTGCCCCA TGAGTATGCG ATTTGGGCAA ATCACTTTAC

1021 ATCTCTAACC TTAGCTGCTT CAGTAAACTG GAAATAGTAA TACACACTGC CTGCGACAGA

1081 GTAGCTATTT CAATAAATGT TAGTTTCCCT GCTTCCCTTT AAAGTATTTA ATAAAGGGTT

1141 AAGTGCTATA CAAGGGGGGT GGGGATATTT TAAGAATGTA TCAAAAGTTT CATAACTTTT

1201 AACAGGAGTT CAAAGTCTAC AGATCATCCT AGTCTCTAAG GCTTAGTCAA CAATGCCTTA

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1261 TTTACTTAA AGTAAAACCT AATAAAAATT AAATATAAA AAATTATATG AGCTTATGGA

1321 CTAATTAGAA GCTT
//

Appendix B.2

Position Q192R; U55882. Human paraoxonase [gi: 1575038]

ORIGIN

1 TTCAATACCT TACACCTTAT ATATTATGTG TGTATGTTTT AATTGCAGTT TGAATGATAT

61 TGTTGCTGTG GGACCTGAGC ACTTTTATGG CACAAATGAT CACTATTTTC TTGACCCCTA

121 CTTACAATCC TGGGAGATGT ATTTGGGTTT AGCGTGGTCG TATGTTGTCT ACTATAGTCC

181 AAGTGAAGTT CGAGTGGTGG CAGAAGGATT TGATTTTGCT AATGGAATCA ACATTTCACC

241 CGATGGCAAG TATGTGAACT CTCTGAAATG TAGTGGATTT ACTCAGATTC TCAGGAG
//

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APPENDIX C.1

เอกสารชี้แจงข้อมูล/คำแนะนำแก่ผู้เข้าร่วมโครงการ
(Patient Information Sheet)

ชื่อโครงการ

1. การศึกษาหาอิทธิพลของสภาวะแวดล้อมในการทำงานต่อความชุกของโรคหัวใจและหลอดเลือดรวมทั้งปัจจัยเสี่ยงด้านพันธุกรรมและปัจจัยเสี่ยงต่างๆในพนักงานไฟฟ้าฝ่ายผลิตแห่งประเทศไทย
2. การศึกษาหาความชุกของโรคตับชนิดต่างๆในพนักงานไฟฟ้าฝ่ายผลิตแห่งประเทศไทยที่มีเอนไซม์ตับ (serum aminotranferase) ผิดปกติ
3. การตายในประชากรไทย: สาเหตุสำคัญ ปัจจัยเสี่ยง การเปลี่ยนแปลงและแนวโน้มในอนาคตการวิเคราะห์และประยุกต์ใช้เชิงสหวิทยาการ
4. การศึกษาเรื่องการถ่ายอุจจาระในประชากรไทย
5. ความสัมพันธ์ของการเกิดโรคมะเร็งตับกับการติดเชื้อไวรัสตับอักเสบชนิดบีและซีในพนักงานการไฟฟ้าฝ่ายผลิตแห่งประเทศไทย

ชื่อผู้วิจัย รศ. ปิยมิตร ศรีธารา, อ.นพ.สายัณห์ ชีพอุดมวิทย์, อ.นพ.สุกิจ แย้มวงศ์, ผศ.สุพจน์ ตุลยาเดชานนท์, ผศ. โฉมศรี โฆษิตชัยวัฒน์, อ.พญ.ธิติยา ศิริสิงห, ผศ.พงศ์อมร บุนนาค, ศ.สุรัตน์ โคมินทร์, ผศ.อดิศักดิ์ ผลิตผลการพิมพ์, รศ.ธันยชัย สุระ, ผศ.รัตนา พากเพียรกิจวัฒนา, ศ.พรทิพย์ โล่ห์เลขา, รศ.อาทิตย์ อังกานนท์, รศ.วินัย วนานุกูล, ผศ.สิทธิ์เทพ ธนกิจจารุ, น.ส.พัชรา แพนพันธ์อ้วน, นพ.ศักดิ์ดา กุสุมภ์, พญ.นงนุช อังกุราภินันท์, พญ.วิภา รัตนชัยวงศ์, นพ. พูนชัย หล่อเจริญผล, นางวัลลภา ปลอดทุกข์, นางเสาวนีย์ อังศุธรารักษ์, น.ส.มาลี อัตตสรรค์สาธิต, ผศ.ทญ.รังสินี มหานนท์, อ.ทพ.ดร.กิตติ ต.รุ่งเรือง, อ.ทพ.สุพจน์ ตามสายลม, อ.ทญ.เกศรินทร์ โรจนสมสิทธิ์ และ คุณจริยา ตันติธรรม

สถานที่วิจัย การไฟฟ้าฝ่ายผลิตแห่งประเทศไทย

ผู้สนับสนุนการวิจัย สำนักงานกองทุนสนับสนุนการวิจัย สภาวิจัยแห่งชาติ

ความเป็นมาของโครงการ

ในปี 2528 ศาสตราจารย์ สมชาติ โลจายะ, ศาสตราจารย์ วิชัย ตันไพจิตร และคณะ ได้ทำการศึกษาร่วมกับการไฟฟ้าฝ่ายผลิตแห่งประเทศไทย โดยได้เก็บข้อมูลทางการแพทย์อย่างละเอียดของพนักงานไฟฟ้าจำนวน 3,499 คน ประกอบด้วยการซักประวัติและตรวจร่างกายอย่างละเอียด และการตรวจทางห้องปฏิบัติการ โดยประชากรที่ศึกษาครอบคลุมประมาณครึ่งหนึ่งของพนักงานทั้งหมด

ในปี 2540 ศาสตราจารย์ วิชัย ตันไพจิตร, รองศาสตราจารย์ ปิยมิตร ศรีธาราและคณะ ได้ติดตามเก็บข้อมูลพนักงานการไฟฟ้าฝ่ายผลิตแห่งประเทศไทยกลุ่มเดิมที่มีชีวิตอยู่ และที่สามารถติดตามมาตรวจได้ จำนวน 2,967 คน โดยได้ซักประวัติ ตรวจร่างกาย และตอบแบบสอบถามเกี่ยวกับลักษณะการถ่ายอุจจาระ รวมทั้งได้

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เพิ่มเติมการตรวจเลือดเพื่อหา สายพันธุกรรม ดีเอ็นเอ เอนไซม์ตับ เชื้อไวรัสตับอักเสบบี และ ซี นอกเหนือจากข้อมูลที่เก็บในปี พ.ศ.2528

ในปี 2541 คณะผู้วิจัยได้ทำการศึกษาหาอิทธิพลของสภาพแวดล้อมในการทำงานต่อความชุกของโรคหัวใจและหลอดเลือดรวมทั้งปัจจัยเสี่ยงต่างๆในพนักงานการไฟฟ้าฝ่ายผลิตแห่งประเทศไทย ระยะที่ 2 จำนวน 3,000 คน โดยประชากรกลุ่มนี้มีสัดส่วนของเพศและอายุเหมือนกับประชากรที่สำรวจในกลุ่มแรกเมื่อปี 2528 ด้วยการเก็บข้อมูลทางการแพทย์ รวมทั้งการเก็บตัวอย่างเลือดเพื่อหา สายพันธุกรรม ดีเอ็นเอ เอ็นไซม์ตับ เชื้อไวรัสตับอักเสบบี และ ซี

การติดตามศึกษาประชากรกลุ่มนี้ต่อเนื่องในระยะยาว เพื่อหาอัตราและสาเหตุการตายจากโรคต่างๆ รวมทั้งอุบัติการณ์ของโรคหัวใจและหลอดเลือด ในระยะเวลา 16 ปีที่ผ่านมา โดยนำมาวิเคราะห์เปรียบเทียบกับปัจจัยเสี่ยงแต่ละชนิดที่เป็นข้อมูลเบื้องต้นเมื่อเริ่มทำการศึกษา จะเป็นประโยชน์ในการช่วยให้ทราบถึงการเปลี่ยนแปลง สาเหตุการตาย อุบัติการณ์ และการดำเนินโรค ที่สัมพันธ์กับปัจจัยเสี่ยงต่างๆของประชากร อันจะนำไปสู่การวางแผนเพื่อควบคุมและป้องกันโรคที่เป็นสาเหตุสำคัญที่ทำให้ประชากรเสียชีวิตหรือเจ็บป่วยอย่างถูกต้องต่อไป

<u>วัตถุประสงค์</u>

1. เพื่อศึกษาถึงสาเหตุการตายที่แท้จริง ในประชากรกลุ่มพนักงานการไฟฟ้าฝ่ายผลิต
2. เพื่อศึกษาปัจจัยเสี่ยงและปัจจัยทางพันธุกรรมต่อการเกิดโรคและการตายจากโรคหัวใจและหลอดเลือด อุบัติเหตุและการบาดเจ็บ มะเร็งตับและมะเร็งอื่นๆ โรคเบาหวานและโรคโภชนาการขาดและเกิน
3. เพื่อศึกษาชีวะกลไกและสาเหตุทางพันธุกรรม ที่ก่อให้เกิดพยาธิสภาพในการตายจากโรคหัวใจและหลอดเลือด มะเร็งตับและมะเร็งอื่นๆ
4. เพื่อศึกษากระบวนการทางสังคมและความสำคัญของสภาพแวดล้อมในการทำงาน ที่จะนำไปสู่การป้องกันการตายจากโรคหัวใจและหลอดเลือด อุบัติเหตุและการบาดเจ็บ มะเร็งตับและมะเร็งอื่นๆ โรคเบาหวานและโรคโภชนาการขาดและเกิน รวมทั้งปัจจัยเป็นอุปสรรคต่อการดำเนินการป้องกัน
5. เพื่อศึกษาลักษณะการถ่ายอุจาระในประเทศไทย
6. เพื่อศึกษาความสัมพันธ์ระหว่างพฤติกรรมการสูบบุหรี่และสาเหตุทางพันธุกรรม กับการเกิดโรคปริทันต์ รวมทั้งศึกษาความสัมพันธ์ระหว่างโรคปริทันต์และโรคทางระบบหัวใจและหลอดเลือด

<u>รายละเอียดที่ผู้เข้าร่วมโครงการทุกคนจะได้รับ</u>

1. การซักประวัติ ทั้งประวัติส่วนตัว ประวัติการเจ็บป่วยและประวัติครอบครัวโดยการสัมภาษณ์และตอบแบบสอบถาม
2. การตรวจร่างกาย ชั่งน้ำหนัก วัดส่วนสูง วัดความดันโลหิตและส่วนประกอบของร่างกาย ได้แก่ ดัชนีมวลกาย เส้นรอบวงเอว เส้นรอบวงตะโพก มวลไขมันและมวลไร้ไขมันในร่างกาย

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3. การเจาะเลือดและเก็บปัสสาวะเพื่อตรวจทางห้องปฏิบัติการ ได้แก่ การตรวจระดับไขมัน น้ำตาลในเลือด เม็ดเลือดแดง และเม็ดเลือดขาว หน้าที่ของตับ ไต กรดยูริก การติดเชื้อไวรัสตับอักเสบชนิดบีและ ซี สายพันธุกรรม ดีเอ็นเอและ BNP (การตรวจการทำงานของกล้ามเนื้อหัวใจ)
4. การถ่ายภาพรังสีทรวงอก
5. การตรวจสภาพของช่องปากโดยทั่วไปและการตรวจสภาพความรุนแรงของโรคปริทันต์

ประโยชน์และผลข้างเคียงที่จะเกิดแก่ผู้ร่วมโครงการ

1. ผู้ร่วมโครงการได้รับการซักประวัติและตรวจร่างกายเพื่อประเมินโรคและปัจจัยเสี่ยงของโรคหัวใจและหลอดเลือด เบาหวาน โรคมะเร็งตับและมะเร็งอื่นๆ โรคทางพันธุกรรมและภาวะโภชนาการขาดหรือเกิน
2. กรณีที่มีการวินิจฉัยโรคเพิ่มเติมจะให้คำแนะนำในการปฏิบัติตัวและส่งต่อให้แพทย์ผู้เชี่ยวชาญเพื่อการตรวจและการรักษาต่อไปหรือถ้ามีปัจจัยเสี่ยงของโรคจะได้รับคำแนะนำในการปฏิบัติตัวและการป้องกันโรค

ผลข้างเคียงที่จะเกิดแก่ผู้ร่วมโครงการ

การเก็บตัวอย่างอาจทำให้ได้รับความเจ็บปวดเพียงเล็กน้อยแก่ผู้ร่วมโครงการหรือเกิดรอยเขียวช้ำบริเวณเจาะเลือด ซึ่งจะหายได้เองภายในไม่กี่วัน ผู้วิจัยจะระมัดระวังให้เกิดภาวะดังกล่าวน้อยที่สุด

การเก็บข้อมูลเป็นความลับ

ถ้าท่านตกลงเข้าร่วมโครงการข้อมูลของท่านจะถูกเก็บเป็นความลับ นอกเหนือจากการบังคับตามกฎหมาย จะมีเพียงแพทย์ เจ้าหน้าที่ ผู้แทนของผู้สนับสนุนการศึกษา คณะกรรมการจริยธรรมที่สามารถดูบันทึกการเจ็บป่วยเพื่อตรวจสอบโครงการนี้ในประเทศไทย การวิเคราะห์ข้อมูลและการรายงานผลการวิจัยจะแสดงเป็นภาพรวมของประชากรที่ศึกษาโดยจะไม่เปิดเผยผลการศึกษาของผู้เข้าร่วมโครงการเป็นรายบุคคล

หมายเหตุ 1. เนื่องจากการตรวจสุขภาพ ปี 2540 ได้มีการตอบแบบสอบถามเกี่ยวกับการถ่ายอุจจาระไปแล้ว ในการตรวจสุขภาพในปี 2545 นี้ จึงไม่มีการตอบแบบสอบถามเรื่องนี้อีก

2. ผู้ร่วมโครงการที่ได้รับการตรวจหาไวรัสตับอักเสบชนิดบีและซีไปแล้ว จะไม่ต้องรับการตรวจหาการตรวจเชื้อดังกล่าวซ้ำอีก

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APENDIX C.2

หนังสือยินยอมโดยได้รับการบอกกล่าวและเต็มใจ

(Informed consent form)

ชื่อโครงการ 1. การศึกษาหาอิทธิพลของสภาวะแวดล้อมในการทำงานต่อความชุกของโรคหัวใจและหลอดเลือดรวมทั้งปัจจัยเสี่ยงด้านพันธุกรรมและปัจจัยเสี่ยงต่างๆในพนักงานไฟฟ้าฝ่ายผลิตแห่งประเทศไทย

2. การศึกษาหาความชุกของโรคตับชนิดต่างๆในพนักงานไฟฟ้าฝ่ายผลิตแห่งประเทศไทยที่มีเอนไซม์ตับ (serum aminotranferase) ผิดปกติ

3. การตายในประชากรไทย: สาเหตุสำคัญ ปัจจัยเสี่ยง การเปลี่ยนแปลงและแนวโน้มในอนาคตการวิเคราะห์และประยุกต์ใช้เชิงสหวิทยาการ

4. การศึกษาเรื่องการถ่ายอุจจาระในประชากรไทย

5. ความสัมพันธ์ของการเกิดโรคมะเร็งตับกับการติดเชื้อไวรัสตับอักเสบชนิดบีและซีในพนักงานการไฟฟ้าฝ่ายผลิตแห่งประเทศไทย

ชื่อผู้วิจัย รศ. ปิยมิตร ศรีธารา, อ.นพ.สายัณห์ ชีพอุดมวิทย์, อ.นพ.สุกิจ แย้มวงศ์, ผศ.สุพจน์ ตุลยาเดชานนท์, ผศ. โฉมศรี โฆษิตชัยวัฒน์, อ.พญ.ธิติยา ศิริสิงห, ผศ.พงศ์อมร บุนนาค, ศ.สุรัตน์ โคมินทร์, ผศ.อดิศักดิ์ ผลิตผลการพิมพ์, รศ.ธันยชัย สุระ, ผศ.รัตนา พากเพียรกิจวัฒนา, ศ.พรทิพย์ โล่ห์เลขา, รศ.อาทิตย์ อังกานนท์, รศ.วินัย วนานุกูล, ผศ.สิทธิ์เทพ ธนกิจจารุ, น.ส.พัชรา แพนพันธ์อ้วน, นพ.ศักดิ์ดา กุสุมภ์, พญ.นงนุช อังกุราภินันท์, พญ.วิภา รัตนชัยวงศ์, นพ. พูนชัย หล่อเจริญผล, นางวัลลภา ปลอดทุกข์, นางเสาวนีย์ อังศุธรารักษ์, น.ส.มาลี อัตตสรรค์สาธิต, ผศ.ทญ.รังสินี มหานนท์, อ.ทพ.ดร.กิตติ ต.รุ่งเรือง, อ.ทพ.สุพจน์ ตามสายลม, อ.ทญ.เกศรินทร์ โรจนสมสิทธิ์ และ คุณจริยา ตันติธรรม

ชื่อผู้ถูกวิจัย……

อายุ……..เลขที่เวชระเบียน……

คำยินยอมของผู้ถูกวิจัย

ข้าพเจ้า นาย/นาง/นางสาว……

ได้ทราบรายละเอียดของโครงการวิจัยตลอดจนประโยชน์ และข้อเสี่ยงที่จะเกิดขึ้นต่อผู้ยินยอมตนให้ทำวิจัยจากผู้วิจัยแล้วอย่างชัดเจน ไม่มีสิ่งใดซ่อนเร้นและยินยอมให้ทำวิจัยในโครงการที่มีชื่อข้างต้น และข้าพเจ้ารู้ว่าถ้ามีปัญหาหรือข้อสงสัยเกิดขึ้นข้าพเจ้าสามารถถามผู้วิจัยได้ และข้าพเจ้าสามารถไม่เข้าร่วมโครงการวิจัยนี้เมื่อใดก็ได้โดยไม่มีผลกระทบต่อการรักษาที่ข้าพเจ้าพึงได้รับ นอกจากนี้ผู้วิจัยจะเก็บข้อมูลเฉพาะเกี่ยวกับตัวข้าพเจ้าเป็นความลับและจะเปิดเผยได้เฉพาะในรูปที่เป็นสรุปผลการวิจัย การเปิดเผยข้อมูลเกี่ยวกับตัวข้าพเจ้าต่อหน่วยงานต่างๆที่เกี่ยวข้อง กระทำได้เฉพาะกรณีจำเป็นด้วยเหตุผลทางวิชาการเท่านั้น

ลงชื่อ..... (ผู้ยินยอมให้ทำวิจัย)

…….. (พยาน)

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คำอธิบายของแพทย์หรือผู้วิจัย

ข้าพเจ้าได้อธิบายรายละเอียดของโครงการ ตลอดจนประโยชน์ของการวิจัย รวมทั้งข้อเสี่ยงที่อาจจะเกิดขึ้นแก่ผู้ยินยอมตนให้ทำวิจัยทราบแล้วอย่างชัดเจนโดยไม่มีสิ่งใดปิดบังซ่อนเร้น

ลงชื่อ..... (แพทย์ผู้ทำวิจัย)

วันที่.....

หมายเหตุ: กรณีผู้ยินยอมตนให้ทำวิจัยไม่สามารถอ่านหนังสือได้ ให้ผู้วิจัยอ่านข้อความในใบยินยอมตนให้ทำวิจัยฟังจนเข้าใจดีแล้ว และให้ผู้ยินยอมตนให้ทำวิจัยลงนามหรือพิมพ์ลายนิ้วมือรับทราบในการให้ความยินยอมดังกล่าวข้างต้นไว้ด้วย

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APENDIX C.3

แบบสอบถาม

แบบสำรวจโครงการ EGAT 2

ส่วนที่ 1 กรุณากรอกข้อมูลในแบบสอบถามหน้า **1-6** โปรด √ ทับข้อที่ท่านเลือกยกเว้นบริเวณข้อความในกรอบ

ชื่อ……..นามสกุล……..

1. หมายเลขบัตรประชาชน □ □□□□ □□□□ □□
2. อายุ □□ ปี
3. เกิดวันที่ □□ เดือน □□ พ.ศ. □□□□
4. เพศ (1) ชาย (2) หญิง
5. สถานภาพ (1) โสด (2) คู่
   (3) ม่าย (คู่สมรสเสียชีวิต) (4) หย่า /แยก
6. การศึกษา (1) มัธยมศึกษาหรือต่ำกว่า
   (2) วิชาชีพ (ปวช, ปวส, อนุปริญญา)
   (3) ปริญญาตรี
   (4) ปริญญาโท
   (5) ปริญญาเอก
7. ที่อยู่ (บ้าน)……
   ……..
   รหัสไปรษณีย์ □□□□□
   โทรศัพท์ (บ้าน) □□□ - □□□□□□□□
8. การปฏิบัติงาน ตำแหน่ง……
   สถานที่ทำงาน ……...
   ……รหัสไปรษณีย์ □□□□□
   โทรศัพท์ □□□ - □□□□□□□ ต่อ □□□□

สำหรับเจ้าหน้าที่

หมายเลขพนักงาน □□□□□□

ตรวจวันที่ □□ เดือน □□ พ.ศ. □□□□

สถานที่ตรวจ ( )

น้ำหนักตัว □□□. □ Kg

ส่วนสูง □□□. □ cm

Waist circumference □□□. □ cm

Hip circumference □□□. □ cm

Triceps skinfold □□□. □ cm

BP.1 □□□ / □□□ Torr

BP.2 □□□ / □□□ Torr

BP.3 □□□ / □□□ Torr

PR1 □□□ / min

PR2 □□□ / min

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9. ระดับรายได้ของครอบครัว (ต่อเดือน)

(1) น้อยกว่า 10,000 บาท (2) 10,000-19,999 บาท

(3) 20,000 – 49,999 บาท (4) 50,000 – 99,999 บาท

(5) ตั้งแต่ 100,000 บาทขึ้นไป

10. การสูบบุหรี่ (1) ไม่เคยสูบบุหรี่

(2) ไม่เคยสูบบุหรี่ แต่ได้รับควันบุหรี่อย่างใกล้ชิด (จากคนอื่นสูบ)

เฉลี่ยครั้งละ □□ นาที วันละ □□ ครั้ง สัปดาห์ละ □ วัน

(3) เคยสูบแต่เลิกแล้ว

(4) ยังสูบอยู่

11. การดื่มสุรา (1) ไม่เคยดื่ม

(2) เคยดื่ม หรือ ยังดื่มอยู่ (กรุณาตอบรายละเอียดข้างล่าง)

รายละเอียดการดื่มสุรา (โปรด √ ทับข้อที่ท่านเลือก)

(1) ดื่มน้อยมาก ไม่เกินเดือนละ 1 ครั้ง อย่างนี้มาตลอด

(2) เคยดื่ม แต่เลิกแล้ว

(3) ยังดื่มอยู่

กรณีที่เคยดื่ม แต่เลิกแล้ว เริ่มดื่มอายุ □□ ปี

I อายุ □□ ปี ถึง อายุ □□ ปี

1. ดื่มเหล้าขาวหรี ยาดองเหล้า ปริมาณ □□□□ ซีซี/ครั้ง ความถี่ □□ ครั้งต่อ (สัปดาห์), (เดือน)
2. ดื่ม วิสกี้ ปริมาณ □□□□ ซีซี/ครั้ง ความถี่ □□ ครั้งต่อ (สัปดาห์), (เดือน)
3. ดื่ม เบียร์ ปริมาณ □□□□ ซีซี/ครั้ง ความถี่ □□ ครั้งต่อ (สัปดาห์), (เดือน)
4. ดื่ม ไวน์ ปริมาณ □□□□ ซีซี/ครั้ง ความถี่ □□ ครั้งต่อ (สัปดาห์), (เดือน)
5. อื่นๆ..... ปริมาณ □□□□ ซีซี/ครั้ง ความถี่ □□ ครั้งต่อ (สัปดาห์), (เดือน)

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II อายุ □□ ปี ถึง อายุ □□ ปี

1. ดื่มเหล้าขาวหรี ยาดองเหล้า ปริมาณ □□□□ ซีซี/ครั้ง ความถี่ □□ ครั้งต่อ (สัปดาห์), (เดือน)
2. ดื่ม วิสกี้ ปริมาณ □□□□ ซีซี/ครั้ง ความถี่ □□ ครั้งต่อ (สัปดาห์), (เดือน)
3. ดื่ม เบียร์ ปริมาณ □□□□ ซีซี/ครั้ง ความถี่ □□ ครั้งต่อ (สัปดาห์), (เดือน)
4. ดื่ม ไวน์ ปริมาณ □□□□ ซีซี/ครั้ง ความถี่ □□ ครั้งต่อ (สัปดาห์), (เดือน)
5. อื่นๆ..... ปริมาณ □□□□ ซีซี/ครั้ง ความถี่ □□ ครั้งต่อ (สัปดาห์), (เดือน)

III อายุ □□ ปี ถึง อายุ □□ ปี

1. ดื่มเหล้าขาวหรี ยาดองเหล้า ปริมาณ □□□□ ซีซี/ครั้ง ความถี่ □□ ครั้งต่อ (สัปดาห์), (เดือน)
2. ดื่ม วิสกี้ ปริมาณ □□□□ ซีซี/ครั้ง ความถี่ □□ ครั้งต่อ (สัปดาห์), (เดือน)
3. ดื่ม เบียร์ ปริมาณ □□□□ ซีซี/ครั้ง ความถี่ □□ ครั้งต่อ (สัปดาห์), (เดือน)
4. ดื่ม ไวน์ ปริมาณ □□□□ ซีซี/ครั้ง ความถี่ □□ ครั้งต่อ (สัปดาห์), (เดือน)
5. อื่นๆ..... ปริมาณ □□□□ ซีซี/ครั้ง ความถี่ □□ ครั้งต่อ (สัปดาห์), (เดือน)

เลิกดื่ม เมื่ออายุ □□ ปี สาเหตุที่เลิก (1) เมาเร็วขึ้น (2) อื่นๆ.....

กรณีที่ ยังดื่มอยู่ เริ่มดื่มที่อายุ □□ ปี

I อายุ □□ ปี ถึง อายุ □□ ปี

1. ดื่มเหล้าขาวหรี ยาดองเหล้า ปริมาณ □□□□ ซีซี/ครั้ง ความถี่ □□ ครั้งต่อ (สัปดาห์), (เดือน)
2. ดื่ม วิสกี้ ปริมาณ □□□□ ซีซี/ครั้ง ความถี่ □□ ครั้งต่อ (สัปดาห์), (เดือน)
3. ดื่ม เบียร์ ปริมาณ □□□□ ซีซี/ครั้ง ความถี่ □□ ครั้งต่อ (สัปดาห์), (เดือน)
4. ดื่ม ไวน์ ปริมาณ □□□□ ซีซี/ครั้ง ความถี่ □□ ครั้งต่อ (สัปดาห์), (เดือน)
5. อื่นๆ..... ปริมาณ □□□□ ซีซี/ครั้ง ความถี่ □□ ครั้งต่อ (สัปดาห์), (เดือน)

II อายุ □□ ปี ถึง อายุ □□ ปี

1. ดื่มเหล้าขาวหรี ยาดองเหล้า ปริมาณ □□□□ ซีซี/ครั้ง ความถี่ □□ ครั้งต่อ (สัปดาห์), (เดือน)
2. ดื่ม วิสกี้ ปริมาณ □□□□ ซีซี/ครั้ง ความถี่ □□ ครั้งต่อ (สัปดาห์), (เดือน)
3. ดื่ม เบียร์ ปริมาณ □□□□ ซีซี/ครั้ง ความถี่ □□ ครั้งต่อ (สัปดาห์), (เดือน)
4. ดื่ม ไวน์ ปริมาณ □□□□ ซีซี/ครั้ง ความถี่ □□ ครั้งต่อ (สัปดาห์), (เดือน)
5. อื่นๆ..... ปริมาณ □□□□ ซีซี/ครั้ง ความถี่ □□ ครั้งต่อ (สัปดาห์), (เดือน)

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III อายุ □□ ปี ถึง อายุ □□ ปี

1. ดื่มเหล้าขาวหรี ยาดองเหล้า ปริมาณ □□□□ ซีซี/ครั้ง ความถี่ □□ ครั้งต่อ (สัปดาห์), (เดือน)
2. ดื่ม วิสกี้ ปริมาณ □□□□ ซีซี/ครั้ง ความถี่ □□ ครั้งต่อ (สัปดาห์), (เดือน)
3. ดื่ม เบียร์ ปริมาณ □□□□ ซีซี/ครั้ง ความถี่ □□ ครั้งต่อ (สัปดาห์), (เดือน)
4. ดื่ม ไวน์ ปริมาณ □□□□ ซีซี/ครั้ง ความถี่ □□ ครั้งต่อ (สัปดาห์), (เดือน)
5. อื่นๆ..... ปริมาณ □□□□ ซีซี/ครั้ง ความถี่ □□ ครั้งต่อ (สัปดาห์), (เดือน)

12. การออกกำลังกาย (1) ไม่ได้ออกกำลังกาย

(2) ออกกำลังกายโดย.....

ออกกำลังกายแต่ละครั้งนาน □□ นาที ความถี่ □□ ครั้ง/สัปดาห์

13. ประวัติการเจ็บป่วย

13.1 ท่านเคยเจ็บป่วยด้วยโรคดังต่อไปนี้หรือไม่ (โปรด √ ทับข้อความที่ท่านเลือก)

โรค	ไม่เคย	เคย	เมื่ออายุ...ปี	การรักษา
เบาหวาน	□	□	□□	.....
ความดันเลือดสูง	□	□	□□	.....
ไขมันในเลือดสูง	□	□	□□	.....
อัมพาต/อัมพฤกษ์	□	□	□□	.....
เส้นเลือดหัวใจตีบ	□	□	□□	.....
กล้ามเนื้อหัวใจตาย	□	□	□□	.....
หัวใจล้มเหลว	□	□	□□	.....
ตับอักเสบ	□	□	□□	.....
นิ่วในถุงน้ำดี	□	□	□□	.....
ตา ตัวเหลือง หรือ ดีซ่าน	□	□	□□	.....
อื่นๆ.....	□	□	□□	.....

14. ประวัติการใช้ยา: ท่านมียาที่ใช้เป็นประจำอยู่หรือไม่

(1) ไม่มี

(2) มี โปรดระบุ.....

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APPENDIX D

The characteristics of subjects

N	Genotype		Sex	Age	BMI	WHR	TC	TG	HDL	LDL	SBP	DBP	Medication
	-108	192											
1	CC	RR	M	41	31	0.97	344	124	46	273	144	94	
2	TT	QQ	M	50	23	0.91	185	116	46.	116	130	80	
3	CC	QQ	M	41	25	0.93	255	170	37	183	127	89	
4	CC	QR	M	49	31	1.01	232	366	47	112	123	80	hypoglycemic drug
5	CT	QR	M	42	24	0.92	263	321	30	168	141	75	Cholesterol lowering drug
6	CT	QQ	M	42	25	0.93	239	187	35	167	134	89	
7	CC	RR	F	38	25	0.83	236	74	68	152	122	77	
8	CT	RR	M	43	27	0.91	259	249	56	153	138	60	Hypoglycemic and diabetes
9	CC	QQ	M	45	19	0.93	201	183	45	119	128	77	
10	CT	RR	M	36	24	0.93	243	103	58	165	156	97	
11	CT	QR	F	39	18	0.80	224	55	55	158	87	62	
12	CT	QR	M	41	28	0.97	239	175	45	160	129	93	
13	CT	QR	F	36	33	0.96	185	83	47	122	114	60	
14	CT	QQ	M	50	23	0.91	239	95	62	158	136	86	
15	CT	QQ	M	50	20	0.83	201	78	56	129	105	70	
16	TT	RR	F	47	30	0.91	205	156	66	107	121	73	
17	CT	QR	F	50	26	0.86	266	119	55	187	110	80	
18	CC	QR	F	40	23	0.87	263	113	78	162	98	64	

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The characteristics of subjects (Continued)

N	Genotype		Sex	Age	BMI	WHR	TC	TG	HDL	LDL	SBP	DBP	Medication
	-108	192											
19	CT	QR	M	47	23	0.93	216	127	42	149	122	86	
20	CT	QQ	M	51	21	0.88	320	281	44	221	140	100	
21	CT	QQ	M	39	19	0.82	263	186	56	169	122	78	
22	CC	QQ	M	46	22	0.89	208	227	51	112	142	85	
23	CT	RR	M	40	22	0.88	212	93	57	137	141	95	
24	CT	QR	M	44	30	0.99	278	337	29	182	116	88	
25	TT	RR	M	43	24	0.90	255	161	42	181	130	101	
26	TT	QR	F	48	25	0.90	255	147	41	185	155	99	
27	TT	RR	M	38	25	0.88	255	104	39	195	138	77	Hypoglycemic drug
28	TT	RR	M	53	35	0.90	193	304	41	91	128	67	
29	TT	RR	M	45	23	0.94	243	317	43	137	147	87	
30	TT	RR	M	47	24	0.91	239	311	45	132	140	98	
31	CT	QR	F	40	25	0.84	220	85	61	142	133	78	
32	CT	QR	M	48	26	0.99	297	324	80	153	124	73	
33	CT	QR	F	43	28	0.89	224	90	46	160	130	76	
34	CC	QQ	M	37	23	0.91	263	306	46	-	109	82	
35	CT	RR	M	45	24	0.93	270	175	72	163	161	108	
36	CT	QQ	F	44	25	0.91	278	158	47	200	121	80	

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The characteristics of subjects (Continued)

N	Genotype		Sex	Age	BMI	WHR	TC	TG	HDL	LDL	SBP	DBP	Medication
	-108	192											
37	CT	QR	F	36	22	0.78	247	84	66	164	130	89	
38	CT	QQ	M	38	23	0.94	178	84	58	103	154	93	
39	CC	QR	M	42	25	0.85	189	87	62	110	115	77	
40	CT	QR	M	38	21	0.88	270	130	46	198	113	71	Aspirin
41	TT	RR	F	42	24	0.99	208	189	57	114	115	74	
42	CC	QQ	M	40	23	0.94	193	120	66	103	124	70	
43	CT	QR	F	53	22	0.88	208	235	43	119	141	78	Hyperglycemic drug
44	CT	QR	F	52	20	0.75	286	58	97	177	103	61	
45	CT	QQ	M	42	24	0.88	201	123	40	136	114	64	
46	CC	QQ	F	35	28	0.91	232	105	61	149	105	78	
47	CT	QR	F	43	25	0.88	290	208	50	198	117	61	
48	CT	QR	M	45	21	0.82	182	124	61	96	139	66	
49	TT	QR	M	40	26	1.00	255	376	46	134	140	91	
50	CT	QR	F	48	27	0.84	209	82	78	114	125	69	
51	TT	QR	M	42	20	0.92	197	281	60	81	149	90	
52	CC	QR	F	54	24	0.86	205	154	48	126	158	83	
53	CT	QR	M	39	19	0.87	224	105	64	139	116	69	
54	CT	RR	M	49	20	0.83	232	174	45	152	119	69	

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The characteristics of subjects (Continued)

N	Genotype		Sex	Age	BMI	WHR	TC	TG	HDL	LDL	SBP	DBP	Medication
	-108	192											
55	CT	QR	M	39	33	0.95	236	127	51	159	162	101	
56	CT	RR	F	55	19	0.86	224	63	74	138	100	66	
57	CT	QQ	M	45	23	0.88	220	231	45	129	117	78	
58	CT	QQ	M	52	22	0.93	216	177	81	99	137	65	
59	CT	QQ	M	43	23	0.96	286	174	52	199	138	80	
60	TT	QR	M	46	27	0.95	224	202	57	127	165	84	
61	TT	QR	F	48	24	0.77	216	56	75	130	110	63	
62	CC	QR	M	51	26	0.95	216	244	39	129	150	94	Aspirin, diuretic drug
63	TT	QR	F	47	23	0.82	216	149	73	114	127	78	
64	TT	QQ	F	47	25	0.72	216	88	68	131	103	64	
65	TT	QR	M	38	17	0.90	185	165	50	103	128	78	
66	CT	QR	M	48	22	0.92	205	105	70	114	107	59	
67	CT	QR	M	42	23	0.92	228	96	74	134	118	78	
68	CT	QQ	M	36	24	0.91	216	188	44	135	124	79	
69	CT	QR	M	35	23	0.85	274	135	45	202	118	63	
70	CC	QQ	M	40	23	0.83	278	242	49	181	115	69	
71	CC	QR	M	51	27	0.98	320	400	39	0	120	75	
72	CT	QR	F	39	22	0.81	208	92	53	137	105	70	

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The characteristics of subjects (Continued)

N	Genotype		Sex	Age	BMI	WHR	TC	TG	HDL	LDL	SBP	DBP	Medication
	-108	192											
73	CC	QQ	M	38	27	0.95	336	215	64	229	100	65	
74	CT	QQ	F	45	18	0.78	212	56	82	119	110	75	
75	TT	QQ	M	44	24	0.86	263	77	63	184	123	85	
76	CT	QR	M	38	23	0.97	228	261	37	138	125	90	
77	CT	QQ	M	42	29	0.95	259	219	46	169	130	70	
78	CT	QQ	M	35	19	0.82	239	126	38	176	114	82	
79	CT	QQ	F	37	21	0.90	232	68	62	156	108	55	
80	CT	QR	F	43	26	0.91	181	91	53	111	110	75	
81	CC	QQ	M	34	23	0.85	255	105	63	171	138	79	
82	TT	QR	F	38	24	0.82	216	69	75	127	115	80	
83	CT	QQ	F	38	23	0.77	243	185	47	159	106	59	
84	CC	QQ	F	38	32	0.96	189	188	51	101	113	71	
85	CT	QR	M	47	23	0.91	108	139	34	47	116	78	
86	CC	QQ	M	43	24	0.89	286	499	39	0	121	69	
87	CT	QR	M	46	25	0.95	297	242	50	199	130	89	
88	CT	QQ	F	39	20	0.79	220	77	67	137	90	65	
89	CC	QR	F	38	19	0.87	197	73	67	115	135	70	
90	CC	QQ	M	43	22	0.89	127	240	29	50	162	101	

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The characteristics of subjects (Continued)

N	Genotype		Sex	Age	BMI	WHR	TC	TG	HDL	LDL	SBP	DBP	Medication
	-108	192											
91	CT	QQ	F	36	19	0.79	158	70	52	92	117	62	
92	CT	RR	M	46	23	0.92	293	118	41	229	115	71	
93	TT	QQ	M	36	26	0.89	236	108	47	166	100	74	
94	CT	RR	M	41	28	0.95	228	177	42	150	125	82	Hyperglycemic drug
95	TT	RR	M	38	26	0.96	270	165	60	177	122	75	
96	CC	RR	F	0	22	0.76	178	52	77	90	109	70	
97	CT	RR	M	48	19	0.79	181	63	71	98	163	82	
98	CC	QR	M	37	26	0.92	332	158	44	256	118	63	
99	CC	QQ	M	39	27	0.92	243	195	36	168	129	84	
100	CT	RR	M	37	21	0.89	208	139	48	132	129	82	
101	CT	RR	M	41	22	0.90	208	90	39	152	122	70	
102	CC	QQ	F	40	20	0.77	224	115	80	121	107	68	
103	TT	RR	M	48	25	0.89	193	184	40	116	161	86	
104	CT	QQ	M	35	22	0.91	224	416	32	0	111	68	
105	CT	QR	M	49	27	1.04	208	147	46	133	133	84	
106	CC	QR	M	45	20	0.88	255	126	60	169	100	61	
107	CC	QQ	M	47	24	0.94	208	142	41	139	149	90	
108	CC	QR	M	47	30	0.92	247	198	50	158	127	70	

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The characteristics of subjects (Continued)

N	Genotype		Sex	Age	BMI	WHR	TC	TG	HDL	LDL	SBP	DBP	Medication
	-108	192											
109	CT	QR	F	39	20	0.79	208	48	109	90	91	55	
110	CT	QR	M	37	23	0.92	232	220	60	127	131	85	
111	CC	QR	M	40	24	0.89	189	156	81	77	179	109	
112	TT	QQ	M	-	26	0.95	239	311	42	136	135	84	
113	CT	QR	M	39	21	0.96	197	34	61	130	129	67	
114	CT	QQ	M	37	22	0.76	174	90	47	108	97	62	
115	CT	QR	M	37	20	0.79	181	92	44	119	131	65	
116	CT	QR	M	36	21	0.92	205	86	68	120	116	75	
117	CT	QQ	M	38	21	0.92	255	71	46	194	93	48	
118	CT	RR	M	40	24	0.89	212	163	35	145	114	65	
119	TT	QQ	M	43	25	0.90	224	222	51	129	139	89	
120	CC	QQ	M	44	27	0.77	324	118	46	255	126	77	Cholesterol lowering drug
121	CC	QQ	M	48	24	0.89	197	113	71	103	146	101	
122	CC	QR	M	46	29	0.91	290	117	58	208	135	76	Hypertensive and diuretic
123	CT	QR	M	44	22	1.04	201	86	84	100	102	67	
124	TT	RR	M	38	24	0.88	243	110	46	175	111	72	
125	CT	QR	M	40	26	0.94	247	165	46	168	144	90	
126	CC	QR	M	37	21	0.92	205	82	51	137	121	78	

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The characteristics of subjects (Continued)

N	Genotype		Sex	Age	BMI	WHR	TC	TG	HDL	LDL	SBP	DBP	Medication
	-108	192											
127	CC	QR	F	36	23	0.82	181	51	73	98	94	64	
128	TT	QR	M	37	20	0.82	286	147	57	199	99	64	
129	TT	QR	M	43	31	1.00	243	139	42	173	153	93	diamicrom
130	CT	RR	M	37	28	0.87	212	165	50	129	134	91	
131	CT	QR	M	38	33	0.96	197	110	69	106	142	91	
132	CT	RR	F	42	19	0.82	313	154	47	235	108	83	
133	CT	QR	M	39	24	0.78	216	136	44	145	121	76	
134	CC	QR	M	37	31	0.96	197	89	47	132	123	78	
135	CT	QR	M	34	24	0.84	305	205	62	202	98	65	
136	CC	QR	M	44	21	0.88	193	299	35	98	100	65	
137	CT	QR	M	39	23	0.90	162	84	50	95	123	86	diuretic
138	CC	QQ	M	41	26	0.90	205	69	64	126	107	69	
139	CT	QQ	M	37	23	0.88	243	241	80	116	115	61	
140	CT	QQ	M	37	17	0.83	181	58	59	111	147	72	
141	CT	RR	M	43	19	0.79	197	310	53	82	101	58	
142	CT	RR	M	37	23	0.88	224	99	44	160	111	56	
143	TT	RR	M	39	22	0.88	228	125	39	164	101	61	
144	CC	QQ	M	37	23	0.88	193	317	33	96	113	68	

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The characteristics of subjects (Continued)

N	Genotype		Sex	Age	BMI	WHR	TC	TG	HDL	LDL	SBP	DBP	medication
	-108	192											
145	CC	QR	M	39	26	0.86	197	119	47	126	130	80	
146	TT	RR	M	45	21	0.86	220	95	63	139	70	45	
147	CC	QQ	M	39	23	0.83	181	59	53	117	93	66	
148	CC	QR	M	36	21	0.84	255	169	53	168	120	80	
149	CT	QQ	M	42	24	0.90	216	158	47	137	117	76	
150	CT	RR	M	43	24	0.89	228	216	40	144	135	73	diuretic
151	CC	QQ	M	40	22	0.84	286	128	42	218	119	73	
152	TT	RR	M	39	23	0.89	247	161	47	167	110	70	
153	CC	QQ	M	42	21	0.84	251	206	47	162	112	64	
154	CC	QQ	M	49	27	0.94	270	201	55	175	131	76	
155	CT	QR	M	39	23	0.87	216	89	101	97	98	51	
156	CT	RR	M	37	23	0.94	201	86	59	124	133	79	
157	TT	QQ	M	51	26	0.99	309	466	39	-	160	102	
158	CT	QR	M	45	25	0.89	255	250	44	161	105	66	
159	CT	QQ	M	51	30	0.93	278	204	39	198	127	82	Cholesterol lowering drug
160	CT	QR	M	36	16	0.83	247	113	84	140	108	66	

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BIOGRAPHY

NAME Mr. Arthit Sripirom

DATE OF BIRTH 19 August 1979

PLACE OF BIRTH Udonthani, Thailand

INSTITUTION ATTENDED Mahidol University, 1998-2002:

Bachelor of Science (Medical Technology)

Mahidol University, 2003-2006:

Master of Science (Toxicology)

RESEARCH GRANT The Post-Graduate Education, Training and Research Program in Environmental Science, Technology and Management under Higher Education Development Project of the Commission on Higher Education, Ministry of Education

HOME ADDRESS 50 Mu 13, Kumpavapi District, Udonthani, 10400, Thailand

E-mail**:** arthit09@hotmail.com

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