การศึกษาหน้าที่และรหัสพันธุกรรมของเอนไซม์ cholesteryl ester transfer protein และ hepatic lipase ในประชากรไทยที่มีระดับ HDL สูง

นางสาว วาณี เปล่งพานิชย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

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ปีการศึกษา 2551

FUNCTIONAL AND GENETIC ANALYSES OF CHOLESTERYL ESTER TRANSFER PROTEIN AND HEPATIC LIPASE IN THAI SUBJECTS WITH HYPERALPHALIPOPROTEINEMIA

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สาเหตุของภาวะ HDL ในเลือดสูง (HALP) มีรายงานว่าเกิดจากความผิดปกติของโปรตีน cholesteryl ester transfer protein (CETP) และ เอนไซม์ hepatic lipase (HL) ซึ่งยังไม่เคยมีการศึกษาในคนไทยมาก่อน จุดประสงค์ของการศึกษานี้เพื่อศึกษาความผิดปกติของโปรตีนทั้งสองในคนไทย และศึกษาการกลายพันธุ์ในยีน *CETP* และ *LIPC* ซึ่งสร้างโปรตีน CETP และ HL ตามลำดับ รวมถึงการศึกษาระดับโปรตีนชนิดต่างๆบน HDL ของคนไข้เปรียบเทียบกับกลุ่มควบคุม การศึกษานี้ประกอบด้วยคนไข้ที่มีระดับ HDL $\geq$ 100 mg/dL จำนวน 38 คน และกลุ่มควบคุมที่มีระดับ HDL ปกติ มีอายุ และ เพศ ใกล้เคียงกับกลุ่มคนไข้จำนวน 38 คน ผลการทดลองพบว่าคนไข้มีระดับ total cholesterol และ HDL สูงกว่ากลุ่มควบคุมอย่างมีนัยสำคัญ (259 $\pm$ 8 และ 234 $\pm$ 6 mg/dL, P=0.05 และ 119 $\pm$ 2 และ 64 $\pm$ 3, P<0.01, ตามลำดับ). ค่าเฉลี่ยของระดับ CETP และ HL activities ในกลุ่มทดลองต่ำกว่ากลุ่มควบคุมอย่างมีนัยสำคัญ (34 $\pm$ 4 และ 44 $\pm$ 3 pmol/μL/hr, P = 0.04; 150 $\pm$ 17 และ 227 $\pm$ 16 nmol FFA/mL/min, P = 0.002 ตามลำดับ) จากคนไข้ 38 คน พบว่ามีคนไข้ 7 และ 3 คนที่จัดว่าการทำงานของ CETP และ HL ผิดปกติ ตามลำดับ ผลการศึกษาในระดับยีนของผู้ที่มีภาวะการทำงานของเอนไซม์ผิดปกติดังกล่าว พบกลายพันธุ์จากที่ตำแหน่ง 1326 บน exon ที่ 15 ของยีน CETP ทำให้เกิดการเปลี่ยนแปลงกรดอะมิโนตำแหน่งที่ 442 จาก asparagine เป็น glycine ซึ่งการเปลี่ยนแปลงนี้พบในคนไข้ที่มีระดับ HDL สูงจำนวน 10 คน คิดเป็น 26% ของคนไข้ทั้งหมด นอกจากนี้ยังพบการขาดหายไปของเบสจำนวน 4 เบสซึ่งก็คือ TCCC บน exon ที่ 9 ของยีน CETP ซึ่งมีไม่เคยมีรายงานมาก่อนจำนวน 1 คน นอกจากนี้ยังพบการกลายพันธุ์ของยีน *LIPC* ในคนไข้ 1 คนโดยมีการเปลี่ยนแปลงกรดอะมิโนจาก glycine ในตำแหน่งที่ 119 เป็น serine ซึ่งไม่เคยมีรายงานมาก่อนเช่นกัน ความรุนแรงในการก่อโรคของความผิดปกตินี้รับการยืนยันจาก evolutionary conservation, predicted damaging function program, and in vitro expression studies สำหรับการศึกษาระดับโปรตีนบน HDL HDL จากกลุ่มคนไข้ และกลุ่มควบคุม กลุ่มละ 7 คน ถูกแยกจากซีรัมโดยใช้ immunoaffinity column จากนั้น ปริมาณโปรตีนบน HDL ของแต่ละคนในกลุ่มทดลองถูกตรวจสอบเปรียบเทียบกับโปรตีนบน HDL ของคนปกติ โดยใช้เทคนิค two-dimensional gel electrophoresis และ mass spectrometry ผลการศึกษาพบโปรตีนบน HDL จำนวน 22 ชนิด อย่างไรก็ตามการศึกษานี้ไม่พบความแตกต่างของปริมาณโปรตีนบน HDL ของคนที่มีภาวะ HDL สูง เทียบกับคนปกติ สรุปผลการศึกษา: ในคนไทยที่มีระดับ HDL สูง พบว่ามีระดับ CETP activity และ HL activity ต่ำกว่ากลุ่มคนปกติ นอกจากนี้ยังพบความเปลี่ยนแปลงในระดับยีนทั้งในยีน *CETP* และ *LIPC* หลายแบบ โดยพบว่า 1 ใน 3 ส่วนของคนไทยที่มีระดับ HDL สูง มีความผิดปกติในยีนใดยีนหนึ่งระหว่างยีน *CETP* และ *LIPC*

ลายมือชื่อนิสิต.....

สาขาวิชา.....ชีวเวชศาสตร์..... ลายมือชื่ออาจารย์ที่ปรึกษา.....

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WANEE PLENGPANICH: FUNCTIONAL AND GENETIC ANALYSES OF CHOLESTERYL ESTER TRANSFER PROTEIN AND HEPATIC LIPASE IN THAI SUBJECTS WITH HYPERALPHALIPOPROTEINEMIA. THESIS ADVISOR: ASST. PROF. WEERAPAN KHOVIDHUNKIT, Ph.D., THESIS COADVISOR: PROF. VORASUK SHOTELERSUK, ASST. PROF. THITI SNABBOON, 65 pp.

Hyperalphalipoproteinemia (HALP), characterized by high plasma HDL level, is primarily caused by mutation in the cholesteryl ester transfer protein (*CETP*) and hepatic lipase (*LIPC*) genes resulting in decreased activities of CETP and hepatic lipase (HL). The cause of Thai HALP is still unknown. The objective of this research is to determine functional and genetic analyses of CETP and hepatic lipase (HL) in Thai HALP subjects and to determine protein composition on HDL of HALP subjects compare with normal controls. Thirty-eight subjects with HDL-cholesterol levels ≥ 100 mg/dL, and thirty-eight age- and sex-matched controls were recruited from an outpatient clinic. Secondary causes of HALP were excluded in all cases. The mean total and HDL cholesterol levels were significantly higher in the HALP group compared with the control group (259 ± 8 vs 234 ± 6 mg/dL, P=0.05 and 119 ± 2 vs 64 ± 3, P<0.01, respectively). The mean CETP and HL activities were significantly lower in the HALP group than in the control group (34 ± 4 *vs.* 44 ± 3 pmol/μL/hr, P = 0.04; and 150 ± 17 *vs.* 227 ± 16 nmol FFA/mL/min, P = 0.002, respectively). Seven and three subjects in the HALP group who had very low CETP and HL activities, respectively, were chosen for further analysis. A mutational analysis study revealed that a D442G missense mutation in exon 15 of the *CETP* gene was present in 10 subjects in the HALP group (26%), but it was not found in the control group. We also identified a novel 4 base-pair deletion mutation (734_737 del TCCC) in exon 9 of the *CETP* gene in one subject in the HALP group. Moreover, we discovered a novel missense mutation in the *LIPC* gene, G119S, in one subject in the HALP group, but not in the control group. The pathogenic role of G119S was supported by evolutionary conservation, its predicted damaging function, and in vitro expression studies. In this study; HDL was isolated by immunoaffinity column. HDL protein of HALP (n=7) and control group (n=7) were separated with two-dimensional gel electrophoresis and identified with mass spectrometry. MS analysis revealed the presence of 22 HDL-associated proteins including all known apolipoproteins and lipid transport proteins. However, there were no significant differences in the quantities of protein on HDL between case and control group, as examined by 2D analysis software. In Conclusions, In Thai subjects with HALP, both CETP and HL activities were significantly lower than those in the control subjects and several mutations in the *CETP* and *LIPC* genes were identified. Approximately one-third of Thai subjects with HALP were caused by either *CETP* or *LIPC* mutations.

Student's signature: …….....

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CONTENTS

Page

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

High-density lipoprotein (HDL) is a group of lipoproteins that play an important role in the development of atherosclerosis. An inverse relationship between plasma HDL levels and the risk of cardiovascular diseases suggests that HDL protects against atherogenesis (1-3). Levels of HDL are controlled by both genetic and environmental factors. Although high level of HDL can be secondary to medications such as estrogen and alcohol, extremely high levels of HDL or hyperalphalipoproteinemia (HALP) are usually due to genetic factors (4).

HDL metabolism is complex and requires a number of plasma enzymes, transfer proteins, and cell surface receptors. One of the main functions of HDL is to transport excess cholesterol from peripheral tissues to the liver for elimination from the body in a pathway known as "reverse cholesterol transport" (RCT)(3;5). The initial step of RCT is the interaction between HDL and peripheral cells such as fibroblasts and macrophages. Free cholesterol is transported out of peripheral cells to apoA-I and HDL via ATP-binding cassette A1 (ABCA1) and ATP-binding cassette G1 (ABCG1). Lecithin:cholesterol acyltransferase (LCAT) subsequently esterifies free cholesterol to form cholesteryl ester (CE). These CE could be transferred to the liver by several mechanisms. Cholesteryl ester transfer protein (CETP) is an enzyme that exchanges CE in HDL with triglyceride (TG) in TG-rich lipoproteins, such as VLDL, IDL and LDL. These lipoproteins are then catabolized in the liver via the LDL receptor. After the CETP-mediated transfer of CE, HDL becomes enriched with TG, which is subsequently hydrolyzed by hepatic lipase (HL). As a result, HDL becomes smaller and is ready for taking up more cholesterol from peripheral tissues. Alterations of these proteins involved in RCT can affect both metabolism and plasma concentrations of HDL in human (6). Deficiency in ABCA1 or LCAT results in low levels of HDL (7), whereas deficiency in CETP or HL leads to high levels of HDL (4).

In Japan, HALP is prevalent and is mainly caused by the genetic mutations in the *CETP* gene resulting in decreased or no activity of CETP (8). An intron 14 splicing

defect (Ivs14+1G>A) and an exon 15 missense mutation (D442G) are the two most common mutations of the *CETP* gene and also the most common cause of HALP in Japan (9;10). Knowledge that CETP deficiency causes HALP has led to the search for an inhibitor of CETP in order to increase HDL levels and modulate the atherosclerotic process. However, a clinical study using torcetrapib, a CETP inhibitor, has been disappointing (11). Nevertheless, the search for a clinically useful CETP inhibitor is still ongoing.

Although HALP is common in the Japanese, information on the cause of HALP outside Japan is relatively scarce. Few studies performed in Caucasians have demonstrated that HALP due to genetic CETP deficiency is rare (12-16). In addition, lower HL activity has also been found in some subjects with HALP (17-22). The objectives of our study were 1) to determine the functional activities and genetic analyses of CETP and HL in Thai subjects with HALP; 2) to determine the differences in the protein composition of HDL between HDL in normal subjects and HDL in HALP subjects.

Hypothesis

We hypothesized that HALP in Thai subjects was associated with deficiencies in CETP and/or HL activities and the genetic mutations were the underlying cause of low enzyme activities. Furthermore, there might be differences in the protein composition of HDL between HDL in normal subjects and HDL in HALP subjects that can be detected by using two-dimensional gel electrophoresis.

CHAPTER II

LITERATURE REVIEW

Hyperalphalipoproteinemia (HALP) is characterized by elevated levels of HDL. HDL metabolism is complex and the level of HDL in plasma is controlled by several enzymes and receptors involved in reverse cholesterol transport pathway (Fig 1).

The first step of reverse cholesterol transport is the interaction between HDL and peripheral cells such as fibroblasts and macrophages, which include aqueous diffusion, lipid-free apolipoprotein membrane microsolubilization, and scavenger receptor class B type I (SR-BI)-mediated cholesterol flux (23-25).

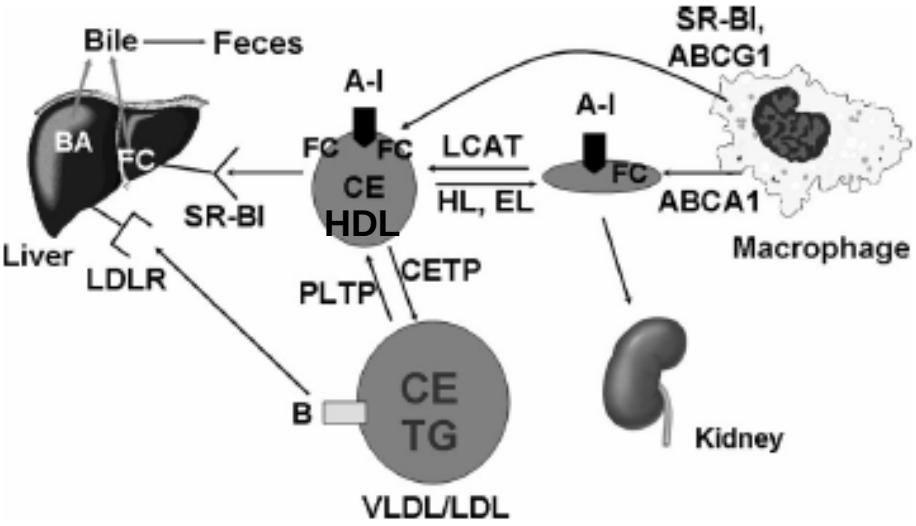

Fig. 1. HDL metabolism and RCT. HDL promotes and facilitates the process of RCT, whereby excess macrophage cholesterol is effluxed to HDL and ultimately returned to the liver for excretion. HDL–cholesteryl ester can be returned to the liver via the liver SR-BI receptor or by transfer to the apoB-containing lipoproteins by the action of CETP. FC indicates free cholesterol; BA, bile acids; CE, cholesteryl ester; PLTP, phospholipid transfer protein ;LDLR, LDL receptor; and TG, triglycerides(26).

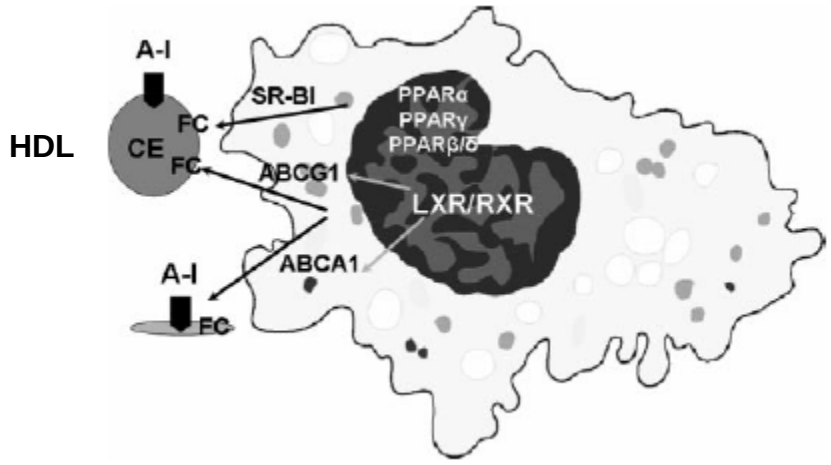

Fig. 2. Pathways of cholesterol efflux from macrophages.
Lipid-poor apoA-I can acquire free cholesterol (FC) from macrophages through an efflux process mediated by ABCA1. Alternatively, mature HDL can promote macrophage cholesterol efflux via the ABCG1 transporter or via SR-BI. ABCA1 and ABCG1 expression is controlled by the nuclear receptor heterodimer LXR/RXR. The PPARs may also influence the cholesterol efflux pathway. CE indicates cholesteryl ester (26).

Alterations in several proteins involved in RCT have been shown to result in HALP.

- **Increasing of apoA-I anabolism**

There are many previous study showed that increased production of apoA-I may be associated with HALP (27;28). However, the mechanism for the apoA-I overproduction is still unknown.

- **Over expression of LCAT**

LCAT is an enzyme critical for HDL metabolism. Deficiency of this enzyme in humans (29) and in mice (30) causes markedly reduced levels of HDL and rapid catabolism of apoA-I and apoA-II (31). Conversely, overexpression of LCAT in mice results in increased HDL levels (32). In humans, the impact of LCAT activity on RCT has not been examined and cholesterol in HDL can be directly transferred to the liver (33).

- Over expression of phospholipid transfer protein (PLTP)

HDL also derives lipids, particularly phospholipid (PL), from other lipoproteins. When triglyceride-rich (TG-rich) lipoproteins undergo hydrolysis of the TG core, surface PLs (and apolipoproteins) are shed and acquired by HDL. Thus, the activity of lipoprotein lipase is inversely associated with HDL levels (34). Lipoprotein-derived PL are transferred to HDL by the PLTP (35). Mice lacking PLTP have a significant reduction in HDL levels (36), and mice over expressing PLTP have increased levels of HDL (37).

- High insulin sensitivity

Insulin-resistant states, which are known to be associated with increased rates of apoA-I catabolism, are also associated with increased HL activity (34). Conversely, High insulin sensitivity may be associated with decreased rates of apoA-I catabolism, decreased HL activity, and increased HDL level.

- SR-BI deficiency

SR-BI is a candidate gene in which loss-of-function might be expected to result in high HDL levels. SR-BI is a cell-surface receptor capable of binding HDL and mediating selective uptake of HDL into cells. In mice, hepatic SR-BI expression appears to be an important overall regulator of RCT: SR-BI overexpression increased RCT, and SR-BI deficiency reduced RCT (38). Lack of the SR-BI pathway may lead to increased of HDL level and atherosclerosis. But in humans, who have CETP pathway, deficiency of hepatic SR-BI may have less importance because cholesterol can be effectively transmitted from HDL and returned to the liver via apoB-containing lipoproteins.

- CETP deficiency

Normally, Rodents lack CETP, and when engineered to express it, they showed reduction in HDL levels (39). In human, who have loss-of-function mutations in both alleles of the *CETP* gene, have high levels of HDL. In addition, their HDL is large, and the turnover of apoA-I is slowed(40). In Japanese, genetic defect of the CETP gene is the most common cause of HALP. Up to now, ten mutations of the CETP gene have been reported in Japanese HALP subjects, including the two most common mutations, an intron 14 splicing defect (Ivs 14+1G>A) and an exon 15 missense mutation (D442G). Cellular expression of mutant cDNA of the missense mutation (D442G) has shown that

the activity was only 30% of the wild type (WT). Moreover, coexpression of WT and mutant cDNA leads to inhibition of WT activity (41). CETP deficiency is considered a state of impared RCT, which may lead to the development of atherosclerosis and cardiovascular risk despite high HDL cholesterol levels (19;42;43). Although CETP mutations are a common cause of HALP in Japan, a study (12) in North American population has found that the mutation of the exon 15 (D442G) in the CETP gene is much less prevalent than in the Japanese population whereas hepatic lipase mutation at exon 6 (C873T) appears to be more prevalent.

- HL deficiency

HL has the ability to hydrolyze both TG and PL in HDL (Figure 1). Thus, inhibition of HL would be expected to reduce HDL remodeling, slow apoA-I catabolism, and increase apoA-I and HDL levels. Ninety mice with targeted disruption of the HL gene have an elevation of plasma HDL when compared with wild-type mice (44). In addition, two missense mutations in the HL gene, substitutions of valine for methionine at amino acid 73 (V73M) and leucine for phenylalanine at amino group while the allele phenylalanine 334 is found in 5.43% of patients and in 2.0% of controls group (17).

Apart from enzymes alterations, there are many phase that the protein composition of HDL also markedly changes. During the reduced CETP activity and high HDL level, increases in apoC-I (45), plasma apo E-rich high-density lipoprotein (46;47), and LCAT (48) are characteristic. So cause of HALP may be the alteration of protein or receptor on HDL. Recently reports had shown proteins composition on HDL. Mass spectrometry (MS) analysis revealed approximate 56 HDL-associated proteins (49). But HDL protein composition in HALP subjects has never been reported. Here we present results of a study on protein compositional analysis of total HDL based on two-dimensional gel electrophoresis (2DE) and mass spectrometry technology.

CHAPTER III

MATERIALS AND METHODS

Materials

1. Chemicals and reagents

CETP and HL activity assays

The CETP activity kit for measuring plasma CETP activity was purchased from Roar Biomedical, Inc. (New York, NY, USA). Glyceryl-tri (1-^{14}C) oleate (100 μCi/mL) and ExoSAP-IT kit were purchased from Amersham Biosciences (Buckinghamshire, England). Ultima Gold was purchased from Perkin Elmer (Waltham, MA, USA).

Mutation analysis and sequencing

Taq DNA polymerase was purchased from Fermentas (Vilnius, Lithuania). Phenol chloroform and ExsoSAP-IT kit were purchased from Amersham Biosciences (Little Chalfont Buckinghamshire, England). Chemical reagents were obtained from Sigma (Steinheim, Germany) or Merck (Damstadt, Germany).

Expression plasmid

Phusion polymerase, EcoR I, and Xho I restriction enzymes were purchased from New England Biolabs (MA, USA). pcDNA3.1 vector and lipofectamine 2000 were purchased from Invitrogen (CA, USA). Site-directed mutagenesis kit was obtained from Stratagene (CA, USA).

Polyclonal antibody production

Imject® Freund's complete adjuvant and modified Lowry assay were purchased from Pierce (Rockford, IL,USA). Copper staining was obtained from Bio-Rad (Hercules, CA, USA).

Chromatographic supports

ImmunoPure Plus immobilized protein A and cyanogen bromide-activated Sepharose 4B were obtained from Pierce (Rockford, IL, USA).

Western blot

A horse-radish peroxidase- conjugated anti-rabbit secondary antibody and chemiluminescence substrates were obtained from Pierce (Rockford, IL, USA).

Electrophoresis reagents and chemicals

Agarose, Glycerol, SDS, coomassie and bromophenol blue were obtained from Sigma (Steinheim, Germany). Protein marker (10-200 KDa) and DNA marker were obtained from Fermentas (Vilnius, Lithuania).

Two Dimensional gel electrophoresis (2DE)

NaCl, KH_2PO_4, EDTA and KBr were obtained from Merck (Darmstadt, Germany). Iodoacetamide, DTT, Triton-X, SDS, CHAPS, trizma base, glycine, TFA, methanol and BSA were acquired from Sigma (Steinheim, Germany). TEMED, 40% acrylamide solution, 2% bis-acrylamide solution and ammonium persulphate were from Bio-Rad (Hercules, CA, USA). Urea and sucrose were from Fluka (Buchs, Switzerland). IPG buffer pH 3–10 NL, IPG's 3–10 NL and DryStrip cover fluid were purchased from Amersham Biosciences (Uppsala, Sweden). Ultrafree centrifugal devices and C18 ZipTip were from Millipore (Bedford, MA). Sequencing-grade modified trypsin was purchased from Promega (Madison, WI). The calibration mixture for peptide mass fingerprinting; des-Argbradykinin, angiotensin I, Glu 1-fibrinopeptide B, neurotensin, adrenocorticotropic hormone (ACTH) clip 1–17, ACTH clip 18–39, and ACTH clip 7–38 with masses: 904.468, 1296.6853, 1570.6774, 1672.9175, 2093.0867, 2465.1989, and 3657.9294, respectively, was purchased from Applied Biosystems (Foster City, CA, USA).

Mass Spectrometry

Acetonitrile (ACN), α-cyano-4-hydroxy-cinnamic acid (HCCA) and trifluor acetic acid (TFA) were purchased from Merck (Damstadt, Germany).

2. Analytical instruments

The following equipments were used in this study: Mastercycler personal PCR (Eppendorf, Germany), Shaking incubator (Daiki Science, Japan), Ready gel ® system (Bio-Rad, U.S.A.), Gel Doc (Bio-Rad, U.S.A.), Smart Spec™3000 (Bio-Rad, U.S.A.), SpeedVac system AES1010 (Savant Instrument, NY), Centrifuge primoR (N.Y.R. ,Thailand), Ettan™ IPGphor II™ (Pharmacia, U.S.A.), Hoefer™ SE 600 Ruby (Pharmacia, U.S.A), Wide mini-sub cell GT (Bio-Rad, U.S.A.), PowerPac™ Basic Power supply (Bio-Rad, U.S.A.), TOF MS model reflex V (Bruker Daltonik GmbH), COBAS, Integra 400 plus automated system (Roche, U.S.A.), Wallac 1420 fluorescence spectrometer (Perkin Elmer , Finland), W-385 ultrasonic liquid sonicator (Ultrasonics Inc., New York, USA) Packard liquid scintillation counter (Packard Instrument, USA), Model 422 Electro-Eluter (Bio-Rad, USA), 112 UV/vis detector (Gilson,USA), N2 reporter (Gilson, USA) and homogenizer.

Methods

1. Screening of HALP subjects

Subjects

Ambulatory subjects with HDL levels ≥100 mg/dL on more than one occasion were recruited from the outpatient clinic of King Chulalongkorn Memorial Hospital. We chose a cutoff level of 100 mg/dL to represent extremely high levels of HDL or HALP as this level approximated 5 standard deviations of mean HDL in our population (50). Secondary causes of HALP, such as long-term use of alcohol, cirrhosis, thyrotoxicosis, nephrotic syndrome, hemodialysis, emphysema, and certain drugs (steroid, insulin, estrogen, fibrate, statin, nicotinic acid, and phenytoin) (4) were excluded in all subjects. A total of thirty eight subjects were included in the HALP group and an equal number of age-matched controls were also recruited. A medical history was obtained and physical examination was performed in all subjects. Informed consent was obtained from each

subject and the study protocol was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Thailand.

Blood samples

Venous blood samples were collected after a 12-hour overnight fast. Postheparin plasma samples were also obtained 15 minutes after intravenous injection of heparin (100 IU/kg body weight). Samples were immediately centrifuged at 3500 g for 10 minutes, and plasma was frozen at –80 C until analyses.

2. Biochemical measurements

Total cholesterol, triglyceride, HDL cholesterol and apoA-I concentrations were determined using enzymatic methods in an automated system **(**COBAS, Integra 400 plus, Roche**)**. LDL-C concentrations were determined by Friedewald's formula**.**

3. CETP activity

CETP activity was determined using a CETP activity kit according to the manufacturer's instructions. Briefly, for each sample assayed, 10 μL of plasma was diluted 1:10 in 90 μL of sample buffer (10 mmol/L Tris, 150 mmol/L NaCl, and 2 mmol/L EDTA, pH 7.4). In a fluorescence-compatible microtiter plate, 20 μL of the diluted plasma was combined with 4 μL of donor and 4 μL of acceptor in a total volume of 200 μL and incubated for 3 hours at 37°C. The assay was read in a fluorescence spectrometer at excitation wavelength of 465 nm and emission wavelength of 535 nm. A standard curve was generated, according to the manufacturer's guidelines, to derive the relationship between fluorescence intensity and mass transfer. Control plasma samples were also run in each plate to account for plate-to-plate variation, and assays were performed in duplicate**.**

4. HL activity

Total lipase, HL, and LPL activities were performed as previously described (51). Briefly, 50 μL of plasma was incubated in duplicate in a total volume of 0.1 mL of the Tris-NaCl buffer containing 0.15 M sodium chloride and heparin 1.5 U (ionic strength

0.15) for 10 min at 27°C. Duplicate samples were also incubated in the same buffer, containing 3 mg protamine sulfate/0.1 mL. After this incubation, 0.9 mL of substrate medium was added. The substrate was prepared within 30 min of use by sonification of the following components: 5 μCi of glyceryl-tri (1-^{14}C) oleate; 113 μmol (100 mg) of triolein; 200 mg of fatty acid-free albumin, and 0.6 mL of 1% (v/v) aqueous Triton X-100 in a total volume of 12 mL of the Tris-NaCl buffer. The sonification procedure was performed in a standardized manner using a W-385 ultrasonic liquid sonicator. The flat tip of the sonicator was centered and inserted below the surface of the assay mixture, contained in a standard 20 mL liquid scintillation vial suspended in a beaker of ice. Sonification was carried out for 1 min at setting 5 (60W) and, after a 15 sec pause, for an additional 30 sec. During the last 5 sec, the output was increased to setting 7.

Incubation was routinely carried out for 60 min at 27°C. For a single assay, 0.1 mL of enzyme extract was added to 0.9 mL of the above mixture and incubated for 1 hr at 37°C. The reaction was terminated by addition of 4 mL of 40:1 isopropanol:3M H_2SO_4. For the extraction of the lipid, 2 mL of H_2O and 5 mL of hexane were added, and the tube was shaken end-to-end on a mechanical shaker for 1 min. Twenty minutes later, a 5 mL aliquot of the hexane phase was added to 1 mL of 0.1 M KOH. On shaking this latter mixture for 10 min, the free fatty acids were extracted into the alkali. The volume of the lower phase was recorded since this phase increased in volume after shaking. One mL of the KOH phase was dissolved slowly with 5 mL of Ultima-gold, and the radioactivity was assayed in a Packard liquid scintillation counter.

5. DNA sequence analysis of *CETP* and *LIPC* genes

Genetic analyses of the *CETP* and *LIPC* genes

Because CETP and HL activities vary from assay to assay and there are no "normal" levels of CETP and HL, we chose the levels below 2 standard deviations of the mean of those in the control groups to represent low activities. Genomic DNA of the HALP subjects with low CETP activity and low HL activity (n = 7 and 3, respectively) were isolated from the whole blood by phenol-chloroform extraction. Each exon of the *CETP* and *LIPC* genes was individually amplified by polymerase chain reaction (PCR). The primers are shown in Table 1.

The primers were designed according to the database of nucleotide sequences of the human *CETP* and *LIPC* genes using bioEdit software and %GC theory. BLAST searcher were performed using the database through the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST) for testing specificity of the primers. In general, PCR was carried out using 0.1 µg of genomic DNA in a buffer containing 10 mmol/L Tris (pH 8.3), 50 mmol/L KCl, 0.2 mmol/L d NTP, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L of each primer, and 0.5 U *Taq* DNA polymerase. PCR conditions were denaturation at 95 C for 5 min, followed by 35 cycles of denaturation at 95 C for 30 sec, annealing at 56 C to 65 C for 30 sec, and extension at 72 C for 1 min, with final extension at 72 C for 10 min. The abundance and quality of DNA fragments were analyzed by electrophoresis on 1% agarose gels containing ethidium bromide and inspected under UV light. PCR products were purified with the ExoSAP-IT kit and sent to Macrogen (Seoul, Korea) for sequencing**.** The standard mutation nomenclature was used according to the Human Genome Variation Society (52).

TABLE 1 Primers used for *CETP* and *LIPC* gene amplification.

Sequence name	Sequence 5’ to 3’
CETP E1-2F	GGA AGG AGG TGA ATC TCT GG
CETP E1-2R	TGC TGC CTT TCC TCC CTG TG
CETP E3-5F	TCT TCC ACC CTC GCC TAG AC
CETP E3-5R	ACC CAC AAG CAT GCC CTG TG
CETP E6-8F	AGA GCC ATG AAC GGT GCC TG
CETP E6-8R	ATC AGG AAT GAG GAG GAG CG
CETP E9F	CTG CAC TCT GGG CTG AAT GC
CETP E9R	TCG TCC TGC TAC ATC TCA GC
CETP E10F	AGA CTT GTG CGA GGT CAC AC
CETP E10R	AGT AGG AGC TGT GTT CGC TG
CETP E11F	GAG AGA GGA GTT CAG GGT AG
CETP E11R	GAG GTG GTG AGA AGG ATC TG
CETP E12-14F	CAT CCT TGC CTC TCC AGT CC
CETP E12-14R	GAA GCT CTG CCT GGG AAG GG
CETP E15-16F	CTG CCG CCA GCG AAA CTC TG
CETP E15-16R	CCG TAC TCC TAA CCC AAC TT
LIPC E1F	GGC AGT AAA GAA AGG GTC TG
LIPC E1R	ACT CTG TCC CAT ACA GTC AG
LIPC E2F	GGC TTG TGC TTG TAG AAG CA
LIPC E2R	TTT TAT GAA CAT GAA CAC GC
LIPC E3F	GGA GCT GGA GAA GGA AGA AG
LIPC E3R	ACT CTC AGA GGA AGG GAA AG
LIPC E4F	AGG GCA CGA AGA ACA GGG TG
LIPC E4R	GGA GTG AGA TCA GTG TGT GAG
LIPC E5F	GCA CCA TGA ACT ACT GTG GT
LIPC E5R	CCG AGC TCG AAT TCC AGA GG
LIPC E6F	GAA CCA AGT GAT CCT CTG AG
LIPC E6R	TTT GGC CAG GGG ACT GCA TC
LIPC E7F	CCA AAC TCT TCC CTC TGT GC
LIPC E7R	CAC CTA GGG GGC TAC ACC TC
LIPC E8F	GCT GTT ACG ACT AAA CTG AT
LIPC E8R	TGA GTA TTA AAT GTG AGA CT
LIPC E9F	GCT CCA CCT AAA ACT TAA TG
LIPC E9R	CAA CAG ATC TAA AAT GGC TC

Mutation screening using PCR-restriction fragment length polymorphism (RFLP) analysis

Two known mutations of the *CETP* gene (D442G and I405V) and a novel deletion mutation (c.734_737delTCCC) were analyzed using PCR-RFLP. The PCR amplification was performed with primers; 5'-CTG CCG CCA GCG AAA CTC TG-3' (sense) and 5'-CCG TAC TCC TAA CCC AAC TT-3' (antisense) for a D442G mutation; 5'-GGG GTT TAG GCA GAA CAG-3' (sense) and 5'- CAT GAC CTC AGG GAT GCC CAC AGC GGT GAT CAT TGA CTG CAG GAA GCT CTG TA -3'(antisense) for a I405V mutation, and 5'-CTG CAC TCT GGG CTG AAT GC -3' (sense) and 5'-TCG TCC TGC TAC ATC TCA GC -3' (antisense) for a c.734_737delTCCC mutation. The amplified PCR products were digested at 37 C overnight with the restriction enzymes *Bgl* I, *Rsa* I, and *NLa* IV for D442G, I405V, and c.734_737delTCCC mutations, respectively. After digestion, the reaction mixture was electrophoresed on 2% agarose gels and DNA fragments were visualized under UV light.

Two known mutations of the *LIPC* gene (V73M and L334F) and a novel missense mutation (c.421G>A or G119S) were analyzed using PCR-RFLP. Exon 3 of the *LIPC* gene was amplified using a pair of primers: 5'-GGA GCT GGA GAA GGA AGA AG -3' (sense) and 5'-ACT CTC AGA GGA AGG GAA AG -3' (antisense). The products were digested with *Pae* I and *BseL* I for detection of V73M and G119S mutations, respectively. For the L334F, exon 7 was amplified using a pair of primers: 5'-TAA ATT TAA AAT CAC TGC TT-3' (sense) and 5' CAC CTA GGG GGC TAC ACC TC-3' (antisense), and the products were digested with *Mse* I. The procedures were also performed in 50 healthy subjects.

6. Pathogenic confirmation of the missense mutation (G119S) of the *LIPC* gene

Construction of expression plasmids

The full length HL cDNA was obtained by PCR amplification of the human liver total RNA using Phusion DNA polymerase. *Eco*R I and *Xho* I restriction sites were added to the primer F: 5' AGC CTG AAT TCC GGG TGA AAC GCC ACC ATG GAC ACA AGT CCC CTG TG 3'and the primer R: 5' TTC ATT CTC GAT CAT CTG ATC TTT

CGC TTT GAT GTT TTA GAC 3'. The PCR product was subcloned into a pcDNA3.1 expression vector. The G119S mutant form was constructed using a site-directed mutagenesis kit according to the manufacturer's instructions. Briefly, *Pfu Turbo* DNA polymerase was used to amplify 50 ng of template DNA (pcDNA3.1-wild type) with mutant sense primer: 5' CAC CCG CCT TGT GAG CAA GGA GGT CGC 3' and mutant antisense primer: 5' GCG ACC TCC TTG CTC ACA AGG CGG GTG 3'. This reaction involved 30 sec of denaturation at 95 C and 15 cycles consisting of 30 sec of denaturation at 95 C, 1 min of annealing at 55 C, and 5 min of extension at 68 C. After digestion of the nonmutated parental DNA template with *Dpn*I, the mutant form of expression vectors was transformed to XL1-blue cells. The correct sequence was confirmed by DNA sequencing.

Expression of the wild-type and mutant HL cDNAs in Vero cells

Wild-type and mutant HL cDNAs were inserted into the expression vector pcDNA3.1. VERO cells, maintained in Dulbecco's modified Eagle's media supplemented with 10% fetal bovine serum, were seeded 1 day prior to transfection at a confluence of 1.3 x 10^6 cells/6-cm petri dish. The cells were transfected with 12 µg of the pcDNA3.1 plasmid DNA constructs using lipofectamine™ 2000. For HL activity, culture media containing heparin (20 U/mL) and cells were collected at 48 h after transfection. Cells were washed in PBS, solubilized in 1 mL of 50 mM NH_3/NH_4Cl (pH 8.1) containing heparin, and sonicated. Media and cell lysates were stored at -70 C until assayed for HL activity. Experiments were performed in triplicate.

pcDNA.3.1/*lac*Z was used as a positive control. After transfection, cells were fixed with 2% formaldehyde and 0.2% glutaraldehyde in 1X PBS for 10 min at room temperature. Plates were rinsed twice with 2.5 mL 1X PBS. Staining solution that contains X-gal was added to the plate and incubated at 37°C. Cells were checked under a microscope for the development of blue color. The number of total cells and blue cells in 3 random fields of view was counted and the average was used to estimate transfection efficiency. Experiments were performed in triplicate.

Bioinformatic studies

Both PolyPhen (http://genetics.bwh.harvard.edu/pph/) and PANTHER (www.pantherdb.org) programs were used to determine dysfunction of the novel missense mutation. In the PANTHER program, substitution position-specific evolutionary conservation (subPSEC) score below -3, which corresponds to a $P_{deleterious}$ of 0.5, has previously been identified as a cutoff point for functional significance (53).

7. Polyclonal antibody production

Isolation of apoA-I

HDL was isolated from pooled human sera or plasma by sequential ultracentrifugation, extensively dialyzed in 5 mM Tris-HCl and 1 mM EDTA, pH 7.4, and concentrated as previously described (54). HDL was electrophoresed in polyacrylamide gels, apoA-I bands identified by copper staining were cut, and proteins were eluted using an electroeluter. Purified human apoA-I was then used to produce antiserum in rabbits.

Production of polyclonal antibodies against human apoA-I

Antibodies against human apoA-I were raised in two New Zealand white rabbits. Before immunization, 1.5 mL of blood was collected pre-immunized serum by bleeding from the marginal vein of the ear. 0.5 mg of human apoA-I mixed with an equal volume of Freund's complete adjuvant was then injected intradermally on the back and proximal limbs of the rabbit (10-ll per site). Immunization was repeated on the 30th day and every 2 weeks afterward. On the 30th day, 0.5 mL of blood was collected and an aliquot was used for checking the titer of antiserum by immunodiffusion assay. The antiserum was harvested.

Radial immunodiffusion assay (RID)

The titer of anti serum against human apoA-I was determined by radial immunodiffusion assay. The 10 × 10 cm slides were filled with agarose gel and the holes were created. 5 μL of rabbit's antiserum diluted to 1:2, 1:4, 1:6, 1:8 and 1:32 were added in outside holes while 5 μL of human apoA-I (200 μg/mL) was added in a central hole. The precipitin rings between outside and inside holes were visualized if there was an appropriate ratio between antiserum and human apoA-I.

Purification of rabbit antihuman apoA-I antibodies

Rabbit antibodies were initially isolated from antiserum using ImmunoPure protein A (cell wall component of *Staphylococcus aureus* which binds specifically to the Fc region of immunoglobulin molecules, especially IgG from rabbit) columns, and a subpopulation of low-affinity antibodies against human apoA-I was further purified using a human HDL protein-Sepharose column. These selected anti human apoA-I antibodies were eluted with 0.2 M acetic acid and 0.15 M NaCl (pH 3.0). The eluate was immediately neutralized to pH 7.4 using 2 M Tris and concentrated using Ultrafree centrifugation devices.

8. Construction of columns for purifying HDL by selected-affinity immunosorption.

The rabbit anti apoA-I antibody was used to construct anti human apoA-I columns by cross-linking to cyanogen bromide-activated Sepharose 4B at 102 mg of Ig per 3 g of gel powder according to the manufacturer's instructions.

9. Isolation of column-purified HDL using human apoA-I immunoaffinity columns.

Human serum was applied to the human apoA-I column and washed with 0.01 M Tris, 0.15 NaCl, 0.04% EDTA, and 0.05% NaN_3 (pH 7.3) at 4°C until there was no detectable absorption at 280 nm. Human apoA-I-containing HDL was eluted with 0.2 M acetic acid and 0.15 M NaCl (pH 3.0) and immediately neutralized with 2 M Tris to pH 7.4 and concentrated using Ultrafree centrifugation devices.

10. Assessment of the purity of HDL

SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out on a 12% separating gel with 4% stacking gel. The protein marker, eluted HDL and flow through were dissolved in sample buffer and heated for 5 min before loading. A constant 150 voltage was set for running the gel electrophoresis until the bromophenol blue dye reached the bottom of the separating gel. Then, the gel was removed and protein bands were visualized by Coomassie blue staining.

Coomassie staining

With Coomassie brilliant blue staining (Coomassie R-250), proteins in the gel were fixed for 1 h in 50% methanol/7% acetic acid, stained for 1 h in 0.1% Coomassie R-250 in 50% methanol/ 1% acetic acid and destained overnight in 30% methanol/1% acetic acid.

11. Western blot analysis

To determine the specificity of the apoA-I polyclonal antibody, eluted HDL were separated by SDS–PAGE and transferred electrophoretically onto a PVDF membrane following a standard protocol. The membrane was incubated overnight at 4^0C with the anti-apoA-I polyclonal antibody (1:100). A horse-radish peroxidase- conjugated anti-rabbit secondary antibody and chemiluminescence substrates were used to determine the immuno-labeled bands by exposure to the X-ray film.

12. Determination of the protein concentration of HDL

Protein concentrations were determined using a Modified Lowry Protein assay according to the manufacturer's protocol. In brief, 100 µL of the protein solution and each standard were added to the labeled test tubes. At 15 -second intervals, 500 µL of Modified Lowry reagent was added to each test tube, mixed, and incubated at room temperature (RT) for exactly 10 minutes. At the end of each tube's 10-minute incubation period, 100 µL of prepared 1X Folin-Ciocalteu reagent was added, immediately vortexed, and incubated at RT for 30 minutes. The color was measured at 750 nm by using a Smart Spec™3000 spectrophotometer. The protein content was estimated from a standard calibration curve using 50-500 µg/mL of bovine serum albumin (BSA) as the standard.

13. 2DE

Samples containing 250 µg proteins each were applied in pH 3–10 NL IPGs by in-gel rehydration for 14 h using low voltage (30 V). The proteins were then focused at 28 000 Vh at maximum voltage of 8000 V. IPGs were either used immediately for second dimension analysis or stored at -80^0C. The second dimension (SDS-PAGE) was

performed by transferring the proteins to a homogeneous 12.5 % gel and run at 20–40 mA for about 5 h.

14. Staining

Proteins in the 2D gels were detected by staining with 0.1% colloidal Coomassie brilliant blue. Proteins were fixed for 1 h in 40% ethanol/10% acetic acid, stained for 5 days in 0.1% colloidal Coomassie R-250 in 50% methanol/ 1% acetic acid and destained overnight in ddH_2O. Spots were then placed in deionized water prior to MS analysis.

15. Quantitative Analysis of Protein Expression

Imagemaster 2D software was used for the matching and analysis of protein spots. The principles of measuring intensity values by 2-D analysis software were similar to those of densitometric measurement. The average mode of background subtraction was used to normalize intensity value, which represents the relative amount of protein. After completion of the spot matching, the normalized intensity values of individual protein spots from each subject were then compared between groups using statistical analysis. Spot volume was compared between control and HALP groups, and $p < 0.05$ were considered statistically significant. Statistical significance was evaluated by use of the Student's *t*-test for comparison of unpaired data, and Pearson's correlation for the relationship between the two groups.

16. Tryptic digestion

All spots that we found were digested with trypsin as described in detail previously (55). The protein spots were excised from the gel with a pipette tip and transferred to small Eppendorf tubes (0.5 mL). The gel pieces were washed twice with 50% acetonitrile/25 mM ammonium bicarbonate, with 100% acetonitrile once and dried in a SpeedVac vacuum concentration system. About 25 µl of trypsin (20 mg/mL in 25 mM ammonium bicarbonate) was added to the gel pieces and the samples were incubated overnight at 37^0C. The supernatant was transferred to a separate tube and the peptides were further extracted from the gel pieces by incubation in 50% acetonitrile/5% TFA for 5 h at room temperature. The supernatants from the two steps were then pooled and dried

in the SpeedVac until complete dryness. If not dissolved in 5 mL 0.1% TFA for further MS preparation, the peptides were stored at -80^{0}C.

17. Mass spectrometry

10 mg solution of HCCA in TA (ACN: 0.1%TFA, 1:2) was produced and mixed. Excess matrix was eliminated by centrifugation (1 min at 14000 rpm), and the clear supernatant was used.Eequal volume of tryptic peptides was mixed with HCCA and 1 µL was applied onto the target. The mass spectra were recorded on a reflector Bruker reflex V delayed extraction MALDI-TOF mass spectrometer equipped with a 2GHz LeCroy digitizer and 337 nm N_2 laser. Instrumental parameters were: positive polarity, acceleration voltage 20kV; IS /2 17 kV; focusing lens voltage 8.90 kV; extraction delay 400 ns. Typically 100 shots were accumulated from three to five different positions within a sample spot. Protein identifications was obtained using MASCOT (MatrixScience) and by searching for matching peptide mass fingerprints in a protein database. The search criteria used were fixed modification (carboxamidomethylation of cysteine), variable modification (methionine oxidation) and considered the accuracy of the experimental to theoretical pI and molecular weight. Protein scores are significant when p value is smaller than 0.05 (P value is the probability that the observed match is a random).

18. Statistical analysis for enzyme activities

Statistical analysis was performed using SPSS (version 12, Chicago, IL, USA). The results are presented as mean ± SEM. Statistical significance was evaluated by use of the Student's *t*-test for comparison of unpaired data, the Chi square test for frequency, and Pearson's correlation for the relationship between the two groups. One-way ANOVA with posthoc analyses was used to compare data among multiple groups. *P* value < 0.05 was considered statistically significant.

CHAPTER IV

RESULTS

Clinical characteristics

Clinical characteristics of the HALP and the control groups are shown in Table 2. Most of the HALP subjects were postmenopausal women. One case in the HALP group had a history of cerebrovascular accident and one case in the control group had history of transient ischemic attack. Body weight, waist circumference, and hip circumference of the HALP group were significantly lower than those of the control group, whereas body mass index and waist:hip ratio were not significantly different. Presence of corneal arcus was not significantly different between the two groups. Plasma concentrations of total cholesterol, HDL cholesterol, and apoA-I in the HALP group were significantly higher than those in the control group (Table 2). In contrast, plasma triglyceride and LDL cholesterol concentrations in the HALP group were significantly lower than those in the control group (Table 2).

We found a significant inverse correlation between plasma HDL cholesterol and triglyceride concentrations ($r = -0.64$, $P < 0.001$). HDL cholesterol concentration was also negatively correlated with body weight ($r = -0.32$, $P = 0.005$), waist circumference ($r = -0.34$, $P = 0.002$), hip circumference ($r = -0.30$, $P = 0.009$), and body mass index ($r = -0.29$, $P = 0.01$).

TABLE 2. Clinical characteristics of subjects in the HALP group and control subjects

Baseline characteristics	Control (n=38)	HALP (n=38)	*P* value
mean age (yr)	56 ± 2	57 ± 2	0.93
male:female (n)	1:37	2:36	0.56
years after menopause	10.2 ± 1.4	9.3 ± 1.8	0.68
SBP/DBP (mmHg)	125 ± 4 / 71 ± 2	123 ± 3 / 73 ± 2	0.81 / 0.37
body weight (kg)	57 ± 2	52 ± 2	0.05
body mass index (kg/m^2)	23 ± 1	22 ± 1	0.09
waist circumference (inch)	30 ± 1	28 ± 1	0.02
hip circumference (inch)	37 ± 1	35 ± 1	0.05
waist: hip ratio	0.82 ± 0.01	0.80 ± 0.01	0.12
Presence of corneal arcus (%)	6/38 (16%)	11/38 (29%)	0.27
total cholesterol (mg/dL)	237 ± 6	258 ± 7	0.03
HDL cholesterol (mg/dL)	65 ± 3	119 ± 2	<0.001
triglyceride (mg/dL)	136 ± 11	72 ± 6	<0.001
LDL cholesterol (mg/dL)	146 ± 6	126 ± 7	0.04
apoA-I (mg/dL)	179 ± 4	215 ± 7	<0.001

HALP, hyperalphalipoproteinemia; SBP, systolic blood pressure; DBP, diastolic blood pressure; values are means ± SEM

Plasma CETP activity

We determined plasma CETP activity in both groups. As shown in Fig. 4, we found that the mean CETP activity in the HALP group was significantly lower than that of the control group (34.1 ± 3.9 *vs.* 43.7 ± 2.6 pmol/μL/hr, $P = 0.04$). A significant inverse correlation between plasma HDL cholesterol concentration and CETP activity was observed ($r = -0.28$, $P = 0.01$). In addition, there was a significant correlation between LDL cholesterol concentration and CETP activity ($r = 0.33$, $P = 0.004$). We did not find a significant correlation between plasma CETP activity and any of the anthropometric parameters.

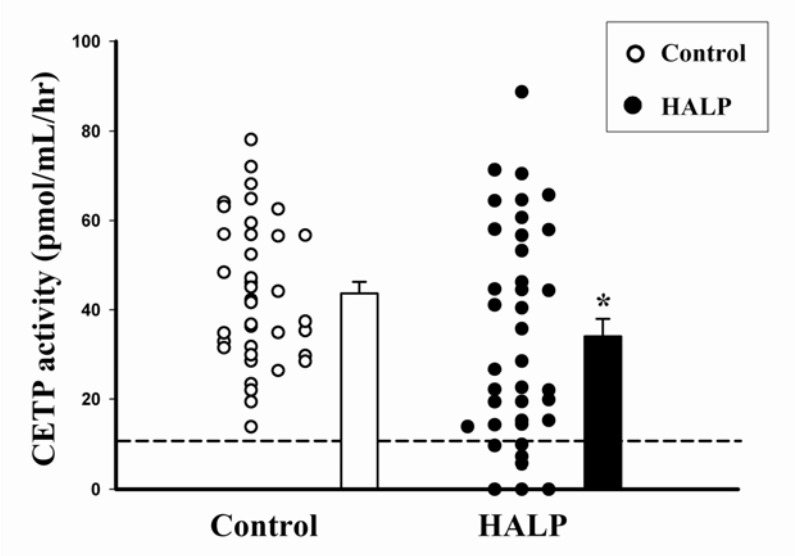

Fig. 3: CETP activity in the control group and the HALP group. *: $P = 0.04$
The dashed line indicates a cutoff point of plasma CETP activity less than 11.7 pmol/μL/hr.

In our study, a cutoff point for plasma CETP activity less than 11.7 pmol/μL/hr, which corresponded to 2 standard deviations below the mean of the CETP activity of the control group, was chosen to indicate an abnormally low CETP. The characteristics of HALP subjects who had normal CETP level and who had CETP deficiency are shown in table 3. We found that mean age, years after menopause and presence of corneal arcus in CETP deficiency group were significantly higher than that of the normal CETP level group. In addition, there was a significantly lower ApoA-I level in the CETP deficiency group.

TABLE 3. Clinical characteristics of subjects in the HALP group who have CETP deficiency and normal CETP activity

Baseline characteristics	Normal CETP activity (n=31)	CETP def. (n=7)	*P* value
CETP activity(pmol/mL/hr)	40.7 ± 3.8	0 ± 4.3	<0.001
mean age (yr)	54 ± 2	68 ± 4	0.009
years after menopause	6.7 ± 1.7	20.6 ± 4.1	0.002
SBP/DBP (mmHg)	121 ± 3 / 73 ± 2	134 ± 10 / 73 ± 3	0.1/0.9
body weight (kg)	52 ± 2	50 ± 5	0.70
body mass index (kg/m^2)	22 ± 1	22 ± 1	0.98
waist circumference (inch)	28 ± 1	29 ± 1	0.59
hip circumference (inch)	35 ± 1	35 ± 1	0.79
waist: hip ratio	0.80 ± 0.1	0.80 ± 0.02	0.30
Presence of corneal arcus (%)	6/31 (19%)	5/7 (71%)	0.005
total cholesterol (mg/dL)	262.6 ± 9	237.8 ± 10	0.20
HDL cholesterol (mg/dL)	118 ± 3	123 ± 6	0.42
triglyceride (mg/dL)	74 ± 7	67 ± 5	0.64
LDL cholesterol (mg/dL)	131 ± 9	102 ± 10	0.13
apoA-I (mg/dL)	221 ± 6	188 ± 22	0.04

HALP, hyperalphalipoproteinemia; SBP, systolic blood pressure; DBP, diastolic blood pressure; values are means ± SEM

Genetic sequence variations of the *CETP* gene

Seven subjects in the HALP group who had very low CETP activity, were chosen for further investigations.

All exons of the *CETP* gene in these seven subjects were sequenced and we found two known genetic variations, D442G and I405V, in two and six subjects, respectively (Table 4). We further analyzed these two variations of the *CETP* gene in the entire group (Table 5). Using PCR-RFLP, the D442G mutation was found in 26% of the HALP group (all of them were heterozygous mutations) but none were found in the control group (P = 0.001). Plasma CETP activity was significantly lower in those who had D442G mutation than those without mutation in the entire group (19.7 ± 5.3 *vs.* 40.5 ± 2.4 pmol/μL/hr, respectively, P = 0.003) or only among 38 subjects with HALP (19.7 ± 5.3 *vs.* 35.8 ± 4.6 pmol/μL/hr, respectively, P = 0.03). However, there were no significant differences in HDL, triglyceride or apoA-I concentrations between those HALP subjects who had D442G mutations and those who did not (data not shown).

In contrast, the I405V variant was found in 71% of the HALP group and 73% in the control group ($P = 0.72$), suggesting that it was not associated with HALP. We found no significant differences in lipoprotein levels or plasma CETP activity among subjects with 405II, 405IV, or 405VV genotypes.

Identification of a novel mutation of the *CETP* gene

In the HALP group, we found a novel heterozygous mutation of the *CETP* gene, c.734_737delTCCC, in one subject (Fig. 5). This mutation occurs in exon 9 and is predicted to result in a premature stop codon 30 amino acids downstream, causing deletion of the C terminus of the protein, which is part of lipid binding site. The proband was a 73-year-old single woman. Beside hypertension, she had no symptoms or signs of cardiovascular diseases. Her HDL cholesterol concentration was 143 mg/dL, and her CETP activity was 7.2 pmol/μL/hr. This novel mutation was found only in this subject but was not found in the rest of the HALP group, the control group, or in 50 healthy subjects.

A further study of the proband's family revealed that her only surviving immediate family member was her 65-year-old brother, who also harbored this mutation. Being a chronic smoker for 44 years with hypertension, he suffered a cerebrovascular accident at age 57 and his HDL cholesterol concentration was 55 mg/dL while on simvastatin 10 mg/d. Unfortunately, his CETP activity was not available.

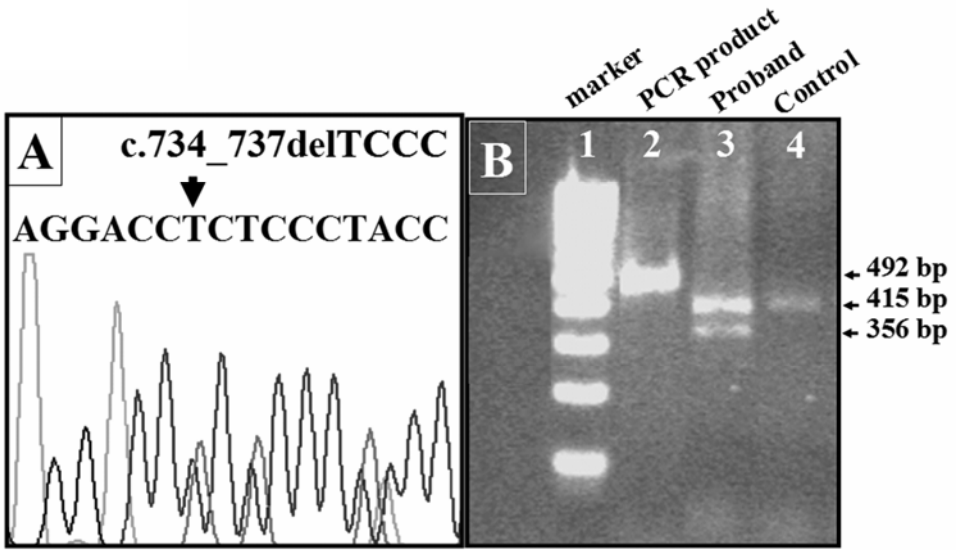

Fig. 4: A: Genomic DNA sequence of exon 9 of the *CETP* gene, showing a novel mutation, c.734_737delTCCC, in one subject in the HALP group. B: PCR-RFLP of the *CETP* mutation. Cleavage of PCR-amplified 492 bp product of exon 9 (lane 2) with *NLa* IV normally results in 415, 51, and 26 bp fragments (lane 4). The presence of the mutant allele is disclosed by appearance of a 356 bp fragment (lane 3). Lane 1 shows molecular size marker.

TABLE 4. *CETP* mutations in subjects in the HALP group who have CETP deficiency

Subject no.	CETP Activity (pmol/mL/hr)	I405V known mutation	D442G known mutation	c.734_737delTCCC novel mutation
1	0	-	√	-
2	0	√	-	-
3	0	√	√	-
4	7.18	√	-	√
5	9.85	√	-	-
6	5.62	√	-	-
7	9.62	√	-	-

TABLE 5. *CETP* mutations in entire group

Mutations	Control(38)	HALP(38)
I405V known mutation	73%	71%
D442G known mutation	0%	26%
c.734_737delTCCC novel mutation	0%	Only one

Total lipase, HL, and LPL activities

Total lipase, HL, and LPL activities were next evaluated in our subjects. We found that postheparin plasma total lipase activity in the HALP group was significantly lower than that in the control group (530 ± 34 *vs.* 645 ± 27 nmol FFA/mL/min, $P = 0.01$). This was primarily due to significantly lower HL activity (150 ± 17 *vs.* 227 ± 17 nmol FFA/mL/min, $P = 0.002$, Fig. 6). LPL activity, however, was not significantly different between the two groups (380 ± 23 *vs.* 418 ± 16 nmol FFA/mL/min, $P = 0.17$).

We found that plasma HL activity was inversely correlated with HDL concentrations ($r = -0.30$, $P = 0.01$). Furthermore, HL activity was correlated with body weight ($r = 0.39$, $P = 0.001$), waist circumference ($r = 0.38$, $P = 0.001$), hip circumference ($r = 0.33$, $P = 0.005$), waist: hip ratio ($r = 0.30$, $P = 0.01$), and body mass index ($r = 0.32$, $P = 0.005$). In contrast, plasma LPL activity was not correlated with any of the lipoprotein concentrations or anthropometric parameters.

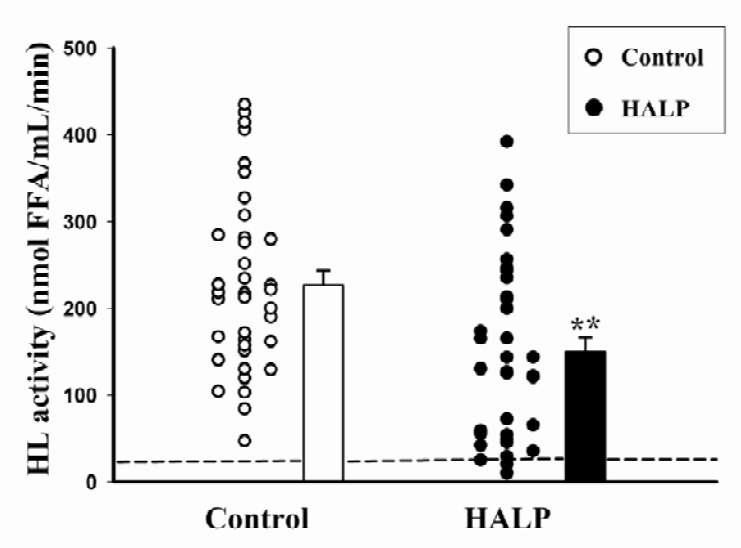

Fig. 5: HL activity in the control group and the HALP group. **: $P = 0.002$
The dashed line indicates a cutoff point of plasma HL activity less than 26.2 nmol FFA/mL/min.

In our current study, a cutoff point for plasma HL activity less than 26.2 nmol FFA/mL/min, which corresponded to 2 standard deviations below the mean of the HL activity in the control group, was chosen to indicate an abnormally low HL activity. The characteristics of HALP subjects who had normal HL activity and who had HL deficiency are shown in table 6. We found that mean age, years after menopause and systolic blood pressure in the CETP deficiency group were significantly higher than those of the group with normal CETP activity.

TABLE 6. Clinical characteristics of subjects in the HALP group who have HL deficiency and normal HL activity

Baseline characteristics	Normal HL activity (n=33)	HL def. (n=3)	*P* value
HL activity(nmol FFA/mL/min)	162.2 ± 17.3	18.6 ± 4.4	<0.001
mean age (yr)	55 ± 2	79 ± 3	0.002
years after menopause	8 ± 2	31 ± 5	<0.001
SBP/DBP (mmHg)	122 ± 3 / 80 ± 10	155 ± 5 / 73 ± 2	0.017/0.35
body weight (kg)	52 ± 2	50 ± 5	0.78
body mass index (kg/m^2)	22 ± 1	22 ± 1	0.93
waist circumference (inch)	30 ± 1	28 ± 2	0.87
hip circumference (inch)	35 ± 1	36 ± 1	0.66
waist: hip ratio	0.80 ± 0.01	0.70 ± 0.03	0.62
Presence of corneal arcus (%)	10/33 (30%)	1/3 (33%)	0.92
total cholesterol (mg/dL)	257 ± 9	270 ± 14	0.65
HDL cholesterol (mg/dL)	118 ± 3	127 ± 8	0.32
triglyceride (mg/dL)	74 ± 6	74 ± 16	0.99
LDL cholesterol (mg/dL)	126 ± 8	129 ± 18	0.92
apoA-I (mg/dL)	216 ± 7	192 ± 35.8	0.34

HALP, hyperalphalipoproteinemia; SBP, systolic blood pressure; DBP, diastolic blood pressure; values are means ± SEM

Genetic sequence variations of the *LIPC* gene

Three subjects in the HALP group had HL activities below 26.2 nmol FFA/mL/min were chosen for further investigations. All exons of the *LIPC* gene in these three subjects were sequenced and we found two known genetic variants, V73M and L334F, and four single nucleotide polymorphisms (SNPs), c.399T>G (NCBI SNP accession number rs690), c.525A>G (NCBI SNP accession number rs6082), c.578A>G (NCBI SNP accession number rs6083), and c.1371C>A (NCBI SNP accession number rs6074).

Using PCR-RFLP, the V73M variant was found in 49% of the HALP group and 57% in the control group (P = 0.35)(Table 7), suggesting that it was not a cause of HALP. In the entire group, we found no significant differences in lipoprotein levels, plasma lipase activities, or anthropometric parameters among subjects with 73VV, 73VM, or 73MM genotypes.

The L334F variant was found in 11% of both the HALP group and the control group (all with heterozygous mutations, P = 1.0)(Table 7), suggesting that it was not a

cause of HALP. We also did not find the difference in HL activity between subjects with or without the L334F mutations.

Identification of a novel mutation of the *LIPC* gene

In the HALP group, we found one subject with a novel heterozygous missense mutation, c.421G>A or G119S, of the *LIPC* gene (Fig. 7). A glycine residue at position 119 of HL is a strictly conserved amino acid across different species and among several related proteins in the lipase superfamily (Fig. 8). It lies proximal to the active site of the enzyme. The proband was a 72-year-old woman. Except for hypertension, she had no evidence of cardiovascular diseases. Her HDL cholesterol was 137 mg/dL, and her HL activity was 25.1 nmol FFA/mL/min. A further study on her family showed that her 54-year-old son also had a similar mutation. His HDL cholesterol concentration was 84 mg/dL. This mutation was not found in the rest of the HALP group, the control group, or 50 healthy subjects (Table 7).

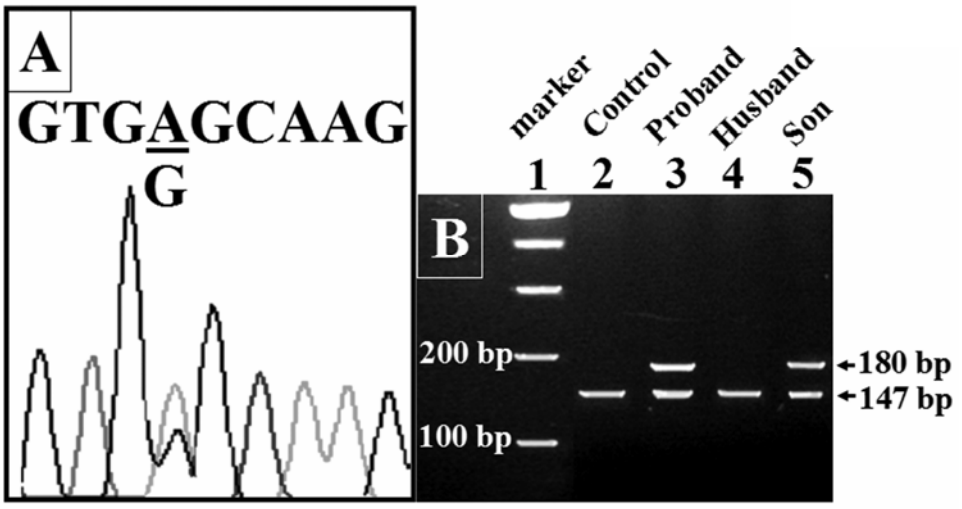

Fig. 6: A: Genomic DNA sequence of exon 3 of the *LIPC* gene, showing a novel missense mutation, G119S, in one subject in the HALP group. B: PCR-RFLP of the *LIPC* mutation. Clevage of PCR-amplified 317 bp product of exon 3 with *BseL* I normally results in 147, 70, 67, and 33 bp fragments (lanes 2 and 4). The presence of the mutant allele is disclosed by appearance of an extra 180 bp fragment (lanes 3 and 5). Lane 1 shows molecular size marker.

TABLE 7. *LIPC* mutations in entire group

Mutations	Control(38)	HALP(38)
V73M known mutation	57%	49%
L334F known mutation	11%	11%
G119S novel mutation	0%	Only one

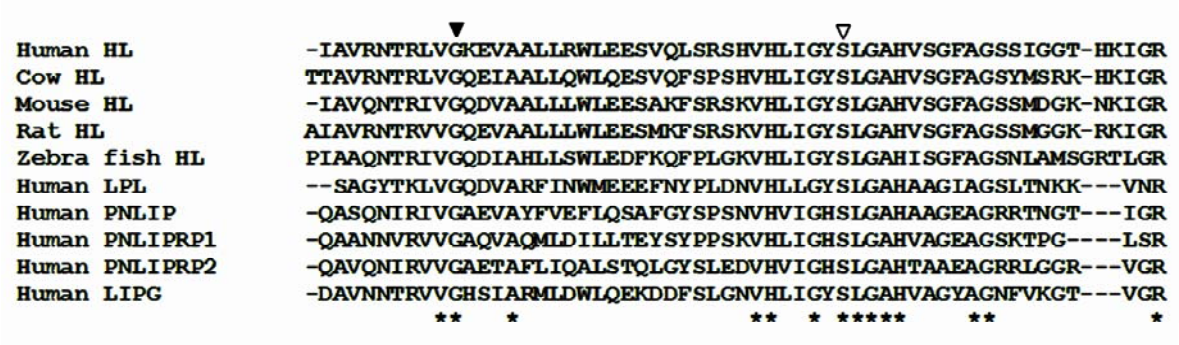

Fig. 7: Amino acid sequence alignment of hepatic lipase (HL) in human, cow, mouse, rat, zebrafish, human lipoprotein lipase (LPL), human pancreatic lipase (PNLIP), human pancreatic lipase-related protein 1 (PNLIPRP1), human pancreatic lipase-related protein 2 (PNLIPRP2), and human endothelial lipase (LIPG). The position of Gly119 is indicated by filled triangle, and the position of Ser146 is indicated by open triangle. Asterisks indicate conserved amino acid residues.

Molecular pathology of the new *LIPC* mutation (G119S)

Because a glycine residue at position 119 of HL is a strictly conserved amino acid across different species and among several related proteins in the lipase superfamily (Figure 8), and it lies proximal to the active site of the enzyme, we speculated that amino acid substitution at this position might be associated with lower HL activity, resulting in high HDL levels. The PolyPhen program predicted that this mutation is probably

damaging. Similarly, the PANTHER program predicted this mutation to be deleterious (subPSEC score of -4.43, and $P_{deleterious}$ of 0.81).

In order to confirm the functional change of this novel G119S mutation of the *LIPC* gene, we expressed this mutant in Vero cells and analyzed HL activities in the media and cell lysates. pcDNA.3.1/*lac*Z was used as a positive control vector and transfection efficiency was 36 %. The results are shown in Table 8 and Fig 9. Cells transfected with wild-type HL cDNA contained significant amount of HL activity both intracellularly and in the culture media. However, HL activity in the lysates of cells transfected with G119S mutant cDNA was 20.4% of that of wild-type HL cDNA (0.38 ± 0.21 *vs*. 1.84 ± 0.48 mU/plate, $P = 0.01$). In addition, HL activity in the media of cells transfected with G119S cDNA was only 6.2% of that wild-type HL cDNA (0.48 ± 0.27 *vs*. 7.65 ± 1.00 mU/plate, $P < 0.001$). These results suggest that the G119S mutation results in the decrease in HL activity.

TABLE 8. HL activity in the cells and media of Vero cells transfected with the wild-type and mutant HL cDNAs

repeats	pcDNA3.1/ Lac Z	Cells			Medium			P1	P2	P3	P4	P5	P6
		pcDNA3.1	HL cDNA	G119S	pcDNA3.1	HL cDNA	G119S						
I	40%	0.17	1.60	0.60	2.33	7.36	0.17						
II	35%	0	2.39	0.33	0.81	6.78	0.58						
III	34%	0	1.52	0.19	0.44	8.80	0.68						
mean	36%	0.06±0.06	1.84±0.28	0.38±0.12	1.19±0.58	7.65±0.60	0.48±0.16	**≤0.001**	**0.01**	**0.55**	**≤0.001**	**≤0.001**	**0.19**

values are means ± SEM

P1 : pc DNA3.1 cells - HL cDNA cells
P2 : HL cDNA cells -G119S cells
P3 : pc DNA3.1 cells - G119S cells
P4 : pc DNA3.1 medium - HL cDNA medium
P5 : HL cDNA medium - G119S medium
P6 : pc DNA3.1 medium - G119S medium

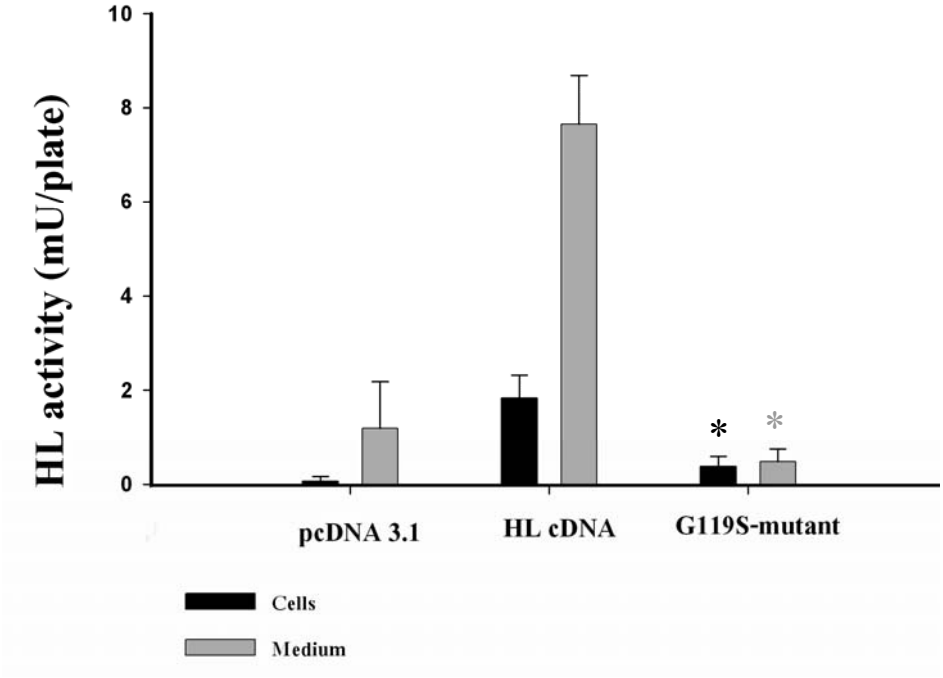

Fig. 8: Expressions of G119S-mutant in the Vero cells. HL activity (mU/plate) was measured in the cell homogenates (dark columns) and in culture medium (gray columns). The means of three measurements are presented. *: *P2*=0.01, *: *P5*<0.001

HDL Proteomics in HALP subjects compare to the control subjects

HDL is heterogeneous lipoprotein particles that contain lipids and a variety of proteins. More than 50 different proteins have been shown to associate with HDL, and each of these proteins plays a distinct role in the function and metabolism of HDL. CETP is an enzyme found on HDL. Mutations of the *CETP* gene lead to lack of CETP protein and activity on HDL. Thus lack of other proteins on HDL might be associated with HALP. In this study, 2 DE and mass spectrometry were used to test this hypothesis.

Because certain HDL-associated proteins can be dissociated from the HDL particles during ultracentrifugation, human apoA-I immunoaffinity columns was constructed and used to isolate HDL from human plasma.

Production of anti-apoA-I polyclonal antibodies

Polyclonal antiserum against apoA-I was obtained from New Zealand white rabbits. Rising of antibody after immunization was checked by immunodiffusion assay (Fig 10A). Total immunoglobulin was purified by protein A column. Then, anti-apoA-I antibodies were purified from total immunoglobulin by HDL protein column and used to construct anti-apoA-I immunoaffinity column.

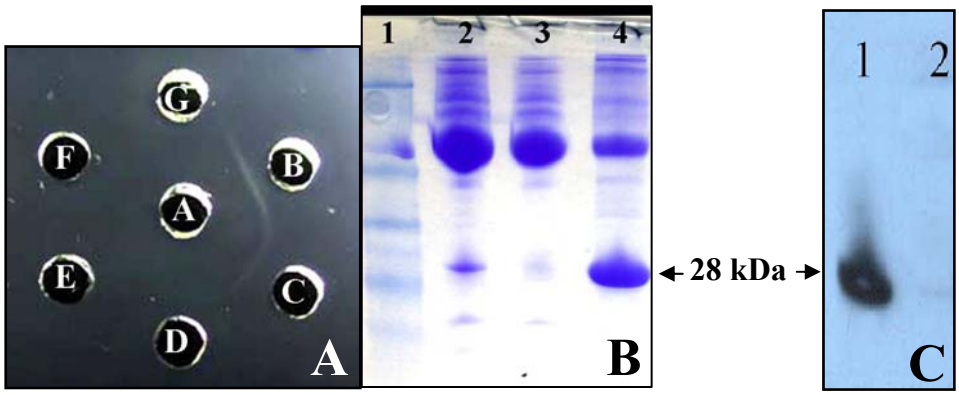

Fig. 9: Immunodiffusion assay (A). A, apoA-I 200 ug/mL; B, undiluted serum; C, diluted serum 1:2; D, diluted serum 1:4; E, diluted serum 1:6; F, diluted serum 1:8; G, diluted serum 1:32. SDS-PAGE (B)**.** 1, Marker; 2, Serum; 3, Flow through; 4, Eluate which shows an apoA-I band (28 kDa). Western blot (C)**.** 1, Eluate; 2, Flow through

The eluted HDL was checked for purity by SDS-PAGE and Western blot (Fig 10B-C). This column was used to purify HDL from sera of patients with HALP (n=7) and control subjects (n=7) who have normal CETP and HL activities.

Changes in HDL-associated proteins in subjects with HALP

To identify changes in proteins on HDL that may be different in HALP subjects, seven subjects from each group were selected as shown in Table 9. Age, CETP activity, HL activity and LPL activity were not significantly different between the two groups. The HDL level in the HALP group was twice higher than that in the control group.

TABLE 9. Characteristics of the selected patients from the HALP group and the control group

characteristics	HALP (n=7)	Control (n=7)	P value
Age	54.5 ± 4.6	55.4 ± 4.0	0.89
HDL (mg/dL)	120.7 ± 7.4	56 ± 4.0	<0.001
CETP activity (pmol/μL/hr)	46.8 ± 7.5	46.8 ± 5.5	0.99
HL activity (nmol FFA/mL/min)	164.2 ± 25.0	161.7 ± 24.4	0.95
LPL activity(nmol FFA/mL/min)	415 ± 38.1	367.2 ± 21.9	0.29

Values are Mean ± SEM

HDL proteins were separated using 2-DE and visualized by colloidal coomassie blue staining (n = 7 in each group). The pattern of protein spots visualized on 2-D gels was reproducible. 22 proteins were identified by MALDI-TOF MS followed by peptide mass fingerprinting. (Fig. 11). However, we found no significant differences in the quantities of each protein on HDL between the HALP group and the control group, as analysed by 2-D analysis software (data not shown).

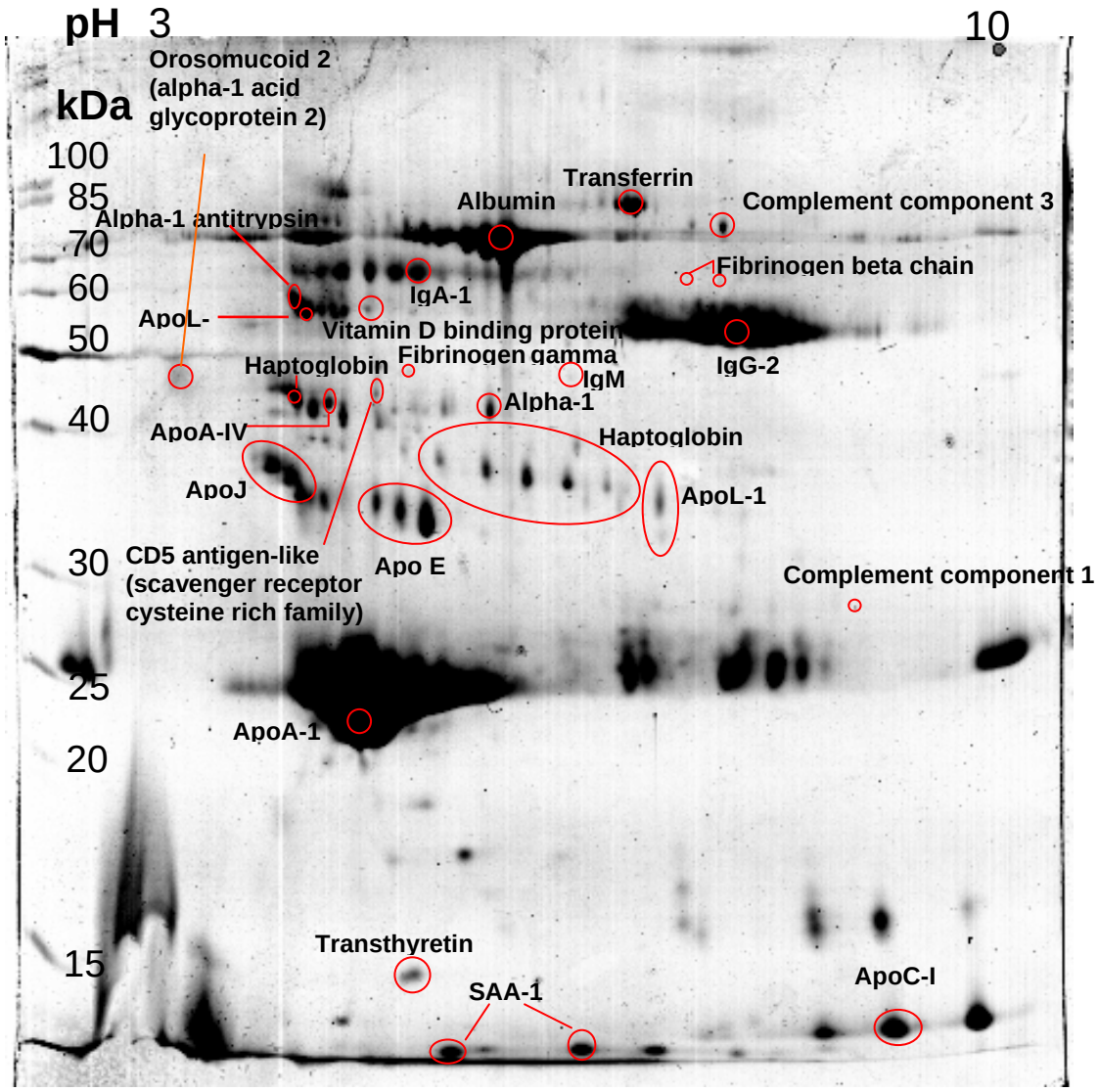

Fig. 10: The representative proteome maps of HDL of a normal control. HDL proteins were separated by 2-D PAGE on the basis of differential isoelectric point (x axis) and molecular weight (y axis). Protein spots were excised and identified by matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry (MS), followed by peptide mass fingerprint.

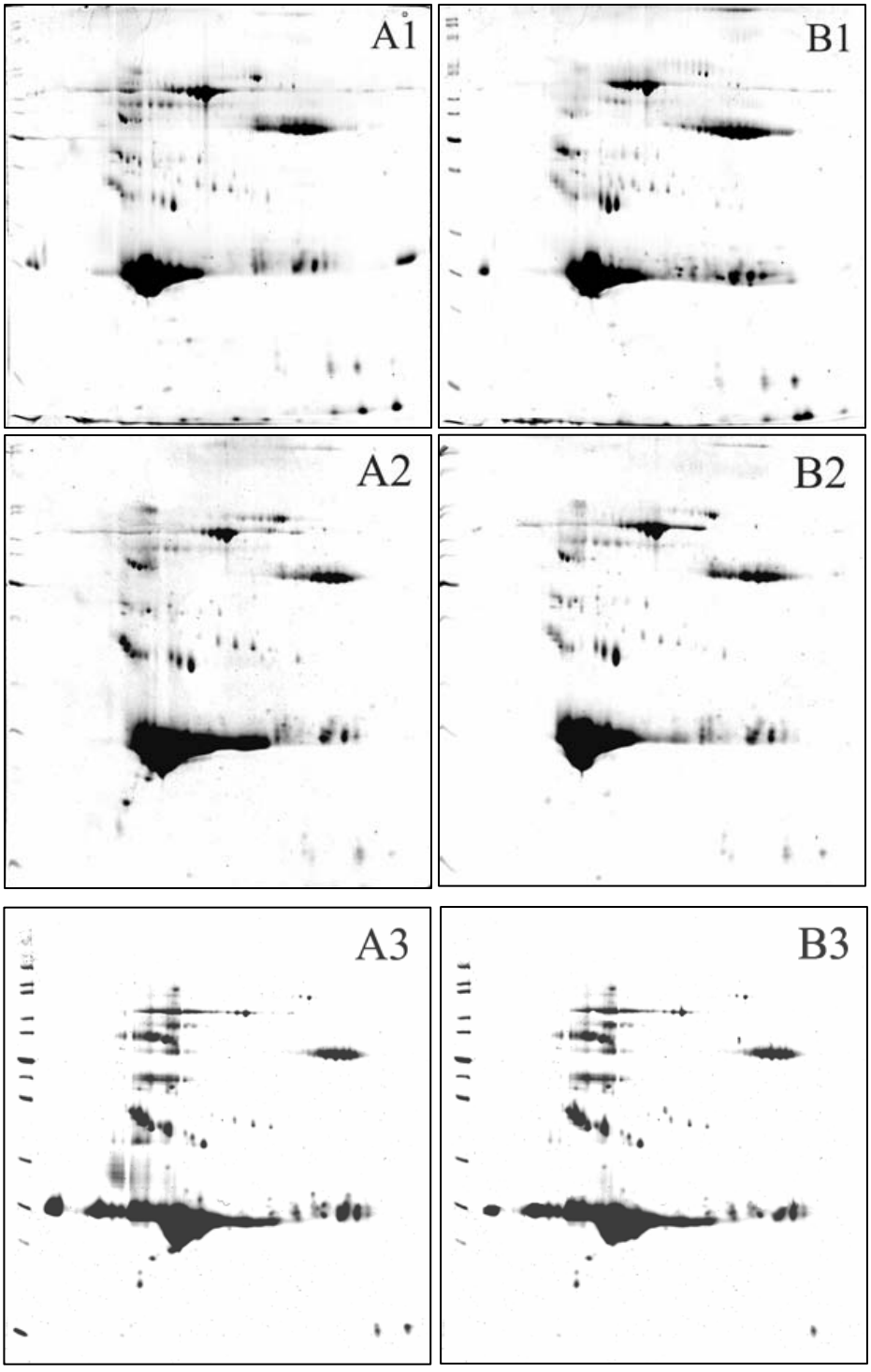

Fig. 11: The individual 2D gels of HDL of normal controls (A1-A7) and HALP subjects (B1-B7).

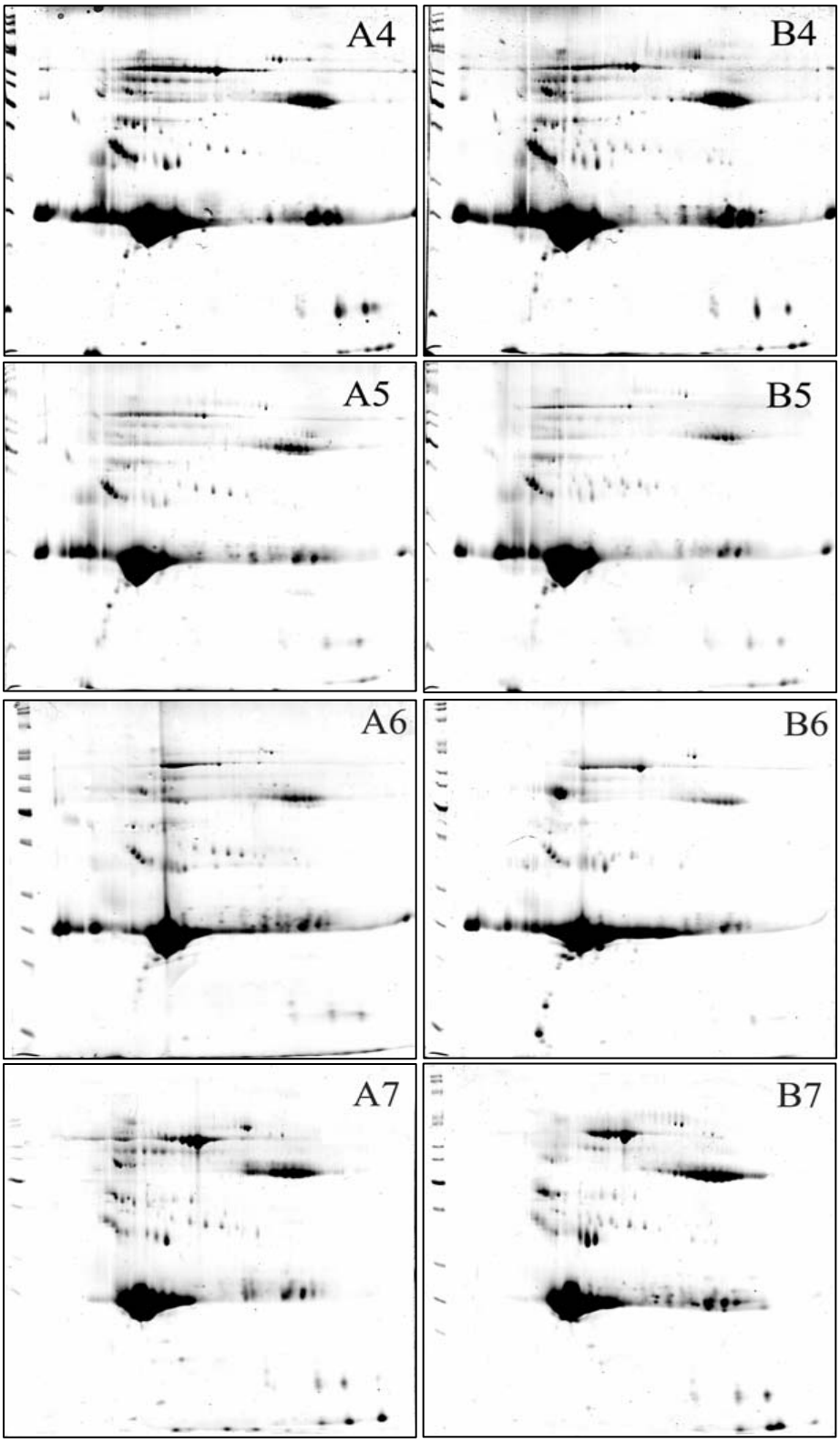
A4
B4
A5
B5
A6
B6
A7
B7

TABLE 10. HDL proteins identified by peptide mass fingerprint

Spot no.	protein	Accession no.	p*I*	Mass (Da)	Masses matched	Amino acid position	Sequence coverage
1	Alpha-1 antitrypsin	gi 942953	5.43	44322	1015.6526 1076.7047 1078.5613 1090.6290 1092.6454 1110.6403 1204.6919 1220.7290 1247.6116 1263.6173 1275.7200 1419.7185 1641.9710 1779.9061 1804.1014 1834.0704 1892.0121 2058.0919 2090.3001 2186.2365 2574.5558 2820.4542	301-310 235-243 275-282 194-201 235-243 291-300 234-243 234-243 224-233 224-233 192-201 223-233 26-39 11-25 260-274 311-328 202-217 137-154 175-191 137-155 102-125 1- 25	52%
2	Albumin	gi4502027	5.92	71317	1149.6993 1226.7270 1468.0419 1640.1419 1899.3211 1911.2381 1932.3378 2045.3998 2490.6611	66-75 35-44 361-372 438-452 170-184 509-524 89-105 397-413 45-65	21%
3	ApoA-I	gi90108664	5.27	28061	1031.4363 1047.4270 1152.5335 1226.4309 1235.5911 1252.5105 1283.4792 1299.4770 1302.5169 1318.5306 1380.6266 1400.5976 1411.6301 1427.6364 1462.7793 1467.7352 1612.7374	141-149 141-149 132-140 1-10 13-23 97-106 108-116 108-116 141-151 141-151 97-107 28-40 107-116 107-116 11-23 119-131 46-59	51%

Spot no.	protein	Accession no.	p*I*	Mass (Da)	Masses matched	Amino acid position	Sequence coverage
					1650.8427	13-27	
					1815.8544	24-40	
					2202.1407	60-77	
4	ApoA-IV	P06727	5.2	43400	1400.6554	52–64	58%
					975.5234	256–264	
					976.5411	156–163	
					983.5715	267–275	
					1083.5442	201–209	
					1102.5842	212–220	
					1104.5932	135–143	
					1196.5747	307–316	
					1215.6821	317–326	
					1231.6747	317–326	
					1235.6807	113–123	
					1258.6802	276–287	
					1258.6802	212–221	
					1287.6708	144–154	
					1311.7359	80–90	
					1350.6649	222–233	
					1352.6847	306–316	
					1558.8170	234–246	
					1574.8153	234–246	
					1634.8509	52–65	
					1805.9295	329–345	
					1927.9756	288–304	
					1994.0076	170–187	
					2084.0141	288–305	
5	ApoC-I	gi4502157	8.01	9326	1003.6603	55-63	39%
					1052.6285	39-47	
					1201.6877	66-74	
					1279.7986	39-49	
					1293.7873	37-47	
					1488.8727	64-74	
					1504.8605	64-74	
6	ApoE	gi178849	5.65	36302	899.1665	51-56	28%
					948.2043	177-185	
					968.2298	199-207	
					1033.1882	270-278	
					1247.1569	34-43	
					1313.2700	259-269	
					1497.3065	210-224	
					1753.3330	208-224	
					2092.3783	91-108	
7	ApoJ	gi338305	5.74	36997	907.5662	201-207	43%
					954.5343	291-298	
					970.5127	291-298	
					1075.6737	76-83	
					1288.7730	187-197	
					1393.8237	44-55	
					1444.8981	186-197	
					1763.0448	168-183	
					1874.2210	270-286	
					1888.2465	29-43	

Spot no.	protein	Accession no.	p*I*	Mass (Da)	Masses matched	Amino acid position	Sequence coverage
					1905.0707	29-43	
					2000.2450	60-75	
					2079.2339	166-183	
					2314.4723	247-269	
8	Apo L-I	gi12232634	5.99	42357	1165.6417	145-155	30%
					1290.6066	306-316	
					1574.7083	306-319	
					1590.8286	276-290	
					1595.7701	219-231	
					1630.7776	291-305	
					2487.2028	241-262	
					2955.4117	64-90	
9	CD5 antigen-like potein	gi5174411	5.28	39603	915.4135	308-314	24%
					993.3838	299-307	
					1147.3851	246-256	
					1545.5021	212-225	
					1565.5080	199-211	
					1747.5545	315-328	
					1761.6470	300-314	
					2063.7339	155-170	
10	Complement component 1	gi87298828	8.83	26933	968.6166	129-136	34%
					1092.5923	178-186	
					1591.8537	216-229	
					1660.8990	164-177	
					1879.9545	142-156	
					2493.3134	137-156	
					2530.3238	194-215	
11	Complement component 3	gi78101267	6.82	71317	1042.6852	260-268	60%
					1139.5736	220-227	
					1370.7953	115-126	
					1389.7539	406-417	
					1470.8690	269-282	
					1511.9360	509-522	
					1520.9089	1 -13	
					1600.0817	571-585	
					1639.9960	140-154	
					1653.9712	83-97	
					1656.0589	140-154	
					1788.1244	322-337	
					1866.1200	243-259	
					1872.2133	441-456	
					1879.1547	204-219	
					1891.2623	387-403	
					1896.1889	81-97	
					1910.2579	98-114	
					1986.2222	545-562	
					1994.2422	242-259	
					2002.1672	545-562	
					2166.2257	186-203	
					2198.3616	365-386	
					2444.6011	487-508	
					2494.4591	612-635	
					2578.6182	418-440	

Spot no.	protein	Accession no.	p*I*	Mass (Da)	Masses matched	Amino acid position	Sequence coverage
					2594.6054	418-440	
					2765.6528	341-364	
					2781.6397	341-364	
					3537.9653	601-635	
12	Fibrinogen beta	gi119625343	8.38	59024	902.4384	472-478	32%
					1030.5544	472-479	
					1239.5781	427-436	
					1535.7807	354-367	
					1544.7650	459-471	
					1560.7960	459-471	
					1684.8344	446-458	
					1785.9590	314-328	
					1941.0419	14-29	
					1951.1581	54-72	
					2127.2987	179-196	
					2390.2522	329-348	
					2401.3979	247-267	
13	Fibrinogen gamma	gi223170	5.54	46823	979.6543	206-212	40%
					1117.6635	248-256	
					1134.6542	257-266	
					1150.6305	257-266	
					1194.6183	6-14	
					1293.8437	163-173	
					1491.9214	96-108	
					1513.8813	109-120	
					1546.0000	391-405	
					1683.1833	233-247	
					2012.1555	339-356	
					2536.5540	63-85	
					2768.6299	128-151	
14	Haptoglobin	gi3337391	6.67	39300	920.6747	111-118	30%
					1225.9599	332-341	
					1544.0297	218-231	
					1845.4919	176-191	
					2763.8202	238-60	
					3845.2305	286-319	
15	IgA1	gi40890038	7.87	51957	931.6030	335-343	17%
					940.5406	291-299	
					1153.6163	396-404	
					1213.6355	386- 395	
					1375.5955	323-334	
					1540.6767	276-290	
					1835.8627	405-421	
16	IgM	gi4467842	6.35	50105	1029.6724	143-150	21%
					1249.7066	132-142	
					1616.9482	377-391	
					1618.0340	154-169	
					1637.9988	224-238	
					1717.0043	301-315	
					1774.1683	323-338	
17	IgG2	gi14030849	7.94	50229	1161.6080	361-370	34%
					1286.7726	345-355	
					1287.7279	126-137	

Spot no.	protein	Accession no.	p*I*	Mass (Da)	Masses matched	Amino acid position	Sequence coverage
					1423.8056	138-151	
					1794.1403	302-317	
					1905.0945	345-360	
					1922.0792	393-409	
					2544.3486	371-392	
					2801.5059	417-439	
					2817.5051	417-439	
					2908.6586	223-248	
18	Orosomucoid2	gi4505529	5.03	23873	994.7498	74-81	44%
					1144.6518	171-179	
					1160.8299	43-51	
					1234.9775	114-123	
					1757.2292	139-153	
					2113.5065	154-170	
					2663.9412	87-108	
19	Serum amyloid A1 (SAA1)	gi40316910	6.28	13581	1456.7585	66-80	66%
					1550.7710	20-33	
					1612.8770	65-80	
					1640.8798	109-122	
					1670.9002	44-57	
					1749.9860	106-121	
					2178.1259	86-105	
20	Transferrin	gi110590599	6.85	76988	1195.6057	101-110	48%
					1211.5874	101-110	
					1249.6656	432-442	
					1273.6509	204-214	
					1276.6683	278-288	
					1283.6655	509-519	
					1317.6424	5 -15	
					1323.7406	294-305	
					1364.7221	520-531	
					1478.8535	310-321	
					1491.8313	276-288	
					1494.8159	310-321	
					1521.7882	350-362	
					1531.8011	662-674	
					1565.9034	625-637	
					1577.8009	454-467	
					1586.8823	566-578	
					1593.8764	454-467	
					1615.9272	204-217	
					1629.8697	86-99	
					1690.0234	237-251	
					1705.8842	473-487	
					1882.0130	215-229	
					1953.1222	550-565	
					2072.0401	412-430	
					2171.3173	122-140	
					2175.2263	378-398	
					2549.5264	230-251	
					2987.5408	494-519	

Spot no.	protein	Accession no.	p*I*	Mass (Da)	Masses matched	Amino acid position	Sequence coverage
21	Transthyretin, chain A	gi443295	5.35	13810	1366.9577 1394.7720 1495.0531 1522.8918 2360.5455 2451.5500 2455.4701 2516.7046 3140.8927	22-34 36-48 22-35 35-48 105-126 81-103 49-70 104-126 49-76	79%
22	Vitamin D-Binding protein	gi139641	5.4	54526	952.6696 1170.9097 1254.9505 1275.8266 1327.0236 1388.9541 1404.9488 1444.8250 1695.2351 2093.2139 2265.3834 2328.6801 2518.4869	277-284 354-363 208-218 219-229 353-363 342-352 342-352 293-303 51-65 371-388 95-114 31-50 66-87	33%

CHAPTER V

DISCUSSION

HALP and *CETP* gene mutations

In Japan, HALP is common, which is primarily due to CETP deficiency. CETP is a key enzyme involved in the transfer of cholesterol ester from HDL to apo B-containing lipoproteins. Lack of CETP results in accumulation of cholesterol-rich HDL and elevation of plasma HDL cholesterol levels. At least ten mutations of the *CETP* gene, resulting in decreased or no CETP activity, have been reported in the Japanese (56), and the two most common mutations are an intron 14 splicing defect (Ivs14+1G>A) and an exon 15 missense mutation (D442G) (7). Outside Japan, however, information on the cause of HALP is relatively scarce. In Chinese, a D442G mutation and a mutation in an intron 1 splice donor site of the *CETP* gene have been identified (57-59). In Caucasians, although there is a strong linkage between a CETP locus and HDL cholesterol concentrations (26), several studies have demonstrated that HALP due to genetic CETP deficiency is rare and the cause of HALP remains unknown (12-16) .

In our study of Thai subjects with HALP, we found that the CETP activity was significantly lower than that in the control group. In addition, we also found a D442G mutation previously observed in the Japanese and the Chinese subjects. Interestingly, an intron 14 splicing defect (Ivs14+1G>A) commonly found in the Japanese was not identified in our subjects. Furthermore, we identified the first deletion mutation in the *CETP* gene. This deletion mutation is predicted to result in a truncated protein lacking exons 10 - 16, which contain a neutral lipid binding site. Although this mutation was also found in the brother of the proband, it is of note that his HDL cholesterol concentration was not high. Unfortunately, his CETP activity was unavailable. A study in Japanese patients with mutations in the *CETP* gene, causing CETP deficiency, has shown that not all of the patients who had mutations displayed HALP (7). In fact, 5.7% and 7.2% of subjects with an Ivs14+1G>A mutation and a D442G mutation, respectively, had HDL cholesterol concentrations lower than 40 mg/dL (7).

Although CETP deficiency is the most common cause of HALP in Japan, it has been reported that 36% of Japanese subjects with HALP (defined as HDL cholesterol level above 100 mg/dL) had normal CETP activity and 34% of subjects with HALP and low CETP activity had no identifiable mutation in the *CETP* gene (7). One of our HALP subjects with undetectable CETP activity also had no identifiable mutation in the *CETP* gene, although we cannot exclude the mutation in the promoter or in the introns.

Our observation that several Thai subjects with HALP had CETP activity comparable to that in the control group is consistent with these findings and suggests that factor(s) other than CETP might be responsible for HALP.

HALP and *LIPC* gene mutations

Beside CETP deficiency, decreased HL activity has been associated with an increase in HDL cholesterol levels (17-22). HL is an enzyme that hydrolyses triglyceride and phospholipids in HDL; therefore, HL activity is another important determinant of plasma HDL cholesterol levels. HL deficiency has been identified in several families, which display variable phenotypes; however, the most consistent finding is an elevation of HDL cholesterol levels (18;22).

In our study, we found that HL activity in the HALP group was also significantly lower than that of the control group. We identified two previously reported genetic variants of the *LIPC* gene, V73M and L334F, in both groups. The association between the V73M variant and types of dyslipidemia is conflicting. One study reported that the V73M variant was present at a higher frequency in patients with combined hypertriglyceridemia and HALP (17), while others showed an increased frequency in patients with familial combined hyperlipidemia (60) or no association with various types of dyslipidemia (61). In our present study, the V73M variant was found at a relatively similar frequency in both control and HALP groups, suggesting that this variant is not associated with HALP. Similarly, the L334F variant was also found in both groups, which excluded this variant as a cause of HALP. The L334F variant was originally found in Finnish families with hepatic lipase deficiency, resulting from compound heterozygosity for the mutations in the LIPC gene, L334F and T383M (62) and L334F and R186H (63). A subsequent study in a larger group of patients, however, showed that

subjects heterozygous for the L334F mutation had HDL cholesterol levels similar to those of subjects without the mutation (64). The results from our study are consistent with these findings and suggest that the L334F variant is not associated with HALP in Thai subjects.

A novel heterozygous missense mutation of the *LIPC* gene, c.421G>A or G119S, was identified in one subject with low HL activity in the HALP group. It is of note that the glycine residue at position 119 of HL is highly conserved across several animal species and among different proteins in the lipase superfamily, such as lipoprotein lipase, endothelial lipase, pancreatic lipase, pancreatic lipase related proteins 1 and 2 (Fig. 4). In addition, Gly119 lies in a conserved helix 3 and is adjacent to Ser 146, which is part of the classical Ser-Asp-His catalytic triad found in several lipase enzymes (65;66). Both the PolyPhen and PANTHER predicted this mutation to be dysfunctional. Our in vitro expression study demonstrated that amino acid substitution at this position was associated with lower HL activity. Due to unavailability of HL antibodies, we do not know whether the lower activity was due to decreased protein mass or mutant protein with inactive enzyme.

HDL proteomic study

CETP is an HDL-associated protein. Lack of CETP on HDL results in HALP, we speculated that comparison of HDL protein components between the HALP subjects and controls might lead to the discovery of biomarker for the diagnosis of HALP. We found no significant differences in the quantities of protein on HDL between the case and control group.

HALP characteristics

It is also interesting that our HALP subjects showed significantly lower triglyceride levels, body weight, and waist circumference than those in the control group. These characteristics are in contrast to several features of the metabolic syndrome, e.g. low HDL cholesterol levels, high triglyceride levels, high waist circumference and obesity (67). Insulin resistance is thought to be the main defect in the metabolic syndrome and is associated with an increased risk for cardiovascular diseases (33). High

activities of CETP and HL have been found in patients with insulin resistance and provide the mechanistic link between insulin resistance and high triglyceride and low HDL cholesterol levels in these patients (68). It is currently unknown whether HALP is associated with higher insulin sensitivity. Furthermore, whether HALP is associated with an increased or decreased risk for cardiovascular diseases is also unclear (19;69;70).

CHAPTER VI

CONCLUSION

1. HALP in Thai subjects is associated with lower CETP and HL activities.
2. The previously known mutation, D442G, and a novel mutation, c.734_737delTCCC, in the *CETP* gene are pathogenic.
3. A novel mutation, G119S, in the *LIPC* gene is pathogenic.
4. Approximately one-third of Thai subjects with HALP are caused by either *CETP* or *LIPC* mutations. These data suggest that HALP is a heterogeneous disorder resulted from various genetic causes.
5. Although our study was not designed to look at environmental factors affecting HDL levels, there is evidence that strong environmental factors, such as smoking or obesity, may influence HDL in subjects with *CETP* and *LIPC* gene mutations. In addition, data from our study and from others suggest that other factors, beside CETP and HL, may be responsible for extremely high HDL phenotype in the population.

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APPENDIX

1. DNA assay reagents

Lysis Buffer 1 (320M sucrose, 10mM Tris-HCl pH 7.5, 5mM $MgCl_2$, 1%tritonX-100)

Sucrose	109.54	g
1M Tris-HCl	10	mL
1M $MgCl_2$	5	mL
100% TritonX-100	10	mL
DW to 1000 mL		

Lysis Buffer 2 (0.75 M NaCl, 0.24 M EDTA pH 8)

5.0 M NaCl	15	mL
0.5 M EDTA pH 8	48	mL
DW to 1000 mL		

Tris-Borate (5X TBE) (0.445M Tris-borate, 0.445M boric acid, 0.01M EDTA)

Tris base	54	g
Boric acid	27.5	g
0.5 M EDTA (pH 8.0)	20	mL
DW to 1000 mL		

2. Expression plasmid reagents

LB agar

Agar	3.75	g
Peptone	2.5	g
Yeast	1.25	g
NaCl	1.25	g
DW to 250 mL		

The reagent was autoclaved and waiting until warm, then ampicillin(100 mg/mL) 250 µl were added

LB Broth

Peptone	0.5	g
Yeast	0.25	g
NaCl	0.25	g

DW to 50 mL

The reagent was autoclaved and waiting until warm, then ampicillin (100 mg/mL) 50 µl was added

SOC Media

Peptone	2.0	g
Yeast	0.5	g
1 M NaCl	1.0	mL
1 M KCl	0.25	mL

DW to 100 mL

The reagent was autoclaved and waiting until warm, then 2M Mg^{2+} 1 µl and 2M glucose 1 µl were added

3. Column reagents

Binding buffer pH 7.5 20X (Tris-buffer saline (TBS))

Tris-base	24.228	g
NaCl	175.32	g

Adjust pH with HCl

DW to 1000 mL

Elution buffer pH 3 (0.2 M acetic acid, 0.15M NaCl)

Glacial acetic acid	11.5	mL
NaCl	8.766	g

DW to 1000 mL

4. Protein assay reagents

4.1 2 DE

Lysis solution (8 M urea, 4% CHAPS, 2% Pharmalyte 3–10)

Urea	19.2	g
CHAPS	1.6	g
Pharmalyte 3–10	800	µl

DW to 40 mL

Rehydration stock solution without IPG Buffer (8 M urea, 2% CHAPS, 0.002% bromophenol blue)

Urea	12	g
CHAPS	0.5	g
Bromophenol blue	50	µl

DW to 25 mL

DTT and IPG Buffer or Pharmalyte were added prior to use. 7 mg DTT were added to 2.5 mL aliquot of rehydration, store in 2.5 mL aliquots at -20 °C.

Bromophenol blue stock solution (1% bromophenol blue, 50mM Tis-base)

Bromophenol blue	100	mg
Tris-base	60	mg

Double distilled H2O to 10 mL

SDS equilibration buffer

(50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.002 % bromophenol blue)

Tris-HCl, pH 8.8	10.0	mL
Urea	72.07	g
Glycerol (87% v/v)	69	mL
SDS	4.0	g
Bromophenol blue (1% solution)	400	µl

Double distilled H2O to 200 mL

This was a stock solution. DTT or iodoacetamide were added before using.

10% SDS

SDS	5.0	g
DW to 50 mL		

10% ammonium persulfate

Ammonium persulfate	0.1	g
DW to 1 mL		

SDS electrophoresis buffer (1X)

(25 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1% SDS)

Tris-base	30.3	g
Glycine	144.0	g
SDS	10.0	g
Double distilled H2O to 10 L		

Agarose sealing solution (0.5%)

SDS Electrophoresis buffer	100	mL
Agarose	0.5	g
Bromophenol blue 0.002% (w/v)	200	µl

4.2 Colloidal Coomassie Staining

5% Coomassie blue G-250

Coomassie Blue G-250	0.5	g
DW to 10 mL		

Colloidal Coomassie Blue G-250 dye stock solution

(10% ammonium sulfate, 1%(w/w) phosphoric acid, 0.1% Coomassie blue G-250)

Ammonium sulfate	50	g
Phosphoric acid 85%(w/w)	6	mL
5% Coomassie blue G-250 stock	10	mL
DW to 500 mL		

Colloidal Coomassie Blue G-250 working solution

(8% ammonium sulfate, 0.8% phosphoric acid, 0.08% Coomassie blue G-250, 20%methanol)

Colloidal Coomassie Blue G-250 dye stock solution	400	mL
Methanol	100	mL

4.3 Western blot

10X Western transfer buffer stock

Tris-base	15.14	g
Glycine	72.1	g
DW to 500 mL		

1X Western transfer buffer with 20% methanol

10X Western transfer buffer stock	100	mL
Methanol	200	mL
DW to 1000 mL, prepare before using		

10X Phosphate buffer saline pH 7.3

NaCl	80	g
KCl	2	g
$Na_2HPO_4.7H_2O$	11.5	g
KH_2PO_4	2	g
DW to 1000 mL		

1X PBS, 0.1% TWEEN

Tween 100%	1	mL
1X PBS to 1000 mL		

5% Non fat dry milk in 1X PBS-TWEEN 0.1%

Non fat dry milk	5	g
1X PBS, 0.1% TWEEN to 100 mL		

BIOGRAPHY

Miss Wanee Plengpanich was born on October 19, 1977 in Pathumtanee Province, Thailand. She graduated with the degree of Master of Science in Zoology from Chulalongkorn University. She has studied for a doctoral degree in Biomedical Sciences at the Graduate School, Chulalongkorn University.

Presentations from this thesis

1. Siriwong S, **Plengpanich W**, Vongthavaravat V, Khovidhunkit W. Deficiency of Cholesteryl Ester Transfer Protein Activity is not a Common Cause of Very High Levels of HDL in Thai People. 87th Annual Meeting of the Endocrine Society, San Diego, CA, USA. June, 2005. (Poster Presentation).

2. **Plengpanich W**, Siriwong S, Vongthavaravat V, Snabboon T, Khovidhunkit W. Hepatic lipase and lipoprotein lipase activities in Thai subjects with hyperalphalipoproteinemia. 88th Annual Meeting of the Endocrine Society, Boston, MA, USA. June, 2006. (Poster Presentation).

3. **Plengpanich W**, Siriwong S, Snabboon T, Khovidhunkit W. Cholesteryl ester transfer protein (CETP) activity and mutations in Thai subjects with hyperalphalipoproteinemia. 17th Annual Meeting of the Endocrine Society of Thailand, Bangkok, Thailand. October 2006. (Oral Presentation).