# STUDIES OF COMMON VARIATIONS IN TWO CANDIDATE GENES OF DYSLIPIDEMIA AND CORONARY ARTERY DISEASE

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# STUDIES OF COMMON VARIATIONS IN TWO CANDIDATE GENES OF DYSLIPIDEMIA AND CORONARY ARTERY DISEASE

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## SUMMARY

Coronary artery disease is a disorder with multiple genetic and environmental factors and dyslipidemia is one of most prominent risk factors. The major purpose of this study is to determine the influence of some of the genetic factors on CAD susceptibility and on plasma lipid traits.

Acyl-CoA: cholesterol Acyltransferase-2 (ACAT2) catalyzes the formation of cholesteryl esters using cholesterol and long-chain fatty acids as substrates. As such ACAT2 is a very important enzyme in the intestinal cholesterol absorption and in the production of apoB-containing lipoproteins in the liver. ACAT2 has been demonstrated to be a potential target for treating coronary artery atherosclerosis in hypercholesterolemic animal model.

In order to explore the effects of genetic variations in the *ACAT2* gene, we screened for variants on its entire coding regions, intron-exon boundaries, and putative promoter region, using denaturing high performance liquid chromatography. A total of 14 polymorphisms were identified. These included three missense mutations, namely c. 41A>G (Gly>Glu) in exon1; c.734C>T (Thr>Ile) in exon7; and c.1291G>T (Ala>Ser) or G>A (Ala>Thr) in exon13; two base changes in putative promoter region (-331C>T and -440G>T), two synonymous exonic base changes (c.609G>T and c. 610C>T in exon6), seven intronic sequence variations, comprising six single base substitutions (IVS1-8C->G; IVS4+172T/G, IVS5-137A/T, IVS9-178G/C, IVS9+37A->T and IVS9+51G->T) and one 48bp insertion. Among these, 3 polymorphisms, 41A>G (Glu$^{14}$Gly), 734C>T (Thr$^{254}$Ile), and IVS4-57_58*ins*48bp, were analyzed for their association with CAD and plasma lipid levels. A total of 2113

subjects, comprising 1228 Chinese, 367 Malays, 518 Indians, were included in this case-control association study. We found these three *ACAT2* polymorphisms showed significant ethnic variations in allele frequencies as well as significantly different effects on plasma lipid levels and CAD risk, though some significances were not observed after Bonferroni correction. In addition, *in vitro* experiments were carried out to determine the expression levels of ACAT2 wild type and mutant proteins and their enzymatic activities in the ACAT-deficient AC-29 cells. The results showed that the enzymatic activity of mutant Glu14Gly was about two times higher compared to that of the wildtype ACAT2, and this increase was mostly due to the higher expression and/or stability of the mutant ACAT2 protein. Our observations suggest that the Glu$^{14}$Gly polymorphism might be very important to ACAT2 protein expression and/or stability.

Another important enzyme, lipoprotein lipase (LPL), the rate-limiting enzyme in hydrolysis of triglycerides in chylomicron and very-low-density lipoprotein particles, was analyzed in this study. LPL has a paradoxical role in the development of atherosclerosis as it can be considered both anti-atherogenic and pro-atherogenic. There is no uniform consensus regarding the association of genetic variations in *LPL* with CAD susceptibility and lipid levels. In addition, it is interesting to evaluate the impact of the combination of IVS6+1594C>T, IVS8+483T>G, and c.1342C>G polymorphisms in Asian populations. Our study showed that the most prevalent CTC haplotype of three *LPL* polymorphisms was consistently associated with increased CAD susceptibility in male Chineses and Indians living in Singapore. In addition, the rare alleles of three individual polymorphisms were also found to lower CAD risk in male Chinese and/or Indians, which is independent of any effect on lipid profile.

## LIST OF ABBREVIATIONS

| | |
|---|---|
| 5'UTR | 5' untranslation region |
| ABC | ATP-binding cassette |
| ACAT | Acyl-CoA enzyme cholesterol transferase |
| ANOVA | Analysis of variance |
| Apo | Apoliproprotein |
| ARE | ACAT-related sterol-esterifying enzymes |
| ARH | Autosomal recessive hypercholeterolemia |
| ATP | Adenosine triphosphate |
| ATP | Adult treatment panel |
| CBS | Cystathionine β-synthase |
| CAD | Coronary artery disease |
| cDNA | Coding deoxyribonucleic acid |
| Cdx-2 | Caudal type homeo box transcription factor 2 |
| CE | Cholesteryl ester |
| CEBP | CCAAT/enhancer binding protein |
| CHD | Coronary heart disease |
| CFLP | Cleavage fragment length polymorphism |
| CVD | Cardiovascular disease |
| DEP | diethyl pyrocarbonate |
| DGGE | Denatureing dradient gel electrophoresis |
| DGAT | Diacylglycerol acyltransferase |
| DHPLC | Denaturing high performance liquid chromotography |
| dNTP | Deoxynucletides (dATP, dCTP, dGTP, dTTP) |
| ER | Endoreticulum |
| FLLD | Familial LPL deficiency |
| HDL | High density lipoprotein |
| HMG-CoA | Hydroxamethylglutaryl-CoA |
| HNF-3b | Hepatocyte nuclear factor-3b |
| HSPG | Heparin sulphate-proteoglycans |
| HWE | Hardy-Weinberg Equilibrium |
| HAEC | human aortic endothelial cell |
| HGVbase | Human Genome Variation database |
| IDL | Intermediate density lipoproteins |
| JSNP | Japanese SNPs |
| LACT | Lecithin:cholesterol acyltransferase |
| LD | linkage disequilibrium |
| LDL | Low density lipoprotein |
| LDLR | Low density lipoprotein receptor |
| LPL | Lipoprotein lipase |
| MEF2A | Myocyte enhancing factor-2 |
| MI | Myocardial infarction |
| Min | Minutes |
| MTP | Microsomal triglyceride transfer protein |
| NCBI | National Center for Biotechnology Information |
| NCEP | National cholesterol education programme |
| NHGRI | National Human Genome Research Institute |
| nsSNP | Non-synonymous single nucleotide polymorphism |
| PBS | Phosphate Buffered Saline |

| | |
|---|---|
| PCR | Polymerase chain reaction |
| RFLP | Restriction fragment length polymorphism |
| SDS | Sodium dodecyl sulphate |
| S | Seconds |
| SNP | Single nucleotide polymorphism |
| SREBP | Sterol regulatory element binding protein |
| SSCP | Single-Strand Conformation Polymorphism |
| STR | Short tandem repeat |
| TSC | the SNP consortium |
| TEAA | Triethylammonium acetate |
| TG | Triglyceride |
| TMD | Transmembrane domain |
| VNTR | variable number of tandem repeats |
| VCAM | Vascular cell adhesion molecule |
| VLDL | Very low density lipoprotein |
| VSMC | Vascular smooth muscle cells |

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# 1. INTRODUCTION AND BACKGROUND

## 1.1. Introduction

Coronary artery disease (CAD) is a complicated disease with multiple genetic and environmental contributions, such as dyslipidemia, hypertension, diabetes mellitus, obesity, cigarette smoking, high-fat and high-cholesterol diet, and physical inactivity. Among these risk factors, dyslipidemia is the most important contributor to CAD susceptibility. Dyslipidemia can be caused by monogenic disorders, such as familial hypercholesterolemia (Brown and Goldstein, 2001), and familial defective apolipoproetin (apo) B (Fisher *et al.*, 1999). Studies on these monogenic disorders have helped to unravel the pathways of cholesterol metabolism regulation and contributed importantly to the understanding of lipid metabolism and atherosclerosis. However, most cases of dyslipidemia are attributed to a combination of many common genetic variations and environmental factors and our understanding of these aspects remains incomplete. Study on such genetic variants of candidate genes in lipid metabolism would contribute towards elucidating the genetic mechanisms of CAD and dyslipidemia.

## 1.2. Background

Given the prominent role of dyslipidemia in atherosclerosis, genetic studies on enzymes which are important to lipid and lipoprotein metabolism have captivated generations of researchers.

A relatively novel protein, acyl CoA: cholesterol acyltransferase (ACAT)2, an enzyme which converts cholesterol into cholesterol esters (CEs) (Chang *et al.*, 1997),

has been shown to be associated with hypercholesterolemia and atherosclerosis using *ACAT2*-/- mice model (Buhman *et al.*, 2000). Furthermore, the deletion of *ACAT2* has been demonstrated to be consistently athero-protective (Willner *et al.*, 2003; Lee *et al.*, 2004). Thus, selective inhibition of ACAT2 would be an important strategy for treatment and prevention of atherosclerosis (Rudel *et al.*, 2005). The ACAT2, also called Soat2, should not be confused with acetyl-Coenzyme acetyltransferase 2, which is also denoted as ACAT2.

Another important protein, lipoprotein lipase (LPL), is the rate-limiting enzyme responsible for the hydrolysis of triglycerides (TGs) in chylomicrons and very low density lipoproteins (VLDL). This hydrolysis functions to clear TGs from the circulation and also provides phospholipids and apolipoproteins to high-density lipoprotein (HDL) cholesterol, therefore, driving the plasma lipids in anti-atherogenic direction. The familial deficiency of LPL may lead to type I hyperlipidemia, characterized by severe hypertriglyceridemia and extremely low HDL levels (Brunzell, 1995). Even heterozygous LPL mutations may result in reduced or loss of LPL activity and increased risk of familial combined hyperlipidemia (Babirak *et al.*, 1992). On the other hand, LPL also has a non-enzymatic molecular bridging function and has been shown to act as a ligand to mediate cellular uptake of lipoproteins and CEs (Stein and Stein, 2003). Furthermore, LPL has been shown to stimulate the proliferation of vascular smooth muscle cells (VSMC) (Mamputu *et al.*, 2000). Hence, LPL may also have pro-atherogenic effects. Whether LPL acts to be anti-atherogenic or pro-atherogenic depends on the tissues specifically expressing LPL (Mead and Ramji, 2002).

Because of their crucial roles in lipid metabolism and atherogenesis, the *ACAT2* and *LPL* are considered as important candidate genes in studies to determine whether they contribute to predisposition for dyslipidemia and atherosclerosis.

Singapore is a small immigrant nation in Southeast Asia comprising different ethnic groups: Chinese (77.4%), Malays (14.2%), Indians (7.2%) and European Caucasians (0.2%). The ancestors of the Chinese were mostly migrants from the coastal regions of southern China. The Malays came from neighboring Malaysia and Indonesia. Most Indian Singaporeans are second, third or even fourth generation descendants of migrants from the southern Indian subcontinent.

Over the past few decades, Singapore has witnessed a high degree of social and political stability and a rapid-growth economy since it obtained independence in 1965. Since 1980, Singapore has been considered a fully urbanized and developed city-state. With the rapid socioeconomic development, some "modern diseases", such as cardiovascular diseases and cancer, have become the major causes of morbidity and mortality in this population. Among them, CAD is the leading cause of death in this population.

Each of the ethnic groups residing in Singapore has contrasting mortalities due to CAD (Hughes *et al.,* 1990[a]; Hughes *et al.*, 1990[b]; Heng *et al.*, 1999), though they reside in the same physical environment and environmental factors are relative constant except their habitual diets, and culturally determined lifestyles. Thus, Singapore offers the advantage for the genetic study of ethnic differences in CAD risk

## 1.3. Objectives of this study

### 1.3.1. Study on *ACAT2* gene

Although *ACAT2* has been shown to be associated with hypercholesterolemia and atherosclerosis in animal models (Buhman *et al.*, 2000), studies on the effects of genetic variants of this gene on CAD susceptibility have not been performed in the general population. There was only one other genetic study which explored the association of two *ACAT2* polymorphisms, Glu14Gly and Thr254Ile, with dyslipidemia in Japanese when this study was initiated in the Singaporean population (Katsuren *et al.*, 2001). In 2003, a similar study on another polymorphic site, IVS1-8G>C, was reported by the same group (Katsuren *et al.*, 2003). Their studies did not reveal any positive association except with plasma apoIII level, which was found to be higher in Thr254Ile heterozygotes (Katsuren *et al.*, 2001). However, no further functional study on the genetic variant was carried out.

Based on the significant effects of ACAT2 on atherosclerosis in *ACAT2*-/- mice (Buhman *et al.*, 2000), it was hypothesized that genetic variants of *ACAT2* gene could exert an important influence on susceptibility to CAD and dyslipidemia in the general population. The Singaporean population is made up of multiple ethnic groups, namely, Chinese, Malays, and Indians. It is also therefore worthwhile to determine if the effect of ACAT2 variants is ethnic-specific.

The following steps were taken to verify these hypotheses:

1. To identify novel polymorphisms of the *ACAT2* gene using denaturing high performance liquid chromatography (DHPLC) and to predict their possible biological function using computational approaches

2. To determine the impact of the *ACAT2* gene polymorphisms on CAD susceptibility and plasma lipid levels in three major ethnic groups in Singapore using population-based case-control association study

    i. To determine the allele frequencies of *ACAT2* polymorphisms between CAD patients and healthy control, as well as between the normolipidemic and dyslipidemic subgroups

    ii. To determine the allele frequencies of the *ACAT2* polymorphisms in the three ethnic groups, and

    iii. To evaluate the effect of these polymorphisms on plasma lipid profiles in different population subgroups,

3. To conduct an *in vitro* functional study of two potential functional polymorphisms in mammalian cells.

### 1.3.2. Study on *LPL* gene

Three common genetic variants, IVS6+1595C>T, IVS8+484T>G, and c.1342C>G of the *LPL* gene, have been reported to be associated with abnormal lipid concentrations and atherosclerosis. Though these three polymorphisms have been extensively studied, there is no uniform consensus on their effects on CAD susceptibility and plasma lipid concentrations. Furthermore, most studies were conducted in Caucasian populations, with only a few that had examined the Asian populations (Shimo-Nakanishi *et al*., 2001; McGladdery *et al*., 2001; Hall *et al*., 2000; Lee *et al*., 2004; Liu *et al*., 2004). In addition, to our knowledge, the combined effect of these three polymorphisms on CAD risk has not been determined in an Asian population. In this study, it was evaluated whether these polymorphisms are associated with CAD risk and with abnormal lipid traits. The combined effect of these three polymorphisms on CAD risk

was also explored in two Asian ethnic groups with contrasting CAD risk, namely, the Indians and Chinese. In addition, the different distribution of allele frequencies between different ethnic groups was compared.

### 1.4. Significance and limitation of the study

CAD is the most common cause of death in developed countries and its prevalence is rapidly increasing in developing countries owing to the burgeoning epidemic of obesity and the aging population. For the treatment of CAD, other than modifying lifestyle risk factors, pharmacological intervention is an important strategy. The determination of specific susceptibility genes and these disease-associated genetic variants will contribute to the development of new drugs. In addition, the identification of high-risk individuals would be important for the efficient prevention of CAD and dyslipidemia. However, although many candidate genes and genetic variants related to CAD and dyslipidemia have been reported, the available data are either inconclusive or inconsistent.

In our study, fourteen polymorphisms were identified in *ACAT2* gene by screening cord blood samples using DHPLC. Our association study showed that a nonsynonymous SNP (nsSNP) in exon 7, 734C>T, was associated with decreased CAD risk while another nsSNP in exon 1, 41A>G, had decreased dyslipidemia in Chinese subjects, after correction for multiple comparisons. It was observed that these two nsSNPs and IVS4-57_58ins48bp (D/I), a 48bp insertin in intron 4, were in strong linkage disequilibrium (LD). The frequency of the most common AC haplotype of these two nsSNPs was significantly increased in dyslipidemic subjects when compared with normolipidemic ones in the Chinese. The CAD+ group had almost

2-fold higher GC haplotype frequency than the CAD- controls in the three ethnic groups, but statistical significance was only attained in the Chinese. However, the normolipidemic subjects had about 3-fold higher GC haplotype frequency than dyslipidemic ones in the Chinese and Malay groups, although the latter did not reach a significant level. The preliminary results from the functional study of these two nsSNPs suggested the 41A>G probably increased ACAT2 enzymatic activity by altering protein expression and/or stability.

The identification of novel polymorphisms in *ACAT* gene would contribute to the SNP database, which is very useful for genetic association studies. Secondly, our association study would provide important information about the impact of *ACAT2* and *LPL* polymorphisms with CAD and dyslipidemia. Furthermore, our preliminary results from *in vitro* study would be helpful for the understanding of the relationship between the structure and function of ACAT2 protein.

The study is not without its limitations, such as the age disparity between case and control subjects and the relatively small sample size, especially after stratification by gender, ethnic groups and lipid profiles. Hence, further study using a larger sample size with well-matched case-control may be needed to confirm our current findings related to association with CAD, dyslipidemia susceptibility and altered plasma lipid profiles. In addition, although the functional study of nsSNPs of *ACAT2* gene in mammalian cell line have shown that the 41A>G (Glu14Gly) variant had significant effects on ACAT2 enzymatic activity, only one colony for each ACAT2 polymorphic type was examined. To verify the results obtained from functional study, at least

three colonies for each ACAT2 polymorphic types should be included in future studies.

# 2. LITERATURE REVIEW

## 2.1. Coronary artery disease

CAD is the leading cause of death and disability in the developed world, with an increasing prevalence (Bonow *et al*., 2002). In the last 30 years, dyslipidemia has been identified as a major modifiable risk factor for CAD (Willerson and Ridker, 2004). In the following sections, the clinical, etiological, and epidemiological aspects of the most prominent risk factor, dyslipidemia, will be reviewed.

### 2.1.1. Definition of CAD

CAD, also called coronary heart disease (CHD) or ischemic heart disease (IHD), is characterized by a "narrowing" of coronary arteries resulting in inadequate blood flow to the heart muscle and leading to angina pectoris, with exertion or at rest, or myocardial infarction (MI), and even sudden death, depending on the severity of obstruction. The narrowing is usually caused by atherosclerosis.

### 2.1.2. Prevalence of CAD

CAD is a major cause of morbidity and is a leading contribution to mortality worldwide, especially in developed countries (Murray and Lopez, 1997). According to the Heart Disease and Stroke Statistics update 2005 from American Heart Association (AHA), CAD caused 1 of every 5 deaths each year and every minute someone will die from CAD in the United States. CAD is responsible for about US$142.1 billion in 2005 in direct and indirect economic costs in the United States (Heart Disease and Stroke Statistics – AHA 2005 Update). With the rapid economic development and urbanization in Singapore, CAD has been the most common cause

of death (Lee *et al*; 2001). Even in developing countries, both CAD associated mortality and the prevalence of CAD risk factors continue to rise rapidly (Okrainec *et al*; 2004). With the aging of population, westernization of the lifestyle in developing countries and the increasing survival rate during acute phases of ischemic disease, which transform acute patients into chronic patients (Viles-Gonzalez *et al*; 2004), CAD will be the single largest cause of disease burden globally by the year 2020. By that year, it is estimated that nearly 40% of all deaths worldwide will be due to cardiovascular disease (CVD), more than twice the percentage of deaths from cancer (Heart Disease and Stroke Statistics – AHA 2005 Update).

### 2.1.3. Pathophysiology and pathogenesis of CAD

Atherosclerosis in the artery wall is the major feature of CAD and is an organized, active, lifelong process. Our views of the pathophysiology of the progressive disease have evolved substantively over the past decades. Before the 1970s, the lipid hypothesis which is based on strong experimental and clinical relationships between hypercholesterolemia and atherosclerosis dominated our thinking. During the 1970s and 1980s, growth factors and the proliferation of smooth muscle cells have been considered to play a prominent role in atherosclerosis. However, at the end of the last century, an increasing number of researchers have considered atherosclerosis an inflammatory disorder (Libby, 2002; Reiss and Glass, 2006).

The accumulation of lipids has a role in inducing and promoting inflammation and atherogenesis. In experimental models, such as atherosclerosis-susceptible *apoE-/-* or *VLDL-/-* mice, the accumulation of lipoprotein particles and their aggregates are initially observed in the subendothelial space (Zhang *et al.*, 1992; Ishibashi *et al.*,

1993). These retained lipoproteins, particularly those modified LDL, elicit a series of biological responses that lead to endothelial dysfunction, which is a critical step in the development of inflammation and atherosclerosis (Gonzalez and Selwyn, 2003). Endothelial dysfunction is caused not only by elevated and modified LDL, but also by other factors, such as elevated plasma homocysteine concentrations, hypertension, infectious micro-organisms, and free radicals. These factors increase the adhesiveness of the endothelium with respect to monocytes, macrophages, and lymphocytes, as well as its permeability to lipoproteins and other constituents. In addition, the injured endothelia also increase the expression of adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), P- and E-selectin (Cybulsky *et al.*, 2001; Libby, 2002; Collins and Cybulsky, 2001). All these responses induce the migration of monocytes, macrophages and T lymphocytes from the blood into the intima of artery wall (Ross, 1999). These monocytes and monocyte-drived macrophages then scavenge the subendothelial lipoproteins and become lipid-laden foam cells, which are the earliest lesions of atherosclerosis. These lesions are not clinically significant, but they may evolve to form fibrous plaques by accumulating lipids, smooth muscle cells and extracellular matrix. These plaques can become increasingly complex and grow large by vascularization, haemorrhage, rupture, ulceration and calcification. The most serious clinical complication is MI or sudden death caused by an acute occlusion due to the thrombus formation.

All these insights suggest that lipids play a crucial role in the development and progression of atherosclerosis and that abnormal level of lipid accumulation is the triggering event in the pathogenesis of atherosclerosis.

### 2.1.4. Genetics of CAD

A few forms of Mendelian diseases that involve premature CAD or atherosclerosis have been identified (Table 2-1). Most of these disorders affect the levels of LDL and HDL cholesterols. Monogenic CAD due to monogenic defects without dyslipidemia syndrome is not common. A few genes with mutation have been identified to be associated with premature CAD. For example, myocyte enhancing factor-2, which encodes a transcription factor, has been shown to be involved in CAD in a single large family with dominant inheritance of CAD and MI (Lusis *et al*., 2004).

Except for a few cases of CAD of monogenic origin, most have been proposed to be caused by common genetic variants with small-to-moderate effects in candidate genes. The magnitude of the effects of genetic factors for CAD has been studied. A cohort study over 36 years in 20,966 Swedish twins has shown that the heritability of death from CAD due to genetic effect was 0.57 amongst male twins, and 0.38 amongst female twins (Zdravkovic *et al*., 2002). Furthermore, genetic factors are in operation throughout the entire life span, though genetic effects appeared to be greater at younger ages. This phenomenon possibly is owing to the increasing variance in environmental factors with age (Watkins and Farrall, 2006). A family history of premature coronary, cerebrovascular or peripheral vascular disease has been well-established risk factor for CAD by the Framingham Heart Study, PROCAM study, and the INTERHEART study, with the odds ratio (OR) ranging from 1.45-2.4 (Lloyd-Jones *et al*., 2004; Assmann *et al*., 2002; Yusuf *et al*., 2004; Watkins and Farrall, 2006).

Although it has been well-accepted that the genetic factors have contributed to CAD susceptibility, the magnitude of their effect and number of contributing effects could not be determined until all CAD-susceptibility genes are identified. Due to the long list of known risk factors and many traits having their own genetic basis, the number of candidate genes could be large. Successful identification of these susceptibility genes for CAD could be dependent on a combination of classical candidate-gene studies and genome-wide positional cloning. Similarly, combined genetic and genomic approaches have the potential to reveal new realms of quantitative heritable variations that influence the biological processes underlying CAD (Watkins and Farrall, 2006).

Table 2-1. Mendelian diseases relevant to premature CAD

| Disease | Mutated gene | Prevalence | Phenotype and Mechanism | Reference |
|---|---|---|---|---|
| Familial hypercholesterolemia | *LDLR* | 1/500 | Decrease in LDL uptake by the live due to the defective binding of LDL by receptor | Brown and Goldstein, 2001 |
| Familial defective ApoB | *ApoB* | 1/3250 | Reduced binding of ApoB to LDLR | Fisher *et al.*, 1999 |
| Sitosterolemia | *ABCG5, ABCG8* | rare | Increased absorption of plant sterols by the intestine | Heimerl *et al.*, 2002 |
| Autosomal recessive hypercholesterolemia | *ARH* | rare | Defective endocyytosis of LDLR in hepatocytes | Garcia *et al.*, 2001 |
| Tangier disease | *ABCA1* | rare | Impaired cholesterol and phospholipids efflux, resulting in very low level of HDL | Rust *et al.*, 1999 |
| | *ApoA1* | rare | Delection or loss-of-function mutation results in the virtual absence of HDL | Matsunaga *et al.*, 1991 |
| Ad CAD1 | *MEF2A* | Rare | A mutation in MEF2A results in dominant vascular disease | Wang *et al.*, 2003 |
| Homocystinuria | *CBS* | Rare | Impaired conversion from homocysteine to cystathionine results in very high homocysteine and severe occlusive vascular disease | Kluijtmans *et al.*, 1999 |

*ABCG*, ATP-binding cassette, subfamily G; *ARH*, autosomal recessive hypercholeterolaemia; *MEF2A*, myocyte enhancing factor-2; *CBS*, cystathionine β-synthase

## 2.2 Genetic epidemiological study of complex disease

### 2.2.1. Genetic variations

Genetic variations refer to the changes in DNA sequence and a variation with an allele frequency of at least 1% in the population is considered as a polymorphism. There are several types of polymorphisms in the genome: single nucleotide polymorphisms (SNPs), tandem repeat polymorphisms, and insertions or deletions, which range from a single base pair to thousands of base pairs in size. SNP refers to a single nucleotide in a locus having two, or sometimes three, forms in the population. There are two classes of SNPs: transition, changing from pyrimidine to pyrimidine or from purine to purine, and transversion, changing from pyrimidine to purine or from purine to pyrimidine. The transition is the most common single base substitution. A tandem repeat polymorphism consists of variable lengths of sequence motifs that are repeated in tandem in a variable copy number. Tandem repeat polymorphisms are subdivided into two subgroups based on the size of the tandem repeat units: micro-satellites (short tandem repeats, STR) and mini-satellites (variable number of tandem repeats, VNTRs). The STR repeat unit consists of only 1-6 base pairs (bp) while the VNTR repeat unit ranges from 10-50bp. The most common microsatellites are dinucleotide, trinucleotide, and tetranucleotide, and they occur once in every 10kb in eukaryotic genomes. Human microsatellites are informative in linkage studies as there are many alleles present at a microsatellite locus.

SNP is the simplest and most common type of genetic variation with one approximately every 180 bp, making up 90% of natural variation in the human genome (Crawford *et al.,* 2005). In the latest release of SNP database (dbSNP) in the National Centre for Biotechnology Information, there are more than 27 million of SNPs

recorded with more than 4 million of SNPs lying within genes (build 125) (Serre and Hudson, 2006). It is believed that these SNPs within genes, especially those within promoter regulatory regions, encoding regions, or splicing sites, are more likely to be deleterious or beneficial to humans than those within intergenic spaces.

For these SNPs within encoding region, they are called non-synonymous SNP (nsSNPs) if they alter the encoded amino acid or synonymous SNPs if they do not change any amino acid. NsSNPs may be the cause of most known inherited monogenic disorders and may be routinely analyzed for diagnostic purposes. However, most SNPs are located in non-coding regions and may have no direct known impact on an individual but are shown to be associated with certain traits. These SNPs have been found to be useful markers in population genetic association studies of complex diseases.

SNPs most often result from a non-repaired error that occurs during DNA replication. The frequency of the error is relatively low ($10^{-8}$ substitution per base per generation), thus, the vast majority of SNPs are inherited rather than de novo mutations. If individuals share the same allele at one position, they are most likely from same ancestor rather than two independent mutations occurring on them (Serre and Hudson, 2006).

A few public SNP database have been established, such as dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP), the SNP consortium (TSC) (http://snp.cshl.org), a database of Japanese SNPs (JSNP database) (http://snp.ims.u-tokyo.ac.jp) and Human Genome Variation database (HGVbase) (http://hgvbase.cgb.ki.se/). The dbSNP aims to catalog variations throughout the

genome, regardless of their functional consequences and was established by the NCBI in collaboration with the National Human Genome Research Institute (NHGRI) (Sherry *et al*., 2001). TSC was established in 1999 as a collaboration of several companies and institutions. Its initial goal was to discover 300,000 SNPs within two years, but to date nearly 1.8 million SNPs had been characterized. JSNP is a database for SNP in Japanese population with emphasis on the identification of SNPs located in genes or in adjacent regions that might influence the coding sequence of genes (Hirakawa *et al*., 2002).

### 2.2.2. The discovery of genetic variations

Direct DNA sequencing is the "gold standard" in the identification of genetic variation. It is an expensive methodology. As such, the cost of identifying sequence variants in a gene can be substantially reduced by prescreening methods, especially when the gene contains multiple exons. The prescreening methods are able to distinguish SNP alleles without identifying the exact position of the SNP and the affected base pair. Each novel SNP would subsequently require sequencing for its confirmation and characterization. Here, several classical pre-screening methods will be reviewed and their principles, advantages and disadvantages, will be briefly discussed.

#### 2.2.2.1. Single-strand confirmation polymorphism (SSCP)

SSCP is based on the principle that a single base change in the DNA sequence can cause single-strand DNA to migrate differently under non-denaturing electrophoresis conditions. SSCP analysis involves the denaturation of the double-strand PCR product, immediate cooling of the denatured DNA, followed by gel electrophoresis under non-denaturing conditions. With the exception of traditional incorporation of

radioisotope labeling and sliver staining, recent advances in SSCP have made the technique more convenient, safe, and have improved efficiency of detection through the application of fluorescent dye-labeled PCR primers, and more recently, capillary-based electrophoresis (Suh and Vijg, 2005). SSCP is a simple technique; however, its sensitivity depends on the fragment size and sequence and the detected fragment should be shorter than 250bp.

#### 2.2.2.2. Cleavage fragment length polymorphism (CFLP)

CFLP is a prescreening method based on the reproducible hairpin duplexes during the self-annealing of single-stranded DNA, the hairpins are cleaved by endonuclease cleavage I at the 5' side of the junctions between the single strand and duplex region (Lyamichev *et al*., 1993). Compared with SSCP, CFLP is more rapid and accurate and permits the analysis of larger fragment. However, the assay time and temperature need to be optimized for each type of DNA fragment to generate reproducible hairpin duplexes (Suh and Vijg, 2005).

#### 2.2.2.3. Denaturing gradient gel electrophoresis (DGGE)

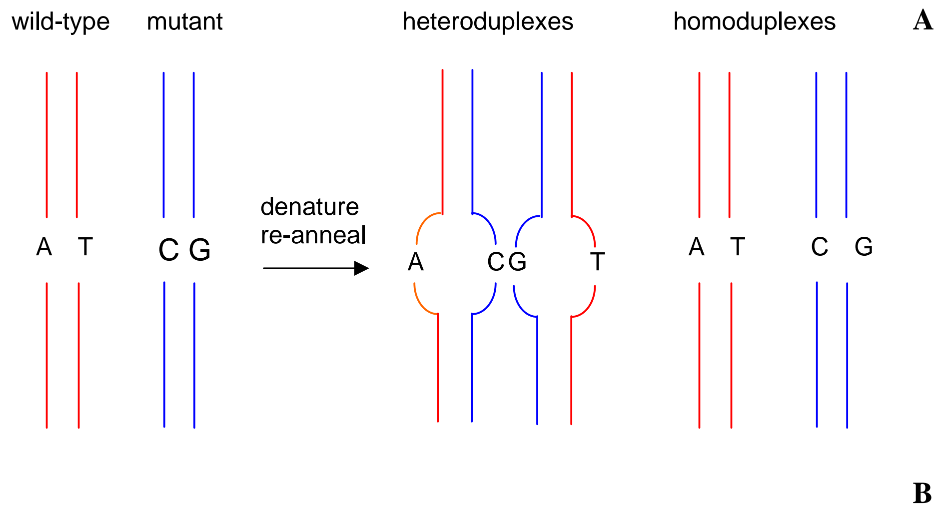
DGGE has been used to separate DNA fragments based on the principle that the mobility of a partially melted double-stranded DNA in polyacrylamine gels increases compared with its complete helical form. DNA fragments with different sequences may have different melting behaviors and will therefore stop migrating at different positions when they are subjected to a gradient of increasingly denaturing conditions. Although DGGE appears to be a relatively popular technique with high sensitivity, some disadvantages greatly hinder its rapid application, such as the designing of primers for optimal PCR amplification, optimal melting behavior of the amplicons

and optimal two-dimensional gel distribution (van Orsouw *et al.*, 1998; Balogh *et al.*, 2004).

#### 2.2.2.4. DHPLC

DHPLC is a relatively recent method that has rapidly gained popularity. Its major advantage is in being an automated alternative to gel-based techniques, requiring no post-PCR sample processing (Suh and Vijg 2005; Xiao and Oefner, 2001; Rudolph *et al.*, 2002).

The basic principle of DHPLC for screening sequence variations is that the DNA helical structure tends to be unstable at or close to its melting temperature, at which 50% of the DNA strand is single-stranded and 50% is double-stranded. The heteroduplexes display different melting properties from their corresponding homoduplexes due to the presence of mismatch base pairs in the former (Figure 2-1 A). The heteroduplexes were formed upon mixing, denaturing, and re-annealing of two or more chromosomes which differ in sequence in a single base pair or the presentation of a short insertion in single copy. As a result, when being analyzed on a reverse-phase column using DHPLC under partially denaturing temperatures, the heteroduplexes elute from the column earlier than the homoduplexes because of their reduced melting temperature (Xiao and Oefner, 2001). As the fragments elute, they are detected by an UV detector and analyzed as chromatograms. Analysis can be performed on individual samples to determine heterozygosity, or on mixed samples to identify sequence variation between individuals (Figure 2-1 B).




B

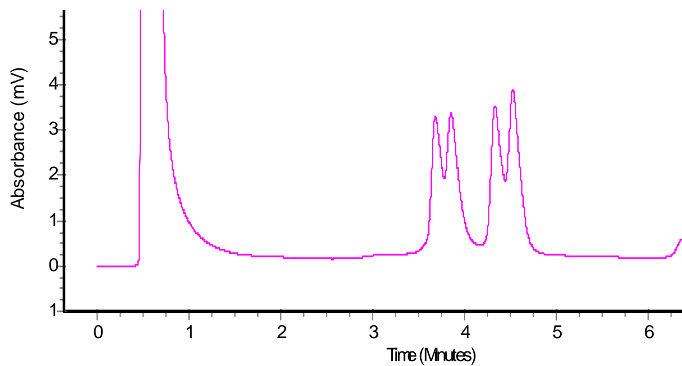



**Figure 2-1**. The principle of the DHPLC. **A**: Heteroduplex formation through hybridization after heating and cooling the PCR products. **B**: the typical DHPLC profile of a heteroduplex

Separation of the two forms of duplex DNAs by DHPLC is based on ionic forces between the negatively charged DNA and the hydrophobic stationary phase, which consists of C (18) chains on non-porous polystyrene-divinylbenzene (PSDVB) particles (2.1± 0.12酸 . The PSDVB particles are coated with positively charged ion-pairing agent triethylammonium acetate (TEAA), acting as a "bridge" molecule between negatively charged DNA fragments and the electrically neutral and hydrophobic stationary phase. The elution of the adsorbed DNA is achieved by an increase in the concentration of organic solvent (acetonitrile) in the mobile phase

(Rudolph *et al.*, 2002). The linear gradient of acetonitrile allows separation of DNA fragments based on size and/or presence of heteroduplexes.

Other than the concentration of acetonitrile, temperature is another factor which affects the retention of DNA fragments (Xiao and Oefner, 2001). Temperature determines the sensitivity of DHPLC, and the optimum can be determined either empirically or predicted using DNA melting analysis software provided with the Transgenomic WAVE DHPLC system.

Compared to the rest of the methodologies for mutation prescreening, DHPLC offers the advantage of being the automated, hand-free alternative to gel-based techniques, requiring no post-PCR ample processing (Suh and Vij, 2005). However, there are some limitations of DHPLC. One major one is the failure to detect homozygous mutants, which could hardly be differentiated from homozygous wildtypes. This limitation can be overcome by mixing wildtypes and homozygous mutants at ratio of 1 to 1 to generate the heteroduplexes during denaturing and re-annealing post-PCR processing. In addition, the primers should be designed to generate a single partially denaturing domain. The optimal temperature could be empirically determined by running the samples at temperatures ranging from 2$^{o}$C above and below the temperature predicted by the WAVE System utility software.

### 2.2.3. Complex diseases

Unlike monogenic diseases which result from mutations in a single gene, complex diseases are influenced by multiple factors, such as environmental influences, gene-environment interactions, and interactions among genetic variants at different loci. Complex diseases, such as CAD, cancer, diabetes, asthma, hypertension,

obesity, and schizophrenia are very common. In addition, complex diseases vary in severity of symptoms, age of onset, and even in their etiological mechanisms. Finally, an important feature of complex diseases is that the contribution of each candidate gene and their genetic variants is small (Tabor *et al*., 2002).

### 2.2.4. Approaches for genetic study of complex diseases

Generally, two approaches are used to map the genes and to identify genetic variations that underlie common diseases and disease-related quantitative traits: linkage studies and association studies.

#### 2.2.4.1. Family-based genome-wide linkage studies

For the last two decades, linkage studies have been the most popular approach for associating genes and genetic variants with human diseases. The genome-wide linkage analysis is used to examine the genotypes of related individuals using numerous evenly distributed polymorphic markers throughout the genome to map the chromosomal regions that are associated with diseases or traits.

Linkage analysis has been successful for mapping genes underlying monogenic diseases (Hirschhorn and Daly, 2005). Genome-wide linkage analyses have also been carried out for many common diseases, such as inflammatory bowel disease (Hugot, *et al*., 2001; Ogura *et al*., 2001; Rioux *et al*., 2001; Stoll *et al*., 2004), schizophrenia (Stefansson *et al*., 2003) and type 1 diabetes (Nistico *et al*., 1996) and quantitative traits, leading to the discovery of variants that contribute to their susceptibility. However, any individual genetic factor generally have small to moderate effect on disease susceptibility. Therefore, only linkage studies with dense marker sites, large

sample sizes, and large pedigrees could yield meaningful results. For example, a linkage study which was able to detect the association of the Pro12Ala variant in the peroxisome proliferative activated receptor-γ gene (*PPARG*) with type 2 diabetes, required a genome scan of over one million affected sib pairs (Altshuler *et al*., 2000). As such, it is clear that most of these well-established disease-susceptibility alleles could never be detected by linkage study. Hence, association studies with increased statistical power should be carried out as a strategic complement to linkage analysis.

#### 2.2.4.2. Association studies

A genetic association study is designed to determine whether genetic variants are associated with the frequency or severity of particular traits. Association studies provide a direct way to unravel the genetic contribution to the etiology of complex disease and help to predict susceptibility of an individual to certain diseases, as well as his or her response to environmental factors including response to drug treatment and adverse drug effects.

Here, two approaches of association studies, namely the candidate gene and the genome-wide approaches, will be reviewed.

##### 2.2.4.2.1. Candidate gene association studies

Candidate gene studies focus on genes that are related to a complex disease based on hypothesis about their etiological role in the pathophysiology. Candidate gene studies are usually conducted with a population-based case-control design. In the candidate-gene approach, the genetic influences on a complex trait are usually studied by carrying out the following: generate hypotheses, identify candidate genes, identify

variants in or near those functional genes or those that are in LD with functional changes, genotype variants in a population, and finally analyze the genotyping data statistically to determine whether there is a correlation between those variants and phenotypes (Tabor *et al.*, 2002; Hirschhorn and Daly, 2005). The two general strategies for analyzing genotyping data are comparing the allelic and genotype frequencies between the samples and allelic association. By means of association studies, variations in candidate gene sequences can be interpreted as providing a protective or a disease-causing effect.

Candidate genes are selected on the basis of different types of information, including available linkage results and results from mouse models of the phenotype. When examining the association of genetic variants in a particular candidate gene with the disease of interest, it is important to select carefully a limited number of SNPs to genotype. In theory, it is desirable to study only those polymorphisms that affect the function or expression of the protein (Tabor *et al*., 2002). However, in most situations, the information on the effects of these genetic variants is not available. Therefore, the information about the location and the type of genetic variants can be used for the selection of polymorphisms. Generally, nsSNPs and nonsense changes that result in premature stop codon are most likely to affect disease susceptibility, and therefore they should be given the highest priority for genotyping in candidate-gene studies (Hirschhron and Daly, 2005; Tabor *et al*., 2002). In addition, the correlation with potential causal variants (LD) and technological considerations including the availability of high-throughput, lower cost pre-selected SNP sets are also the criteria of polymorphism selection (Newton-Cheh and Hirschhorn, 2005).

Although candidate gene association study takes advantage of both great statistical power and biological understanding of the disease, it has been subjected to criticisms. The most common problem is the lack of reproducibility; many candidate gene association studies have not been replicated in subsequent independent studies. The possible reasons for the discrepant findings are listed as follows: 1) Studies might differ in the selected population and true association in one population may not be true in another due to heterogeneity in genetic and environmental background; 2) The definition of phenotype might be different, which leads to the different inclusion/exclusion criteria of subject; 3) Type I error could arise from statistical fluctuation and the widespread, inappropriate use of *P*-value below 0.05 as a criterion for declaring association; 4) Inconsistent results could be due to technical errors in genotyping; 5) False association could arise from performing data analysis of quantitative traits if appropriate transformation to achieve normality of non-Gaussian traits and removal of outliers are not carried out. In addition, population stratification, confounder publication bias, and disease misclassification are all potential problems of association study.

**2.2.4.2.2. Genome-wide association studies**

Although candidate gene association studies have been proposed as a powerful means of identifying the common variants that underlie complex disease, it relies on the biological hypotheses generated or the fundamental location of the candidate genes within a previously determined region of linkage. When these information and the fundamental physiological defects of a disease are unknown, the candidate-gene approach will be unable to fully explain the genetic basis of the disease. In the absence of convincing evidence regarding the function or location of the causal genes,

the unbiased and comprehensive genome-wide association approach is a preferred option. Genome-wide association studies involve genotyping a sufficiently comprehensive set of variants in a large patient sample and surveying most of the genome for causal genetic variants without having to guess the identity of the causal genes (Collins *et al.*, 1997). Due to the expense and labor involved, genome-wide studies have not been applied widely and only a few reports are available (Ozaki and Tanaka, 2006; Duerr *et al*., 2006; Donfack *et al*., 2006; Fung *et al*., 2006). However, with the development of high-throughput genotyping technologies and the determination of LD patterns on a genome-wide scale through the HapMap project (Need and Goldstein, 2006; de Bakker *et al*., 2006; Conrad *et al*., 2006; The International HapMap Consortium, 2003), more genome-wide association studies will be carried out and should greatly advance our understanding of the genetic basis of common diseases and complex traits.

#### 2.2.4.2.3. Association tests

To observe the association of genotypes with disease status, the most common analysis of SNP genotypes and case-control status at a single locus is to test the null hypothesis of no association between rows and columns of the 2x3 matrix that contains the counts of the three genotypes among cases and controls (Balding, 2006). In terms of association of allele with disease, the 2x2 contingency table is used to test the null hypothesis of no association. Considering the effect of confounding factors, linear and logistic regression methods would be advantageous as they are able to adjust these confounding variables (Clayton and McKeigue, 2001).

For the association with quantitative traits, analysis of variance (ANOVA) is a statistical method that is generally used. Linear regression is an alternative tool to

ANOVA by achieving a reduction in degrees of freedom from 2 to 1 by assuming a linear relationship between mean value of the trait and genotype (Balding, 2006). In either case, the traits have to be normally distributed and with an equal variance.

Haplotype is a popular strategy in association studies with multiple loci involved. In regions of little recombination, haplotype can capture the correlation structure of SNPs. More importantly, haplotype can capture the combined effects of tightly linked variants (Balding 2006). We can interpret haplotye as complete data and genotype as incomplete data from the statistical viewpoint, as we can extract all genotype data from haplotype, while the revere is not the case. Therefore, it is generally more useful to consider the association based on haplotype or diplotype rather than genotype (Ito *et al*., 2004). However, haplotype poses the problem of not being observed directly without involvement of family members. They must be inferred and some uncertainty could arise in phase inference and the uncertainty may cause some loss of information. However, the information loss is usually small when LD between markers is strong (Balding, 2006).

#### 2.2.4.2.4. Data analysis of association studies

Other than a well-designed study, appropriate statistical methods are very crucial to minimize the chance of false positives. First of all, data quality is of paramount importance. Testing for Hardy-Weinberg equilibrium (HWE) using a Pearson goodness-of-fit test should be done before processing the analysis of association (Balding, 2006). Rather than genotyping error, deviation from HWE can be due to population stratification, inbreeding, selection, and gene drift (Balding, 2006).

If multiple loci have been genotyped for association studies, haplotypes should be more informative than genotypes. The haplotype frequencies can be inferred using softwares, such as SNPHAP (Adkins, 2004), PHASE (Stephen *et al*., 2001), and SNPAlyze from Dynacom (Kanagawa, Japan).

Whether these polymorphisms of interest are in LD should be examined when multiple loci are analyzed. LD, simply defined, is the nonrandom association of alleles at linked loci (Jorde, 2000) and it describes the tendency of alleles to be inherited together more often than would be expected under random segregation. The measure of LD, *D* quantifies disequilibrium as the difference between the observed frequency of a two-locus haplotype and the expected frequency if the alleles were segregating at random (Ardlie *et al.*, 2002). *D*, one of the simplest measures of LD is equal to $P_{AB}$-$P_A$ X $P_{B,}$, where $P_{AB}$ is the observed frequency of the haplotype of alleles A and B, and $P_A$ is the frequency of allele A at the first locus and $P_B$ is the frequency of allele B at the second locus. The most common measures are the absolute values of *D'* (Lewontin, 1964), and $r^2$ (also denoted by $\Delta^2$) (Hill and Robertson, 1968). The absolute value of *D'* is determined by dividing *D* by its maximum possible value, given allele frequencies at two loci, while $r^2$ is formed by dividing $D^2$ by the product of the four allele frequencies at the two loci. The case of *D'* = 1 or $r^2$ = 1 is known as complete LD. However, *D'* values that are near to 1 provide a useful indication of minimal historical recombination, but intermediate values should not be used for comparisons of the extent of LD. When $r^2$ is used as a pairwise measure of LD for association studies, values above 1/3 might indicate sufficiently strong LD is present between two loci (Ardlie, 2002; Jorde, 2000).

## 2.3. Dyslipidemia

### 2.3.1. Lipids and lipoproteins

The major plasma lipids are cholesterol and TGs. Cholesterol is an essential structural component of cell membranes and is the precursor of steroid hormones and vitamin D while TGs are energy sources. These lipids are transported as components of the lipoprotein particles in the plasma, from sites of synthesis and absorption to sites of uptake. These lipoprotein particles contain a hydrophobic core of CEs and TGs, with a hydrophilic surface coat consisting of phospholipids, free cholesterol, and apolipoproteins, which provide plasma lipoproteins with structural stability and solubility. According to their densities, lipoproteins can be classified into four major subtypes: chylomicron (<0.95g/ml), VLDL (0.95-1.006g/ml), LDL (1.019-1.063g/ml) and HDL (1.063-1.210g/ml). Chylomicrons are produced for the purpose of transporting dietary TGs and cholesterol absorbed by intestinal epithelia, while VLDLs are formed to transport endogenously derived TGs to extra-hepatic tissues. In addition to TGs, VLDLs contain some cholesterol and CEs and the apoproteins, apoB-100, apoC-I, apoC-II, apoC-III and apoE. IDLs are derived from TG depletion of VLDLs. IDLs can be taken up by the liver for reprocessing or upon further TG depletion, be converted to LDLs. LDLs are the primary plasma carriers of cholesterol for delivery to all tissues from the liver and apoB-100 is the exclusive apolipoprotein of LDLs. HDL facilitates the reverse cholesterol transport from peripheral cells to the liver.

### 2.3.2. Definition of dyslipidemia

Dyslipidemia describes a range of disorders that include both abnormally high and low lipid and lipoprotein levels, as well as disorders in the composition of these particles. The National Cholesterol Education Programme (NCEP) Adult Treatment

Panel (ATP) III defines dyslipidemia as an elevation of serum TGs, presence of small LDL particles and low HDL-cholesterol level. No standard and non-expensive methodologies are available for the measurement of small LDL particle and approximately 70% of total plasma cholesterol is partitioned into LDL, thus, the measurement of total plasma cholesterol concentration is a surrogate of LDL level. In practice, dyslipidemia is defined as total plasma cholesterol (TC) $\geq$ 240mg/dl (6.15mmol/L) or TG $\geq$200mg/dl (2.25mmol/L).

Dyslipidemia is a heterogeneous group of disorders which can be classified in several ways. According to etiology, dyslipidemia can be classified into primary and secondary dyslipidemia. Based on the biochemical phenotype, dyslipidemia can be classified into (1) mainly hypercholesterolemia, (2) a combined increase in cholesterol and TGs and (3) mainly hypertriglyceridemia.

### 2.3.3. Dyslipidemia and CAD

Dyslipidemia is a prominently important risk factor for CAD. In this thesis, two main types of dyslipidemia, namely, hypercholesterolemia and hypertriglyceridemia are reviewed.

The casual relationship between cholesterol and atherosclerosis has been extensively surveyed from three aspects, epidemiological studies, clinical trials, and pathological studies, by Steinberg (2002; 2004; 2005[a], 2005[b], and 2006). In population studies, the Framingham Heart Study (Kannel *et al*; 1961), the Seven Countries Study (Keys, 1970 and 1980), and a cohort study in three large cohorts (Stamler *et al.,* 2000) have provided the evidences linking hypercholesterolemia to CAD. Numerous clinical

trials with larger number of subjects have conclusively shown that cholesterol-lowering treatment reduces both coronary events and total mortality (Libby *et al*., 2000; NCEP, ATPIII, 2002; Yokoi, *et al*., 2005; Ballantyne, 2005; Huse *et al*., 2006). Moreover, the pooled results of many clinical trials using different cholesterol-lowering regimens indicated that for every 10% reduction in plasma cholesterol level, CAD mortality will be reduced by 15% (Gould *et al.*, 1998); patients who received statin treatment demonstrated a 20% to 30% reduction in death and major cardiovascular events compared with patients who received placebo (Ross *et al.*, 1999). Other than these epidemiological studies and clinical trials, pathological studies also showed that the early lesion of atherosclerosis consists of subendothelial accumulation of foam cells. It was nearly 100 years ago that cholesterol had been found to cause arterial lesions by the Russian investigators, Antisckkow and Chaltow (Anitschkow anf Chalatow, 1913). The trigger event for atherosclerosis is the accumulation of oxidized LDL, which stimulates the underlying endothelial cells to produce a number of pro-inflammatory molecules (Ross, 1999), which then lead to foam cell formation and atherosclerosis development.

In summary, the causal relationship between blood cholesterol and coronary artery atherosclerosis is generally accepted. Cholesterol is not only a component of atherosclerotic plaques but also is an initiator of atherosclerosis (Steinberg, 2002). Therefore, cholesterol-lowering treatment is one of the most promising therapeutic targets in the prevention of coronary atherosclerosis, especially those with hypercholesterolemia.

The impact of hypertriglyceridemia on CAD risk has long been a matter of intense debate. However, during the last decade, a considerable number of evidences have been gathered to support hypertriglyceridemia as an independent risk for CAD (Hokanson and Austin, 1996; Assmann *et al.,* 1996; Yarnell *et al.,* 2001). Clinical trials also demonstrated the benefits of triglyceride lowering alone on clinical or cardiovascular outcomes (Fruchart *et al*., 2004). In addition, a meta-analysis of prospective studies conducted in the Asia-Pacific region also suggested that serum TGs are an important and independent predictor of CAD and stroke risk in this region (Patel *et al*., 2004)

**2.3.4. Genetics of dyslipidemia**

Dyslipidemia can be monogenic disorders or a part of a complex genetic disease. Some monogenic dyslipidemia, such as familial hypercholesterolemia and sitosterolemia, are found to be associated with atherosclerosis. Studies on these monogenic disorders have provided important insights into the fundamental biology of cholesterol metabolism. For example, studies of familial hypercholesterolemia led to the discovery of LDLR and the elucidation of the feedback control of cholesterol synthesis (Brown and Goldstein, 2001). In addition, the study of monogenic diseases can lead to successful drug therapies for atherosclerosis. For instance, the discovery of LDLR led to development of statin drugs, which significantly reduce cholesterol and CAD mortality. Statins are used as routine medication not only for the treatment of CAD by tens of millions of CAD patients but also for the prevention of CAD by the healthy. Last but not least, monogenic diseases also provide a logical basis for candidate gene study for the more common forms of dyslipidemia (Lusis *et al.*, 2004). However, monogenic dyslipidemia can

only account for a small percentage of atherosclerosis and most dyslipidemia are regarded as complex diseases involving common variations in candidate genes.

Over the years, many genes encoding apolipoproteins, lipoprotein receptors, and enzymes involved in plasma lipid metabolism have been evaluated to examine the possible associations with levels of plasma lipids and lipoproteins. Some of those associations observed are convincing and have been replicated. For example, three common apoE alleles exhibit marked effects on plasma cholesterol level in Western populations, accounting for 4% or more in total plasma cholesterol variation and increased atherosclerosis risk (Lusis *et al.,* 2004) Furthermore, the apoE genetic variation is also associated with type III hypercholesterolemia and >95% of type III hyperlipoproteinemia individuals have the E2/2 phenotype. However, only $1/50^{th}$ of E2/2 individuals manifest the overt disease (Mahley and Rall, 1995; Breslow, 2000). These studies also implied that lipid disorders are complex diseases and can be influenced not only by genetic but also by environmental factors. Identification of these genetic factors and quantifying their effects are an area of great interest and the objective of much current research.

### 2.4. Acyl-coenzyme A: cholesterol acyltransferase (ACAT)

ACAT (EC2.3.1.26) catalyzes the formation of CEs by using cholesterol and long-chain fatty acids as substrates, and ATP and coenzyme A (CoA) as co-factors. The esterification of cholesterol serves several important functions in cholesterol metabolism: 1) Addition of long-chain fatty acids to cholesterol reduces its solubility in the cell membrane phospholipid bilayer and promotes CEs to be stored as lipid droplets within the cytoplasm (Rudel and Shelness, 2000). This process prevents

toxic accumulation of free cholesterol in various cell membrane fractions. 2) The cholesterol esterification is required for the intestinal cholesterol absorption, since 75-80% of cholesterol absorbed undergoes esterification before incorporation in chylomicrons (Klein and Rudel, 1983). 3) CEs are also required for the synthesis and secretion of apoB-containing lipoproteins (e.g., VLDL) by the liver (Buhman *et al.*, 2000). 4) ACAT has a role in the accumulation of CEs in macrophages and vascular tissue, an event that is central to foam cell formation and atherosclerosis.

There are two forms of ACAT, ACAT1 and ACAT2, which catalyze the formation of CEs using cholesterol and fatty acids. However, their distribution, physical structures and physiological functions in cholesterol metabolism are distinct from one another. Another enzyme catalyzing the formation of CEs, lecithin cholesterol acyltransferase (LCAT), is noteworthy. LCAT transfers an acyl chain from phosphatidylcholine (lecithin) to cholesterol. Furthermore, the CEs provided by LCAT are responsible for the maturation of HDL particles (Chang et al., 1997) and have anti-atherosclerotic potential (Lee *et al*., 2004). Before reviewing these characteristics, the identification of ACAT2 is described below.

### 2.4.1. Identification of ACAT2

The first evidence suggesting that at least two forms of ACAT exist was obtained by Kinnunene and his colleagues in early 1988. They found that ACAT activity in pancreas was inhibited by 94% while hepatic ACAT activity was only inhibited by 6% (Kinnunen *et al*., 1988) by chemically modifying an active-site histidine residue. In 1996, two ACAT-related sterol-esterifying enzymes (Are1p and Are2p) were simultaneously but independently identified in yeast (Yang *et al.*, 1996; Yu *et al.*,

1996). However, convincing evidence that at least two mammalian intracellular cholesterol esterifying enzymes must exist came from *ACAT1*-/- mice, as the esterification activity was absent in the adrenal glands, but not significantly reduced in the liver and small intestine (Meiner *et al.*, 1996). In 1998, the immunodepletion using anti-human ACAT1 monoclonal antibody further supported this hypothesis. After immunodepletion, ACAT activity was inhibited by 80% in adult human liver, adrenal glands, macrophages and kidneys, while ACAT activity was only inhibited 20% in human intestine (Lee *et al.*, 1998). All these studies led to the identification of a second mammalian ACAT isoform, designated ACAT2, in monkeys, mice, and humans (Anderson *et al.*, 1998; Cases *et al.*, 1998; Oelkers *et al.*, 1998).

**2.4.2. Distribution and function of ACAT2**

Whereas ACAT1 is ubiquitously present in a variety of tissues and cell types in mammals, ACAT2 is only expressed in the small intestine and liver (Lee *et al.*, 1998; Oelkers *et al.*, 1998; Chang *et al.*, 2000; Sakashita *et al.*, 2000). Even in same tissue, the cell types of their expressions are also different. In the liver and intestine, ACAT2 is confined to hepatocytes and enterocytes, which secrete apolipoprotein B-containing lipoproteins, while ACAT1 is present in numerous other cell types, such as Kupffer cells and macrophages (Lee *et al.*, 2000; Parini *et al.,* 2004). It is clear that the intestine and liver are the only tissues to express ACAT2 under normal conditions and enterocytes and hepatocytes are the only cell types to abundantly express ACAT2 but do not express detectable amounts of ACAT1 (Rudel *et al.*, 2005).

The different distribution of these two ACATs determines their different physiological roles. ACAT2 facilitates cholesterol absorption in the intestine and the

incorporation of atherogenic CEs into apoB-containing lipoproteins (Buhman *et al.*, 2000), while ACAT1 mainly contributes to the foam cell formation in the early stage of atherosclerosis development (Meiner *et al.*, 1996; Chang *et al.*, 1997). The distinct functions of these two ACAT enzymes in cholesterol metabolism determine their different contributions to atherosclerosis development and render them tissue-specific targets for drug development.

### 2.4.3. ACAT2 and atherosclerosis

Due to the specific distribution of ACAT2 and its role in cholesterol absorption in the intestine, and in secretion and assembly of apoB-containing lipoproteins in the liver, the relationship between ACAT2 and atherosclerosis has attracted great interest among researchers. In 2000, a study in *ACAT2*-/- mice indicated that the deficiency of *ACAT2* results in a reduction in CE synthesis in the small intestine and liver, which in turn limits intestinal cholesterol absorption, cholesterol gallstone formation, and the accumulation of CEs in the plasma apoB-containing lipoproteins (Buhman *et al.*, 2000). In order to examine the contribution of *ACAT2* to atherosclerosis, *ACAT2*-/- mice were crossed with *apoE*-/- mice to generate double-knockout mice (Willner *et al.*, 2003). The results from this study suggested that it is the amount of ACAT2-derived CEs present in the core of these lipoproteins and not the number of apoB-containing lipoproteins that plays a critical role in the development of atherosclerotic lesions (Willner *et al.*, 2003). Further evidence for the atherogenic role of ACAT2 was obtained from studies in *LDLR*-/- mice with deletion of ACAT2, LCAT, or both enzymes together (Lee *et al.*, 2004). This study demonstrated that only LCAT and ACAT2 are responsible for synthesis of plasma CEs and the CEs provided by LCAT and ACAT2 have opposite atherosclerotic potential (Lee *et al.*,

2004). These data further supported the importance of ACAT2 in the development of atherosclerosis since this enzyme facilitates intestinal cholesterol absorption and incorporation of atherogenic CEs into apoB-containing plasma lipoproteins, which then appear to accumulate in the aortic intima and promoting atherosclerotic lesion development.

Although ACAT1 is the enzyme responsible for the formation of CEs in macrophages (Meiner *et al.*, 1996), ACAT1 deficiency did not prevent the development of atherosclerotic lesions in either *apoE*-/- or *LDLR*-/- mice (Accad *et al.*, 2000; Yagyu *et al.*, 2000). Instead, severe side effects, such as extensive cutaneous xanthomatosis, were observed (Accad *et al.*, 2000; Yagyu *et al.*, 2000). Furthermore, macrophage ACAT1 deficiency even promotes lesion formation (Fazio *et al.*, 2001). Together, these studies suggested that complete ACAT1 deficiency has been varyingly problematic.

All information available about ACAT1 and ACAT2 suggests that it would be beneficial to selectively inhibit ACAT2 to decrease atherogenicity of plasma lipoproteins without causing severe side effects and affecting normal membrane function to the body with the availability of adequate ACAT1 function (Rudel *et al.*, 2005).

### 2.4.4. ACAT2 protein structure

Human ACAT2 protein contains 522 amino acid residues and exhibits 48% sequence identity with ACAT1 at their carboxyl termini. The molecular weight of ACAT2 is 46 kDa, which is bigger than the predicted molecular size (Chang *et al.*, 2000).

Some studies have been performed to unravel the relationship between the structure and function of ACAT and its related protein, especially regarding the activity and substrate-binding site. Joyce *et al.* (2000) used mutagenesis methods to demonstrate that the conserved serine in the 245$^{th}$ residue of ACAT2 may be essential for ACAT activity. Guo *et al.* (2001) also suggested the importance of the conserved serine for enzyme activity and stability in the ACAT1 and Are2p, an ACAT-related-enzyme in yeast. Furthermore, Guo *et al.* (2001) also found that the mutation of a tyrosine or the tryptophan in the conserved FYxDWWN motif either decreased or nullified enzyme activity. Because the motif containing the conserved serine is absent from DGAT, a member in the acyltransferase family, they speculated that the conserved serine of (H/Y)SF may play a role in sterol binding and the FYxDWWN domain may mediate the binding of acyl-CoA. However, Lin *et al.* (2003) confirmed that the conserved serine is important for ACAT protein stability but not essential for ACAT catalytic activity, as they found that the mutagenesis of ACAT protein from serine to leucine or to alanine caused the mutant protein to be expressed at a much lower level. Instead, they found that a conserved histidine (His434) may be essential for ACAT2 catalysis. In addition, Guo *et al.* (2001) reported that the 31 amino acid residues at the amino-terminal region in Are2p, the ACAT-related-enzyme (Are)2, may mediate the enzyme activity in a negative fashion using the truncation approach, and suggested that it may be true for ACATs in general. However, whether the amino terminal amino acid residues have important biological function is unclear.

### 2.4.5. *ACAT2* genomic organization and its regulation of ACAT expression

The *ACAT2* gene (NT_029419) maps to chromosome 12 (12q13.13) and spans slightly over 18 kb. It is much smaller than the *ACAT1* genomic DNA, which is about 200kb in length (Li *et al.*, 1999). *ACAT2* comprises 15 coding regions and exon 1 contains 5'-untranslated region (5'-UTR) (AF332857, AF332858).

Most genes involved in the cholesterol metabolism pathway, such as *LDLR* and *HMG-CoA reductase* genes, are regulated by the sterol response element binding protein (SREBP) (Brown and Goldstein, 1986 and 1997). These gene promoters contain a 10-base-pair sterol-regulatory element and SREBP binds this element to mediate these gene expressions. However, the sterol-regulatory element could not be identified within the *ACAT1* and *ACAT2* promoters (Li *et al.*, 1999; Song *et al.*, 2001). These findings indicated that both *ACATs* are not regulated by the SREBP mode.

The 5'-flanking region *ACAT2* gene contains many potential *cis*-acting elements for multiple potential transcriptional regulatory factors, such as Cdx-2, HNF-3b, Sp1 and CEBP, but lack TATA and CCAAT boxes (Song *et al.*, 2001). There are studies to show that Cdx-2, HNF-1 alpha and beta play important regulatory roles in *ACAT2* gene expression (Song *et al.*, 2003; Pramfalk *et al.*, 2005). In addition, analysis of the promoter activity of serially deleted 5'-flanking regions of *ACAT2* suggested that there might be positive regulatory elements between -1299 to -768 (the transcription start site is designated as +1) (Song *et al.*, 2001).

### 2.4.6. Genetic analysis of *ACAT2*

Although the *ACAT2* gene certainly qualifies as a candidate gene for hypercholesterolemia and atherosclerosis, genetic association study of this gene has only been carried out in Japanese population (Katsuren *et al.*, 2001 and 2003). They used single-strand conformation polymorphisms (SSCP) to identify 4 polymorphisms and found the association of the heterozygotes of Thr254Ile with plasma apoCIII concentrations in 91 dyslipidemic subjects (Katsuren *et al.*, 2001). Whether there is any association of *ACAT2* polymorphisms with CAD susceptibility is still unknown. Furthermore, the association of the *ACAT2* polymorphisms with dyslipidemia and plasma lipid parameters is worth investigating in other non-Japanese populations. Genetic studies on this gene may offer the opportunity to identify the determinants of hypercholesterolemia and atherosclerosis and help to unravel certain structural and functional features.

## 2.5. Lipoprotein lipase (LPL)

LPL (EC 3.1.1.34) is a member of the lipase super-family that includes hepatic lipase, pancreatic lipase and LPL itself (Hide *et al.*, 1992). Research carried out over the past two decades has not only established the central role of LPL in the overall lipid metabolism and transport but also identified novel, non-catalytic functions of the enzyme. Abnormal LPL expression has also been found to be associated with atherosclerosis, dyslipidemia and diabetes.

### 2.5.1. Function and localization of LPL

LPL is a crucial enzyme in the removal of plasma TG-rich lipoproteins, such as chylomicrons and VLDL from the circulation, and provides non-esterified fatty acids

and 2-monoacylglycerol for tissue utilization. After the hydrolysis process, the chylomicrons in the intestine are converted to smaller remnants and transported into the liver. In the case of VLDL, the lipoprotein particles become smaller IDLs and LDLs after the hydrolysis of its TG component by LPL. This process also results in the generation of surface remnants, phospholipids and apolipoproteins, which give rise to HDLs. Therefore, LPL is critical for the formation of HDL particle (Lewis and Rader, 2005).

Other than its role in the hydrolysis of the TG component in chylomicrons and VLDL, LPL also fulfills other important functions. For example, LPL can function as a non-catalytic bridge to facilitate the uptake and cellulization of plasma lipoproteins and CEs through specific receptors, such as LDL receptor, and VLDL receptor (Pentik鋓en *et al*., 2002; Merkel *et al*., 2002[b]; Stein and Stein, 2003; Loeffler *et al*., 2006). In addition, LPL has been shown to stimulate the proliferation of VSMCs (Mamputu *et al*., 2000). Thirdly, LPL has been reported to have differential effects on several inflammatory pathways, such as tumor necrosis factor-alpha and interferon-gamma -mediated inflammatory cytokine signal transduction pathways in human aortic endothelial cells (HAECs) (Kota *et al.,* 2005). These inflammatory pathways are known to be important in atherosclerosis. Finally, LPL reduces the secretion of apoE from macrophages, which could significantly influence apoE accumulation in arterial vessel wall lesions (Mead *et al*., 2002).

Adipose and muscle cells are the major sources of LPL synthesis. LPL is synthesized and secreted in catalytically active form by adipocytes and myocytes and then transported to the luminal surface of the capillary endothelium. The

physiological function in capillary endothelium is to hydrolyze the TG component of chylomicrons and VLDL and IDL particles on the luminal side. The LPL in capillary endothelium drives the lipoprotein profiles in a non-atherogenic direction with increase in HDL levels. The vast majority of the total LPL in the body is located in capillary endothelium. However, LPL is also found in the arterial endothelium and most of the arterial LPL is derived from monocyte-derived macrophages and macrophage-derived foam cells and smooth muscle cells (Wang *et al*., 2006). The LPL on the arterial intima can lead to the retention of LDL in these structures by acting as a molecular bridge. Therefore, the arterial LPL has been suggested to have a role in atherogenesis. Taken together, the localization of LPL is curicial to determine whether its role in the development of atherosclerosis is predominantly pro-atherogenic or anti-atherogenic.

### 2.5.2. LPL and atherosclerosis

#### 2.5.2.1. The anti-atherogenic effects of LPL

LPL expressed in adipose tissue and muscle is believed to be anti-atherogenic because it aids the clearance of circulating lipoprotein particles and the formation of HDL. The anti-atherogenic effect of LPL has been backed by clinical and experimental evidences. Firstly, LPL-deficient patients were reported to develop premature atherosclerosis (Benlian *et al.*, 1996). Furthermore, individuals with heterozygous LPL mutations which lead to decreased enzyme activity (e.g. Asn291Ser, Gly188Glu, and Asp9Asn) or reduced expression (e.g. -93T>G in the promoter) have been reported to be predisposed to hyperlipidemia and premature atherosclerosis (Reymer *et al.*, 1995; Gilbert *et al*., 2001; Wittrup *et al.*, 2006; Goldberg and Merkel 2001; Frikke-Schmidt *et al*., 2006). The Ser447Ter, a naturally occurring mutation, has

been proven to increase LPL catalytic activity (Ross *et al.,* 2005). Most studies have suggested that this beneficial mutation was associated with decreased plasma TG levels, higher HDL and reduced risk of CAD (Rip *et al.,* 2006). Finally, administration of the compound NO-1886, a LPL activator, may contribute to improving atherosclerosis by increasing HDL cholesterol or influence the expression of inflammatory cytokines (Nakamura *et al*., 2006; Cai *et al*., 2006).

#### 2.5.2.2. The pro-atherogenic action of LPL

In contrast to LPL expressed in adipose tissue and muscle, the enzyme expressed in cells of the arterial wall, particularly macrophages and smooth muscle cells, is proposed to be pro-atherogenic (Babaev *et al.*, 1999 and 2000). Zilversmit was the first to propose that chylomicron and VLDL remnants produced by LPL would contribute to the development of atherosclerosis (Zilversmit, 1973). LPL induces the formation of atherogenic lipoprotein remnants by the following mechanisms. Firstly, LPL converts TG-rich lipoproteins to small and dense particles with enrichment in their CE contents and such remnants are taken up readily by macrophages (Zilversmit, 1995; Lindqvist *et al.*, 1983). In addition, LPL has been shown to act synergistically with sphingomyelinase in the lesion to enhance the association of LDL and the highly atherogenic lipoprotein(a) [Lp(a)] to the vascular wall (Tabas *et al.*, 1993; Mead *et al.*, 1999). Finally, additional functions of LPL, including stimulation of smooth muscle cell proliferation, adhesion of monocytes to endothelial cells and regulation of gene expression, may trigger other pro-atherogenic events (Mamputu *et al.*, 1997; Mamputu *et al.*, 2000; Renier *et al.*, 1994). It has been shown that the suppression of LPL expression attenuated pro-inflammatory cytokine secretion in THP-1 macrophages (Qiu *et al*., 2006).

### 2.5.3. The organization of LPL protein and gene

Mature LPL protein contains 448 amino acid residues. LPL consists of two structurally distinct regions, a larger amino-terminal domain (residues 1-312) and a smaller carboxyl-terminal end (residues 313-448). The C-terminus appears to be implicated in the uptake of lipoproteins by cell surface receptors whereas the N-terminus is important for the catalytic activity (Mead *et al.*, 2002).

The *LPL* gene is located on chromosome 8p22, spanning about 35 kb, and contains 10 exons separated by 9 introns. Exon 1 encodes the 5'–UTR while exon 10, notably longer than the others, encodes the entire 3'-UTR (Enerback and Gimble, 1993; Raisonnier *et al.*, 1995). *LPL* gene expression is regulated by transcriptional and post-transcriptional control (Mead *et al.*, 2002).

### 2.5.4. Genetic analysis of *LPL*

Because of the physiological importance of LPL in the catabolism of TG-rich lipoprotein particles and its possible role in lipid-related pathologies, LPL is an important candidate gene for association studies on atherosclerosis risk in the general population. About 100 natural-occurring polymorphisms have been identified in the *LPL* gene, of which 80% occur in coding regions and the others in non-coding regions (Murthy *et al.*, 1996). There are 61 missense mutations, most of which are located on exons 5 and 6; 12 nonsense mutations, 10 frameshift mutations or small insertions/deletions, 3 gross mutations, 8 splicing mutations, and 4 promoter variants (Merkel *et al.*, 2002[a]). Some functional mutations can influence LPL function in different ways, such as affecting catalytic activity, dimerization, secretion, and heparin binding (Razzaghi *et al.*, 2000), leading to altered lipid profiles. Some of

them have been associated with familial LPL deficiency (FLLD) and chylomicronemia. For instance, Gly188Glu has been identified in almost 50% FLLD in European and Indian ancestry (Mailly, *et al.*, 1997) and two families with type I hyperlipidemia (Paulweber *et al.*, 1991). Pro207Leu is the most common cause of familial chylomicronemia in French Canadians (Ma *et al.*, 1992). However, most of these functional LPL mutations are rare, either restricted to families with LPL deficiency or isolated geographic regions. For example, Gly188Glu, Asn291Ser, and Asp9Asn are common in some Western populations, such as the French Canadian population, but not in the Asian general population (Yoshida *et al.*, 2000; Yamana *et al.*, 1998: Yang *et al.*, 2003). In this review, only three most common genetic variants with modest effects on lipids profiles are surveyed.

The c.1342C>G, a C to G transversion, results in a premature stop codon truncating the LPL protein by two amino acids and it is usually defined as Ser447Ter or S447X. The c.1342C>G is a very common variant, occurring at a frequency approximately 20% in general population and has been reported to be the only variant with increased LPL activity (Merkel *et al.*, 2002[a]). Results from association studies suggested that the 1342G carriers had a beneficial lipid profile (Kuivenhoven *et al.*, 1997; Razzaghi *et al.*, 2000; Groenemeijer *et al.*, 1997; Arai *et al.*, 2005; Souverein *et al.*, 2005; Brousseau *et al.*, 2004; Morabia *et al.*, 2003; Chen *et al.*, 2001; Humphries *et al.*, 1998) and a reduced risk of atherosclerosis (Humphries *et al.*, 1998; Clee *et al.*, 2001; Arca *et al.*, 2000; Shimo-Nakanishi *et al.*, 2001). However, inconsistent results have been reported (Morrison *et al.*, 2002; Jemaa *et al.*, 1995; Fidani *et al.*, 2005).

Two variations, IVS6+1595C>T and IVS8+484T>G, in intronic regions, have been most extensively studied. The IVS6+1595C>T polymorphism, a C>T transition, can be identified by the cleavage of restriction enzyme *Pvu*II (Chamberlain *et al.*, 1991; Nicklas *et al.*, 2000), hence, it is usually defined as *Pvu*II polymorphism. The IVS8+484T>G polymorphism is a T>G transversion and can be identified by restriction enzyme *Hind*III (Heizmann *et al.*, 1991), so it is also known as *Hind*III polymorphism. The rare G allele of the IVS8+484T>G polymorphism has consistently been shown to be associated with lower TGs and/or elevated HDL-cholesterol, and the impact may be modulated by other environmental factors, such as smoking and physical activity (Senti *et al.*, 2001; Corella *et al.*, 2002; Whiting *et al.*, 2005; Reilly *et al.*, 2005). In addition, the IVS8+484T>G polymorphism has been shown to influence CAD susceptibility and the progression of atherosclerosis in several studies (Jemaa *et al.*, 1995; Taylor *et al.*, 2004; Socquard *et al.*, 2006). The results on the association of the IVS6+1595C>T polymorphism with plasma lipids have been inconsistent; increased TGs in T allele carriers were reported in some studies (Chamberlain *et al.*, 1989; Galton *et al.*, 1994; Duman *et al.,* 2006) but not in others (Peacock *et al.*, 1994; Jemaa *et al.*, 1995). Although the IVS6+1595C>T polymorphism has been reported to be associated with the severity of CAD in Australian whites (Wang *et al.*, 1996), no association was found with CAD in most studies in other populations (Galton *et al.*, 1994; Taylor *et al.*, 2004; Duman *et al.,* 2006).

The three polymorphisms, c.1342C>G , IVS8+484T>G, IVS6+1595C>T, have been found to be in LD in most populations (Chamberlain *et al.*, 1989; Hallman *et al.*, 1999; Razzaghi *et al.*, 2000). Furthermore, most IVS8+484T>G-associated effects could be

explained by the LD between the IVS8+484T>G and c.1342C>G (Humphries *et al*., 1998; Talmud and Humphries, 2001).

As mentioned above, although there have been many studies to examine the effects of *LPL* genetic variants, these results are inconsistent. Furthermore, most studies were carried out in Caucasian populations and only a few studies have examined the impact of these polymorphisms in Asian populations (Shimo-Nakanishi *et al*., 2001; McGladdery *et al*., 2001; Hall *et al*., 2000; Radha *et al*., 2006) and in Singaporeans (Lee *et al*., 2004; Liu, *et al*., 2004). To our knowledge, there are no large-scale studies to explore their combined effects of these three polymorphisms on CAD risk and lipid profiles in an Asian population. Therefore, other than individual effects of these polymorphisms, the combined effects should be investigated.

# 3. MATERIALS AND METHODS

## 3.1. Subjects

For polymorphism screening of the *ACAT2* gene, umbilical cord blood was collected from 336 consecutive newborns at the National University Hospital, Singapore. Informed consent was obtained from mothers of the neonates.

For the association studies, the CAD+ subjects were selected from patients who had been admitted to Singapore National Heart Center for coronary artery bypass graft surgery. All the patients had more than 50% stenosis in at least one of the major coronary arteries as revealed by angiography. The controls were recruited from workers sent by their employers to attend routine health screening. This screening included blood hemoglobin estimation, urine analysis for albumin and sugar, chest X-ray and resting electro-cardiogram. Subjects with any abnormal test results or who had a history of heart illnesses, stroke, diabetes or hypertension were excluded. All subjects gave informed consent.

A total of 809 CAD patients (CAD+) were included in the *ACAT2* gene association study. These comprised 526 Chinese, 137 Malays and 146 Asian Indians. A total of 1304 controls (CAD-) comprising 702 Chinese, 230 Malays and 372 Indians were studied.

However, only male Chinese and male Indians were studied in our *LPL* gene association study. Since the *LPL* gene has been extensively studied in many populations, we are interested to examine the genetic effect in male Chinese and

Indians, which have contrasting mortalities from CAD and Malays have intermediate mortality from CAD (Hughes *et al.*, 1990[a] and 1990[b]). The CAD+ patient samples comprised only 899 Chinese males and 302 Indian males and the CAD- controls comprised 538 Chinese and 305 Indians.

This study was approved by the Research and Ethics Committee, National University Hospital, which is the predecessor of the Institutional Review Board .

## 3.2. DNA analysis

### 3.2.1. DNA Extraction

After blood was collected, the white blood cells and plasma were separated by centrifugation at 1500g for 15 min. The white blood cells were used for DNA extraction and the plasma was freezed at -20$^{o}$C for the measurement of lipids. Afterwards, genomic DNA was isolated from white blood cells using Parzer's method (Parzer and Mannhalter, 1991). Five ml of blood sample was lyzed using 10ml of lysis buffer (0.32 M sucrose, 5mM $MgCl_2$, 0.01M Tris-HCl, 1% Triton X-100, pH 8.0) and then centrifuged for 10 min at 3000g. The supernatant was discarded and the pellet was re-suspended in 14ml of NaCl/EDTA solution (10mM NaCl, 10mM EDTA, pH8.0) and centrifuged as before. Then the pellet was incubated with 210痞of 20% sodium sarcosyl, 150痞of 7.5M ammonium acetate, 2.1ml of 6M guanidium hydrochloride and 60痞of 10mg/ml proteinase K at 60 $^{o}$C for 2-5min until the solution was clear. This digested mixture was cooled to 0$^{o}$C on ice and DNA was precipitated using 10ml of cold absolute ethanol. The precipitated DNA was dried at room temperature and then dissolved with 100痞of TE (10mM Tris-HCl, 1M EDTA, pH 8.0) and stored at 4$^{o}$C.

### 3.2.2. Primer design for polymorphism screening of *ACAT2* gene

The DNA sequence of the *ACAT2* gene was obtained from a public database (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=16163027; accession no. NT_030118). For the polymorphism detection, the regions of interest, such as the coding regions, the intron-exon boundaries, 5'-UTR, and the putative promoter region, were screened. Primers to amplify these regions in separate amplification reactions were designed using the program at http://seq.yeastgenome.org/cgi-bin/web-primer. The primer sequences and their related information are shown in Table 3-1. For the region, exon 9 and its flanking intronic boundary, an additional upstream primer with 20bp of GC content (CGCCCGCCGCCGCCCGCCGC) at the 5' end was used to increase the resolution (Marsh *et al.*, 2001; Narayanaswami and Taylor, 2001). All primers were purchased from Qiagen (Tokyo, Japan) or Alpha DNA (Montreal, Canada).

**Table 3-1**. PCR primers and DHPLC conditions

| Regions | Forward primer<br>Reverse primer | PCR Product Length (bp) | Annealing Tm (°C)(A-B | DHPLC Gradient* | DHPLC Tm (°C) |
|---|---|---|---|---|---|
| Promoter region (a) | 5'-TCTCCAGATGGCAATGGAAG-3'<br>5'-TCTGTGAGGCTTGATGGCTTG-3' | 384 | 60-53 | 59-65%B 3min | 60-62 |
| Promoter region (b) | 5'-AGGAACCCAGCAAAGAGGAAC-3'<br>5'-TGAGCATGTAGGCAGAAAGG-3' | 349 | 60-53 | 58-64%B 3min | 57-59 |
| Promoter Region (c) | 5'-CGTGAGTAGCACAGTGCCAA-3'<br>5'-TTGTCCCACTCAGCTCAGGTG-3' | 439 | 61-54 | 60-66%B 3min | 60-62 |
| Exon1 | 5'-CAGATAACCTATCGCACTCCC-3'<br>5'-CTTCTCTCCATAGCGCATCTC-3' | 323 | 62-56 | 60-66% B 3 min | 61-63 |
| Exon 2 | 5'-GTCTGCAGAACCCCAATTCC-3'<br>5'-AGCCGAGATTGTGCCAATGC-3' | 268 | 63-57 | 57-63%B 3 min | 60-64 |
| Exon 3 | 5'-CTGGTGAATGAAAGGATGGCTG-3'<br>5'-CTGATAAAGCTGGCAAGGGCAC-3' | 290 | 61-54 | 57-63%B 3 min | 62-64 |
| Exon 4 | 5'-TTGGCTCCCAAGTATTGACC-3'<br>5'-CTGGGGATGAGAAATGAGG-3' | 227 | 61-54 | 55-61%B 3min | 60-62 |
| Exon 5 | 5'-TGGAAGTTCTGGATCGCTAG-3'<br>5'-GTTGGGAGCAATGTTGGTAC-3' | 355 | 62-55 | 51-60%B 5 min | 61-65 |
| Exon 6 | 5'-CAGACTGAAGAGGAAGGGGAC-3'<br>5'-CCCCTCTGCTGAGATGGC-3' | 399 | 63-56 | 55-64%B 5 min | 61-64 |
| Exon 7 | 5'-CGATTTGCTTAAAGCCACACAGC-3'<br>5'-AAGAGGACTGAGTTCACAACCGG-3' | 164 | 62-56 | 51-57%B 3 min | 59-61 |
| Exon 8 | 5'-CTTACTAATCCACCCCTCTCATTC-3'<br>5'-GAGGGGAGAGTAGAAGGGTAAG-3' | 185 | 63-53 | 49-58%B 4.5 min | 60-63 |
| Exon 9 | 5'-CGTCCCAGGCTGAACAGAGAACAACA-3'<br>5'-ATGAGTCACAGGTCCACCCTT-3' | 205 | 63-52 | 53-59%B 3 min | 61-63 |
| Exon 10 | 5'-TGCTGGGCCAGAGGTCAATG-3'<br>5'-CACAGCTGCTCTAGCCCTAC-3' | 239 | 64-60 | 55-61%B 3 min | 62-65 |
| Exon 11 | 5'-GCCTCTTGTCCCCAACCAC-3'<br>5'-CACCCAACCTGGCTACCATC-3' | 205 | 63-56 | 52-58%B 3min | 61-64 |
| Exon 12 | 5'-GAACCCAGATGCTTGCTTACCTC-3'<br>5'-GAGAGCCCCTATTAACTGTGAGAG-3' | 202 | 63-56 | 53-59%B 3min | 61-63 |
| Exon 13 | 5'-GAGGAGCTCAGGGAGACTTAC-3'<br>5'-AGGCCTCCCACCATCAGTCTG-3' | 275 | 63-56 | 56-62%B 3min | 60-63 |
| Exon 14 | 5'-CACTCCTGGAGCTGGAATGAC-3'<br>5'-GGACTGGCTGTTGGTCTGTG-3' | 251 | 63-54 | 56-62%B 3 min | 61-63 |
| Exon 15 | 5'-CTTGGGGGTGATGGACTC-3'<br>5'-GTTTTGCAGGCAGAGAACTTGG-3' | 197 | 63-54 | 54-60%B 3 min | 59-62 |

*: buffer B percentage (start gradient - stop gradient).

### 3.2.3. PCR amplification

All amplification reactions were carried out in a 40μl of mixture containing 100ng of genomic DNA, reaction buffer, 0.2 mM each dNTP, 10% dimethyl sulfoxide, 40 pmol of each primer and 0.4U of DynaZyme II (Finnzymes, Espoo, Finland). The reaction mixture was denatured at 94°C for 2 min, followed by an initial 14 cycles of denaturing at 94°C for 30s and annealing from a range of temperatures as given in Table 3-1 at ramping rate of 0.5°C per cycle. The subsequent 20 cycles were run at annealing temperature B°C (Table 3-1). The final extension step is at 72°C for 10 min. The reactions were carried out in the T-GRADIENT Thermocycler (Biometra, Goettingen, Germany).

## 3.3. Survey of genetic variant in *ACAT2* gene from public resources

Before screening the regions of interest, known SNPs have been identified using the program at http://www.ncbi.nlm.nih.gov/projects/SNP/snp_blastByOrg.cgi. Other SNP resources, such as TSC at http://snp.cshl.org, JSNP at http://snp.ims.u-tokyo.ac.jp, and HGVbase at http://hgvbase.cgb.ki.se/, were also explored. We aimed to identify novel polymorphisms when we screened these regions using DHPLC.

## 3.4. DHPLC analysis

DHPLC was carried out using the Transgenomic WAVE® DNA Fragment Analysis system (Transgenomic, Omaha, USA). The amplified DNA fragments of interest were injected (3-10μl) automatically from the 96-well autosampler onto the DNAsep cartridge. They were then eluted at a constant flow rate of 0.9ml/min with a linear acetonitrile gradient that has been determined by the WAVEMaker software (Transgenomic) based on their size and GC. The gradient was achieved by combining

buffer A (0.1M triethylammonium acetate, pH 7.0) and buffer B (0.1M TEAA and 25% acetonitrile in water, pH 7.0). The DNA fragment elution was monitored with a UV detector at 260nm and displayed as chromatography using the Transgenomic WAVE MAKER$^{TM}$ software, and the chromatograms were compared and analyzed.

The processing time for each sample was about 7 min, comprising a 2.2-min lag for the detector, a 0.5-min sample loading with a 5% decrease in Buffer B, a 3-min linear gradient step with a slope of 5% decrease in Buffer B per minute, a 0.5-minute cleaning stage using 75% acetonitrile, and a 0.9-min equilibration before next injection. Homo- and hetero-duplex peaks were detected between the initial injection peak, which is produced by residual nucleotides and primers in the sample mixture, and the washing peak. The washing peak is produced by the acetonitrile wash at the end of each analysis.

PCR products were initially analyzed under non-denaturing conditions (50$^{o}$C) to assess the quality of the PCR products. A single sharp peak was taken as indication for pure PCR products that could be used for subsequent polymorphism screening at partial denaturation temperatures. The WAVE System utility software predicted the range of partial denaturation temperatures based on the DNA fragment sequence. The sample was run at 2$^{o}$C above and below the predicted temperature. Given that heteroduplexes are less stable, they denatured earlier than the homoduplexes and will be eluted first.

A minimum of 48 samples for each of three ethnic groups (Chinese, Malay and Indian) were screened by DHPLC. In order to increase the chance of heteroduplex formation, eight individual samples were pooled together by mixing same amount of PCR products from each individual sample. The mixture and all the individual samples were denatured at 95$^{o}$C for 4 min, and then gradually re-annealed by decreasing the temperature to 25 蜗 over a period of 45 min in a thermal cycler. The pooled samples

were screened first and if a heteroduplex peak was detected in the DHPLC profile, the corresponding 8 individual samples were then screened to find the mutant samples. The DHPLC gradient and the range of partial denaturation temperature are displayed in Table 3-1.

### 3.5. Sequencing

Samples that generated abnormal DHPLC heteroduplex patterns were further sequenced using ABI3100 to confirm the presence of the altered sequence. For this purpose, the genomic DNA from the samples with atypical DHPLC profiles were amplified using PCR and purified via Qiaquick gel extraction kit (Qiagen, Tokyo, Japan ) following agarose gel electrophoresis (if more than one band was observed by UV) or Qiaquick PCR purification kit (Qiagen, Tokyo, Japan). The purified PCR products were used as templates for sequencing with the ABI Prism BigDye Terminator cycle sequencing kit (Applied Biosystems, USA). For cycle sequencing, each 20µl sequencing reaction comprises 20ng of template, 3.2 pmol of primer, 2µl of Terminator Ready Reaction mix and 3µl of 5x sequence buffer. The reaction mixture was denatured at 94蛹l for 5 min and then 25 cycles of denaturing at 96蛹l for 10 s, annealing at 50蛹l for 5 s, and extension at 60蛹l for 4 min After 25 cycles, the reaction was rapidly chilled to 4蛹l The reaction products were precipitated by the ethanol/sodium acetate method. Firstly, 20µl of a mixture of 3.0µl of 3M sodium acetate (pH 4.6), 14.5µl of deionized water and 62.5µl of non-denatured 95% ethanol was added to each sequencing sample. After a minimum 15 min of incubation at room temperature, the products were the centrifuged for 20 min at 14,000 rpm. After removing the supernatant, the pellet was washed in 250µl of 70% ethanol and then re-centrifuged for 10 min at 14,000 rpm. The samples were finally air dried for 5

min. After the clean-up step, 15μl of HiDi-formamide (ABI) was added to each sample and then samples were loaded into 96 well optical microplate and ran on the ABI PRISM® 3100 Genetic Analyzer. The sequencing data was analyzed using the ABI Genetic Analyzer Data Collection Software.

In case the polymorphic sites were located at the extreme end of the fragment of interest, each fragment was sequenced bi-directionally using forward and reverse primers in two separated reactions.

**3.6. Prediction of biological impact of polymorphisms**

Among the polymorphisms that were found in this study, two SNPs were located in the putative promoter region. However, it is unlikely that these two SNPs affect ACAT2 gene regulation, as the deletion of the fragment containing these two SNPs did not significantly change the promoter activity (Song *et al.*, 2001). Thus, further functional predictions of these two polymorphisms using computational methods were not needed. Hence, only these polymorphisms within encoding region were examined using these approaches as follows.

I. Two substitution matrix scores, GRANTHAM (Grantham, 1974) and BLOSUM62 (Henikoff and Henikoff, 1992), were used to identify any amino acid changes and to assess their potential to be deleterious to the protein. Variations involving two amino acids are given the same weight irrespective of their positions in the protein. As such, these substitution scores are merely based on physicochemical change but are not position-specific. Amino acid changes with GRANTHAM scores of more than 100 are considered radical

changes and increasingly associated with disease (Balasubramanian *et al.*, 2005). The BLOSUM62 matrix defines amino acid substitution as conservative and non-conservative changes. Conservative changes are those having a positive or neutral value on the matrix, whereas non-conservative changes are those having a negative score. Non-conservative changes are more likely to be disease-causing mutations (Henikoff and Henikoff, 1992).

II. Multiple sequence alignment was used to identify conserved amino acid positions. It is known that an amino acid substitution occurring at a conserved position is likely to affect the function of protein. Five mammalian ACAT2 protein sequences from human (NP_003569), monkey (O_77759), mouse (NP_666176), rat (NP_714950) and dog (XP_543637) were aligned using CLUSTAL W version 1.82 software program. All orthologs were at least 65% identical to human ACAT2 protein sequence. In addition, the sequences of six acyltransferase (ACAT1 NP_003092, ACAT2 NP_003569, DGAT1 NP_036211, DGAT2 NP_115953, Are1P NP_009978 and Are2p NP_014416) were compared to investigate whether these polymorphic loci are conserved in the family.

III. Polymorphism Phenotyping (PolyPhen) (http://genetics.bwh.harvard.edu/pph/), a position-specific phylogenetic approach, was employed to evaluate the evolutionary conservation of these polymorphic sites. PolyPhen is based on the analysis of profile scores and various structural parameters to discriminate between disease-causing mutation and neutral substitution. An amino acid variant is predicted to affect function or structure of the protein if the

substitution is at a functional site, or is not compatible with the context of amino acid substitution at the position in the family of homologous proteins, or changes hydrophobicity and electrostatic charge, or affects solubility, or involves a proline residue in the α-helix, or affects protein-ligand interactions (Sunyaev *et al.*, 2001). The profile score, known as position-specific-independent count (PSIC), is a logarithmic ratio of the likelihood that a given amino acid occurring at a particular site, to the likelihood of this amino acid occurring at any site. A large PSIC difference indicates that the substitution is rarely or never observed in the protein family and probably affects protein functions (Sunyaev *et al.*, 2001). A variant is predicted to be possibly damaging if PSIC is more than 1.5 (Ramensky *et al.*, 2002).

### 3.7. Genotyping of *ACAT2* gene Polymorphisms

Three polymorphisms, 734C>T, 41A>G, and D/I, were genotyped. The genotyping method for 734C>T was carried out as described previously (Katsuren *et al.,* 2001). In brief, the presence of the point mutation (ATC to ACC) was verified by digesting the PCR amplicons generated with primers 5'-CAG ATC TTA CAC TCT GCC TGC CTC T-3' and 5'-TGC ACC TGC TGG CTT CAT TCA GTC A-3' with the *Bam*HI restriction enzyme.

The 41A>G genotype was determined using mutagenic primers and restriction enzyme via restriction fragment length polymorphism (RFLP). A pair of primers was designed to introduce a *Bsr*I restriction site (ACTGGN/) by changing a base from A to T: forward primer: 5'-GCC CGT CTG CGT CTG CAG AGG ACT G-3' and reverse primer: 5'-AGG CCA CAG CTC TGA CAT AGC-3'. The amplified 269-bp

PCR product was digested with *Bsr*I enzyme followed by gel electrophoresis. The PCR amplicon of the rare G allele (GAA to GGA) is cleaved into 243-bp and 26-bp fragments.

The D/I was first found and genotyped in this study. The D/I polymorphism had an amplicon size difference of 48bp between the I allele with the 48bp insertion and the D allele without the insertion and as such, can be directly genotyped by visualization on a 2% agarose gel after PCR amplification with a pair of primers: 5'-GCT GGA AGT TCT GGA TCG CTA-3' and 5'-TAA CCA AAG GGG GAA CCT GT-3'.

### 3.8. Genotyping of three *LPL* gene polymorphisms

The methods used in genotyping of IVS8+484T>G and IVS6+1595C>T polymorphisms have been previously described (Ahn *et al.*, 1993). The genotyping of c.1342C>G was carried out according to Kuivenhoven *et al.* (1997). The corresponding primers and annealing temperatures for PCR are shown in Table 3-2. The PCR products were digested using appropriate restriction enzymes (Table 3-2). The digestion reactions were incubated at $37^{o}C$ overnight. After digesting the amplified fragments, the resultant products were analyzed using 2% agarose gel.

**Table 3-2**. Primers and conditions for genotyping three *LPL* polymorphisms.

| SNP | Primers | Annealing Tm ($^0$C) | Restriction enzyme | Products sizes (bp) |
|---|---|---|---|---|
| IVS6+1595C>T | 5'-GTGGGTGAATCACCTGAGGTC-3'<br>5'-TAGAGGTTGAGGCACCTGTGC-3' | 64 | *PvuII* | CC: 858<br>CT: 592<br>TT: 266 |
| IVS8+484T>G | 5'-TTTAGGCCTGAAGTTTCCAC-3'<br>5'-CTCCCTAGAACAGAAGATC-3' | 60 | *Hind*III | TT: 1300<br>TG: 700<br>GG: 600 |
| c.1342C>G | 5'-TACACTAGCAATGTCTAGGTGA-3'<br>5'-TCAGCTTTAGCCCAGAATGC-3' | *61* | *Mnl*I | CC: 285<br>CG: 248<br>GG: 203 |

### 3.9. Estimation of plasma lipid levels

This assay was done by the technician in our laboratory. Venous blood was drawn from subjects after an overnight fast of at least 10 hours. Plasma was separated from blood cells by centrifugation and stored at -20$^o$C until lipid analysis.

TC and TGs were analyzed by colorimetric enzymatic methods on a COBAS Mira autoanalyzer (Roche, Basel Switzerland) using the manufacturer's reagent kits (cholesterol kit 2016630, TG kit 2016647, Roche Diagnostics, Mannheim, Germany).

Cholesterol was determined using a commercial cholesterol oxidase method by a series of enzymatic reactions (Allain *et al.*, 1974). The principle of the reaction is as follows. CEs are hydrolyzed to free cholesterol by cholesterol esterase. Free cholesterol is then oxidized by cholesterol oxidase producing hydrogen peroxide which forms a red chromophore when combined with 4-aminophenazone and phenol. The chromophore formed is measured at photometrically 520 nm at 37$^o$C and is directly proportional to the cholesterol concentration of the sample.

TGs were measured using a colorimetric enzymatic assay kit. In this method (McGowan *et al.*, 1983), the TGs are hydrolyzed by lipase to produce free glycerol. The liberated glycerol is then reacted with glycerol kinase followed by oxidization to dihydroxyacetone phosphate and hydrogen peroxide. The free glycerol is determined by enzymatic, colorimetric reaction of peroxide and 4-aminophenazone to form a colored product, the concentration of which is directly proportional to the amount of TGs present in the sample.

HDL-cholesterol quantification was performed by using the above-described cholesterol method after precipitation of non-HDL cholesterol (LDL and VLDL) with polyanions and magnesium chloride.

LDL-C was calculated by the Friedewald formula (Friedewald *et al.* 1972):

LDL-C = TC- HDL-C – TG/2.2 (all measured in mmol/L) when TG<4.49mmol/L. When TG is over 4.49mmol/L, the estimation of LDL-C by this method is not accurate.

ApoA1 and apoB were estimated by an immunoturbidometric assay on the same machine using the Tina quant apoA Unisys (no. 03032612122) and Tina quant apoB Unisys from Roche Diagnostics (Mannheim, Germany).

Lipoprotein (a) [Lp(a)] was estimated by enzyme-linked immunosorbent assay (ELISA) using commercially available kits [TintElize Lp(a), cat. no. 610220, Biopool, Umeå, Sweden].

### 3.10. Statistical analysis

Allele frequencies were estimated by the gene-counting method. Before performing other data analysis, Pearson's chi-square test was used to test HWE for each locus. Haplotypes were constructed using the Phase Standard analysis software version 2.0.2 available at http://archimedes.well.ox.ac.uk/pise/. Haplotype frequencies of each group were estimated by the Expectation-Maximization algorithm using the SNPAlyze software version 4.0 from Dynacom (Kanagawa, Japan). Diplotypes were obtained by combining genotype information from different polymorphic sites. The Z-test was used to determine significant differences in allele frequencies between the cases and controls and between the different ethnic groups. Odds ratios (OR) are presented with 95% confidence intervals (CI) when significant associations were observed. LD between polymorphic sites was assessed by $\Delta$ (Hill and Robertson, 1968). The lipid profiles were significantly different between different ethnic groups and gender. As such we further stratified the subjects by ethnicity and gender. In order to accurately evaluate the influences of *ACAT2* gene variants on plasma lipid profiles, CAD-subjects within each ethnicity were further stratified into normolipidemic (TC<6.15mM and TG<2.25mM) and dyslipidemic (TC>=6.15mM or TG>=2.25mM) group according to the NECP ATP III criteria (NCEP ATPIII, 2002). Allele, haplotype and diplotype frequencies between cases and controls as well as between normolipidemic and dyslipidemic subjects and between ethnic groups were compared.

The association of plasma lipids with genotypes and diplotypes was investigated only in the CAD- subjects, as the lipid levels of the cases (CAD+) would have been affected by treatment with lipid-lowering drugs. The lipids were presented as means

± SD unless otherwise stated. Due to the skewed distribution of TG and Lp(a) in all populations, their values were transformed by natural logarithm prior to using parametric tests.

Three different models were used in this association study: 1) dominant (wildtypes vs. heterozygotes and mutant homozygotes), 2) additive (wildtypes vs. heterozygotes vs. mutant homozygotes), and 3) recessive (wildtypes and heterozygote vs. mutant homozygotes). Confounding factors such as age, smoking and body mass index (BMI) were included as covariates in the analysis of covariance (ANCOVA) model. The lipid levels between diplotypes were compared by carrying out post hoc multiple comparisons using Tukey's honestly significant difference (HSD) test.

Most analysis was carried out using the SPSS software version 11. Statistical significance was defined by P value of 0.05 or less. Bonferroni correction was used to adjust the p value according to the number of tests performed.

### 3.11. Cell line, vector and reagents

The AC-29 cell line, an ACAT-deficient Chinese hamster ovary cell mutant (Cadigan *et al.* 1988), was kindly provided by TY Chang's lab (Department of Biochemistry, Dartmouth Medical School, US). The vector pRS426GP and pCR3.1/*ACAT2* (Oelkers *et al*., 1998; Liang *et al*., 2004) were obtained from Dr Yang (Department of Biochemistry, National University of Singapore). The pcDNA3.1/His expression vector containing Xpress epitope (Asp-Leu-Tyr-Asp-Asp-Asp-Lys) and the monoclonal antibody against Xpress were from Invitrogen (California, US). Antipain, chymostatin, leupeptin, fetal bovine serum, and Nile red, were purchased from Sigma

(St. Louis, US). Geneticin (G-418 sulfate), Ham's F-12 and DMEM/F-12 medium, and anti-Xpress were from Invitrogen (California, US). [9,10(n)-$^{3}$H]oleic acid and anti-mouse Ig were from Amersham Biosciences (Hilleroed, Denmark).

### 3.12. Cell culture

The AC-29 cells were grown as monolayers in Ham's F-12 medium supplemented with 10% fetal bovine serum, 2mM L-glutamine, 1% Eagle's vitamins and 100 units/ml of Penicillin and 100 µg/ml of Streptomcyin in 5% $CO_2$ incubator at 37$^{o}$C.

### 3.13. Expression of various ACAT2 proteins

#### 3.13.1. Construction of various ACAT2 gene expression plasmids

The expression vector pcDNA3.1/His was digested using restriction enzymes, *Eco*RI and *Not*I, at 37$^{o}$C overnight. The digested product was then purified using the gel purification kit from Qiagen (Tokyo, Japan).

The *ACAT2* cDNA insert was excised from recombinant pRS426GP/*ACAT2,* which was constructed from vector pRS426GP and pCR3.1/*ACAT2*, using *Eco*RI and *Not*I restriction enzymes. As the pcDNA3.1/His expression vector contained an ATG start codon, the *ACAT2* gene's start codon and the upstream sequence between the start codon and the *EcoR*I cutting site were deleted using site-directed mutagenesis (Stratagene, California, USA). The mutagenic primers were designed using Stratagene's recommended program at http://labtools.stratagene.com/QC. The two complimentary oligonucleotides which contained the desired mutations were synthesized using pRS426GP/*ACAT2* as the template and 5'-TTT GAA AAT TCA AGG AAT TCG AGC CAG GCG GGG C-3' and 5'-GCC CCG CCT GGC TCG AAT TCC TTG AAT TTT CAA A-3' as sense and anti-sense primers, respectively. The

synthesis reaction was carried out in thermal cycler (T-GRADIENT, Biometra, Goettingen, Germany) with a denaturing step at 95$^{o}$C for 30 s followed by 18 cycles of denaturing at 95$^{o}$C for 30 s, annealing at 55$^{o}$C for 1 minute and extension at 68$^{o}$C for 10 min. After the mutant strand synthesis, the parental DNA template was digested by specific *Dpn*I endonuclease at 37$^{o}$C for 1 hour. The selected mutation-containing synthesized DNA was then transformed into XL1-Blue super-competent cells (Stratagene, California, USA). The generated plasmids were purified using the miniprep kit (Qiagen, Tokyo, Japan) and the deletion of the ATG start codon and upstream sequence was confirmed by DNA sequencing. The resultant *ACAT2* insert was ligated with the expression vector pcDNA3.1/His between the *Eco*RI and *Not*I sites. The generated construct pcDNA3.1/His/*ACAT2* was confirmed using PCR amplification and DNA sequencing.

In this study, two nsSNPs, Glu14Gly and Thr245Ile, were explored for their effects on the ACAT2 enzyme activity as well as on gene and protein expression. For this purpose, the wildtype and three mutant *ACAT2s*, consisting of two variants carrying one mutant, Gly14 or Ile245, and one variant carrying both mutants, Gly14Ile245, were constructed. The recombinant pcDNA3.1/His/*ACAT2*, which contained the wild type *ACAT2*, designated as wild type pcDNA3.1/His/*ACAT2* (WT), was used as the template to generate various mutant *ACAT2s* using Stratagene's QuickChange site-directed mutagenesis kit. The construction procedure is briefly described here: 1) The ACAT2 Gly14 was generated using the wild type pcDNA3.1/His/*ACAT2* and oligonucleotides 5'-GCA GAG GAC AG**G** AGG GCT GGG AGG-3' and 5'-CCT CCC AGC CCT **C**CT GTC CTC TGC-3' as sense and anti-sense primers, respectively. The resultant ACAT2 was designated ACAT2 Gly14. 2) The ACAT2 Ile254 was

produced using the wild type pcDNA3.1/His/*ACAT2* as the template and oligonucleotides 5'-GCT GTG CCT GGG A**T**C CTT CGT GCC AGA C-3' and 5'-GTC TGG CAC GAA GGA TCC CA**G** GCA CAG C-3' as sense and anti-sense primers, respectively. The resultant ACAT2 was designated as ACAT2 Ile254. 3) The recombinant pcDNA3.1His*ACAT2* with mutant amino acid residues at position 14 and 254 was produced using pcDNA3.1/His/*ACAT2* Gly14Thr254 as the template and 5'-GCT GTG CCT GGG A**T**C CTT CGT GCC AGA C-3' and 5'-GTC TGG CAC GAA GGA TCC CA**G** GCA CAG C-3' as sense and anti-sense primers, respectively. The resultant was designated as pcDNA3.1/His//*ACAT2* Gly14Ile254. The sequences of the various pcDNA3.1/His/*ACAT2*s recombinants were confirmed by DNA sequencing.

### 3.13.2. Transfection of AC-29 with various pcDNA3.1/His/*ACAT2*

The AC-29 cells were transfected with various pcDNA3.1/His/*ACAT2* using the calcium phosphate transfection kit (Invitrogen, California, US). The unmodified pcDNA3.1/His vector was also used to transfect the AC-29 cells to serve as a negative control. The procedure used is briefly described below. The day before transfection, the AC-29 cells were plated at the density of $1.5x10^6$ cells in 10ml of Ham's F-12 medium in a 100mm-diameter Petri dish. This would produce cells that would be 50-60% confluent on the day of transfection. On the day of transfection, the medium was changed 3-4 hours prior to transfection and the cells were further incubated at 37°C. Twenty µg of DNA was mixed with 36µl of 2M $CaCl_2$ in a final volume of 300µl and the mixture was added slowly to 300µl of 2x HBS (HEPES Buffered Saline) in 1-2 min and incubated at room temperature for 30 min in order to form a fine DNA $CaPO_4$ coprecipitate. The precipitate was added dropwise to the

medium containing the AC-29 cells, which were then incubated in the 37$^{o}$C $CO_2$ incubator overnight. The medium was then removed and the cells were washed twice with 1xPBS. Three ml of 10% DMSO in 1xPBS was added into the Petri dish and incubated at room temperature for exactly 2.5 min. After the DMSO shock treatment, fresh medium was added and the cultures were incubated at 37$^{o}$C in the 5% $CO_2$ incubator for another 48 hours.

**3.13.3. Selection of stable transformants**

After the cells were maintained in non-selective media for 2 days post-transfection, they were scraped off using a scraper and transferred to be grown in selective medium containing 500痢ml of G-418 for 2 weeks. Discreet G-418 resistant colonies were seen after 2 weeks. Single colonies were picked up and transferred into a 6-well plate for subsequent propagation.

**3.13.3. Selection of positive stable transformants**

After the G-418 resistant cells were grown for 2 weeks, they were examined for their cytoplasmic CEs under an inverted phase-contrast microscope (ULWCD 0.30, Olympus, Tokyo, Japan). The cytoplasmic lipid droplets were further examined using a differential interference contrast microscope (BX60; Olympus, Tokyo, Japan) after staining with 100 痢ml Nile red. Nile red is a highly fluorescent compound which preferentially partitions into hydrophobic environments such as intracellular neutral (Greenspan and Fowler, 1985[a] and 1985[b]) lipid droplets. In positive transformants containing pcDNA3.1/His/*ACAT2*, the CEs would be synthesized leading to a large accumulation of intracellular CEs. In contrast, in the negative cells, in which the G-418 resistant gene might be expressed but the target *ACAT2* gene was not expressed, no obvious Nile red-staining lipid drops were observed.

The procedure of Nile red staining was described as below. The monolayers were washed twice with 1xPBS and incubated with 1ml non-enzymatic dissociation solution (Sigma, St. Louis, US) per well at 37$^{o}$C for 10 min. The cells were then transferred into a sterile tube and then stained using Nile red (100-150ng/ml). After Nile red staining, cytoplasmic CE lipid droplet content was examined using differential interference contrast microscope. Only colonies containing abundant cytoplasmic lipid droplets were re-cultured in 6-well plate containing selective medium.

### 3.14. *Ex vivo* ACAT activity assay

This activity assay was described by Chang *et al*. previously (1986). The cells were plated in 25cm$^{2}$ flask and grown to 80% confluence. Two hours before pulse, medium was changed and the cells were incubated in a 37$^{o}$C water bath capping the flask tightly. The cells were pulsed with $^{3}$H-oleate BSA and the flask was incubated in 37$^{o}$C water for 30 min. The medium was then removed and the cells were rinsed five times, each with 5 ml of cold 1xPBS. The cells were lyzed using 0.2 M NaOH at room temperature 40 min and then neutralized by adding 3M HCL and $KH_2PO_4$. The cellular lipids were extracted with chloroform-methanol (2:1) after adding cholesteryl oleate as carrier and [$^{14}$C] cholesterol as internal standard. The various lipids were then separated using thin layer chromatography and the different lipid bands identified using iodine vapor. The bands of cholesterol ester and cholesterol were excised from the sheet and dissolved in scintillation cocktail. The ACAT enzyme activity was determined by calculating the incorporation of labeled oleate into CEs and normalized with the concentration of the internal standard, [$^{14}$C] cholesterol, and total proteins. The enzymatic activities were expressed as means ± standard deviations (SDs) of triplicate assays over at least three separate experiments.

### 3.15. Quantitative reverse transcription (RT) PCR

Total RNA was prepared using Trizol ® (Invitrogen, California, US) following the manufacturer's instructions. Cells were grown in a 25cm$^2$-flask to 70-80% confluence and then lyzed with the addition of 2.5ml of Trizol and incubation for 5 min at room temperature. The cell lysates were put into 10ml tubes and 0.5ml of chloroform was added. The tubes were capped tightly and shaken vigorously by hand for 15s and incubated at room temperature for 3 min. The tubes were then centrifuged at 12,000 x g for 15 min at 4$^o$C, and the supernatant was transferred to fresh tubes containing 1.25ml of isopropyl alcohol and incubated at room temperature for 10 min. The mixture was centrifuged at 12000 x g for 10 min at 4$^o$C to get the RNA pellet. This RNA pellet was washed again with 3 ml of 75% ethanol, vortexed and centrifuged for 5 min at 7500 x g at 4$^o$C. The pellet was briefly dried and 50痞 of RNase-free water was added for re-constitution.

The total RNA extracted from cells grown to 70-80% confluence was used as template to run real-time RT-PCR to determine whether the expression of wild type and various mutant *ACAT2* mRNA were different. Before doing quantitative the real-time RT-PCR, RT-PCR was carried out first using the OneStep RT-PCR kit (Qiagen, Tokyo, Japan) to confirm the expression of *ACAT2* mRNA in the AC-29 cells. The oligonucleotides, 5'-CCC AGT TTC TCC AGC TAC CT-3'and 5'-AAG ACA GGA ACA CAG AGG CG-3', were used as *ACAT2* gene-specific primers in the OneStep RT-PCR and the follow-up real-time RT-PCR amplification.

The real-time RT-PCR amplification was performed to determine the mRNA expression of the various *ACAT2s* using LightCycle RNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany). Each of 20痞reaction contained 3.25mM

$Mn(OAc_2)$, 6痞SYBR Green I, 0.3然each primer, and 450ng RNA template. The real-time RT-PCR was started with an initial reverse transcription at 61°C for 20 min, followed by a denaturation step of 95°C for 30 s and 40 cycles of amplification (95°C for 1s, 57°C for 4s followed by 72°C 13s). After completion of the amplification samples were subjected to a melting curve analysis to test the product specificity. The reaction was carried out in LightCycler capillary tubes. The LightCycler apparatus measured the fluorescence of each sample in every cycle at the end of the annealing step. The Second Derivative Maximum Method was used for the determination of the crossing point (Cp) automatically for the individual samples. A housekeeping gene, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was included as a reference gene. A pair of primers, 5'-TTC TGG CAA AGT GGA AGT TGT TG-3' and 5'-ATG GTG ATG GCC TTC CCG TT-3', was used to amplify *GAPDH* gene. The relative quantification, of the wild type and various mutants, was normalized by *GAPDH* gene. To ensure that the correct product was amplified in the quantification reaction, all samples were visualized on 2% agarose gel electrophoresis. The quantitative reverse transcription was done in triplicate over three experiments. The t-test was employed to test the statistical significance of fold changes in gene expression of mutant *ACAT2* relative to wildtype.

### 3.16. Western blot

When cells reached 70%-80% confluence in the 75cm$^2$ flask, they were lyzed with 0.5-1ml of a cocktail consisting of 1x Phosphate Buffered Saline (PBS) containing 2% SDS and proteinase, and then centrifuged at 14,000rpm at 4°C for 20 min. The supernatant was kept as the total soluble protein. Protein concentration was determined using the method by Bradford (1976). The total soluble protein (15ug) was incubated with 5x loading buffer at 37°C for 15 min and subjected to

SDS-polyacrylamide gel electropherosis (SDS-PAGE) using a 12% Tris-glycine gel. The separated proteins were electrotransferred to a nitrocellulose membrane. The membrane was incubated in 15ml of 5% non-fat milk at $4^{o}C$ overnight. After blocking, the blot was incubated with primary antibody, Anti-Xpress (Invitrogen, California, US), at 1:5000 dilutions in Tris Buffered Saline (TBS) at room temperature for 1 hour. After washing with TBST (TBS with 0.5% Tween-20) three times and TBS once, the blot was then subjected to blotting with as secondary antibody, anti-mouse Ig, at 1:10,000 dilutions in TBS, with 2.5% non-fat milk, at room temperature for 1 hour. After the second round of washing, the membrane was subjected to the enhanced chemiluminescent detection kit (ECL, Amersham Biosciences). Densitometric analysis of western blot signals was conducted to compare the different expression levels of the various recombinant ACAT2 proteins in the tranfected AC-29 cells.

# 4. STUDY OF *ACAT2* GENE

## 4.1. Introduction

In this study, the putative promoter, 5'-UTR, encoding regions, and intron-exon boundaries of *ACAT2* gene were screened for genetic polymorphisms with DHPLC. After identifying polymorphisms in this gene, the possible biological significances of three nsSNPs, 41A>G (Gly>Glu) in exon1, 734C>T (Thr>Ile) in exon7, and c.1291G>T (Ala to Ser) or G>A (Ala to Thr) in exon 13, were predicted using computational approaches. The association of three polymorphisms, 41A>G, 734C>T, and one 48bp insertion (D/I), with CAD and dyslipidemia was examined in the three ethnic groups (Chinese, Malay and Indian) in Singapore. The nsSNP, c.1291G>T or G>A, is very rare and only one heterozygote was found among 348 samples. It was therefore not included in the association study. The effects of two nsSNPs, 41A>G and 734C>T, on ACAT2 function were further investigated by *in vitro* functional study using the AC-29 cell line, a mutant Chinese hamster ovary (CHO) cell line deficient in ACAT activity (Cadigan *et al.*, 1988).

## 4.2. Results

### 4.2.1. Polymorphism screening

#### 4.2.1.1. Survey of known genetic variants in *ACAT2* gene

Before screening for novel *ACAT2* gene polymorphisms, known SNPs were first identified using public SNP resources. By searching these public SNP databases, such as NCBI SNPdatabase, TSC, JSNP, and HGVbase, a total of 10 SNPs were identified among these regions of interest (Table 4-1).

#### 4.2.1.2. Polymorphism screening

After the systematic screening of these regions of interest in *ACAT2* gene with DHPLC using cord blood DNA samples from 336 neonates, distinct elution profiles were detected for some DNA fragments of interest (shown in Figure 4-1). The corresponding samples were amplified by PCR and sequenced to verify these variants. The sequencing results are shown in Figure 4-2. A total of 14 polymorphisms were detected, 9 of them were novel and are indicated by their corresponding SubSNP (ss) numbers in the NCBI SNPdatabase (Table 4-1). These included three missense mutations [c.41A>G (Gly>Glu) in exon1, c.734C>T (Thr>Ile) in exon7, and c.1291G>T ss5607249 (Ala to Ser) or G>A (Ala to Thr) ss6313900 in exon13], two single-base changes in the putative promoter region (-331C>T ss9807253 and -440G>T ss9807252), two silent mutations (c.609G>T ss4480613 and c. 610C>T ss4480614 in exon6), seven single-base changes in intronic regions, comprising six single-base substitutions (IVS1-8C>G; IVS4+172T/G ss4329267, IVS5-137A/T ss4329269, IVS9-178G/C ss5606370, IVS9+37A>T and IVS9+51G>T), and one 48bp insertion (D/I ss4329268). Information of all the novel polymorphisms is available at http://www.ncbi.nlm.nih.gov.libproxy1.nus.edu.sg/SNP/snp_viewTable.cgi?handle=NUSPAE.

The DHPLC profile for most polymorphisms showed two different, homozygous and heterozygous, patterns (Figure 4-1), while the D/I polymorphism presented three distinct profiles: wild type homozygotes (DD), heterozygotes (DI), and mutant homozygotes (II) (Figure 4-3). The wildtype and mutant homozygotes of D/I polymorphism each gave one sharp peak in their profiles, except that the retention

time of the wildtype was longer than that of the mutant homozygotes. The heterozygotes of D/I however showed an elution profiles quite different from those of the two homozygotes.

**Table 4-1**. *ACAT2* gene polymorphisms found in the regions of interest in this study

| Polymorphisms | Position | Type | RefSNP (rs#) | Found in this study | Novel variant |
|---|---|---|---|---|---|
| 41A>G[a] | exon | Glu>Gly | 9658625 | yes | |
| 734C>T[a] | exon | Thr>Ile | 2272296 | yes | |
| 609G>T | exon | Silent mutation | 3219199 | yes | novel |
| 610C>T | exon | Silent mutation | 3219200 | yes | novel |
| c. 1291G>T or A | exon | Ala>Ser (or Thr) | 4244355 | yes | novel |
| -331C>T | promoter | SNP | 2280698 | yes | novel |
| -440G>T | promoter | SNP | 6413499 | yes | novel |
| IVS1-8C>G | intron | SNP | 17551115 | yes | |
| IVS9+37A>T[b] | intron | SNP | 2280696 | yes | |
| IVS9+51G>T[b] | intron | SNP | 711315 | yes | |
| IVS4+172T/G | intron | SNP | 3093945 | yes | novel |
| IVS5-137A/T | intron | SNP | 3093947 | yes | novel |
| IVS9-178G/C | intron | SNP | 4151119 | yes | novel |
| IVS4-57_58INS48bp | intron | insertion | 3093946 | yes | novel |
| -74A>G | 5'-UTR | SNP | 2280698 | no | |
| -1297G>A | promoter | SNP | 6413499 | no | |
| -1283G>A | promoter | SNP | 1053167 | no | |
| -1327A>C | promoter | SNP | 1053153 | no | |
| -1382A>C | promoter | SNP | 1053149 | no | |

a and b: Variations reported by Katsuren *et al.* (2001) and Haga *et al.* (2002), respectively. Those polymorphisms underlined were searched from SNP databases.

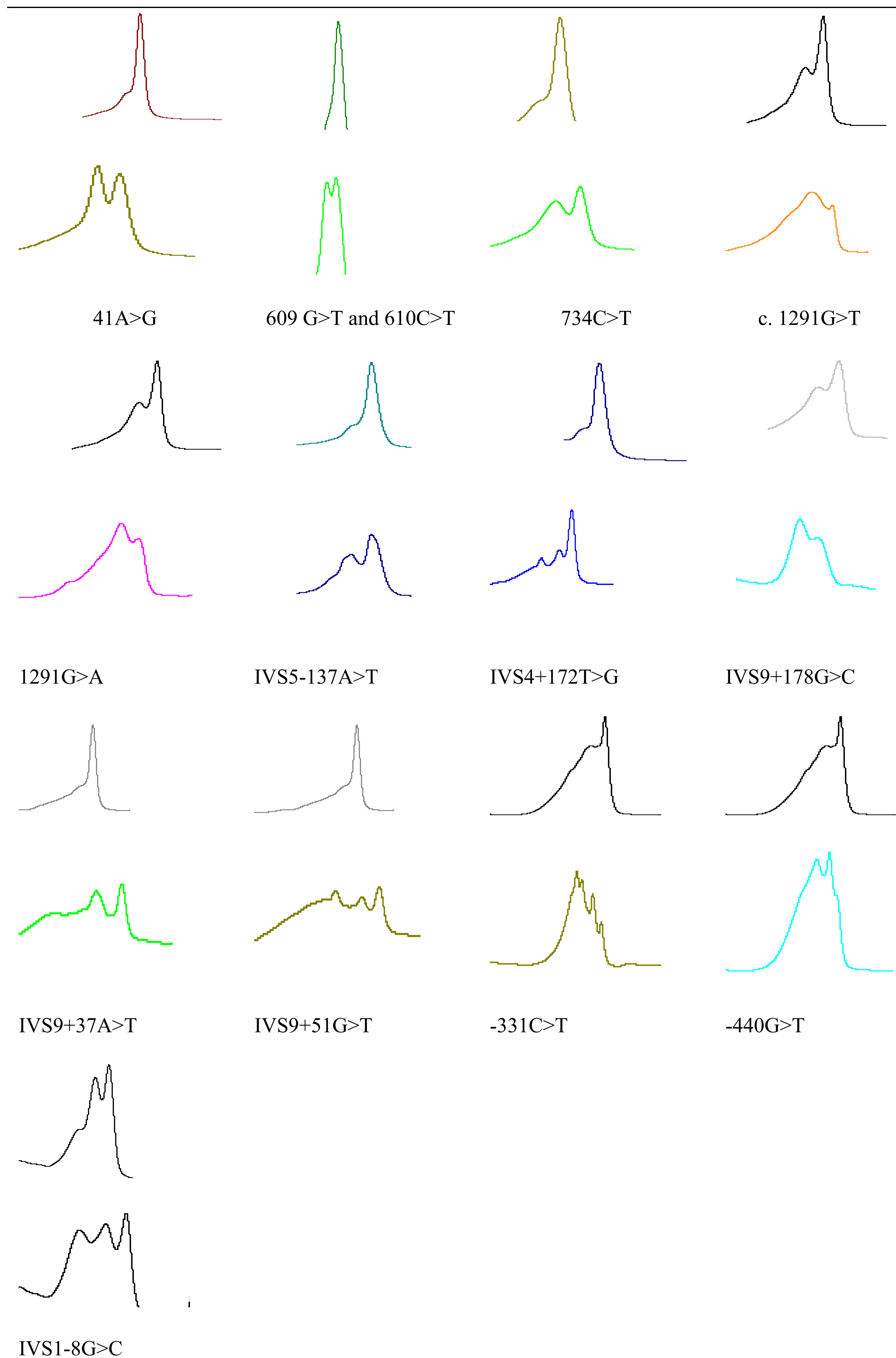



**Figure 4-1.** DHPLC profiles of *ACAT2* polymorphisms. For each polymorphism, the upper pattern is from wild type homozogotes and the lower from the mutant heterozygotes.

41AA
609GG/610CC
734CC
41GG
609GT/610CT
734TT
1291GG
1291GG
IVS5-137AA
1291GT
1291GA
IVS5-137TT
IVS4+172TT
IVS9+178GG
IVS9+37AA
IVS4+172GG
IVS9+178GC
IVS9+37AT

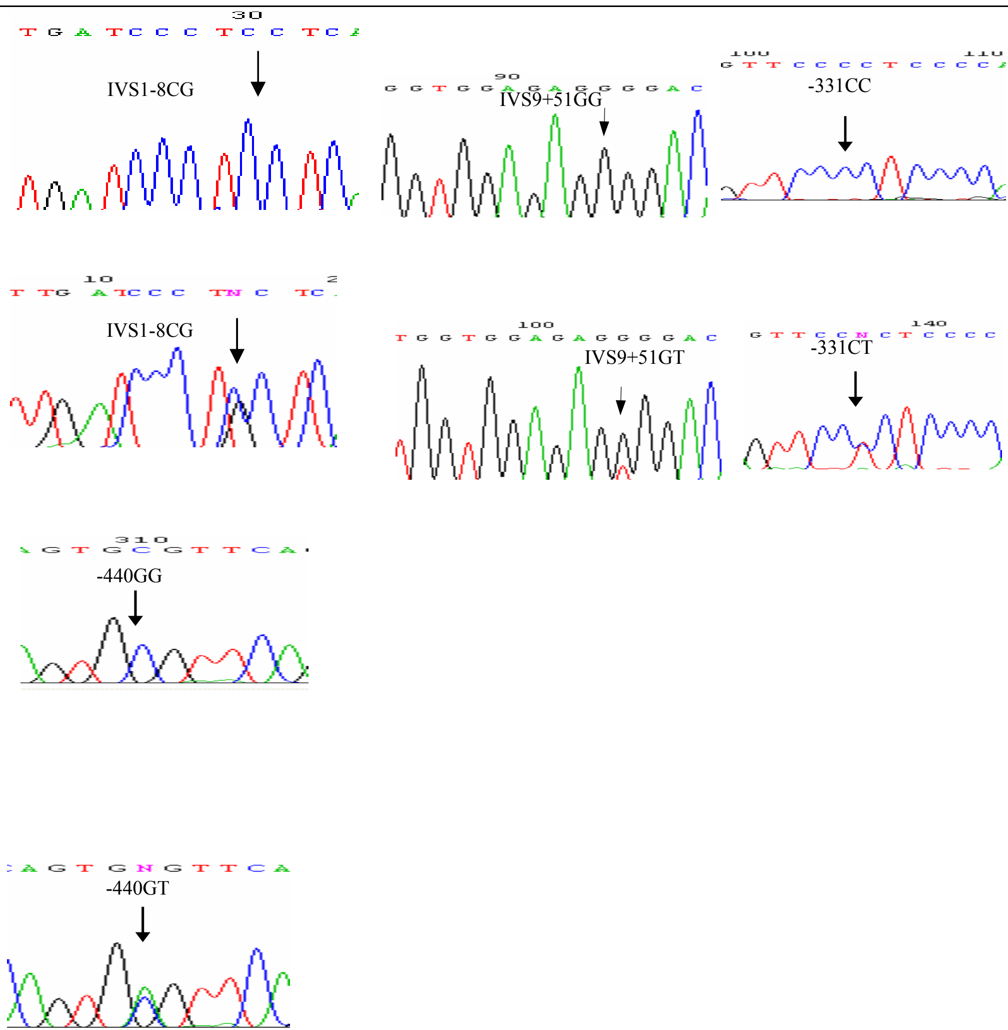



**Figure 4-2.** DNA sequencing results of *ACAT2* polymorphisms detected in this study. The corresponding polymorphisms were marked with arrow.

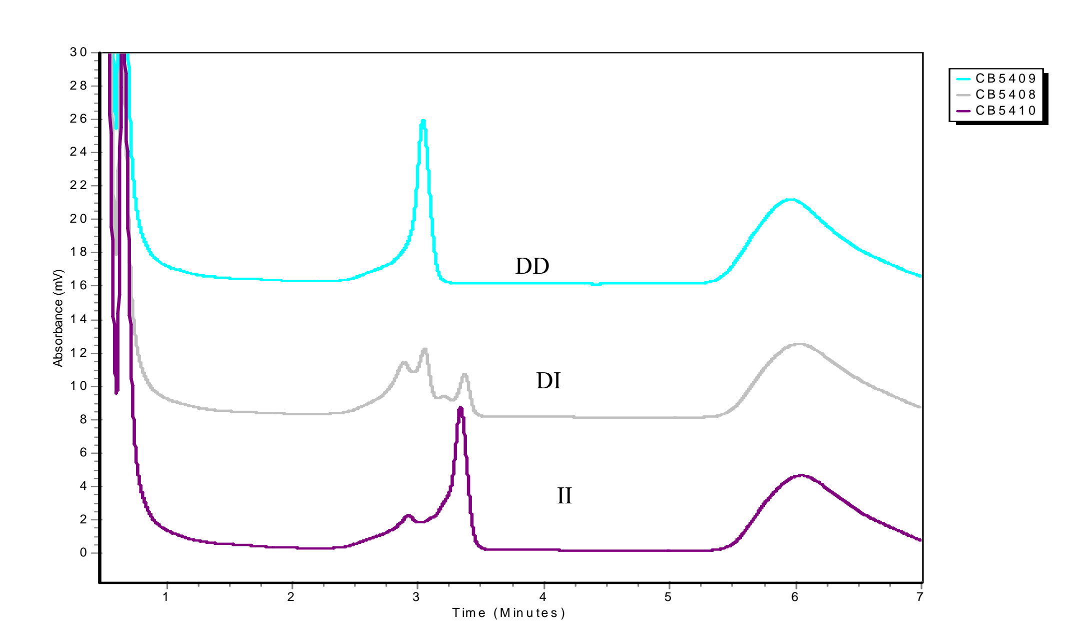



**Figure 4-3.** DHPLC profile of *ACAT2* D/I polymorphism. The upper one that gives one sharp peak and emerges earlier is wildtype homozygote and the lowest one emerging later is mutant homozygote and the middle one giving three peaks is heterozygote.

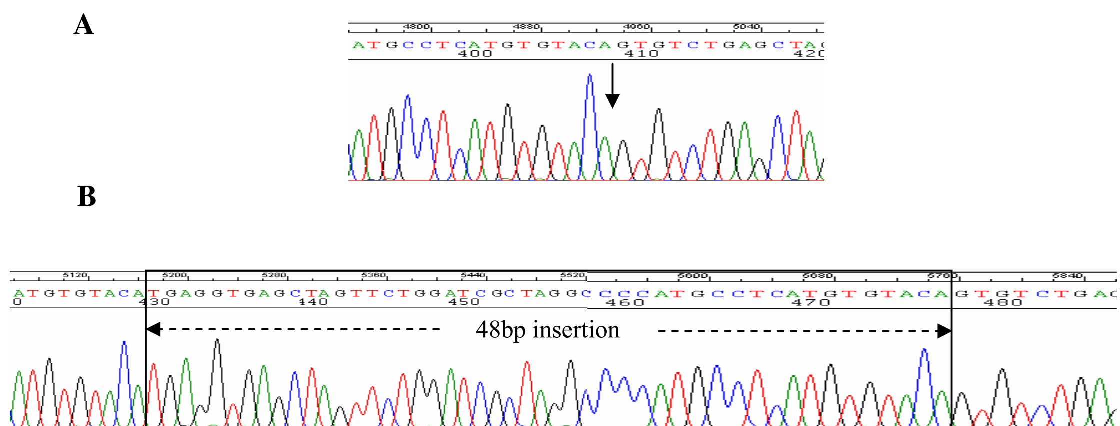



**Figure 4-4**. Sequencing result of *ACAT2* D/I. A: wild type DD; B: mutant homozygotes II; Arrow in **A** indicates the site where the 48bp inserts.

### 4.2.1.3. Improved efficiency of mutation screening using modified primers

Two polymorphisms, IVS9+37A>T and IVS9+51G>T, in the flanking region of exon 9, were reported in the NCBIs dbSNP. However, in the study, none of them was detected when normal primers were used to amplify the fragment of interest, as the corresponding DHPLC profile generated a broad unresolved peak (Figure 4-5 A). The melting profiles of the fragment predicted using WAVEMAKER software is shown in Figure 4-6 A. The two polymorphisms are located in the high melting domain, which is predicted to partially denature at 62$^oC$. The temperature range from 60$^oC$ to 63$^oC$ was chosen to detect the sequence variants. However, the peaks became broad and the variants were poorly discernible when running at 62$^oC$ (Figure 4-5A). As a result, the polymorphism IVS9+37A>T was not detected when running at predicted temperatures.

In order to change the melting property of the DNA fragment and to improve the resolution of hetero- and homoduplex peaks in the elution profile, a modified primer with a 20bp GC-clamp was used. After the modification, the IVS9+37A>T was able to be detected and the resolution of IVS9+51G>T was significantly improved (Figure 4-6B. Therefore, it was proved that application of GC-clamp modified primers can increase the resolution of the heteroduplexed fragments.

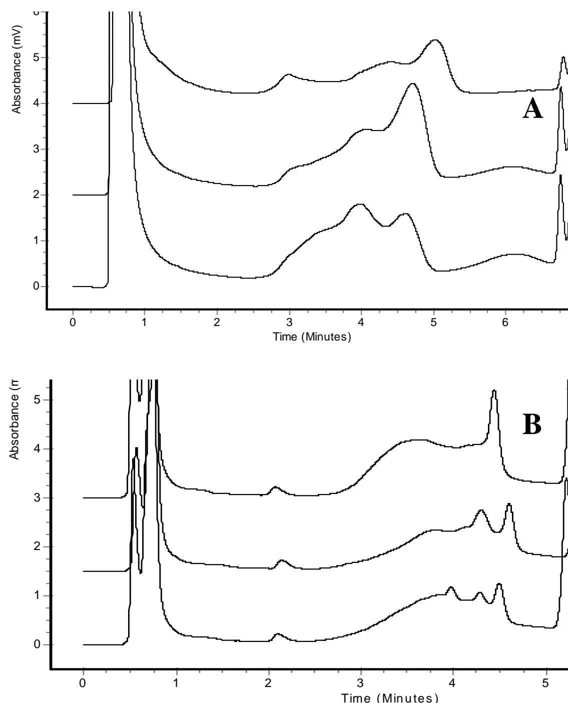



**Figure 4-5.** DHPLC profiles of IVS9+51G>T (lowest) and IVS9+37A>T (middle) and the wild type (upper) amplified with primers with non-clamped (A) and with GC-clamp (B).

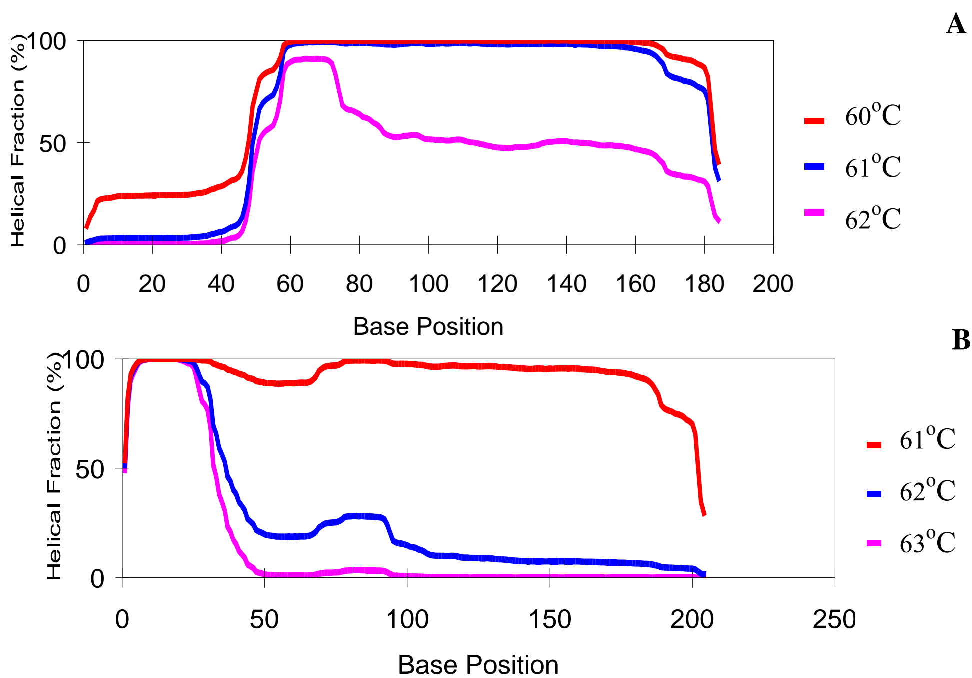



**Figure 4-6.** Predicted melting profile of the fragment containing exon 9. The upper panel shows the fragment amplified with the non-GC-clamp primers and the lower panel shows one amplified with GC-clamp primers.

#### 4.2.1.4. Factors affecting DHPLC elution profiles

The DHPLC profiles of some fragments of interest showed artefact peaks or shoulders in front of the elution peak. In order to find out the possible reasons causing the artefact peak or shoulder, two factors were examined: the methods of extracting DNA (Parzer's method or phenol: chloroform method), and the amount of template used. The salts used for DNA extraction in Parzer' method may not be removed completely and thus it may affect the DNA quality and PCR amplification reaction. However, the results showed that the artefact peaks or shoulders could be decreased by using less amount of template for PCR amplification (Figure 4-7), while DNA extraction methods had no significant effect on the DHPLC profile.

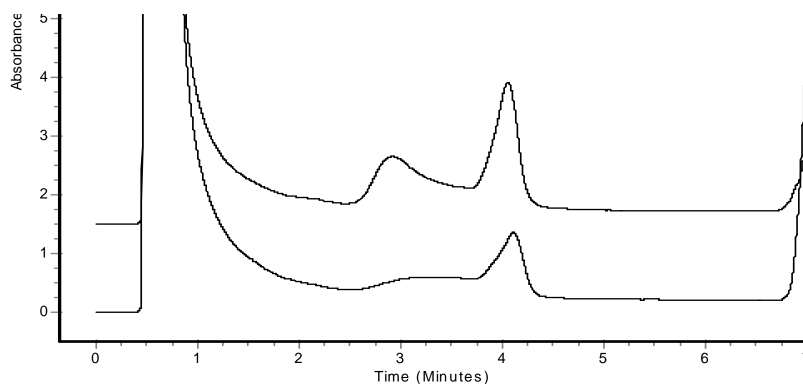



**Figure 4-7.** DHPLC profile of the fragment containing *ACAT2* gene exon 8. The upper profile is the elution profile of the amplicon using 0.6µl template (about 100ng) in 20µl reaction and the lower profile for the amplicon using 0.3µl template (about 50ng) in same volume of reaction. For both cases, 5µl of PCR product was used to run DHPLC.

#### 4.2.1.5. Prediction of functional implications of *ACAT* polymorphisms

Among the 14 identified polymorphisms, only three polymorphisms, 41A>G (Glu14Gly) in exon1, 734C>T (Thr254Ile) in exon7, and c.1291G>T (Ala431Ser) or

G>A (Ala431Thr) in exon13, are nsSNPs. The possible biological significances of the three nsSNPs were predicted with different approaches.

When evaluating whether there is any difference in the physiochemical properties between wildtype and mutant amino acids, two substitution scoring matrices, GRANTHAM and BLOSUM62, were applied. The results showed that the GRANTHAM D values obtained for Glu14Gly, Thr254Ile, and Ala431Ser (or Thr), were 98, 89, and 99 (58), respectively. Variations with values >100 were more likely to affect disease susceptibility. However, the distinction between disease-causing mutations and neutral mutations is not clear. The BLOSUM62 scores of these substitutions were -2, -1, and 1 (-1), respectively. The amino acid change with a value less than -1 was probably a disease-causing mutation. Taken together, these evaluations suggested that the Glu14Gly may have greater biological significance than Thr254Ile and Ala431Ser (or Thr).

Other than the influence on the physiochemical properties, the evolutionary conservation was also examined by using an interspecies alignment. The protein sequences of the ACAT2 protein from human, monkey, mouse, rat, and dog, were compared. The comparison indicated that the Gly14 and Ala431 are identical in the five species while the Thr254 was not conserved (Figure 4-8). The Thr254 was present in human, monkey and dog while the Ile254 occurred in mouse and rat. It suggested that the Thr254 was less likely to affect protein function while the other two amino acid residues were evolutionarily conserved, at least in the five species, and thus, probably had some biological significance.

```
                    Glu14Gly

Human      -MEPGGARLRLQRTEGLGGERERQPCG-DGNTETHRAPDLVQWTRHMEAVKAQLLEQAQG 58
Monkey     -MEPGGARLRLQRTEGPGGEREHQPCR-DGNTETHRAPDLVKWTRHMEAVKAQLLEQAQG 58
Mouse      EMQPKVPQLR--RREGLGEEQEKGARGGEGNARTHGTPDLVQWTRHMEAVKTQFLEQAQR 58
Rat        -MEPKAPQLR--RRERQGEEQENGACG-EGNTRTHRAPDLVQWTRHMEAVKTQCLEQAQR 56
Dog        -MEPKATRLR--RGEGPRGEQEDRPSG-EGEPPSGGA----ESWEVLEVVKTQLLEHAQG 52
           *:*  .:** * *   *:*  .   :*:. :  :    :  . :*.**:* **:***

                        Thr254Ile

Human      EQVRFLMKSYSFLREAVPGTLRARRGEGIQAPSFSSYLYFLFCPTLIYRETYPRTPYVRW 294
Monkey     EQVRFLMKSYSFLREAVPGTLRARRGEGIQAPSFSSYLYFLFCPTLIYRETYPRTPYIRW 298
Mouse      EQVRLLMKSYSFLRETVPGIFCVRGGKGISPPSFSSYLYFLFCPTLIYRETYPRTPSIRW 298
Rat        EQVRFLMKSYSFLRETVPGIFCVRGGKGICTPSFSSYLYFLFCPTLIYRETYPRTPSIRW 296
Dog        EQVRLLMKSYSFLREALPGTLCARVGEGMQAPSFSSYLYFLFCPTLIYRKTYPRTPNVRW 292
           ****:*********::** : .* *:*: .*****************:****** :**

                     Ala431Ser

Human      GARARGVAMLGVFLVSAVAHEYIFCFVLGFFYPVMLILFLVIGGMLNFMMHDQRTGPAWN 474
Monkey     GAQARGVAMLGVFLVSAVAHEYIFCFVLGFFYPVMLILFLVIGGMLNFMMHDQHTGPAWN 478
Mouse      GRRARGVAMLGVFLVSAVVHEYIFCFVLGFFYPVMLMLFLVFGGLLNFTMNDRHTGPAWN 478
Rat        GRQGRGAAMLGVFLVSALVHEYIFCFVLGFFYPVMLILFLVVGGLLNFTMNDRHTGPAWN 476
Dog        GGRARGAAMLAVFLVSAVVHEYIFCFVLGFFYPVMLILFLVIGGLMNFMMHDRHTGPAWN 472
           * :.**.***.******:.*****************:****.**::** *:*::******
```

**Figure 4-8.** Multiple alignments of ACAT2 protein orthologs from Homo sapiens (NP_003569), Africa green monkey (O77759), Mus musculus (NP_666176 ), Rattus norvegicus (NP_714950), and Canis familiaris (XP_543637) using CLUSTAL W ver.1.82. Highlights are nsSNPs found in current study. "*": identical residues; ":": conserved residues; ".": semi-conserved.

In addition, multiple sequence alignments were conducted in the acyltransferase family and 6 family members (ACAT1, ACAT2, Are1p, Are2p, DGAT1, and DGAT2) were chosen. This comparison showed that only the 14$^{th}$ residue is semi-conserved and the other two amino acid residues (254$^{th}$ and 431$^{th}$) are not. The 254$^{th}$ residue is most variable, and four different amino acids occurred among these members with isoleucine occurring in DGAT1. The 431A>S or T is less variable, with alanine to be found in ACAT, DGAT, and Are1p and serine in Are2p. At the 14$^{th}$ amino acid residue, glutamic acid was found in ACAT2 and DGAT2, glutamine in Are2p and arginine in ACAT1, Are1p and DGAT1. Although glutamic acid is not completely conserved at the 14$^{th}$ residue, no glycine was found at this site in the homologous

family. Therefore, it is likely that the substitution of glutamine to glycine affects ACAT2 function or structure.

Whether these nsSNPs affect protein structure and function was also predicted by PolyPhen. Analysis of the Glu14Gly variant with PolyPhen yielded a PSIC of 1.575, suggesting that it is possibly damaging while the other two variants were predicted to be neutral changes.

**Figure 4-9**. Multiple alignments of acyltransferase family members: ACAT1 (NP_003092), ACAT2 (NP_003569), Are1P (NP_009978), Are2p (NP_014416), DGAT1 (NP_036211), and DGAT2 (NP_115953).. Highlights are nsSNPs found in current study. "*": identical residues; ":": conserved residues; ".": semi-conserved.

### 4.2.2. Association studies of *ACAT2* gene

#### 4.2.2.1. Genotyping of three polymorphisms

The individual genotype for three polymorphisms, 41A>G, 734C>T, and D/I, were determined based on the number and size of DNA bands on DNA gel image (Figure 4-10).

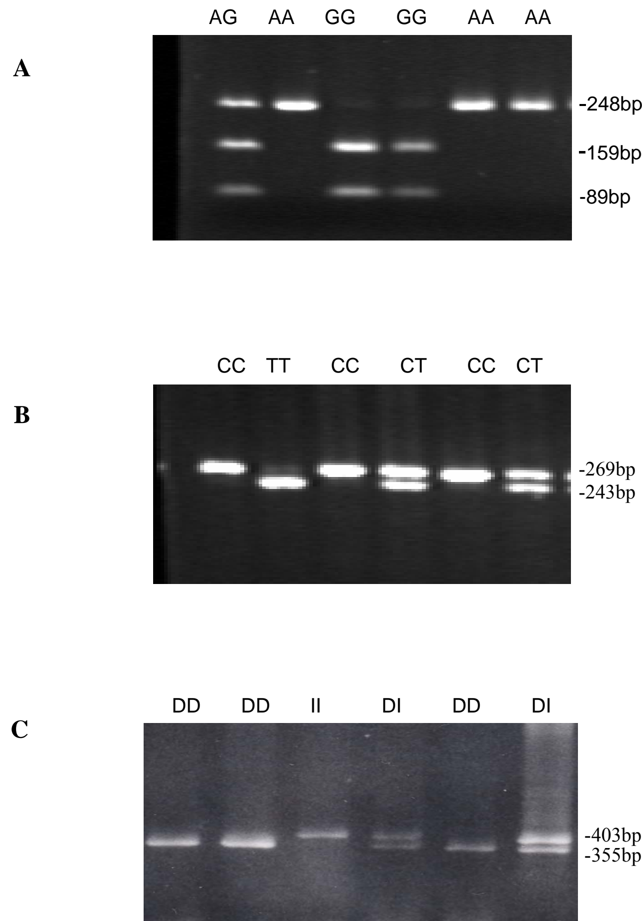



**Figure 4-10**. Genotyping results of three *ACAT2* gene polymorphisms. A: 41A>G; B: 734C>T; C: D/I

#### 4.2.2.2. Population Demographics

The characteristics of the study population are summarized in Table 4-2. Healthy controls were selected from workers who underwent pre-employment medical check-up, and they were significantly younger than the CAD+ subjects in all ethnic

groups (P<0.0005). The controls had significant lower BMI than the CAD+ subjects in Chinese males (P<0.001) and females (P<0.0005) as well as Malay females (P<0.001) but not in the Indians. However, this significance did not exist after adjusting for age. Cigarette smoking was more prevalent among males than females in three ethnic groups. The percentage of smokers was significantly higher in the CAD+ than the CAD- in the Chinese and male Indians but not in the Malays**.** Three parameters, age, BMI, and smoking, are known to influence CAD susceptibility and plasma lipid profiles, so all of them were included as confounders in the association tests. Significantly higher levels of HDL-C, apoA1 and lower levels of Lp(a) were seen in the CAD+ subjects as compared to the CAD- subjects. Smoking is known to decrease HDL-C level, thus, its effect was adjusted when the HDL-C level was compared between the CAD+ and CAD- subjects. Lower levels of TC, LDL-C, TG and apoB were observed in male CAD+ in all ethnicities compared with the male CAD- subjects (P<0.05 or less). The same trend was also seen in the females. These observations are most likely due to the effects of lipid-lowering drugs and adoption of healthy lifestyle in the CAD+ subjects. In view of such confounding effects on the lipid levels, the CAD+ patients were not included when carrying out ANOVA to see whether the genotypes and diplotypes were associated with lipid levels.

**Table 4-2.** Demographics of the Singaporean subjects. Age, BMI and plasma lipid profiles are presented in mean ± SD.

| | Males | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Chinese | | | Malays | | | Indians | | |
| Variables | CAD- n = 389 | CAD+ n = 402 | P | CAD- n = 188 | CAD+ n = 106 | P | CAD- n =215 | CAD+ n = 119 | P |
| Age (years) | 39±14 | 59±9 | <0.0005 | 39±9 | 57±10 | <0.0005 | 43±14 | 59±11 | <0.0005 |
| BMI (kg/m$^2$) | 23.41±3.62 | 24.30±3.59 | 0.306 | 25.32±4.15 | 25.63±3.35 | 0.265 | 24.43±3.89 | 24.75±3.48 | 0.898 |
| Smokers* (%) | 30.23 | 59.95 | <0.0005 | 60.96 | 57.58 | 0.619 | 19.16 | 52.68 | <0.0005 |
| TC (mM) | 5.69±1.32 | 4.53±1.11 | <0.0005 | 5.89±1.25 | 4.63±1.10 | <0.0005 | 5.86±1.18 | 4.41±1.16 | <0.0005 |
| HDL-C (mM) | 1.26±0.33 | 0.96±0.27 | <0.0005** | 1.16±0.26 | 0.89±0.26 | <0.0005** | 1.02±0.27 | 0.91±0.23 | <0.001** |
| LDL-C (mM) | 3.92±1.34 | 2.92±0.85 | 0.016 | 4.26±1.22 | 3.56±0.85 | 0.003 | 4.26±1.20 | 3.51±1.16 | <0.0005 |
| TG (mM) | 2.03±1.98 | 1.79±1.00 | 0.035 | 2.16±1.42 | 1.92±0.94 | 0.582 | 2.24±1.58 | 1.63±0.76 | <0.0005 |
| ApoA1 (mg/dl) | 140.64±24.01 | 117.49±20.81 | <0.0005 | 128.43±17.70 | 114.25±19.82 | <0.0005 | 132.08±23.83 | 112.33±22.27 | <0.0005 |
| ApoB (mg/dl) | 108.65±30.68 | 101.57±29.53 | <0.001 | 122.85±28.75 | 102.29±28.20 | <0.0005 | 129.70±32.17 | 101.05±29.16 | <0.0005 |
| Lp(a) (mg/dl) | 12.95±15.90 | 21.73±20.77 | <0.0005 | 11.82±11.18 | 22.37±18.14 | <0.0005 | 18.30±19.17 | 29.39±25.04 | <0.0005 |

**Table 4-2(Continued)** Demographics of the Singaporean subjects. Age, BMI and plasma lipid profiles are presented in mean ± SD

| | Females | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Chinese** | | | **Malays** | | | **Indians** | | |
| Variables | CAD-<br>n = 313 | CAD+<br>n = 124 | P | CAD-<br>n =42 | CAD+<br>n = 31 | P | CAD-<br>n =157 | CAD+<br>n = 27 | P |
| Age (years) | 35±12 | 62±9 | <0.0005 | 29±12 | 61±7.78 | 0.0005 | 38±13 | 61±8 | <0.0005 |
| BMI (kg/m$^2$) | 22.14±3.82 | 23.91±3.34 | 0.447 | 23.27±6.08 | 28.03±3.95 | 0.260 | 24.73±4.77 | 25.22±4.90 | 0.261 |
| Smokers* (%) | 3.19 | 16.81 | <0.0005 | 4.76 | 10.34 | 0.327 | 1.91 | 7.69 | 0.148 |
| TC (mM) | 5.54±1.20 | 4.61±1.24 | <0.0005 | 5.09±1.04 | 4.68±1.20 | 0.121 | 5.26±1.08 | 4.04±1.11 | <0.0005 |
| HDL-C (mM) | 1.55±0.41 | 1.04±0.32 | <0.0005** | 1.39±0.33 | 0.99±0.31 | <0.0005** | 1.27±0.34 | 0.96±0.29 | <0.0005** |
| LDL-C (mM) | 3.61±1.20 | 3.57±0.99 | 0.543 | 3.35±0.97 | 3.64±0.91 | 0.552 | 3.60±1.10 | 2.99±0.64 | 0.082 |
| TG (mM) | 1.36±0.79 | 1.75±0.80 | <0.0005 | 1.04±0.64 | 1.82±1.02 | <0.0005 | 1.46±1.11 | 1.68±0.73 | 0.314 |
| ApoA1 (mg/dl) | 151.97±23.93 | 126.73±24.56 | <0.0005 | 146.38±18.11 | 119.13±22.51 | <0.0005 | 140.08±26.36 | 122.78±21.90 | 0.002 |
| ApoB (mg/dl) | 92.43±26.66 | 98.71±28.65 | 0.031 | 97.12±20.56 | 98.42±26.25 | 0.811 | 108.89±27.45 | 90.67±25.02 | 0.002 |
| Lp(a) (mg/dl) | 17.76±21.60 | 25.23±24.35 | 0.004 | 13.26±11.20 | 21.44±17.44 | 0.031 | 22.16±20.12 | 27.68±30.83 | 0.250 |

*: Smokers and ex-smokers form one category while non smokers constitute another. **: Adjusted by smoking.

#### 4.2.2.3. Genotype and allele frequencies

The genotype distributions for the three polymorphisms are shown in Table 4-3 and 4-4. All genotype frequencies did not deviate from the expected Hardy-Weinberg proportions. In terms of the association of the genotypes with CAD and dyslipidemia susceptibility, two genetic models, dominant and recessive, were used. In the case of dominant model, no association was detected in the three ethnic groups. Under the assumption of recessive model, the 734C>T and the 41A>G were found to be associated with CAD risk in the Chinese, with heterozygotes and mutant homozygotes being more prevalent in the CAD+ subjects, (P=0.0051, and P=0.0104, respectively) and the D/I in Indians (P=0.015). However, only the association of the 734C>T had statistical significance after multiple comparison correction (OR=1.49; 95% CI: 1.43 – 1.55).

The distribution of allele frequencies of the three polymorphisms in different ethnic groups was investigated before carrying out the test of their association with disease. The results showed that the allele frequencies of the 734C>T and D/I were significantly different among the three ethnic groups (Table 4-3). The 734T and I alleles were the lowest in Indians, followed by Malays, and Chinese. Unlike the 734C>T and D/I, whose distributions were significantly different among the three ethnic groups, 41A>G showed different allele frequency only between the Chinese and Indians. In terms of association with CAD susceptibility, the 734T frequency was significantly lower in the CAD+ patients than in the CAD- controls in the Chinese (0.26 in CAD- vs. 0.20 in CAD+, P = 0.003; OR = 0.72; 95% CI: 0.57 – 0.90). The statistical significance was still observed after Bonferroni correction. In Indians, the I allele was associated with increased CAD risk (0.17 in CAD+ vs. 0.10

in CAD-, P = 0.01; OR= 1.89; 95% CI: 1.65-2.18). However, this significance did not remain after Bonferroni correction.

Considering the effect of confounders such as age, BMI and smoking, the binary logistic regression model was applied with these confounders as covariates. It was observed that the association of the 734C>T polymorphism with CAD was still significant after adjusting for these confounding effects (P=0.039). In fact, age and smoking were not significant confounding factors in the Chinese but BMI was. In the Indians, smoking and age were the significant confounders. However, the association of the D/I polymorphism with CAD remained significant in the presence of smoking and age in the model. After Bonferroni correction, all these significant associations did not remain.

There was no significant difference in genotype and allele frequencies of 734C>T and D/I between the normolipidemic and dyslipidemic subgroups in all three ethnic groups. Comparing normolipidemic and dyslipidemic individuals in the three ethnic groups, significantly higher G allele frequency was observed in the normolipidemic Chinese (P=0.008). The analysis under the assumption of recessive genetic model also showed that the AG+GG genotype was more prevalent in the normolipidemic subjects than the dyslipidemic subjects (P=0.0059). However, these significant differences did not exist after multiple comparison correction.

#### 4.2.2.4. Linkage disequilibrium among these three polymorphisms

The genomic distance between 41A>G and 734C>T is 12.5kb, with D/I being located between them. The D/I is closer to 41A>G (2.2kb) than to 734C>T (10.3kb). Linkage

disequilibria of different degrees were found between these three polymorphisms in the various subgroups (Table 4-5). The 734C>T was found to be in very strong LD with D/I in the CAD+ (Δ> = 0.64, P<0.0005) in three ethnic groups and in the CAD- group (Δ>0.90, P<0.0005). Strong LD was also observed between 41A>G and 734C>T and between 41A> G and D/I.

#### 4.2.2.5. Multi-loci case-control analysis

Other than the single-locus analysis, two forms of multi-loci association tests, using haplotypes and diplotypes, were also conducted to evaluate the combined effects of different polymorphic sites. Due to the nearly complete LD between D/I and 734C>T, the D/I was not included in the haplotype and diplotype analysis. Only subjects with complete genotype data at the two loci were examined in the two-locus association study. The final subjects consisted of 580 Chinese (158 CAD+ vs. 422 CAD-), 156 Malays (45 CAD+ vs. 111 CAD-) and 202 Indians (51 CAD+ vs. 151 CAD-).

The results of the 4 haplotypes for these two polymorphic sites are summarized in Table 4-6. The differences in haplotype frequencies between the CAD+ and CAD- subjects and between the normolipidemic and dyslipidemic groups were evaluated by permutation test. In all three ethnic groups, the GC haplotype was about two times higher among the CAD+ patients than among the CAD- controls. However, statistically significant difference was attained only in the Chinese (Permutation P=0.032; OR = 1.85; 95%CI: 1.38-2.48) but not in Malays and Indians, which was most likely due to the smaller sample sizes in these two ethnic groups. The comparison of the haplotype frequencies between the normolipidemic and the dyslipidemic groups showed that the GC haplotype was more frequent in the

normolipidemic subjects than in the dyslipidemic subjects (Permutation P=0.012; OR = 0.32; 95%CI: 0.14-0.76) in the Chinese while the AC haplotype was higher in the dyslipidemic group as compared to the normolipidemic group (Permutation P = 0.023; OR = 1.44; 95%CI: 1.29-1.59). The lower GC haplotype in the dyslipidemic subjects was not expected; however, the result is still reasonable, as dyslipidemia is only one of many risk factors for CAD.

Diplotypes were determined by combining genotype information from the 41A>G and 734C>T polymorphic sites. All possible combinations of eight diplotypes were present in the Chinese group while the Malays and Indians had only seven diplotypes. There was no significant difference in diplotype frequencies between the CAD+ and CAD- subjects, as well as between the normolipidemic and dyslipidemic groups in all three ethnic groups.

**Table 4-3**. Genotype and allele frequencies (freq) of the *ACAT2* polymorphisms in the three Singaporean ethnic groups.

| | | **Chinese** | | **Malays** | | **Indians** | |
|---|---|---|---|---|---|---|---|
| | | CAD- | CAD+ | CAD- | CAD+ | CAD- | CAD+ |
| 734C>T n (%) | CC | 274 (56) | 240 (65) | 97 (66) | 71(73) | 162 (83) | 87 (86) |
| | CT | 182 (37) | 110 (30) | 45 (31) | 23 (23) | 29 (15) | 14 (14) |
| | TT | 37 (7) | 19 (5) | 4 (3) | 4 (4) | 4 (2) | 0 (0) |
| | T freq | 0.26†‡ | 0.20 | 0.18– | 0.16 | 0.09– | 0.07 |
| P | T freq | 0.003 | | 0.563 | | 0.386 | |
| | CC+CT vs. TT | 0.165 | | 0.563 | | 0.305 | |
| | CC vs. CT+TT | 0.005 | | 0.325 | | 0.493 | |
| 41A>G n (%) | AA | 367 (68) | 175 (66) | 100 (72) | 48 (70) | 231 (78) | 56 (72) |
| | AG | 164 (30) | 84 (32) | 34 (25) | 18 (26) | 61 (20) | 20 (25) |
| | GG | 9 (2) | 4 (2) | 4 (3) | 3 (4) | 5 (2) | 2 (3) |
| | G freq | 0.17† | 0.17 | 0.15 | 0.17 | 0.12† | 0.15 |
| P | G freq | 0.960 | | 0.604 | | 0.342 | |
| | CC+CT vs. TT | 0.878 | | 0.308 | | 0.609 | |
| | CC vs. CT+TT | 0.010 | | 0.663 | | 0.267 | |
| D/I n (%) | DD | 313 (54) | 198 (60) | 128 (62) | 64 (63) | 182 (83) | 91 (72) |
| | DI | 223 (38) | 105 (32) | 72 (35) | 34 (33) | 34 (15) | 30 (23) |
| | II | 45 (8) | 25 (8) | 6 (3) | 4 (4) | 4 (2) | 6 (5) |
| | I freq | 0.27†‡ | 0.24 | 0.20– | 0.21 | 0.10– | 0.17 |
| P | I freq | 0.156 | | 0.773 | | 0.011 | |
| | CC+CT vs. TT | 0.945 | | 0.638 | | 0.119 | |
| | CC vs. CT+TT | 0.058 | | 0.918 | | 0.015 | |

–, – : Significant difference in allele frequencies between ethnic groups (P<0.01).

**Table 4-4** Genotype and allele frequencies (freq) of 734C>T, D/I and 41A>G in the normolipidemic (Normo) and dyslipidemic (Dys) subjects.

| | | **Chinese** | | **Malays** | | **Indians** | |
|---|---|---|---|---|---|---|---|
| | | Normo | Dys | Normo | Dys | Normo | Dys |
| 734C>T n (%) | CC | 170 (54) | 107 (60) | 53 (70) | 45 (62) | 93 (85) | 70 (81) |
| | CT | 117 (37) | 65 (36) | 20 (27) | 25 (35) | 14 (13) | 15 (17) |
| | TT | 29 (9) | 8 (4) | 2 (3) | 2 (3) | 2 (2) | 2 (2) |
| | T freq | 0.28 | 0.23 | 0.18 | 0.20 | 0.08 | 0.11 |
| P | T freq | 0.085 | | 0.662 | | 0.317 | |
| | CC+CT vs. TT | 0.054 | | 0.967 | | 0.943 | |
| | CC vs. CT+TT | 0.223 | | 0.294 | | 0.366 | |
| 41A>G n (%) | AA | 208 (64) | 156 (75) | 53 (74) | 47 (71) | 136 (81) | 95 (74) |
| | AG | 112 (32) | 50 (24) | 18 (25) | 16 (24) | 30 (18) | 31(24) |
| | GG | 7 (2) | 2 (1) | 1 (1) | 3 (5) | 2(1) | 3 (2) |
| | G freq | 0.19* | 0.13* | 0.14 | 0.17 | 0.10 | 0.14 |
| P | G freq | 0.008 | | 0.492 | | 0.140 | |
| | CC+CT vs. TT | 0.295 | | 0.270 | | 0.451 | |
| | CC vs. CT+TT | 0.006 | | 0.782 | | 0.133 | |
| D/I n (%) | DD | 183 (53) | 127 (56) | 49 (64) | 62 (62) | 90 (84) | 75 (82) |
| | DI | 134 (38) | 87 (38) | 24 (32) | 35 (35) | 16 (15) | 15 (16) |
| | II | 31 (9) | 14 (6) | 3 (4) | 3 (3) | 1 (1) | 2 (2) |
| | I freq | 0.28 | 0.25 | 0.20 | 0.20 | 0.08 | 0.10 |
| P | I freq | 0.257 | | 0.944 | | 0.488 | |
| | CC+CT vs. TT | 0.226 | | 0.731 | | 0.474 | |
| | CC vs. CT+TT | 0.463 | | 0.737 | | 0.629 | |

* Significant difference in allele frequency (P<0.01)

**Table 4-5**. Linkage disequilibria between the 3 polymorphisms of *ACAT2* gene

| | Chinese | | Malays | | Indians | |
|---|---|---|---|---|---|---|
| | CAD- | CAD+ | CAD- | CAD+ | CAD- | CAD+ |
| LD between | Δ=0.98 | Δ=0.67 | Δ = 0.96 | Δ=0.71 | Δ = 0.91 | Δ=0.64 |
| 734C>T and D/I | P<0.0005 | P<0.0005 | P<0.0005 | P=0.009 | P<0.0005 | P=0.086 |
| LD between | Δ = 0.52 | Δ = 0.42 | Δ = 0.65 | Δ = 0.41 | Δ = 0.62 | Δ = 0.68 |
| 734C>T and | P<0.0005 | P<0.0005 | P<0.0005 | P=0.004 | P<0.0005 | P<0.0005 |
| LD between D/I | Δ = 0.52 | Δ = 0.37 | Δ = 0.75 | Δ = 0.58 | Δ = 0.59 | Δ = 0.53 |
| and 41A>G | P<0.0005 | P<0.0005 | P<0.0005 | P<0.0005 | P<0.0005 | P<0.0005 |

Δ: Linkage disequilibria correlation coefficient.

**Table 4-6** Haplotype frequencies for 41A>G and 734C>T in CAD+ and CAD- subjects as well as in normolipidemic and dyslipidemic various groups.

| | | | **Chinese** | | | |
|---|---|---|---|---|---|---|
| Haplotype | CAD- | CAD+ | Permutation P* | Normo | Dys | Permutation P* |
| AC | 0.698 | 0.707 | 0.763 | 0.671 | 0.745 | **0.023** |
| AT | 0.129 | 0.113 | 0.482 | 0.133 | 0.122 | 0.689 |
| GC | 0.042 | 0.075 | **0.032** | 0.057 | 0.019 | **0.012** |
| GT | 0.131 | 0.106 | 0.279 | 0.139 | 0.115 | 0.325 |
| | | | **Malays** | | | |
| Haplotype | CAD- | CAD+ | Permutation P* | Normo | Dys | Permutation P* |
| AC | 0.791 | 0.751 | 0.483 | 0.796 | 0.785 | 0.869 |
| AT | 0.047 | 0.071 | 0.421 | 0.058 | 0.037 | 0.350 |
| GC | 0.047 | 0.093 | 0.126 | 0.067 | 0.0278 | 0.106 |
| GT | 0.115 | 0.085 | 0.470 | 0.078 | 0.151 | 0.097 |
| | | | **Indians** | | | |
| Haplotype | CAD- | CAD+ | Permutation* | Normo | Dys | Permutation P |
| AC | 0.853 | 0.843 | 0.877 | 0.862 | 0.839 | 0.613 |
| AT | 0.031 | 1.92E-10 | 0.153 | 0.045 | 0.009 | 0.071 |
| GC | 0.044 | 0.0784 | 0.164 | 0.045 | 0.043 | 0.781 |
| GT | 0.072 | 0.078 | 0.842 | 0.041 | 0.110 | 0.103 |

*Permutation test significance levels for individual haplotype frequency comparisons between the CAD+ and CAD- groups.

#### 4.2.2.6. Association of single-locus genotype with lipid traits in the CAD- subjects

In terms of the association with plasma lipid profiles, three models, dominant, additive, and recessive, were applied when three different genotypes were present. For normolipidemic Malay and Indian females, no mutant heterozygote was observed. Therefore, only additive model was used in these two subgroups. Except for the normolipidemic Malay and Indian females, there was no significant association to be found using any genetic model in other subjects. As such, only the data for normolipidemic female subjects is presented in details.

##### 4.2.2.6.1. c.734C>T

There was no significant difference in lipid profiles between the CAD+ and CAD- subjects as well as between the normolipidemic and dyslipidemic subgroups in the Chinese. The correlation of genotypes with plasma lipid traits in female normolipidemic Malays and Indians is shown in table 4-7. The normolipidemic female Malays with the CT genotype had significantly lower apoA1 level than those with the CC genotype (P=0.007). In the normolipidemic female Indians, significantly decreased plasma apoA1 (P=0.027), increased apoB (P=0.007) and Lp(a) (P=0.011) levels were observed in the CT heterozygous than the CC individuals. These analysis were adjusted with age, BMI, and smoking. No TT homozygote was found in the two female normolipidemic groups. However, these statistical significances were not observed after multiple comparison correction in the two subgroups. The CT carriers had a trend towards increased LDL-C compared with CC individuals, but this is not statistically significant.

#### 4.2.2.6.2. D/I

ANOVA with age, BMI and smoking as covariates showed that the effects of this polymorphism on apoA1 in female normolipidemic Malays and on Lp(a) in female Indians were very similar to that of 734C>T (Table 4-8). The phenomenon is not surprising as both polymorphisms are in strong linkage disequilibrium. However, the statistically significance was only observed on Lp(a) level in Indian females (P=0.004), with DI heterozyotes being associated with increased Lp(a) level. In addition, elevated LDL was found in DI heterozygotes comparing with the DD carriers in Malay females (P=0.013). Actually, the trend was the same as that of 734C>T on LDL in this Malay group. However, all these significances did not remain after Bonferroni correction.

#### 4.2.2.6.3. c.41A>G

In female normolipidemic Indians (Table 4-9), the heterozygous carriers of the 41A>G had higher TC than AA homozygotes. The rare GG homozygotes were not found in female Malays and female Indians.

### 4.2.2.7. Association of diplotypes with lipid traits

Diplotypes were determined by combining genotype information from the 41A>G and 734C>T sites. All possible combinations of eight diplotypes were present in Chinese group while Malays and Indians had only seven diplotypes. There was no significant difference in diplotype frequencies between CAD+ and CAD-, as well as between normolipidemic and dyslipidemic groups in all three ethnic groups. The association study of diplotypes and lipid traits was conducted only in CAD- subjects and only positive results were present here (Table 4-10). There were only 4

diplotypes in normolipidemic Malay and Indian females and 1 diplotype (AA/CT) was omitted due to small sample size in the Malays. In normolipidemic Malay females, only apoA1 level was found to be significantly different between AG/CT and AA/CC as well as AG/CC (P=0.0031 and P=0.0035 respectively). In the Indian females, the AA/CT carriers had significantly higher level of apoB relative to the wild type AA/CC homozygotes (P=0.006). After multiple comparison correction, the association did not exist any more.

**Table 4-7.** Genotypic lipid levels (Mean ± SD) of the 734C>T in the healthy normolipidemic Chinese, Malay and Indian females.

| | Chinese | | | | Malays | | | Indians | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | CC | CT | TT | | CC | CT | P | CC | CT | |
| | n=97 | n=58 | n=16 | P | n=18 | n=7 | | n=49 | n = 7 | P |
| TC(mM) | 4.67±0.71 | 4.60±0.66 | 4.95±0.49 | 0.212 | 4.32±0.70 | 4.70±0.37 | 0.287 | 4.67±0.79 | 4.76±0.62 | 0.238 |
| LDL-C(mM) | 2.75±0.73 | 2.86±0.71 | 3.00±0.47 | 0.715 | 2.59±0.70 | 3.20±0.46 | 0.131 | 2.95±0.81 | 3.26±0.72 | 0.069 |
| HDL-C(mM) | 1.56±0.31 | 1.44±0.38 | 1.50±0.20 | 0.529 | 1.29±0.22 | 1.23±0.26 | 0.566 | 1.29±0.31 | 1.09±0.22 | 0.147 |
| ApoA1(mg/dl) | 148.29±22.11 | 143.77±23.98 | 144.27±25.56 | 0.784 | 150.22±14.63 | 131.29±13.10 | 0.007 | 137.76±25.96 | 114.71±17.23 | 0.027 |
| ApoB(mg/dl) | 79.74±18.18 | 76.62±17.28 | 81.55±14.96 | 0.267 | 87.17±12.78 | 93.67±13.10 | 0.345 | 94.88±17.25 | 107.86±18.49 | 0.007 |
| lnTG* | 0.02±0.39 | 0.06±0.41 | 0.09±0.40 | 0.697 | 0.07±0.37 | 0.29±0.28 | 0.204 | 0.06±0.38 | 0.04±0.29 | 0.765 |
| TG** | 1.02 | 1.06 | 1.09 | | 1.07 | 1.34 | | 1.06 | 1.04 | |
| lnLp(a)* | 2.41±0.94 | 2.30±0.93 | 2.58±0.84 | 0.595 | 2.51±0.57 | 2.94±0.72 | 0.191 | 2.65±0.71 | 3.42±0.82 | 0.011 |
| Lp(a)** | 8.50 | 9.97 | 12.18 | | 12.30 | 18.92 | | 14.15 | 30.57 | |

*: lnTG and lnLp(a): natural logarithmic transformed TG and Lp(a), respectively.
**: TG and Lp(a) levels are presented in geometric means.
Age, BMI and smoking were included as covariates in the ANOVA.

**Table 4-8.** Genotypic lipid levels (Mean ± SD) of the D/I in the healthy normolipidemic Chinese, Malay and Indian females.

| | Chinese | | | | Malays | | | Indians | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | DD | DI | II | P | DD | DI | P | DD | DI | P |
| | n=106 | n=61 | n=16 | | n=18 | n=11 | | n=68 | n = 11 | |
| TC(mM) | 5.08±0.69 | 4.93±0.71 | 5.23±0.58 | 0.224 | 4.74±0.78 | 5.19±0.58 | 0.110 | 4.76±0.66 | 4.57±0.66 | 0.705 |
| LDL-C(mM) | 3.09±0.69 | 3.09±0.77 | 3.24±0.55 | 0.709 | 2.89±0.70 | 3.55±0.56 | 0.013 | 3.07±0.70 | 3.06±0.68 | 0.678 |
| HDL-C(mM) | 1.59±0.32 | 1.52±0.31 | 1.59±0.29 | 0.475 | 1.36±0.25 | 1.35±0.28 | 0.903 | 1.27±0.29 | 1.19±0.22 | 0.409 |
| ApoA1(mg/dl) | 151.17±20.89 | 149.30±20.55 | 144.87±23.25 | 0.582 | 149.88±13.46 | 142.30±15.00 | 0.187 | 130.11±19.24 | 124.45±18.26 | 0.368 |
| ApoB(mg/dl) | 82.35±17.16 | 80.09±15.29 | 85.50±14.78 | 0.372 | 89.56±13.63 | 101.10±15.18 | 0.049 | 94.32±19.20 | 95.45±19.24 | 0.510 |
| lnTG* | -0.055±0.36 | 0.0013±0.37 | -0.0069±0.32 | 0.632 | -0.136±0.34 | -0.196±0.27 | 0.628 | 0.0808±0.36 | 0.0016±0.26 | 0.493 |
| TG** | 1.07 | 1.00 | 1.01 | | 1.15 | 1.22 | | 1.08 | 1.00 | |
| lnLp(a)* | 2.40±0.93 | 2.30±0.92 | 2.43±0.68 | 0.817 | 2.55±0.53 | 2.31±0.76 | 0.402 | 2.45±0.63 | 3.23±0.63 | 0.004 |
| Lp(a)** | 11.02 | 9.97 | 11.36 | | 12.81 | 10.07 | | 15.56 | 25.28 | |

*: lnTG and lnLp(a): natural logarithmic transformed TG and Lp(a), respectively.
**: TG and Lp(a) levels are presented in geometric means.
Age, BMI and smoking were included as covariates in the ANOVA.

**Table 4-9.** Genotypic lipid levels (Mean ± SD) of the 41A >G in the healthy female normolipidemic Chinese, Malays and Indians

| | Chinese | | | | Malays | | | Indians | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | AA | AG | GG | P | AA | AG | P | AA | AG | P |
| | n=115 | n=63 | n=5 | | n=19 | n=9 | | n=65 | n = 19 | |
| TC(mM) | 5.09±0.65 | 4.97±0.70 | 5.01±0.62 | 0.578 | 4.61±0.81 | 4.66±0.77 | 0.899 | 4.84±0.69 | 5.06±0.71 | 0.042 |
| LDL-C(mM) | 3.17±0.71 | 3.14±0.67 | 2.93±0.62 | 0.777 | 2.81±0.68 | 2.99±0.65 | 0.620 | 3.24±0.77 | 3.37±0.82 | 0.208 |
| HDL-C(mM) | 1.60±0.38 | 1.55±0.35 | 1.70±0.39 | 0.489 | 1.45±0.33 | 1.33±0.31 | 0.347 | 1.24±0.31 | 1.28±0.26 | 0.660 |
| ApoA1(mg/dl) | 151.83±23.33 | 151.70±22.91 | 145.60±28.99 | 0.788 | 148.44±15.28 | 137.89±19.60 | 0.156 | 107.33±9.00 | 104.37±15.57 | 0.659 |
| ApoB(mg/dl) | 83.30±17.17 | 89.02±17.45 | 82.20±14.76 | 0.099 | 86.39±13.39 | 93.67±21.64 | 0.255 | 97.19±22.38 | 104.17±16.29 | 0.070 |
| lnTG* | -.0054±0.43 | -.032±0.36 | -.0114±0.30 | 0.955 | -0.21±0.39 | -0.24±0.35 | 0.789 | 0.016±0.38 | 0.06±0.52 | 0.382 |
| TG** | 0.99 | 0.97 | 0.99 | | 0.81 | 0.76 | | 1.02 | 1.06 | |
| lnLp(a)* | 2.47±0.93 | 2.40±0.79 | 2.65±1.21 | 0.841 | 2.34±0.54 | 2.51±0.61 | 0.634 | 2.63±0.71 | 2.46±0.84 | 0.387 |
| Lp(a)** | 11.82 | 11.02 | 14.15 | | 10.38 | 12.30 | | 13.87 | 11.70 | |

*: lnTG and lnLp(a): natural logarithmic transformed TG and Lp(a), respectively.
**: TG and Lp(a) levels are presented in geometric means.
Age, BMI and smoking were included as covariates in the ANOVA.

**Table 4-10.** Diplotypic lipid levels in normolipidemic Malay and Indian females.

| | Malays | | | Indians | | | |
|---|---|---|---|---|---|---|---|
| | AA/CC | AG/CC | AG/CT | AA/CC | AG/CC | AA/CT | AG/CT |
| | n=13 | n=4 | n=4 | n=37 | n=6 | n=3 | n=3 |
| TC (mM) | 4.71±0.92 | 4.96±0.91 | 4.65±0.32 | 4.56±0.81 | 4.70±0.50 | 4.98±0.85 | 4.53±0.52 |
| LDL-C (mM) | 2.83±0.77 | 2.99±0.79 | 3.16±0.56 | 2.84±0.87 | 2.86±0.64 | 3.33±1.09 | 3.05±0.45 |
| HDL-C (mM) | 1.45±0.36 | 1.52±0.25 | 1.19±0.31 | 1.30±0.37 | 1.38±0.38 | 1.26±0.21 | 1.02±0.045 |
| ApoA1(mg/dl) | 150.08±15.39[a] | 152.50±15.55[b] | 125.50±16.34[c] | 133.00±23.53 | 130.60±7.27 | 125.67±9.45 | 108.33±21.60 |
| ApoB (mg/dl) | 88.67±12.45 | 101.50±23.76 | 94.25±14.98 | 92.30±18.92[d] | 97.83±16.43 | 115.0±28.62[e] | 99.33±3.51 |
| TG | 0.87 | 0.99 | 0.71 | 1.04 | 1.07 | 0.97 | 1.01 |
| InTG | -0.13±0.44 | -0.01±0.10 | -0.34±0.38 | 0.04±0.34 | 0.07±0.61 | -0.04±0.40 | 0.01±0.15 |
| Lp(a) | 12.68 | 11.13 | 16.44 | 14.59 | 11.02 | 33.45 | 25.53 |
| InLp(a) | 2.54±0.57 | 2.41±0.66 | 2.80±0.61 | 2.68±0.68 | 2.40±1.08 | 3.51±1.20 | 3.24±0.68 |

[a] significantly higher than [c] (P=0.031)
[b] significantly higher than [c] (P=0.035)
[d] significantly lower than [e] (P<0.006)
Age, BMI and smoking were included as covariates in the ANOVA.

### 4.2.3. Functional analysis of two nsSNPs of *ACAT2* gene

#### 4.2.3.1. Expression of ACAT2 in AC-29 cell

In contrast to pcDNA3.1 vector-transfected cells, *ACAT2*-transfected cells contained abundant lipid droplets, which were seen under inverted or differential interference contrast microscope or fluorescence microscope after Nile red staining (Figure 4-11).

The mRNA expression was verified by reverse transcription PCR using *ACAT2*-specific primers and *GAPDH*-specific primers. The amplification of *ACAT2* was only detected in ACAT2 transfected cells, but not in parent AC-29 cells and in the cells transfected with vector alone (Figure 4-12 A). With regards to the mRNA expression levels of wild type *ACAT2* and the other three variants, reverse transcription real-time PCR showed that there was no significant difference in *ACAT2* gene expression level between wildtype *ACAT2* and mutant *ACAT2*s, when normalized by reference gene *GAPDH* (Gly14/WT: 1.32 ± 0.22, P=0.13; Ile254/WT: 0.94±0.14, P=0.57; Gly14Ile254/WT: 1.10±0.22, P=0.64). The western blot results however showed that the ACAT2 protein was expressed about two-fold higher level in mutants with Gly14 when compared to that of wildtype and the mutant with Ile254 (Figure 4-12 B).

#### 4.2.3.2. ACAT2 activity assay

The measurement of ACAT enzyme activity was carried out in triplicate and their average was used for data analysis. The ACAT2 activity assay showed that the rate of CE biosynthesis in intact cells transfected with the Gly14 mutants was higher than that in cells transfected with wildtype or the Ile254 mutant only (Figure 4-13).

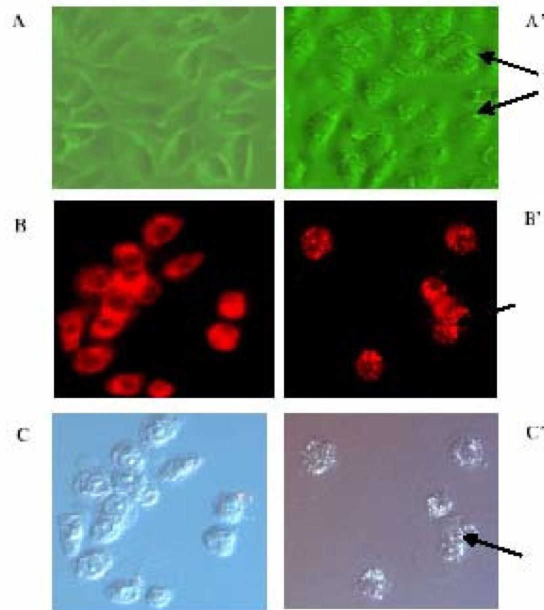



**Figure 4-11**. Cells viewed with inverted microscope (**A** and **A**') and Nile red-staining with Texas red (**B** and **B**') and differential infraction contrast (**C** and **C**'). *ACAT2*-transfected cells (**A'**, **B'** and **C'** was filled with numerous lipid droplets while no obvious lipid droplets were seen in control cells (vector-transfected cells, **A, B** and **C**). Arrows indicated lipid droplets.

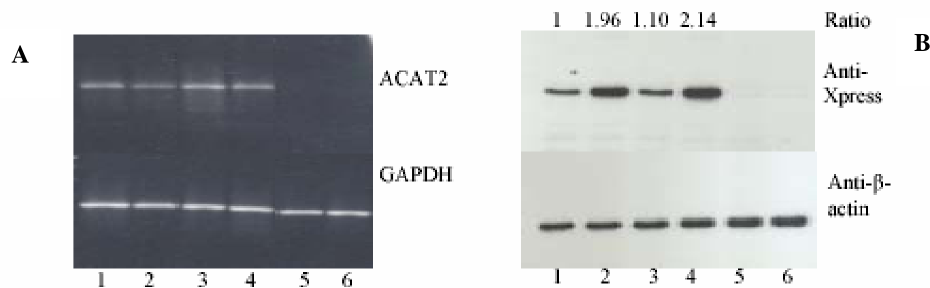



**Figure 4-12.** *ACAT2* mRNA and protein expression in AC-29 cells. Lane 1: wildtype *ACAT2*; Lane 2: *ACAT2*-Gly14; Lane 3: *ACAT2*-Ile254; Lane 4: *ACAT2*-Gly14Ile254; Lane 5 and 6: negative controls (vector alone-transfected AC-29 cells and untransfected AC-29). Ratio: the expression level ratio of various mutant *ACAT2* to wildtype *ACAT2*. (**A**) The amplified *ACAT2* products were detected in *ACAT2*-transfected cells but not in control (cells transfected with vector alone and untransfected AC-29 cells). (**B**) Western blot analysis using anti-Xpress and anti-β-actin as primary antibody and quantitations of ACAT2 bands by densitometric analysis. The ACAT2 protein was detected in *ACAT2*-transfected cells.

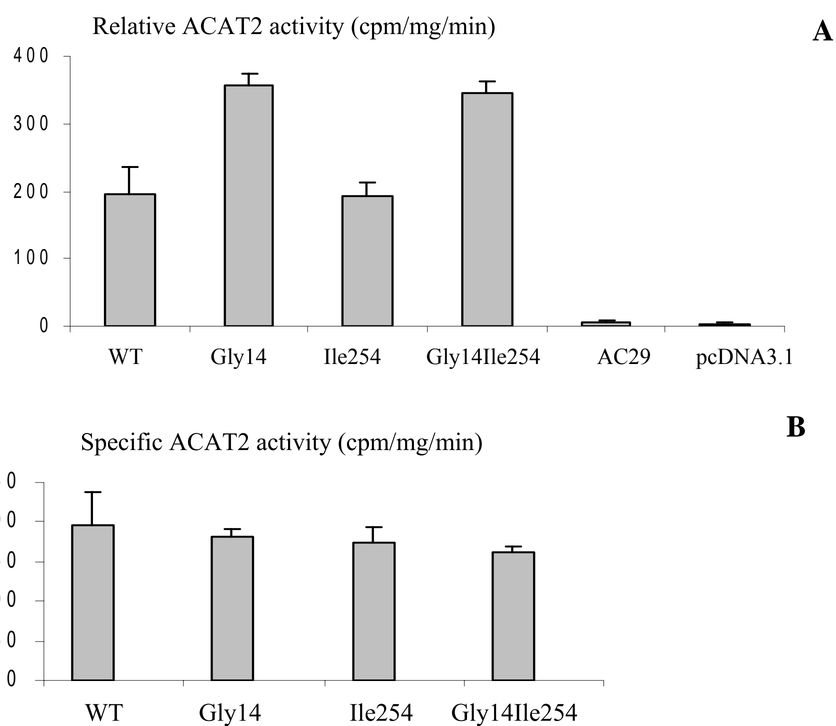



**Figure 4-13.** ACAT2 enzyme activity analysis of wild type and mutant ACAT2s. (**A**) The relative rates of cholesteryl ester biosynthesis in intact cells expressing various ACAT2 indicated. (**B**) Normalized ACAT2 activity of various ACAT2 by different ACAT2 protein expression levels.

**4.3. Discussion**

ACAT2 has been identified as an important enzyme in the development of hypercholesterolemia and as a therapeutic target for treatment of hypercholesterolemia and coronary artery atherosclerosis (Buhman *et al.*, 2000; Chang *et al.*, 2000; Rudel *et al.*, 2005). It was hypothesized that the variations in *ACAT2* gene may be implicated in hypercholesterolemia and CAD susceptibility as well as related phenotypes. In order to test this hypothesis, the regulatory regions and the entire coding region of *ACAT2* gene, were screened for sequence variations. This is the initial step for subsequent association study to investigate the effect of *ACAT2* gene polymorphisms on hypercholesterolemia and CAD susceptibility.

For the detection of *ACAT2* gene polymorphisms, a highly sensitive and economic method, DHPLC, was used. After screening about 300 cord blood samples, a total of fourteen polymorphisms were found. Of these polymorphisms, two were single-base changes in the putative promoter region; five were sequence variants located in exonic regions: three were nsSNPs and two were silent mutations; and seven were sequence variants, comprising six single base substitutions and one 48bp-insertion, within intronic regions. Among these seven sequence variants, five SNPs, 41A>G, 734C>T, IVS9+37A>T, IVS9+51G>T, IVS1-8C>G, had been reported previously (Katsuren *et al.*, 2001 and 2003; Haga *et al.*, 2002), and other nine polymorphisms were novel and have been submitted to the NCBIs SNP database.

DHPLC has been documented to be a highly sensitive method for the detection of point mutation and small insertions/deletions (Xiao and Oefner 2001; O'Donovan *et al.*, 1998; Liu *et al*; 1997; Gross *et al.*, 1999; Jones *et al.*, 1999), which also has been

proven in the study. In this study, all known polymorphisms within the coding region and all intronic-exonic boundaries had been detected. However, within the 5'UTR or putative promoter region, some SNPs, such as -74A>G, -1279G>A, -1283G>A, -1327A>C, and -1382A>C, had been reported in the NCBIs SNP database but were not found in current study. It was postulated that these undetected polymorphisms may occur only in certain population or at quite low frequencies. For example, -1279G>A, -1283G>A, -1327A>C, and -1382A>C were found in Caucasians, and the -1279G>A occurs at a very low frequency (0.005 for rare A allele).

In addition, it was proven that the use of primers with a GC clamp is able to improve the efficiency of mutation detection. The mechanism behind is described as follows. At higher temperature, double-strand DNA dissociates into single-strand DNA, leading to peak broadening when passing through DHPLC column. The addition of a GC-clamp at one end of the fragment should stabilize the DNA and raise the temperature at which the double-strand DNA is denatured into single-strand DNA to ensure a sharp peak even at higher temperature (Narayanaswami and Taylor, 2001). The use of GC-clamped primers greatly enhances the sensitivity of detection of mutant hetero- and homoduplex peaks in DHPLC profiles compared to non-GC-clamped primers. Thus, when screening sequence variants, the incorporation of appropriate GC-clamped primers provides a simple method of altering the melting behavior and increases the resolution of homo- and heteroduplex peaks.

For a tri-allele polymorphism, c.1291G>T or A in exon 13, the two kinds of mutant heterozygotes (GT and GA) showed very similar DHPLC profiles, and direct

sequencing detected different base substitutions (G>T or A). This polymorphism occurs at a very low frequency, as only one heterozygote of each was found among the 348 samples screened. In order to exclude the possibility that this substitution was introduced during *Taq* DNA polymerase amplification, re-amplification by *Pfu* DNA polymerase with high fidelity was carried out. The same heteroduplex profile can be generated from the amplicon obtained using the *Pfu* DNA polymerase and the base substitution was also confirmed by DNA sequencing. Thus, this polymorphism is a naturally occurring genetic variant with a very low frequency in general population.

Among the polymorphisms identified in this study, two polymorphisms were identified in the putative promoter region. However, previous study suggested that the fragment containing the two polymorphic sites was not important for promoter activity (Song *et al.*, 2001). For the polymorphisms found in intronic regions, none of them was located within splicing sites or branch sites and thus it is unlikely to have biological influence. So only those located in the coding region are considered for function analysis to evaluate their biological significance.

The GRANTHAM D values of the Glu14Gly, Thr254Ile and Ala431Ser were close to 100, while the D value of Ala431Thr was 58. The BLOSUM scores of the former triad were negative and only the Ala431Thr has a positive value. Recent studies on sequence variants in G-protein-coupled receptors using substitution matrix suggested that variations with GRANTHAM scores >100 or BLOSUM scores<-1 are increasingly associated with disease-causing mutations (Balasubramanian *et al.*, 2005). However, these substitution matrix scores did not consider position-specific

information and whether these substitution matrixes are useful for predicting function is still controversial (Miller and Kumar, 2001; Ng and Henikoff, 2001; Leabman *et al.*, 2003). Interspecies sequence alignment and acyltransferase family members sequence comparison were carried out to define the evolutionary conserved sites. The Glu14 and Ala431 were conserved in Homo sapiens, Africa green monkey, Mus musculus, Rattus norvegicus, and Canis familiaris but not in all acyltransferase family. The Thr254 was not conserved both interspecies and in the acyltransferase family. PolyPhen demonstrated that the Glu14Gly was possibly damaging. Combining all the predicted results, the Glu14Gly may have a functional role.

Other than these prediction approaches, literature review on ACAT2 also gave us some information on the possible functional roles of these polymorphic sites. For example, the 254$^{th}$ amino acid residue is very close to an important amino acid residue, serine, at the 245$^{th}$ residue. The mutation from serine to leucine mutation at the 245$^{th}$ residue led to loss of ACAT2 enzyme activity (Joyce *et al.*, 2000; Lin *et al.*, 2003). It was proposed that this 245$^{th}$ residue may be very important to ACAT2 protein expression or stability. In addition, the amino acid residues at the amino-terminal region in ACAT family have been suggested to negatively mediate the enzyme activity (Guo *et al.,* 2001). Therefore, it is likely that the The254Ile and Glu14Gly polymorphisms may have some biological function.

With the information on the potential biological significance of The254Ile and Glu14Gly polymorphisms, it is instructive to investigate their effect of these two polymorphisms on disease susceptibility in Singaporean population using a

case-control association study. The D/I polymorphism was also included in this study, as a long insertion is uncommon among sequence variations.

The findings from this association study indicated that common genetic variants within the *ACAT2* gene were associated with altered plasma lipid levels and CAD risk in Singaporean population. The heterozygote 734C>T was associated with decreased apoA1 and/or increased apoB and Lp(a) in healthy female normolipidemic Malays and Indians. The 41A>G heterozygotes had significantly higher TC level and a trend towards increased apoB in the Indian females. It was found that the 734C>T was in almost complete LD with D/I polymorphism and in very strong LD with 41 A>G. As such, very similar associations were observed for the 734C>T and D/I with plasma lipid levels. However, all these associations with lipids were not observed after strict Bonferroni correction.

The T allele of 734C>T was significantly lower in the Chinese CAD+ patients with multiple comparison correction. In addition, the analysis using the recessive model also showed that the Chinese CAD+ patients had lower CT+TT genotypes compared with the CAD- controls. These results suggest that the CT+TT genotypes and T allele may be protective against CAD in the Chinese, although no corresponding protective effect on plasma lipid levels was observed. In the other two ethnic groups, Malays and Indians, the T allele frequency was also slightly higher in the CAD- subjects compared to the CAD+ patients but did not reach a significant level**.** No significant association was observed between the 734C>T polymorphism and dyslipidemia in any ethnic group. This was consistent with that of the study done by Katsuren *et al.* (2001) in the Japanese. The allele frequency of 734C>T in the

Chinese subjects in the study was also similar to those reported in the Japanese study. In the normolipidemic Indian and Malay females, the heterozygotes of 734C>T had decreased apoA1 and /or increased apoB and Lp(a) levels, but no significant difference was detected in the dyslipidemic subjects. The effects of 734C>T on plasma lipid traits were probably explained by the function of ACAT2 in the synthesis and secretion of apoB-containing lipoproteins and in intestinal cholesterol absorption. As for the effect of ACAT2 on apoA1 level, it probably results from an increase in LCAT activity, which may complementarily increase if ACAT2 activity is lower in the T allele carriers. Such compensatory increase in LCAT activity due to ACAT2 activity deficiency was observed in $ACAT2^{-/-}$ $ApoE^{-/-}$ mice (Willner *et al.,* 2003).

Katsuren's study in the Japanese (2001) showed that 734C>T heterozygotes in normolipidemic and dyslipidemic subjects had significantly higher apoC-III levels as compared to the other two homozygous genotypes (CC and TT). There was no significant difference in apoA1 and apoB levels between different genotypes. It should be noted that these association of the 734C>T polymorphism with lipids may be spurious, as the significance was not observed after strict multiple test correction.

As for the 41A>G, there was no difference in allele frequencies of this nsSNP between the CAD+ and CAD- subjects in all three ethnic groups. However, when the CAD- subjects were stratified into normolipidemic and dyslipidemic subgroups, the G allele frequency was significantly higher in normolipidemic subjects than in dyslipidemia subjects in Chinese but not in Malays and Indians. It appears that the G allele was protective against dyslipidemia in the Chinese, but this effect was not

consistent across ethnic groups. However, this effect was not detected after adjusting multiple tests.

The 41A>G heterozygotes had higher TC and apoB in the normolipidemic female Indians compared to the AA homozygotes. Together with the higher G allele frequency in the dyslipidemic Indians, it suggested that the G allele may be associated with an increase in ACAT2 activity, resulting in the corresponding increase in the intestinal cholesterol absorption and the secretion of apoB-containing lipoproteins.

The impact of the D/I polymorphism on plasma lipid profiles was quite similar to that of the 734C>T. In addition, significant effect of the D/I polymorphism on LDL level was observed among the normolipidemic Malay females, with DI genotype being associated with increased LDL level. Increased LDL was also found in heterozygous carriers of the 734C>T, but the increase did not reach statistical significance. The lack of statistical significance is most likely due to the smaller sample size of the heterozygotes of the 734C>T polymorphism in this group. Although the functions of introns are still controversial, it is not likely that the intronic D/I polymorphism exerts its effect through any change in the ACAT2 enzyme structure or serves any regulatory role. As such, any D/I-related association may be due to its LD with a functional site.

In order to assess the combined effects of multiple polymorphisms, the association testing using haplotyes and diplotye was carried out. As the D/I site is in almost complete LD with 734C>T, it was deemed not sufficiently informative for inclusion in the haplotype and diplotype analysis. Therefore included only two loci, 41A>G and

734C>T, for haplotype and diplotype analysis were included. In the haplotype case-control study, it was observed that in the Chinese, the GC haplotype was almost two times more frequent in the CAD+ patients than in the CAD- controls but three times more frequent in the normolipidemic group than those who are dyslipidemic. It was noted that the association of haplotype with CAD does not rule out the possibility of its association with lower atherogenic lipid levels since the latter is only one of the many risk factors of CAD. It is clear that Malay individuals with diplotypes bearing the GC haplotype (AG/CC) have significantly higher protective plasma apoA1 levels while the Indian subjects with the AT-bearing diplotype (AA/CT) have the highest atherogenic apoB levels. The probable small 'protection' conferred by the GC haplotype in terms of its association with favorable levels of lipids could be overridden by other more pronounced effects from genes that are in LD with other CAD candidate genes located both upstream and downstream of *ACAT2* (12q13). These include the genes encoding the LDL receptor-related protein 6 (12p13.3-p11.2), apoB mRNA editing enzyme catalytic polypeptide 1 12p13.1, LDL, oxidized, receptor 1 (12p13-p12) and scavenger receptor class B, member 1 (12q24.31), though the latter might be far from *ACAT2* gene. In the vicinity of the *ACAT2* gene are also those related to immune response, such as interleukins. They are known to play important roles in CAD, which is, in many ways, considered as an inflammatory disease.

The association study of diplotypes and plasma lipid levels showed that, in female normolipidemic Malays, the AG/CT diplotype had the lowest level of apoA1 than the AA/CC and AG/CC diplotypes. It is therefore apparent that the lower apoA1 is likely to be due to the T allele. This is consistent with the single-locus analysis of

734C>T. In the female Indians, the AA/CT had a significantly higher level of apoB than the AA/CC. Since the AA genotype was kept constant in the two diplotypes, the higher apoB is more likely to be attributed to the 734C>T site. However, the effects of 734C>T on Lp(a), and 41A>G on TC were only observed in single-locus analysis but not with diplotypes. This might be due to the reduced sample sizes of the diplotype analysis as only samples with both sites successfully genotyped were included.

The lack of significant association between the polymorphisms and plasma lipid levels in the dyslipidemic subgroups may be due to the following. Firstly, the contributions of a single gene or a few polymorphisms are expected to be very small. Therefore, their effects are very likely to be masked by a myriad of other environmental factors, such as diet, physical activity and lifestyle. In statistical analysis, BMI, age and smoking were included as covariates in the ANOVA models to adjust for their confounding effects. Secondly, there are many other genes involved in lipid metabolism. Genes such as those coding for apolipoproteins, LPL and LDL receptors confer great effects on lipid levels and CAD risks. However, these were not taken into account in the statistical analysis although their influences on plasma lipid levels may mask that of 734C>T and 41A>G. Thirdly, the T and G alleles may exert their influences only by interacting with other genes or environmental factors. Fourthly, statistical analysis with the small sample sizes in Malays and Indians might not have sufficient power to reliably detect a true association. Last but not least, it does not exclude the possibility of Type I error, as all these associations with lipids were not observed any more after Bonferroni correction for multiple comparisons.

The 734C>T polymorphism was shown to be associated with CAD risk in the Chinese Singaporean. In addition, the 41A>G and 734C>T were found to be correlated with altered lipid profiles in female normolipidemic Malays and Indians, but the significance was not observed after Bonferroni correction. Thus, further experiments were carried out to verify whether these two nsSNPs were really functional variants and their underlying mechanism. Meanwhile, the experimental study also verifies the validity of the computational predictions for these nsSNPs.

The wild-type and three mutant ACAT2 proteins (Gly14, Ile254, and Gly14Ile254) as well as the empty vector were expressed in an AC-29 cell line, in which ACAT activity was absent. The functional analysis was performed by measuring the expression levels of *ACAT2* genes and their proteins, and their relative enzyme activities of various polymorphic forms.

The preliminary results from *in vitro* experiments indicated that the relative catalytic activity of ACAT2-Gly14 was almost 2-fold higher compared to that of ACAT2-Glu14. There was no significant difference between ACAT2-Thr254 and ACAT2-Ile254. The higher activity of ACAT2-Gly14 was obviously due to the higher protein expression of ACAT2 bearing Gly14. No significant difference was detected after performing western blot to normalize the activities with their corresponding protein expression levels. There was no significant difference in *ACAT2* mRNA expression between wildtype and various variants, suggesting that a post-transcriptional mechanism may mainly account for the difference in ACAT2 protein expression levels. Thus, the current functional study suggested that the

Glu14Gly polymorphism was important to ACAT2 protein expression and/or protein stability while the Thr254Ile might be a non-functional polymorphism.

The association study suggested that the Thr254Ile mutant was anti-atherogenic in Chinese (He *et al*., 2005). In addition, the Glu14Gly affected the TC level in female normolipidemic Indians while the Thr254Ile altered apoA1 and/or apoB and Lp(a) concentrations. The possible reasons for the different effects in different subgroups were stated (He *et al*., 2005). The effect of the Glu14Gly on plasma cholesterol concentration in female normolipidemic Indians was consistent with the higher expression level in the experimental study. As for the Thr254Ile, its expected influence from this *in vitro* experimental study is opposite to that of epidemiological study. This might be explained by the following reasons. First, the functional effect of the Thr254Ile might be too small to be detected in the experimental system in AC-29 cells, but might become observable in a specific human tissue or upon exposure to relevant environmental factors. Second, other factors such as the cell type and DNA constructs used for this study might affect experimental outcomes. It is known that different cell types and DNA constructs might exert different regulatory influences that may affect the function of a genetic variant (Rebbeck *et al*., 2004). Finally, the inconsistency may be due to some inherent bias in epidemiological investigations, such as insufficient statistical power and multiple comparisons in the statistical analyses, which could produce false positive inferences (Rebbeck *et al*., 2004). The experimental study in AC-29 cell line also supported the computational prediction that the Glu14Gly was conserved and probably have biological implication while the Thr254Ile was not.

We also should be aware that these results are very preliminary, as all these experiments, including real-time RT PCR, western blot, and activity assay, were done with only one colony for each polymorphic form. Therefore, before making a conclusion, all of these assays should be repeated with at least three colonies for each polymorphism type in future work.

In conclusion, a total of 14 *ACAT2* gene polymorphisms were identified in this study. The population study showed that the three polymorphisms were associated with CAD susceptibility in an ethnic-specific manner, and the statistical significance was not observed any more except for 734C>T in the Chinese after multiple test correction. The 43A>G and 734C>T were correlated with altered plasma lipid profiles in female normolipidemic Malays and Indians, but the significance did not exist after Bonferroni correction. For the functional characterization of the two nsSNPs, preliminary results suggested that the Glu14Gly might be important to ACAT2 protein expression and/or stability, implying that the Glu14Gly might clinically be related with CAD and hypercholesterolemia. However, further study including at least three colonies for each polymorphic form is needed to confirm this finding.

# 5. ASSOCIATION STUDY OF THREE LIPOPROTEIN LIPASE POLYMORPHISMS IN CHINESE AND ASIAN INDIANS

## 5.1. Introduction

LPL has a paradoxical role of being anti-atherogenic and pro-atherogenic, which is dependent on the tissues expressing LPL. Plasma LPL confers decreased risk of atherosclerosis while arterial wall LPL is associated with increased risk (Clee *et al*., 2000).

Although there are many variants in the *LPL* gene, most of the functional LPL ones are rare and either or restricted to families with LPL deficiency or isolated in geographic regions (Brunzell, 1995; Santamarina-Fojo *et al*., 1991). Three common genetic variants, the IVS6+1595C>T polymorphism in intron 6, the IVS8+484T>G polymorphism in intron 8, and the c.1342C>G in exon 9, are widely studied. Some results showed that these polymorphisms are associated with altered lipid profiles (Razzaghi *et al*., 2000; Morabia *et al*., 2003; Gerdes *et al*., 2005) and cardiovascular diseases susceptibility (Yang *et al*., 2004; Taylor *et al*., 2004). However, inconsistent finding have been reported (Fidani *et al*., 2005; Spence *et al*., 2003; Brousseau *et al*., 2004). These association studies were carried out mainly in Caucasian populations and a few studies have been done to examine the impact of these polymorphisms in the Asian population (Shimo-Nakanishi *et al*., 2001; Lee *et al*., 2004; McGladdery *et al*., 2001; Hall *et al*., 2000). To our knowledge, the combined effect of these three polymorphisms on CAD risk has not been investigated in any Asian population. Thus in this study, the impact of the haplotypes formed by the three polymorphisms on CAD susceptibility in two Asian ethnic groups in

Singapore, namely the Indians and Chinese, was examined. The correlations of the individual polymorphisms with CAD risks and plasma lipid traits were evaluated in this population.

## 5.2. Results

### 5.2.1. Demographic characteristics of subjects

The demographic characteristics of the CAD+ patients and the healthy (CAD-) controls are presented in Table 5-1. The healthy controls were younger than the CAD+ patients and the smoking rate was significantly lower in the healthy group compared to the CAD+ group in both ethnic groups. The Chinese CAD+ patients had higher BMI than the respective controls. In terms of lipid parameters, anti-atherogenic lipid traits, such as HDL and apoA1, were significantly higher in healthy individuals than the CAD+ patients in both ethnic groups. However, atherogenic TC and apoB were significantly lower in patients than controls in both ethnic groups. TGs were also significantly lower in the CAD+ patients compared with healthy controls in Indians. All CAD+ patients were put on lipid lowering medications and advised to go on a restricted diet and adopt a healthier lifestyle. The lower levels of TC and apoB in the patients, along with lower TG values in Indian patients, were therefore mainly due to a regime of controlled diet and drug therapy.

**Table 5-1**. Demographics of male Chinese and Indian subjects

| Variables | Chinese | | | Indians | | |
|---|---|---|---|---|---|---|
| | CAD- n = 538 | CAD+ n = 899 | *P* | CAD- n = 305 | CAD+ n = 302 | *P* |
| Age (years) | 41±14 | 58±9 | <0.0005 | 43±14 | 57±10 | <0.0005 |
| BMI (kg/m$^2$) | 23.47±3.45 | 24.15±3.50 | 0.066† | 24.51±3.93 | 24.68±3.24 | 0.576† |
| Smokers* (%) | 25.47 | 30.50 | 0.025 | 17.10 | 25.52 | 0.008 |
| TC (mg/dl) | 224.26±50.63 | 202.70±55.21 | <0.0005 | 228.50±46.05 | 198.88±50.86 | <0.0005 |
| HDL-C (mg/dl) | 48.72±12.68 | 33.96±11.67 | <0.0005** | 39.661±10.74 | 30.79±10.64 | <0.0005)** |
| LDL-C (mg/dl) | 143.57±46.91 | 136.31±52.86 | 0.011 | 154.00±41.95 | 136.37±48.47 | <0.0005 |
| TG (mg/dl) | 175.16±173.30 | 165.59±95.75 | 0.568 | 189.56±97.07 | 132.64±89.37 | 0.003 |
| ApoA1 (mg/dl) | 141.27±23.26 | 116.87±37.65 | <0.0005 | 132.69±23.22 | 110.38±39.93 | <0.0005 |
| ApoB (mg/dl) | 111.98±31.35 | 104.91±31.80 | <0.0005 | 129.23±31.49 | 106.15±30.66 | <0.0005 |
| Lp(a) (mg/dl) | 12.31±15.54 | 22.03±22.48 | <0.0005 | 18.41±19.09 | 31.16±31.20 | <0.0005 |

*: Smokers form one category while non smokers and ex-smokers constitute another.
Adjusted for the confounding effect of †age and**smoking.

### 5.2.2. Genotyping results of three *LPL* polymorphisms

The genotyping gel pictures are shown in Figure 5-1.

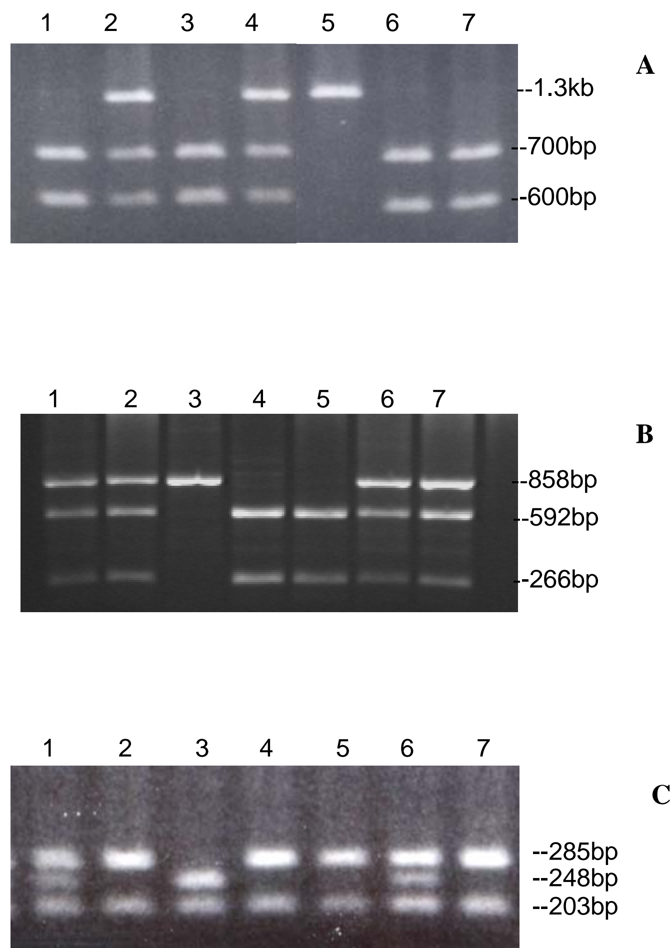



**Figure 5-1.** Genotyping of three polymorphisms. **A**: IVS8+484T>G. Lane 1,3,6, and 7: TT Lane 2 and 4: TG; Lane 5: GG. **B**: IVS6+1595C>T, Lane 1, 3, 6, and 7: CC; Lane 2 and 4: CT; Lane 5: TT. **C**: c.1342C>G. The PCR product contains two *Mnl*I cutting sites, one of which is polymorphic site. Lane 2, 4, 5 and 7: CC; Lane 1 and 6: CG; Lane 3: GG.

### 5.2.3. Distribution of three *LPL* polymorphisms

All genotype frequencies were in accordance with HWE (P>0.05), with the exception of the IVS6+1595C>T polymorphism in the Indian controls (P=0.033). The test of HWE is very sensitive to rare homozygous genotype. In this case, had there been 64 instead of 66 individuals with the TT genotype, the distribution would have been

within Hardy Weinberg expectation. Therefore this departure is attributed to chance rather than an indication of a violation of Hardy-Weinberg assumptions. This is also supported by the fact that the other two *LPL* and three *ACAT2* gene polymorphisms in the same subjects are well within Hardy-Weinberg expectations.

Table 5-2 and 5-3 show the distribution of genotype and allele frequencies of the IVS6+1595C>T, IVS8+484T>G and c.1342C>G polymorphisms between the CAD+ patients and controls as well as between dyslipidemic and normolipidemic subgroups in the Chinese and Indians, respectively. No significant difference in genotype and allele frequencies of the IVS6+1595C>T polymorphism was observed between the healthy and CAD+ subjects in both ethnic groups after Bonferroni correction. The major T allele of the IVS8+484T>G polymorphism was significantly higher in the CAD+ patients compared to the healthy controls in the Chinese (OR = 1.34, P = 0.0063) and in Indians (OR = 1.47, P = 0.0014). However, no significant difference in genotype frequencies was found in both ethnic groups under dominant and recessive models. The C allele frequency of the c.1342C>G polymorphism was higher in the CAD+ patient in both ethnic groups but the statistically significant difference was only observed in the Chinese (OR = 1.72, P = 0.0034). Furthermore, mutant homozygotes were significantly higher in the CAD+ patients compared with the healthy controls. Different distributions of allele frequencies of the IVS8+484T>G and IVS6+1595C>T polymorphisms were found between Chinese and Indians controls; however, there was no significant difference in the allele frequencies of the c.1342C>G between the two ethnic groups. When stratifying the healthy controls into dyslipidemic and normolipidemic subgroups and comparing the distribution of allele frequencies and genotypes, no significant association of allele and genotype frequencies with dyslipidemia was found in the two ethnic groups. In dyslipidemic subjects, the T allele was present more frequently in Chinese compared to Indians. Significant LD with $\Delta$ ranging from 0.34 to 0.60 was observed in all loci-pairs for both ethnic groups (Table 5-4).

### 5.2.4. Hapolotype distribution

The distribution of haplotypes in the CAD+ patients and control groups is present in Table 5-5. All eight of the expected haplotypes were observed. The most predominant haplotype is the CTC haplotype which constitutes more than 50% of the population followed by the TTC and TGC haplotypes in both ethnic groups. The frequency of the most prevalent haplotype, CTC, is also significantly and consistently elevated in the CAD+ patients than the healthy controls in both Chinese and Indians (P=0.0096 and P=0.0011, respectively). The OR of having CAD for individuals with the CTC 'risk' haplotype vs. those without is 1.4 (95% CI 1.1 to 1.8) for the Chinese and 2.0 (95% CI 1.3 to 3.0) for the Indians. The TGG haplotype is 'protective' with a significantly lower frequency in the Chinese CAD+ patients compared to that of healthy controls. However, this association is not significant in the Indians. Instead, another 'protective' TGC haplotype has a significantly higher frequency in the healthy controls.

The haplotype distribution was also compared between dyslipidemic and normolipidemic subgroups in the two ethnic groups (Table 5-6). Although there were 8 expected haplotypes present before stratifying healthy controls into dyslipidemic and normolipidemic subgroups, only 6 and 7 haplotypes were reported in the two subgroups in the Chinese and in the Indians, respectively. It may be because that haplotypes with low frequency are not reported by the software. No significant association was observed between haplotype distribution and dyslipidemia in both ethnic groups.

**Table 5-2**. Distribution of *LPL* polymorphisms in male CAD+ patients and controls

| | | Chinese | | Indians | |
|---|---|---|---|---|---|
| | | CAD- | CAD+ | CAD- | CAD+ |
| IVS6+1595C>T | CC | 183 | 351 | 93 | 103 |
| | CT | 236 | 412 | 121 | 142 |
| | TT | 67 | 106 | 66 | 41 |
| | T freq | 0.38† | 0.36 | 0.45† | 0.39 |
| P | T freq | 0.030 | | 0.041 | |
| | CC vs. CT+TT | 0.323 | | 0.484 | |
| | CC+CT vs. TT | 0.401 | | 0.005 | |
| IVS8+484T>G | TT | 252 | 421 | 133 | 132 |
| | TG | 155 | 208 | 100 | 69 |
| | GG | 33 | 27 | 30 | 15 |
| | G freq | 0.25† | 0.20 | 0.30† | 0.23 |
| P | G freq | **0.0063** | | **0.0014** | |
| | TT vs. TG+GG | 0.0214 | | 0.0209 | |
| | TT+TG vs. GG | 0.0158 | | 0.096 | |
| c.1342C>G | CC | 246 | 474 | 131 | 171 |
| | CG | 68 | 86 | 46 | 38 |
| | GG | 6 | 0 | 3 | 3 |
| | G freq | 0.12* | 0.08* | 0.14 | 0.10 |
| P | G freq | **0.0034** | | 0.0902 | |
| | CC vs CG+GG | 0.019 | | 0.064 | |
| | CC+CG vs. GG | **0.0011** | | 0.840 | |

† and ‡: Significant difference in allele frequencies between ethnic groups ($P<0.05$)
*: Significant difference in allele frequencies between cases and controls.

**Table 5-3**. Distribution of LPL polymorphisms in dyslipidemic and normolipidemic subjects

| Polymorphism | | Chinese | | Indians | |
|---|---|---|---|---|---|
| | | Dys | Normo | Dys | Normo |
| IVS6+1595C>T | CC | 84 | 102 | 45 | 48 |
| | CT | 117 | 116 | 66 | 55 |
| | TT | 30 | 37 | 36 | 30 |
| | T freq | 0.39* | 0.38 | 0.47* | 0.43 |
| IVS8+484T>G | TT | 120 | 134 | 71 | 62 |
| | TG | 76 | 77 | 52 | 48 |
| | GG | 13 | 20 | 17 | 13 |
| | G freq | 0.25 | 0.26 | 0.31 | 0.30 |
| c.1342C>G | CC | 132 | 114 | 67 | 64 |
| | CG | 35 | 33 | 25 | 21 |
| | GG | 3 | 3 | 0 | 3 |
| | G freq | 0.12 | 0.13 | 0.14 | 0.15 |

**Table 5-4.** Linkage disequilibria correlation coefficient (Δ) between two loci in male Chinese and Indians

| | Chinese | | | Indians | | |
|---|---|---|---|---|---|---|
| | IVS6+ 1595C>T | IVS8+ 484T>G | c.1342C>G | IVS6+ 1595C>T | IVS8+ 484T>G8 | c.1342C>G |
| IVS6+ 1595C>T | - | 0.57 | 0.53 | - | 0.46 | 0.55 |
| IVS8+ 484T>G | 0.52 | - | 0.34 | 0.49 | - | 0.38 |
| c.1342C>G | 0.44 | 0.60 | - | 0.37 | 0.49 | - |

In each race, values for CAD patients are above the diagonal and values for controls are below the diagonal.

**Table 5-5**. Haplotype frequencies for three polymorphisms in the CAD+ patients and control subjects

**Chinese**

| IVS6+1595C>T | IVS8+484T>G | c.1342C>G | Overall | CAD- | CAD+ | Permutation P* |
|---|---|---|---|---|---|---|
| C | T | C | 0.606 | 0.632 | 0.563 | **0.0096** |
| C | T | G | 0.004 | 0.003 | 0.005 | 0.713 |
| C | G | C | 0.028 | 0.016 | 0.048 | **0.001** |
| C | G | G | 0.005 | 0.004 | 0.005 | 0.779 |
| T | T | C | 0.165 | 0.169 | 0.158 | 0.566 |
| T | T | G | 0.005 | 0.003 | 0.008 | 0.202 |
| T | G | S | 0.107 | 0.106 | 0.109 | 0.855 |
| T | G | G | 0.080 | 0.067 | 0.104 | **0.017** |

**Indians**

| IVS6+1595C>T | IVS8+484T>G | c.1342C>G | Overall | CAD- | CAD+ | Permutation P* |
|---|---|---|---|---|---|---|
| C | T | C | 0.511 | 0.576 | 0.451 | **0.0011** |
| C | T | G | 0.010 | 0.005 | 0.013 | 0.312 |
| C | G | C | 0.051 | 0.043 | 0.060 | 0.319 |
| C | G | G | 0.004 | 0.008 | 0.000006 | 0.110 |
| T | T | S | 0.193 | 0.181 | 0.205 | 0.425 |
| T | T | G | 0.011 | 0.008 | 0.015 | 0.415 |
| T | G | S | 0.113 | 0.087 | 0.136 | **0.042** |
| T | G | G | 0.107 | 0.092 | 0.121 | 0.231 |

* P values were obtained from Z-tests to compare haplotype frequencies between the case and control groups.

**Table 5-6.** Haplotype frequencies for three polymorphisms in dyslipidemic and normolipidemic subjects

**Chinese**

| IVS6+1595C>T | IVS8+484T>G | c.1342C>G | Overall | Dys | Normo | Permutation P* |
|---|---|---|---|---|---|---|
| C | T | C | 0.564 | 0.586 | 0.536 | 0.2496 |
| C | T | C | 0.052 | 0.037 | 0.071 | 0.088 |
| C | G | G | 0.005 | 0.005 | 0.005 | 0.955 |
| T | T | C | 0.163 | 0.160 | 0.168 | 0.800 |
| T | G | C | 0.102 | 0.106 | 0.096 | 0.692 |
| T | G | G | 0.114 | 0.105 | 0.124 | 0.508 |

**Indians**

| IVS6+1595C>T | IVS8+483T>G | c.1342C>G | Overall | Dys | Normo | Permutation P* |
|---|---|---|---|---|---|---|
| C | T | C | 0.457 | 0.443 | 0.480 | 0.517 |
| C | T | G | 0.018 | 0.007 | 0.022 | 0.264 |
| C | G | C | 0.057 | 0.074 | 0.038 | 0.167 |
| T | T | C | 0.201 | 0.229 | 0.162 | 0.137 |
| T | G | C | 0.135 | 0.112 | 0.160 | 0.209 |
| T | G | G | 0.132 | 0.135 | 0.128 | 0.861 |
| T | T | G | 0.00000 | 6.15E-1 | 9.76E-3 | 0.208 |

### 5.2.5. Association with plasma lipid levels

ANOVA was carried out to examine the correlation of these *LPL* polymorphisms with lipid profiles using age, BMI and smoking as covariates. These results are shown in Table 5-7 and Table 5-8. In Chinese, a significant association was found between the IVS6+1595C>T polymorphism and plasma HDL level (P = 0.003), with the minor T allele being associated with elevated HDL, after Bonferroni correction. The same trend of increased HDL level was also observed in c.1342C>G carriers but did not attain statistical significance, which is most probably due to the small sample size of mutant homozygotes for c.1342C>G.

When the healthy controls were classified into dyslipidemic and normolipidemic subgroups, the same effect of IVS6+1595C>T on HDL was still observed in normolipidemic subjects but not in dyslipidemic subjects in Chinese. No significant effect was seen in the Indians.

**Table 5-7.** Genotypic lipid levels (Mean ± SD) of three polymorphisms in the healthy Chinese males

| | | n | TC(mg/dl) | LDLC(mg/dl) | HDLC(mg/dl) | ApoA1(mg/dl) | ApoB(mg/dl) | lnTG* | TG** | lnLp(a)* | Lp(a)** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| IVS6+1595C>T | CC | 183 | 220.22±54.03 | 137.61±48.34 | **47.08±10.16**[†] | 139.47±24.89 | 110.97±33.18 | 5.00±0.62 | 195.89 | 2.15±0.92 | 8.58 |
| | CT | 236 | 231.65±49.36 | 144.02±46.29 | **48.41±10.86**[†] | 142.43±21.68 | 110.69±30.50 | 4.95±0.54 | 166.34 | 2.17±0.89 | 8.76 |
| | TT | 67 | 224.67±47.38 | 142.22±43.00 | **51.58±11.74**[†] | 145.13±19.45 | 114.81±29.07 | 4.96±0.51 | 162.52 | 2.24±0.89 | 9.39 |
| IVS6+483C>T | TT | 252 | 221.42±52.74 | 140.41±49.25 | 48.42±12.54 | 140.02±22.42 | 112.44±32.21 | 4.99±0.53 | 173.91 | 2.18±0.89 | 8.85 |
| | TG | 155 | 227.57±48.34 | 147.52±46.01 | 50.65±12.04 | 145.36±22.39 | 114.16±31.11 | 4.94±0.53 | 161.25 | 2.25±0.97 | 9.49 |
| | GG | 33 | 218.20±52.46 | 139.82±48.78 | 48.84±12.47 | 141.90±22.28 | 110.45±28.33 | 4.88±0.52 | 148.27 | 1.99±0.64 | 7.32 |
| c.1342C>G | CC | 246 | 230.04±52.95 | 155.16±49.92 | 46.62±10.57 | 139.42±21.49 | 117.82±31.59 | 4.97±0.52 | 169.01 | 2.15±1.01 | 8.58 |
| | CG | 68 | 226.70±42.56 | 147.70±42.37 | 47.41±11.15 | 146.05±19.53 | 110.65±30.27 | 4.98±0.54 | 169.88 | 2.03±1.01 | 7.61 |
| | GG | 6 | 223.00±43.94 | 132.00±52.76 | 51.40±10.62 | 145.60±31.17 | 108.50±23.74 | 5.05±0.48 | 171.50 | 2.14±1.08 | 8.50 |

*: lnTG and lnLp(a): natural logarithmic transformed TG and Lp(a), respectively.
**: TG and Lp(a) levels are presented in geometric means.
[†] Significant difference (P=0.003).

**Table 5-8.** Genotypic lipid levels (Mean ± SD) of three polymorphisms in the healthy normolipidemic Chinese males

| | | n | TC(mM) | LDLC(mM) | HDLC(mM) | ApoA1(mg/dl) | ApoB(mg/dl) | lnTG* | TG** | lnLp(a)* | Lp(a)** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | CC | 100 | 4.87±0.76 | 3.24±0.79 | **1.26 0.24_** | 137.35±23.64 | 95.56±25.31 | 0.20±0.37 | 1.22 | 2.19±0.96 | 8.93 |
| IVS6+1595C>T | CT | 116 | 4.94±0.76 | 3.31±0.78 | **1.30 0.28_** | 141.50±20.20 | 92.62±21.02 | 0.16±0.39 | 1.17 | 2.11±0.81 | 8.24 |
| | TT | 37 | 5.04±0.91 | 3.30±0.97 | **1.40 0.27_** | 146.56±18.97 | 98.82±24.16 | 0.19±0.37 | 1.21 | 1.93±0.77 | 6.89 |
| | TT | 132 | 4.88±0.77 | 3.24±0.82 | 1.28±0.26 | 139.88±20.37 | 95.46±22.51 | 0.21±0.35 | 1.23 | 2.18±0.86 | 8.85 |
| IVS8+484T>G | TG | 77 | 5.03±0.79 | 3.35±0.80 | 1.30±0.27 | 142.32±22.25 | 97.14±22.79 | 0.17±0.41 | 1.19 | 2.11±0.97 | 8.25 |
| | GG | 20 | 4.98±0.96 | 3.33±1.08 | 1.31±0.22 | 141.55±15.50 | 100.15±23.02 | 0.16±0.44 | 1.17 | 1.95±0.72 | 7.03 |
| | CC | 114 | 5.07±0.75 | 3.58±0.71 | 1.24±0.29 | 138.57±21.07 | 99.41±22.94 | 0.20±0.38 | 1.22 | 1.94±0.98 | 6.96 |
| c.1342C>G | CG | 33 | 5.01±0.69 | 3.37±0.65 | 1.28±0.38 | 147.47±19.68 | 93.60±20.16 | 0.20±0.37 | 1.22 | 1.82±1.06 | 6.17 |
| | GG | 3 | 5.84±0.14 | 4.24±0.4 | 1.29±0.30 | 129.00±16.09 | 119.00±21.00 | 0.40±0.24 | 1.49 | 2.86±0.78 | 17.46 |

*: lnTG and lnLp(a): natural logarithmic transformed TG and Lp(a), respectively.
**: TG and Lp(a) levels are presented in geometric means.
**†:** Significant difference in HDL levels between different IVS6+1595C>T genotypes (P=0.008)

## 5.3. Discussion

We have characterized the distribution and association of three *LPL* polymorphisms with the respective plasma lipid profiles, CAD susceptibility, and the severity of CAD in male Chinese and Asian Indians living in Singapore. The major outcome of this study is the finding of different distribution of allele and haplotype frequencies of these three polymorphisms between the CAD+ patients and controls, suggesting that these polymorphisms may confer some effects on CAD risks in both ethnic groups. No significant difference was found in allele and haplotype frequency distribution between normolipidemic and dyslipidemic subgroups. Regarding the effect on lipid profiles by individual polymorphisms, it was found that, in the Chinese, the IVS6+1595C>T polymorphism carriers had significantly higher HDL-cholesterol, and this increase in HDL-cholesterol level was also observed in the c.1342C>G polymorphism carriers but not reaching statistically significant level. No association was found between the IVS8+484T>C polymorphism and lipid levels. In addition, these three polymorphisms were found to be in strong LD with each other.

Our results showed that the three polymorphisms are very common in the Chinese and Indians living in Singapore. Among them, the IVS6+1595C>T polymorphism is the most prevalent, followed by the IVS8+484T>G and c.1342C>G. The C allele frequency of the c.1342C>G polymorphism (0.12) in the Chinese is in agreement with a previous study in Singapore (Lee *et al*., 2004). However, its higher frequency in the Indians relative to the reported frequency can be explained as follow. It was not stated whether all of their subjects were healthy. If CAD+ subjects had been included, then the difference would be reasonable, as here generally the CAD+ subjects had lower allele frequency than healthy controls (Table 5-2).

The case-control association analysis based on the distribution of allele frequencies indicated that the rare G allele of IVS8+484T>G was associated with decreased CAD risk in both ethnic groups, while the rare T allele of IVS6+1595C>T and the rare G allele of c.1342C>G was associated with lower CAD risk in Chinese and Indian, respectively. As such, these polymorphisms may have anti-atherogenic effects or may be in LD with another functional site.

The c.1342C>G polymorphism results in a truncated LPL protein. It had been demonstrated that the c.1342C>G polymorphism can increase LPL enzymatic activity and non-enzymatic activity, which then enhance TG-rich lipoproteins conversion and LDL removal (Nierman *et al*., 2005). These might contribute to the decreased risk of cardiovascular diseases. In contrast, the IVS6+1595C>T and IVS8+484T>G polymorphisms are sequence variants in non-coding region. Thus, it has been proposed that the effect of the IVS8+484T>G and IVS6+1595C>T polymorphisms on CAD risk was due to their LD with the functional variant, c.1342C>G. However, the effect of c.1342C>G could not explain all the IVS8+484T>G- and IVS6+1595C>T-related association. For example, in single-locus analysis, both common alleles of these two polymorphisms were correlated with higher CAD risk while no association was seen between the c.1342C>G and CAD risk in Indians. In haplotype analysis, the effect of IVS8+484T>G was more apparent, which was independent of c.1342C>G.

In order to examine the combined influence of these three polymorphisms, the haplotype was constructed and the haplotype frequencies were compared between cases and

controls. It was suggested that the most common CTC haplotype was consistently atherogenic in Chinese and Indians, while the TGG and CGC haplotypes were associated with lowered CAD risk in the Chinese and the TGC haplotype was protective from CAD in the Indians. The opposite effects of the CTC and CGC haplotypes on CAD susceptibility in the Chinese suggests that the anti-atherogenic effect of G allele of the IVS8+484T>G polymorphism is independent of the c.1342C>G polymorphism. In addition, in the Indians, the CTC haplotype was pro-atherogenic while the TGC haplotype was anti-atherogenic, although the same C allele was present in the c.1342C>G polymorphic site. Furthermore, it is known that the IVS6+1595C>T polymorphism is located in intron 6 and no functional element has been identified thus far in this intron. On the other hand, though the IVS8+484T>G polymorphism is within intronic region, a regulatory element encompassing the IVS8+484T>G site was reported to be important for LPL transcriptional activity (Enerback *et al*., 1992). Taken together, the IVS8+484T>G polymorphism may be a functional site by itself, which is consistent with the Razzaghi *et al.*'s studies (2000).

We also found that the T allele of IVS6+1595C>T increased plasma HDL-cholesterol in Chinese and this effect was probably due to its LD with a functional site, such as c.1342C>G. A similar increasing trend in HDL level was also found in homozygous GG carriers of the c.1342C>G, but it is not statistically significant. The lack of statistical significance may be due to too few individuals with the GG genotype, as the higher HDL level in G allele carriers was reported in a previous study with large sample size in Singapore (Lee *et al*. 2000). In addition, although this study showed a significant effect of T allele of IVS6+1595C>T on lipid profiles in the Chinese, this polymorphism

was not associated with CAD risk. One possible reason is that its impact on HDL level is not sufficiently strong to cause any significant difference in CAD susceptibility.

We did not observe any significant differences in allele and haplotype frequencies between normolipidemic and dyslipidemic subgroups. As such, these polymorphisms may influence atherosclerosis susceptibility through other mechanisms rather than through altering lipid profiles. Due to the dual role of LPL in atherosclerosis, an independent association of these polymorphisms with CAD risk is not unexpected despite the lack of significant association with circulating lipid levels. Furthermore, many genetic and environmental factors have been suggested to modulate the effects of *LPL* polymorphisms on CAD risk and lipid profiles, such as smoking, practical activities, age, race, sex, and oestrogen (Lee *et al*., 2004; Senti *et al*., 2001; Holmer *et al.*, 2000; Hallman *et al*., 2001). Thus, it is also possible that the interaction between LPL and these factors might have masked the small influence of LPL on lipid traits.

In conclusion, it was shown that the most prevalent CTC haplotype of the three *LPL* polymorphisms, IVS6+1595C>T, IVS8+484T>G and c.1342C>G, was consistently associated with increased CAD susceptibility in male Chinese and Indians living in Singapore. The finding is that the rare alleles of the three individual polymorphisms decreased CAD risk in male Chinese and/or Indians and the protection against CAD is independent of any effects on lipid profiles.

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