THE EFFECT OF C677T AND A1298C MUTATIONS ON METHYLENETETRAHYDROFOLATE REDUCTASE GENE ON PLASMA HOMOCYSTEINE CONCENTRATION IN THAI CAD PATIENTS

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THE EFFECT OF C677T AND A1298C MUTATIONS ON METHYLENETETRAHYDROFOLATE REDUCTASE GENE ON HOMOCYSTEINE CONCENTRATION IN THAI CAD PATIENTS.

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ABSTRACT

Hyperhomocysteinemia has been identified as an independent risk factor for coronary artery disease (CAD). Methylenetetrahydrofolate reductase (MTHFR) is an enzyme in the remethylation cycle in homocysteine metabolism which catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. Two common polymorphisms of the MTHFR gene, C677T and more recently reported A1298C polymorphisms may contribute to hyperhomocysteinemia. In the present study, we determined the prevalence of two common MTHFR polymorphisms, C677T and A1298C, in 98 healthy control subjects and 199 CAD patients. MTHFR genotypes were analyzed by polymerase chain reaction followed by *Hin*f I and *Mbo* II digestions for C677T and A1298C, respectively. It was found that the T allelic frequency of C677T mutation on the MTHFR gene was 0.12 in healthy control subjects and 0.16 in CAD patients. The C allelic frequency of the A1298C was 0.27 in healthy control subjects and 0.31 in CAD patients. The homozygous of 1298CC genotype was more significantly highly prevalent in CAD patients (10.55%) than in healthy control subjects (2.04%). Here we found that 1298CC individuals had a 5.663-fold increased risk for CAD. Mean plasma homocysteine concentrations in heterozygous 677CT genotype (13.75±4.70 μmol/L) and homozygous 677TT genotype (50 μmol/L) in CAD patients were higher than wild type (12.75±4.62 μmol/L) but these differences were not significant ($p>0.05$). The only A1298C mutation has no effect on the increased level of homocysteine. The combined heterozygosity 677CT/1298AC MTHFR variant was, however, correlated with the elevation of the level of homocysteine, it was significantly different in healthy control subjects ($p=0.0156$) but in CAD patients it was not. It was concluded that C677T and combined heterozygous 677CT/1298AC MTHFR gene variants may be involved in increasing the level of homocysteine concentration. Moreover, individuals with MTHFR homozygous 1298CC genotype may be at an increased risk for CAD. However, the association of these genotypes was not correlated with CAD.

KEY WORDS : C677T/ A1298C/ HYPERHOMOCYSTEINEMIA/ HOMOCYSTEINE/ METHYLENETETRAHYDROFOLATE REDUCTASE/ MUTATION

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ผลของการเปลี่ยนแปลงของยีนเมธิลีนเตตระไฮโดรโฟเลท รีดักเทสที่ตำแหน่ง C677T และ A1298C ต่อระดับโฮโมซีสเตอีนในคนไทยที่เป็นโรคหัวใจ (THE EFFECT OF C677T AND A1298C MUTATIONS ON METHYLENETETRAHYDROFOLATE REDUCTASE GENE ON HOMOCYSTEINE CONCENTRATION IN THAI CAD PATIENTS)

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บทคัดย่อ

ภาวะของระดับโฮโมซีสเตอีนสูงในกระแสเลือดเป็นภาวะเสี่ยงต่อการเกิดโรคหัวใจ เอนไซม์เมธิลีนเตตระไฮโดรโฟเลท รีดักเทสเป็นเอนไซม์ที่ทำหน้าที่ในวงจรรีเมธิลเลชั่นของวิถีโฮโมซีสเตอีน ซึ่งเอนไซม์นี้จะทำหน้าที่กระตุ้นปฏิกิริยาของการเปลี่ยน 5,10-เมธิลีนเตตระไฮโดรโฟเลทไปเป็น 5-เมธิลเตตระไฮโดรโฟเลท ยีนที่ทำหน้าที่ควบคุมการผลิตและการทำงานของเอนไซม์นี้คือ ยีนเมธิลีนเตตระไฮโดรโฟเลท รีดักเทส ความหลากหลายของยีนที่พบได้บ่อยในยีนนี้คือ การกลายพันธุ์ของยีนที่นิวคลีโอไทด์ตำแหน่ง 677 โดยการเปลี่ยนเบส ไซโตซีนเป็นไธมีน ซึ่งส่งผลให้เกิดการเปลี่ยนแปลงของกรดอะมิโนจาก อะลานีนเป็นวาลีน และการกลายพันธุ์ของยีนที่ นิวคลีโอไทด์ตำแหน่ง 1298 โดยการเปลี่ยนเบสอะดินีนเป็นไซโตซีน ทำให้กรดอะมิโนเปลี่ยนจากกลูตาเมทเป็นอะลานีน การกลายพันธุ์ของยีนทั้งสองตำแหน่งนี้ส่งผลต่อการเกิดภาวะระดับโฮโมซีสเตอีนสูงในเลือด ในงานวิจัยนี้ได้ทำการศึกษาเปรียบเทียบถึงความถี่ของการเกิดการกลายพันธุ์ดังกล่าวในยีนนี้ และผลกระทบของการกลายพันธุ์ของยีนต่อการเพิ่มขึ้นของระดับโฮโมซีสเตอีนในเลือด ในคนปกติของไทยจำนวน 98 คน และคนไข้โรคหัวใจจำนวน 198 คน โดยใช้วิธี PCR-RFLP พบว่า ความถี่ของอัลลีล T ของการกลายพันธุ์ที่ตำแหน่ง 677 ในคนปกติเท่ากับ 0.12 และในคนไข้โรคหัวใจเท่ากับ 0.16 ส่วนความถี่ของอัลลีล C ของการกลายพันธุ์ที่ตำแหน่ง 1298 ในคนปกติเท่ากับ 0.27 และในคนไข้โรคหัวใจเท่ากับ 0.31นอกจากนี้ยังพบว่าการกลายพันธุ์แบบโฮโมไซกัสที่ตำแหน่ง 1298 (1298CC) มีความเสี่ยงสูงถึง 5.663 เท่าที่อาจเป็นสาเหตุของโรคหัวใจ การกลายพันธุ์ที่ตำแหน่ง 677 มีแนวโน้มในการทำให้ระดับโฮโมซีสเตอีนในเลือดเพิ่มสูงขึ้น แต่การกลายพันธุ์ที่ตำแหน่ง 1298 ไม่ส่งผลกระทบต่อการเพิ่มขึ้นของระดับโฮโมซีสเตอีน นอกจากนี้การกลายพันธุ์แบบรวมกันของยีนทั้งสองตำแหน่งแบบเฮทเทอโรไซกัส (677CT/1298AC) ยังทำให้ระดับโฮโมซีสเตอีนสูงกว่ายีนปกติ(677CC/1298AA) อย่างมีนัยสำคัญ (p=0.0156) ในคนปกติ จากการศึกษานี้สามารถสรุปได้ว่า การกลายพันธุ์ของยีนนี้ที่ตำแหน่งนิวคลีโอไทด์ 677 และ การกลายพันธุ์แบบรวมกันของยีนทั้งสองแบบเฮทเทอโรไซกัสส่งผลกระทบต่อการเพิ่มขึ้นของระดับโฮโมซีสเตอีนในเลือด นอกจากนี้การกลายพันธุ์ที่ตำแหน่ง 1298 แบบโฮโมไซกัส (1298CC) ยังพบว่าก่อให้เกิดความเสี่ยงในการเกิดโรคหัวใจ แต่อย่างไรก็ตามความผิดปกติของยีนเมธิลีนเตตระไฮโดรโฟเลท รีดักเทสทั้งสองตำแหน่งนี้ไม่มีความสัมพันธ์กับการเกิดโรคหัวใจ

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LIST OF ABBREVIATONS

A	Adenine
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartate
bp	base pairs
CAD	Coronary artery disease
CHD	Coronary heart disease
CVD	Cardiovascular disease
°C	degree Celcius
C	Cytosine
cDNA	complementarydeoxyribonucleicacid
CBS	Cystathionine β-synthase
DNA	Deoxyribonucleic acid
DEL	deletion
dNTP	deoxynucleotide 5'-triphosphate
EDTA	ethylenediamine tetraacetic acid
ER	endoplasmic reticulum
G	Guanine
Gly	Glycine
Glu	Glutamine
g	gram
PCR	Polymerase Chain Reaction
Pro	Proline
RFLP	Restriction Fragment Length Polymorphism
SD	Standard Deviation
Ser	Serine

LIST OF ABBREVIATONS (continued)

T	Thymine
Thr	Threonine
Tyr	Tyrosine
V, Val	Valine

CHAPTER I

INTRODUCTION

Homocysteine is a sulfur containing amino acid formed during the metabolism of methionine, an essential amino acid derived from dietary protein. Moderately increased plasma concentration of homocysteine have been shown to be an important risk for atherosclerosis, including coronary artery disease (CAD), cerebrovascular disease and peripheral vascular disease[1,2,3].

Hyperhomocysteinemia can be acquired as the results of dietary defiencies of folate[4], vitamin B_{12} and/or vitamin B_6[5] which are vitamin cofactors for the optimal function of methylenetetrahydrofolate reductase and cystathionine β-synthase. Deficiencies in the absorption or transport of these vitamins can also cause hyperhomocystinemia. Increases in homocysteine concentrations are often the results of decreased activity of key enzymes involved in ether of homocysteine metabolisms i.e. methionine symthase, cystathionine β-synthase and methylenetetrahydrofolate reductase. The defective gene can promote the abnormal activity of the enzymes[1].

Cystationine β-synthase (CBS) deficiency is the most common inherited form of hyperhomocysteinemia. The homozygous form can be associated with plasma homocysteine concentrations up to 400 μmol/L[6]. But it occur in only 1 in 200,000 births[6]. Heterozygote is the most likely not an important cause of hyperhomocysteinemia in vascular disease[7]. An alteration in the gene encoding the enzyme methylenetetrahydrofolate reductase (MTHFR) leads to hyperhomocysteinemia[1,7,8]. MTHFR is the regulating enzyme in the remethylation pathway in homocysteine metabolism, by catalizing the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is the methyl group donor in the B_{12} dependent remethylation of homocysteine to methionine.

Defective of MTHFR gene variant is reduce MTHFR activity. A milder deficiency of MTHFR, with 50% residual enzyme activity is indicated that associated with elevated homocysteine and CAD[8-11].

The most common mutation of MTHFR, C677T mutation, leading to the substitution of alanine to valine residue at position 226 was described[1,8,12,13]. The presence of this common mutation was shown to be correlate with increase MTHFR thermolability, reduced specific activity and subsequently in raised homocysteine levels[8]. Recently, a second common mutation in the MTHFR gene was described[14-19]. In this mutation, an A to C transition at nucleotide 1298(A1298C) leads to glutamate to alanine substitution in the MTHFR protein. The effect of this A1298C mutation on the MTHFR activity and homocysteine concentrations is less than that observed for the C677T mutation.

In this present study, the C677T and A1298C mutations in the MTHFR gene were investigated in Thai CAD patients undergoing coronary angiography by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The exon 4 and exon 7 of the MTHFR gene were amplified and such polymorphisms identified by *Hin*f I and *Mbo* II digestion, respectively. The combination of C677T and A1298C mutations were done to looking for the effect on plasma homocysteine concentration.

OBJECTIVE

1. To study the frequencies of C677T and A1298C mutations in Thai CAD patients and normal control subjects.

2. To study the effect of C677T and A1298C mutations of the MTHFR gene on plasma homocysteine concentration in Thai CAD patients and normal control subjects.

3. To study the relationship between C677T and A1298C mutations of MTHFR gene on plasma homocysteine concentration and risk to CAD.

CHAPTER II

LITERATURE REVIEW

1. Homocysteine

Homocysteine is a circulating sulfur amino acid formed by demethylation of the essential amino acid methionine, and it can be irreversibly degraded by cystathionine-β-synthase (CBS) gene. Alternatively, homocysteine may be remethylated to conserve methionine, a process requiring an adequate function of several enzymes. Methionine synthase (MS) remethylates homocysteine in the presence of methyl-cobalamine as a cofactor and the cosubstrate 5-methyltetrahydrofolate. Production of 5-methyltetrahydrofolate requires both an adequate supply of reduced folate and proper function of the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR). Dysfunction enzyme or inadequate amounts of cofactors may therefore result in elevated concentration of homocysteine[1,4-5,10]. Homocysteine is an important intermediary in the metabolic pathways for both methionine and cysteine. At least four different enzyme systems regulate its concentration in body fluids via synthesis and degradation pathways. In the early 1960s, an autosomal recessive inherited disease called homocystinuria was identified[20]. A severe deficiency of either of two enzymes, CBS or MTHFR, resulted in pronounced elevations of homocysteine in the blood (200-400μM/L) and urine[6]. A presentation of homocystinuria was severe vascular disease existing in early childhood with thromboembolic disease of vital organs often occurring before the age of 30. In the late 1960s, it was postulated that elevated homocysteine levels might contribute to premature coronary artery disease (CAD) in the general population as well. Several studies demonstrated that the hyperhomocysteinemia might be considered as an independent risk factor for cardiovascular disease[8-11,21-27]. Hyperhomocysteinemia

has been identified as independent risk factor for cerebral[6,25-26,28], coronary[3,29-32]and peripheral atherosclerosis[6], although the pathological mechanism of this risk factor is not fully understood. High plasma levels of homocysteine are also associated with an increased risk of neural tube defect[17,33-34], Alzheimer's disease, and loss of cognitive functions[6,34].

Homocysteine, the demethylated derivative of methionine, is an important branch-point metabolite in metabolism of methionine; an essential amino acid derived from dietary protein. Homocysteine is a sulfur-containing amino acid with a sulfhydryl group which was presented in figure 2-1. This sulfhydryl group makes it susceptible to oxidation at physiological pH, thereby forming disulfides with other thiols such as homocysteine thiolactone; which will aggregate with low density lipoprotein (LDL) to promote the development of atherosclerotic lesion.

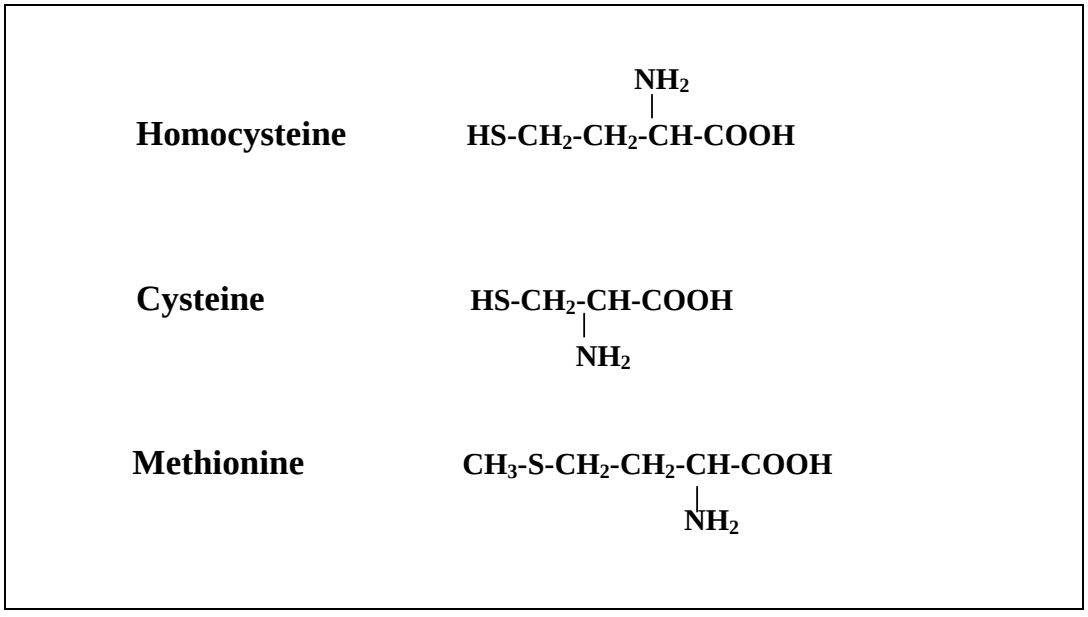

Figure 2-1 Chemical structure of homocysteine, cysteine and methionine[35]

The "elevated blood homocysteine" is used to define the combined pool of homocysteine. When homocysteine is transported out of cells into circulation, it reacts with other compounds containing sulhydryl (-SH) or disulfide (-S-S-) groups. As a result of these reactions almost all of the homocysteine in circulation is converted to a disulfide (oxidized) form. Less than 1 percent of total plasma homocysteine is found as the free-SH form. The disulfide forms include the symmetrical dimer homocysteine and mixed disulfides with cysteine and cysteine-containing plasma proteins (Figure 2-2). In fact, over 70 percent of circulating homocysteine is carried as a mixed disulfide by plasma proteins[36-38].

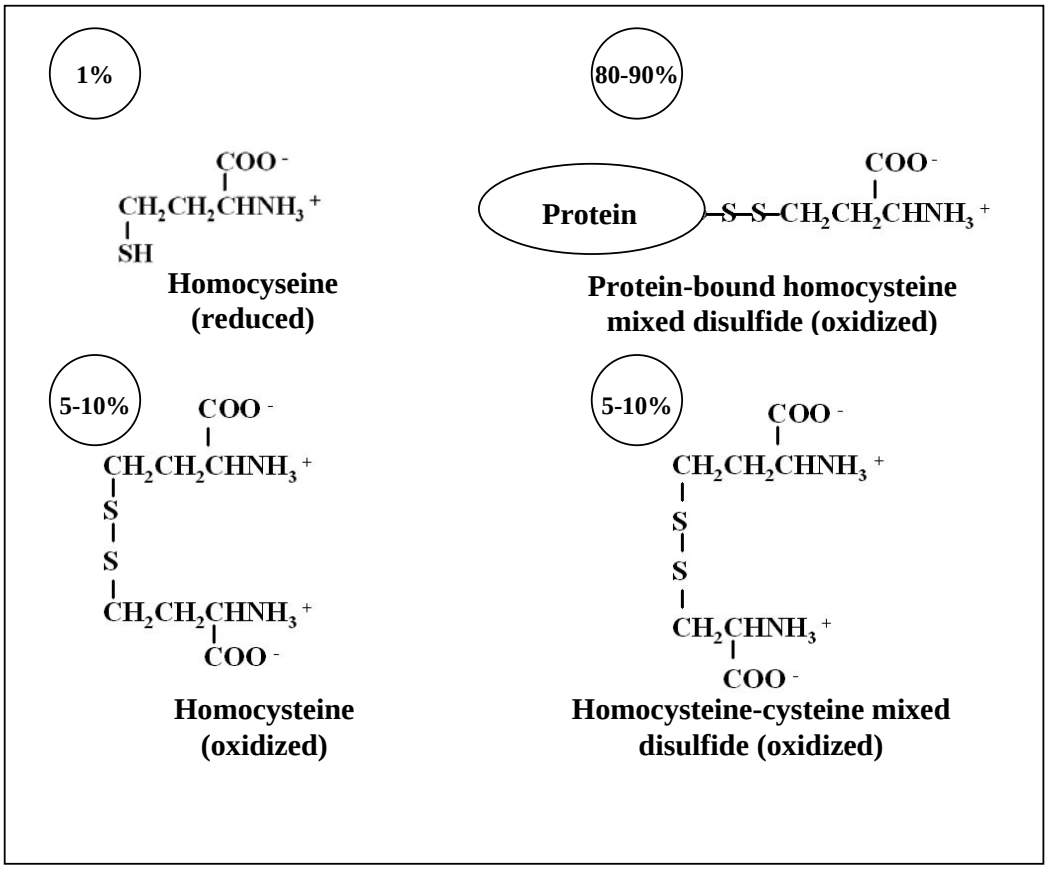

Figure 2-2 The circulating forms of homocysteine that make up plasma total homocysteine[39]

2. Homocysteine metabolism

Homocysteine is a sulfur-containing amino acid that is formed during metabolism of methionine, an essential amino acid derived from dietary protein. Homocysteine is metabolized by one of two pathways: remethylation and transulfuration[6] (Figure 2-3). The essential amino acid methionine is activated by ATP to form S-adenosylmethionine (SAM) in a reaction catalyzed by S-adenosylmethionine synthetase (EC 2.5.1.6). SAM serves primarily as a universal methyl donor via reactions catalyzed by a variety of methyl transferases and involving a variety of acceptors. SAM is essential for the biosynthesis of several cellular components including creatinine, epinephrine, carnitine, phospholipids, proteins, DNA and RNA. The byproduct of these methylation reactions is S-adenosylhomocysteine (SAH) which is hydrolyzed by S-adenosylhomocysteine hydrolase (EC 3.3.1.1), thus generating adenosine and homocysteine. The homocysteine can be salvaged to form methionine or transulfurate to form cysteine.

In the remethylation cycle, homocysteine is recycled to methionine in a reaction catalyzed by methionine synthase or MS (EC 2.1.1.13)[40]. MS is widely distributed in mammalian tissues and contains vitamin B_{12} as an essential cofactor. Homocysteine acquires a methyl group from 5-methyltetrahydrofolate that is catalyzed from 5,10-methylenetetrahydrofolate by the enzyme methylenetetrahydrofolate reductase or MTHFR (EC1.7.99.5)[40]. The other homocysteine methyltransferase, betaine-homocysteine methyltransferase (EC 2.1.1.5), is present only in liver and kidney of humans and requires betaine as the methyl donor. This reaction uses preformed methyl groups because betaine is derived from choline, which is either obtained from the diet or synthesized through successive S-adenosylmethionine dependent methylations of phosphatidylethanolamine. Homocysteine enter the transulfuration pathway when methionine is excessive or cysteine synthesis is required. In this reaction, homocysteine is condensed with serine to form cystathionine catalyzed by vitamin B_6-dependent enzyme cystathionine β-synthase or CBS (EC 4.2.1.22)[40]. Cystathionine is subsequently hydrolyzed to yield cysteine by the enzyme cystathionase (EC 4.4.1.1). Cysteine can be either incorporated into glutathione or further metabolized to sulfate and excreted in the urine. Every tissue posses the methionine cycle, thereby each can remethylate homocysteine. But

transulfuration which is the means for catalyzing homocysteine, can occur only in liver, kidney, small intestine and pancreas[41].

3. Hyperhomocysteinemia

Hyperhomocysteinemia has received increasing attention during the past decade and induced pathologic changes in the arterial wall and, thus, is strongly associated with an increased risk of atherosclerosis, including coronary artery disease[6,27-28], cerebrovascular disease[29-33], and peripheral vascular disease[6]. Hyperhomocysteinemia can be acquired as the result of dietary deficiencies of folate, vitamin B_{12} and /or vitamin B_6[4-5]. These nutrients are necessary cofactors for the optimal function of methylenetetrahydrofolate reductase and cystathionine β-synthase[4-5]. Deficiencies in the absorption or transport of these vitamins can also cause hyperhomocysteinemia. Increases in homocysteine concentrations are often the result of decreased activity of key enzymes involved in either of homocysteine metabolisms. The most common inherited form of hyperhomocysteinemia results from an alteration in the gene encoding the enzyme MTHFR.

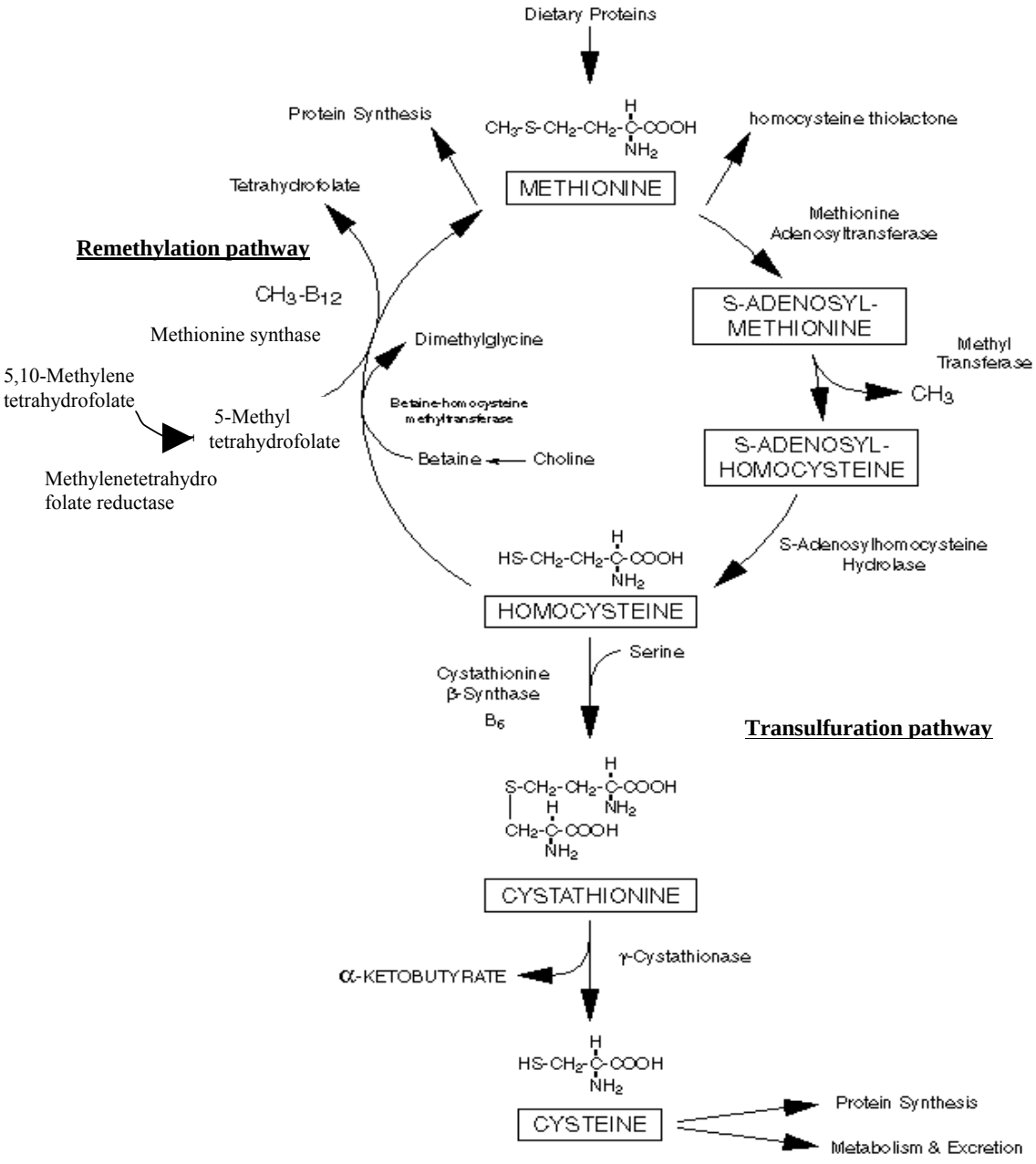

Figure 2-3 Metabolism of Homocysteine : Homocysteine metabolism composed of two main pathways ; remethylation and transulfurationpathway. The remethylation pathway reversed homocysteine to methionine and transulfuration pathway conversed homocysteine to cystathionine. Both pathways stabilize the plasma level of homocysteine.

4. Cause of hyperhomocysteinemia

There are several factors that influenced the level of homocysteine. It is classified into 2 groups : non-genetic and genetic risk factors.

4.1 Non-genetic factors

Non-genetic factors compose of :

- **Age and gender** : Homocysteine concentrations increased with age[42-44]. Men have higher basal homocysteine level than women and the level of homocysteine in women who have postmenopausal state could be reduced by hormone replacement therapy[44-45].

- **Nutritional status** : Nutritional deficiencies in the vitamin cofactors (folate, vitamin B_{12} and vitamin B_6) required for homocysteine metabolism may generate hyperhomocysteinemia[46]. As noted above, de novo synthesis methionine methyl groups required both vitamin B_{12} and folate coenzymes, whereas transulfuration required vitamin B_6. Recent studies have reported that treating hyperhomocysteinemia patients with vitamin supplementation (with folic acid, vitamin B_6 and vitamin B_{12}) is generally effective in reducing homocysteine concentrations[6,45].

- **Renal diseases** : Plasma homocysteine levels increase with elevating in serum creatinine. The levels are significantly increased in patients with moderate renal failure and rise steeply in terminal uremia[47-48]. The rise in plasma homocysteine levels in patients with renal failure is attributed to loss of renal parenchymal uptake and metabolism of plasma homocysteine rather than to decrease urinary excretion of homocysteine[6,49].

- **Smoking** : It interferes with the synthesis of pyridoxal phosphate, and it has recently been reported that smokers have significantly lower pyridoxal phosphate concentrations than nonsmokers[6]. Nygard *et al.*[44] suggested that the heavy smoker had plasma homocysteine levels 12% higher than control subjects who have never smoked.

- **Carcinoma** : Hyperhomocysteinemia have been reported in association with several types of carcinoma such as colorectal[6].

- **Medications** : Several drugs and toxins involved in various process leading to hyperhomocysteinemia depletes folate and causes a transient increase in

plasma homocysteine concentrations. Phenytoin also interferes with folate metabolism and may cause mild hyperhomcysteinemia. Theophylline, a phosphodiesterase inhibitor, may cause hyperhomocysteinemia by antagonizing the synthesis of pyridoxal phosphate[6,45].

4.2 Genetic factor

Elevations in plasma homocysteine are caused by genetic defects in the enzymes involed in homocysteine metabolism, CBS, MS and MTHFR[29,33]. The deficiencies of enzymes may cause the accumulation of homocysteine in circulation and finally promotes high level of homocysteine[15,50].

5. Genetic defects in homocysteine metabolism

Hyperhomocysteinemia results from congenital or acquired defects in the pathways of homocysteine metabolism. The most severe form, homocystinuria, is an inborn error of metabolism presenting largely in children[6-7]. It is most often a result of a deficiency of CBS, the first enzyme in the transsulfuration pathway[6-7,51-53]. However, other severe enzymatic deficiencies can also produce this phenotype, including those of MTHFR[1,7-8,54], MS([55], and enzymes involved in vitamin B_{12}[54] metabolism, the enzymes in the remethylation pathway.

5.1 Mutations in methionine synthase gene

MS gene is located on 1q43[56] on chromosome 1 which encoded enzyme 5-methyltetrahydrofolate-homocysteine-S-methyltransferase or methionine synthase. This enzyme has the function to transfer the methyl group. In homocysteine cycle, MS catalyzes the remethylation of homocysteine to form methionine which requires vitamin B_{12} as a cofactor (Figure 2-3). Many researches reported that MS deficiency related with mental retardation, macrocytic anemia and homocysteinuria[57]. Without functional methionine synthase, homocysteine cannot be converted to methionine. As a result, homocysteine builds up in the bloodstream and methionine is depleted. The most common mutation in MS gene is an A to G substitution, 2756A→G, which converts an aspartate to a glycine[58].

Other mutations are

1. 4-bp Deletion

Four bp deletion at codon 1675 in the MS gene results in a frameshift and thereby stop codon. This mutation induces homocysteine-megaloblastic anemia.

2. 3-bp Deletion

Three bp deletions at codon 1675 identify a 1726 deletion TTG mutation results in the loss of a highly conserved leucine at position 576. This mutation presents in heterozygous state and induces homocysteine-megaloblastic anemia.

3. D919G

This polymorphism is associated with risk of colorectal adenoma.

4. A366G

This polymorphism is associated with increased risk for spina bifida.

5.2 Mutations in cystathionine β-synthase gene

CBS enzyme converts homocysteine to cystathionine in the trans-sulfuration pathway of the methionine cycle and requires pyridoxal 5-phosphate as a cofactor (Figure 2-3). The other two cofactors involved in remethylation pathway of methionine include vitamin B_{12} and folic acid. CBS gene spans 30 kb and consists of 19 exons on chromosome 21[59]. CBS gene defect leads to hyperhomocysteinemia, the inborn errors of homocysteine metabolism[6]. Patients with homocysteinuria have mental retardation, osteoporosis[60], increasing homocysteine, enhancing thromboxane biosynthesis, abnormal rapid turnover of platelets, fribinogen and plasminogen[59,61,62-63]. Homozygousity mutation for CBS is the most common cause with the frequency of homocystinuria ranging from 1 in 58,000 to 1 in 1,000,000 but with marked geographic difference[31]. Heterozygosity for CBS presents less than 1 % of the general population[31]. The substitution of T to C at nucleotide position 833 (T833C) is a highly prevalent mutation in the CBS gene found in homocytinuric patients with decreased CBS enzyme activity. In tandem with the T833C mutation, a 68-base pair insert (844 ins 68) has also been identified in exon 8[64]. The presence of this T833C/844INS68 has added to the complexity of the genetic regulation of homocysteine metabolism and associated with coronary artery disease[64-65]. The second most common mutation of CBS gene is a G to A transition at nucleotide 919

(G919A) resulting in a serine for glycine at amino acid residue 307[66-67]. This mutation has been associated with pyridoxine nonresponsieveness. Patients with the T833C mutation can respond to pyridoxine therapy, whereas, patients with the G919A mutation do not respond to pyridoxine therapy[68].

Other mutations are :

1. T191M

The substitution of C to T at nucleotide 572 on exon 5.

2. R266K

G-to-A transition at nucleotide 797 substitution.

3. P145L

C-to-T transition at nucleotide 434 substitution of proline at position 145 with leucine.

4. A114V

C-to-T transition at nucleotide 341 substitution.

5.3 Mutations in methylenetetrahydrofolate reductase

MTHFR catalyzed the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. The human MTHFR gene is composed of 11 exons and located on 1p36.3[69-70]. Decreasing of MTHFR activity is one of the most frequent causes which results in inadequate production of 5-methyltetrahydrofolate that is required for the remethylation of homocysteine to methionine so the level of homocysteine is accumulated[71-73]. There are several studies reported about the polymorphisms of MTHFR which induced hyperhomocysteinemia[8,17,74-76]. In 1991, a thermolabile variant of MTHFR was discovered and found to be correlated with coronary artery disease[9]. MTHFR gene has been investigated and found to have several polymorphisms and mutations. Nowadays, many researchers found many mutations in this gene. C677T and A1298C mutations on exon 4[1,8,19] and exon 7[17-19] respectively, of the MTHFR gene have been associated with MTHFR deficiency[73,75]. C677T is the most common mutation, leading to the substitution of alanine to valine residue at position 226. The presence of this common mutation was shown to correlate with increased MTHFR thermolability and reduced specific activity[8-9]. Recently, a second common mutation was described; an A to C transition

at base pair 1298 which substitutes a glutamate for an alanine[1,17]. The A1298C is also associated with increased homocysteine and reduced enzymatic activity, although to a lesser extent than C677T[77]. In addition, other MTHFR mutations which cause hyperhomocysteinemia are

1. G482A mutation (Arginine to Glutamine)
2. A983G mutation (Serine to Aspatate)
3. T1027G mutation (Tyrosine to Glycine)
4. C1084T mutation (Arginine stop to codon)
5. T1059C mutation (no change amino acid)
6. G1793A mutation (Arginine to Glutamate)

Three main enzymes, CBS, MS and MTHFR regulated plasma homocysteine level. Nevertheless, hyperhomocysteinemia causing by CBS and MS enzyme was found in rare case among normal population from many studies. Whereas, many investigators suggested that MTHFR gene defect is the cause of hyprhomocysteinemia and found to be common in normal population all over the world. Thus, this study will investigate the effect of these two mutations, C677T and A1298C, of the MTHFR gene in coronary artery disease subjects.

5.3.1 C677T mutation

The MTHFR gene was mapped to chromosome region 1p36.3. A common missense mutation C to T transition at nucleotide 677 (C677T) on exon 4 of the MTHFR gene, leading to the substitution of alanine to valine residue at position 226[1,24]. The C677T was shown to correlate with increased MTHFR thermolability and decreased specific enzyme activity (Table 2-1). This mutation is in the region encoding the N-terminal catalytic domain that could indirectly affect flavin adenine dinucleotide (FAD) cofactor and destabilize the quaternary structure[18]. Individual homozygous for the mutation have significantly elevated plasma homocysteine level because of 50% reduction of the activity of enzyme MTHFR. From the studies of the C677T mutation in cardiovascular disease patients and controls, many researchers concluded that the C677T polymorphism may be a risk factor for coronary artery disease[1,45]. The homozygosity for this frequent mutation in the MTHFR gene is associated with a 3-fold increase in risk for premature cardiovascular disease in

patients with mild hyperhomocysteinemia even without other known risk factors such as hypertension, hyperlipidemia or diabetes[12]. Klerk *et al.*[78] performed a metaanalysis of the risk of coronary heart disease related to the C677T polymorphism. Individuals with the C677T genotype have a significant higher risk of coronary heart disease, particularly in the setting of low folate status. These results supported that the high homocysteine levels is causally related to increased risk of coronary heart disease. The common C677T mutation in the MTHFR gene shows ethnic difference with a high allele frequency[79]. This mutation has relatively high frequency throughout the world[79]. The allele frequency of the substitution was a 0.30 in Caucasian and 0.10 in African-americans[79].

5.3.2 A1298C mutation

The second common mutation of MTHFR gene is A1298C in exon 7 which results in a substitution of glutamate for an alanine residue at codon 429[17-18,77]. The A1298C mutation is found in the C-terminal regulatory domain which is S-adenosyl-methionine-regulatory domain of the enzyme[75]. The binding of S-adenosyl methionine (SAM) results in conformational changes within the MTHFR enzyme that inhibit the enzyme activity[75]. The A1298C mutation, like the C677T mutation, results in a decrease in MTHFR activity that is more pronounced in the homozygous (CC) than in the heterozygous (AC) or normal (AA) states, and does not result in a thermolabile protein[80-82]. The effect of the only A1298C mutation on the level of homocysteine is lesser than the C677T.

The frequency of the A1298C allele was significantly higher in CAD (0.304) than in controls (0.199)[15,83]. Genotype frequencies of the MTHFR A1298C polymorphism were shown in Table 2-2. Allele 1298C showed a significant association with early-onset CAD both in homozygote and in heterozygous carriers[80-81]. Hyperhomocysteinemia is commonly inherited with homozygosity for the C677T mutation of the methylenetetrahydrofolate reductase gene (MTHFR), or with compound heterozygosity for the C677T and A1298C mutations of the MTHFR gene. Many studies commented that only A1298C mutation is not influenced on increasing the level of homocysteine or lower blood folate concentrations however, is associated with reduced *in vitro* enzyme activity (~ 40% to 50% reduction), higher

plasma concentrations of homocysteine and decreased plasma folate levels[15]. *In vitro* studies, the observed of activity for the A1298C polymorphism (~65% of control) is clearly higher than that observed for the C677T mutation (~40% of control), suggesting that homocysteine metabolism might not be significantly disrupted by the A1298C polymorphism alone[18]. The MTHFR C677T and A1298C sites are 2.1 kb apart and have been found to be in strong linkage disequilibrium. Combined heterozygosity of the A1298C mutation with other MTHFR mutations including C677T has been associated with increased total plasma homocysteine levels[84-87]. Type of genotypes can be classified as

- **Heterozygous MTHFR C677T:** The patient has only one copy of the MTHFR C677T mutation and is negative for the A1298C mutation. This mutation is associated with intermediate levels of enzyme activity, but no increase in plasma homocysteine levels. It has not been correlated with coronary artery diseases or venous thrombosis.
- **Heterozygous MTHFR A1298C:** The patient has one copy of the MTHFR A1298C mutation and is negative for the C677T mutation. This is associated with decreased levels of enzyme activity, but no increase in plasma homocysteine levels. It has not been correlated with coronary artery diseases or venous thrombosis.
- **Homozygous MTHFR C677T:** The patient has two copies of the MTHFR C677T mutation. This is associated with increased plasma homocysteine levels and an increased risk for atherosclerotic coronary artery diseases and venous thrombosis.
- **Homozygous MTHFR A1298C:** The patient has two copies of the MTHFR A1298C mutation. This is associated with decreased levels of enzyme activity, but no increase in plasma homocysteine levels. It has not been correlated with coronary artery diseases or venous thrombosis.
- **Compound Heterozygous MTHFR C677T/A1298C:** The patient has one copy of the MTHFR C677T and the other of the A1298C mutation. This is associated with increased plasma homocysteine levels, a risk factor for atherosclerotic coronary artery diseases and venous thrombosis.
- **Compound Homozygous MTHFR C677T/A1298C:** The patient has two copies of the MTHFR C677T mutation and two copies of the A1298C mutation. This is associated with increased plasma homocysteine levels and an increased risk for atherosclerotic coronary artery diseases and venous thrombosis.

- **Homozygous MTHFR C677T/Heterozygous A1298C:** The patient has two copies of the MTHFR C677T mutation and one copy of the MTHFR A1298C mutation. This is associated with increased plasma homocysteine levels and an increased risk for atherosclerotic coronary artery diseases and venous thrombosis.
- **Homozygous MTHFR A1298C/Heterozygous C677T:** The patient has two copies of the MTHFR A1298C mutation and one copy of the MTHFR C677T mutation. This is associated with increased plasma homocysteine levels, a risk factor for atherosclerotic coronary disease and venous thrombosis.

In addition, the C677T and A1298C mutations in the heterozygous or homozygous states correlate with reduced enzyme activity[80-87]. On the other hand, only individuals homozygous for C677T mutation or compound heterozygous for the C677T/A1298C mutations have significantly elevated plasma homocysteine levels[84-87].

Table 2-1 Correlation between MTHFR C677T genotypes and enzyme activity, thermolability and plasma homocysteine level [1]

Genotype	C/C N=19	C/T N=9	T/T N=12
Specific activity [a,b] (mmol CH_3O/mg protein/hr)	22.9±1.7 (11.8-33.8)	15.0±0.8 (10.2-18.8)	6.9±0.6 (2.6-10.2)
Residual activity after heating [a,b] (%)	66.8±1.5 (55-76)	56.2±2.8 (41-67)	21.8±2.8 (10-35)
Plasma homocysteine [a,c] (M) (after fasting)	12.6±1.1 (7-21)	13.8±1.0 (9.6-20)	22.4±2.9 (9.6-42)
Plasma homocysteine [a,c] (M) (post-methionine load)	41.3±5.0 [d] (20.9-110)	41±2.8 (29.1-54)	72.6±11.7 [e] (24.4-159)

[a] one-way anova $p<.01$

[b] paired t test for all combinations $p<.01$

[c] paired t test $p<.05$ for TT group versus CT group or CC group ; $p>.05$ CT versus CC group

[d] n=18

[e] n=11

Table 2-2 Genetic frequencies of the MTHFR A1298C polymorphism frequencies in different populations [75]

Study area and ethnic group	Total no.	Genotype (no.)			C allele frequency	References
		AA	AC	CC		
Africa						
South Africa, Black indigenous *	114	70	39	5	0.21	Gebhardt *et al.*, 2001
Asia						
China*	360	242	113	5	0.17	Song *et al.*, 2001
China*	166	111	50	5	0.18	Shen *et al.*, 2001
Japan*	243	159	75	9	0.19	Matsuo *et al.*, 2001
Europe						
Austria*	389	184	168	37	0.31	Fodinger *et al.*, 2001
Crete*	125	57	55	13	0.32	Zetterberg *et al.*, 2002
Germany	280	128	123	29	0.32	Geisel *et al.*, 2001
Germany*	174	88	68	18	0.30	Stegmann *et al.*, 1999
Germany, Caucasians	981	433	443	105	0.33	Meisel *et al.*, 2001
Netherlands*	403	179	186	38	0.33	van der Put *et al.*, 1998
Netherlands*	120	45	64	11	0.36	Lachmeijer *et al.*, 2001
Netherlands*	565	250	258	57	0.33	Lievers *et al.*, 2001
Poland*	521	316	180	25	0.22	Szczeklik *et al.*, 2001

* Case-control study. Only polymorphism frequencies for the control group are included in this table.

Table 2-2 Genetic frequencies of the MTHFR A1298C polymorphism frequencies in different populations (continued) [75]

Study area and ethnic group	Total no.	Genotype (no.)			C allele frequency	References
		AA	AC	CC		
Poland*	100	55	41	4	0.24	Domagala *et al.*, 2002
United Kingdom*	114	49	54	11	0.33	Skibola *et al.*, 1999
United Kingdom*	200	93	83	23	0.32	Weimels *et al.*, 2001
United Kingdom	394	211	151	32	0.27	Dekou *et al.*, 2001
Middle East						
Israel, Jewish	397	178	168	51	0.34	Friedman *et al.*, 1999
North America United States						
Ashkenazi Jewish	149	80	57	12	0.27	Rady *et al.*, 2002
Caucasians	159	70	75	14	0.32	Rady *et al.*, 2002
Hawaii, Caucasians*	171	86	65	20	0.31	Rady *et al.*, 2002
Hawaii, Japanese descent*	395	244	136	15	0.18	Rady *et al.*, 2002
Midwest, unspecified ethnicities*	329	164	139	26	0.29	Hanson *et al.*, 2001
Texas, Caucasians*	554	265	249	40	0.30	Shen *et al.*, 2001
Male physicians*	344	153	159	32	0.32	Chen *et al.*, 2002
Canada*	129	69	49	11	0.28	Ray *et al.*, 2001
Canada*	119	43	67	9	0.36	Isotalo *et al.*, 2000

* Case-control study. Only polymorphism frequencies for the control group are included in this table

6. Association between hyperhomocysteinemia and atherosclerosis

An elevated plasma homocysteine concentration has recently been identified as an independent risk factor for atherosclerosis[1-3,45]. The association between elevated concentrations of plasma homocysteine and peripheral vascular arterial disease has been shown clinically.

6.1 Homocysteine-induced oxidative stress

Because homocysteine is a thiol, it can undergo autooxidation and oxidation with other thiols. In several investigations hyperhomocysteinemia causes endothelial cell damage[45,88-89]. The toxic effects of hyperhomocysteinemia on endothelial cells can result from the formation of reactive oxygen derived substances, appears in process of auto- oxidation of homocysteine to be a major cause of damage[45,88-89]. Most of the occurring reactive oxygen species (ROS) is hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\bullet -}$). Auto-oxidation of homocysteine may produce hydrogen peroxide (Figure 2-4). Indirect oxidative effects of hyperhomocysteinemia may include generation of superoxide anion from uncoupled endothelial nitric oxide synthase (eNOS), xanthine oxidase, or NAD(P)H oxidase, downregulation of auto-oxidant enzymes and depletion of intracellular glutathione. In the presence of transition metals such as copper (Cu) or iron (Fe), hydrogen peroxide and superoxide anion may react to form hydroxyl radical ($^{\bullet}OH$). Hydroxyl radical may promote the generation of lipid peroxyl radicals ($LOO^{\bullet}$), and both superoxide and lipid peroxyl radicals may react rapidly with endothelium-derived nitric oxide ($^{\bullet}NO$) to produce peroxynitrite ($ONOO^-$) or lipid peroxynitrites (LOONO), respectively (Figure 2-4). This endothelial cell damage can be prevented by an antioxidant catalase and an intact nitric oxide production pathway in endothelial cells and may play an important role in protecting against homocysteine damage by formation of non-toxic adduct S-nitro-homocysteine[88].

Nitric oxide (NO) released from normal endothelial cell can detoxify homocysteine[90]. Nitric oxide serves as an endothelium-dependent vasodilator and an antithrombotic agent in the vasolature because NO forms adducts with several classes of biologic compound, one of which is the sulfhydryl functionality, to form thionitrites or S-nitrothiols (S-NO-Homocysteine) (Figure 2-5). The S-nitrothiols is a protein

vasodilator and antiplatelet agent by decreasing in the expression of fibrinogen-binding sites on the platelet surface and reduction[91]. In contrast to homocysteine, S-nitrosothiols does not support hydrogen peroxide generation nor undergo conversion to homocysteine-thiolactone. The releasing of endothelial derived NO inhibits the vascular smooth muscle cell migration and proliferation (Figure 2-6a). However, the high level of homocysteine can destroy the endothelial cell. When endothelial cell dysfunctions, the production or bioavailability of NO is attenuated because of inability to sustain adequate elaboration of NO in the presence of the elevations of homocysteine[90] (Figure 2-6b).

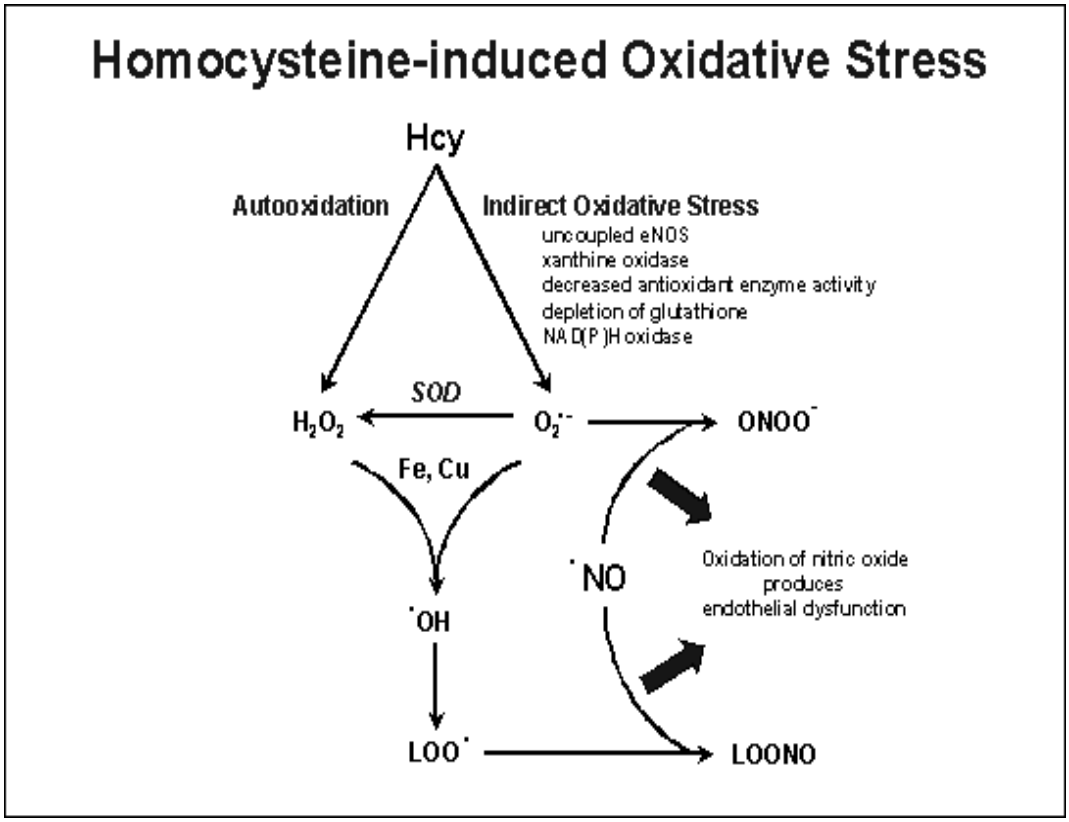

Figure 2-4 Schematic diagram of the effect of homocysteine oxidation on endothelial dysfunction[92]

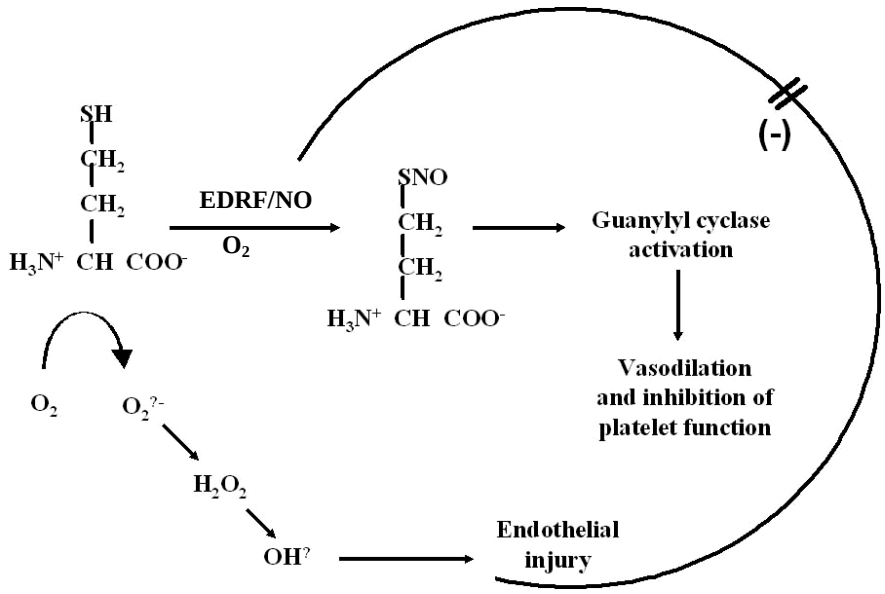

Figure 2-5 Schematic diagram of the conversion of homocysteine to S-nitroso-homocysteine, which serves as a vasodilator and inhibitor of platelet function via activation of guanylyl cyclase[91].

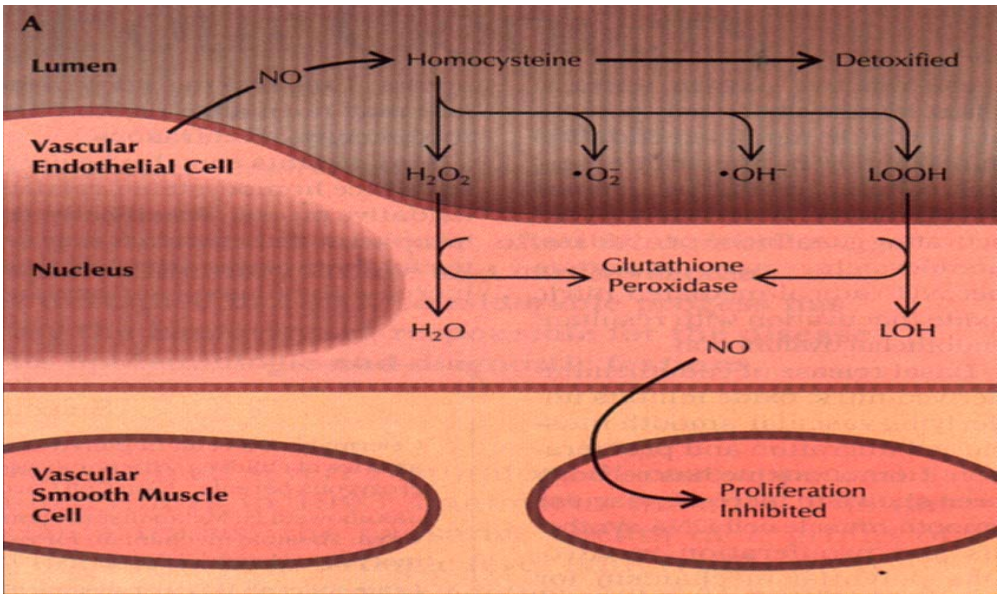

Figure 2-6a Circulating homocysteine is detoxified by NO which is released from endothelial cell. This reaction also inhibits the vascular smooth muscle proliferation and migration[45].

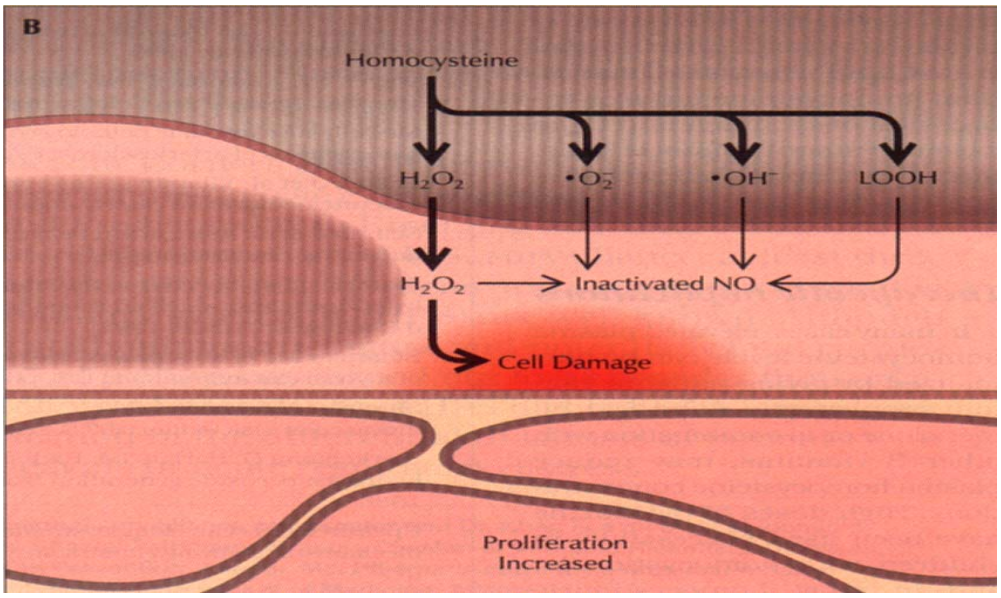

Figure 2-6b NO may be inactivated by intraendothelial reactive oxygen species, leading to endothelial damage and smooth muscle cell proliferation[45].

7. Homocysteine and endothelial dysfunction

Atherosclerosis which is associated with hyperhomocysteinemia, results from endothelial dysfunction and injury followed by platelet activation and thrombus formation[88]. Homocysteine is rapidly auto-oxidized when added to plasma, forming homocysteine, mixed disulfides and homocysteine thiolactone (Figure 2-7). The reactive oxygen species (ROS) including superoxide and hydrogen peroxide, are formed during the auto-oxidation of homocysteine. Both of these oxygen-derived molecules have been linked to endothelial toxicity[45]. Superoxide-dependent formation of the hydroxyl radical has been shown to initiate lipid peroxidation, oxidation of low-density lipoprotein (LDL). The excess of homocysteine promotes the homocysteine thiolactone which is a major byproduct of deregulated homocysteine metabolism[45] (Figure 2-8). Homocysteine thiolactone reacts with LDL to produce LDL - homocysteine thiolactone aggregates which are taken up by macrophages and subsequently incorporated into foam cells in early atherosclerotic plaques. In plaques, homocysteine thiolactone acrylates proteins and enhances oxidative process in the vessel, thereby generating atherothrombosis. Homocysteine may induce the expression and secretion of chemokines in vascular endothelial cells. Production of these chemokines by stimulated endothelial cells would attract monocytes and neutophils to sites of vascular injury where they could take up residence in the intimal space (Figure 2-9). White blood cells (WBC) such as monocytes and neutophils which flow through blood vessels normally have random contact with vascular endothelial cells. When endothelial cells are damaged from injurious agents, the WBCs begin to roll along, and adhere to, the endothelial surface. Homocysteine may speed the progression of vascular disease by stimulating production of monocyte and neutrophil chemoattractants, MCP-1 and IL-8, in the vascular endothelium. Secretion is targeted to the bottom site of the cell, thereby establishing a concentration gradient for chemotaxis. Once attached, monocytes migrate between endothelials and become resident in the vascular intimal space[39]. Here they are transformed into macrophages, engulf oxidized LDL, and become foam cells.

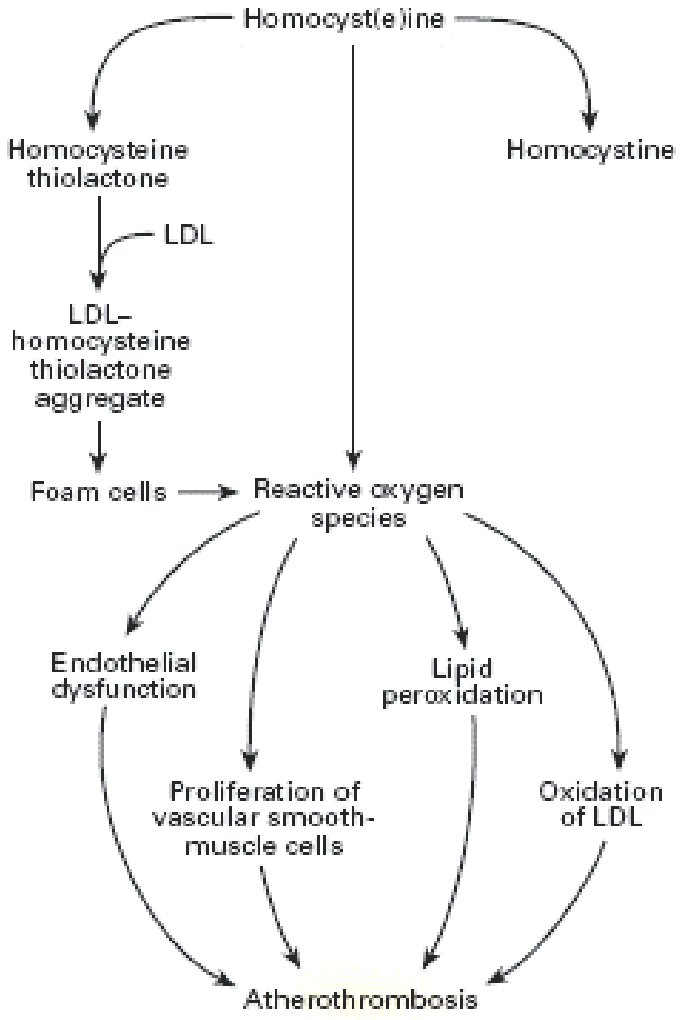

Figure 2-7 The postulated adverse vascular effects of homocysteine[93]

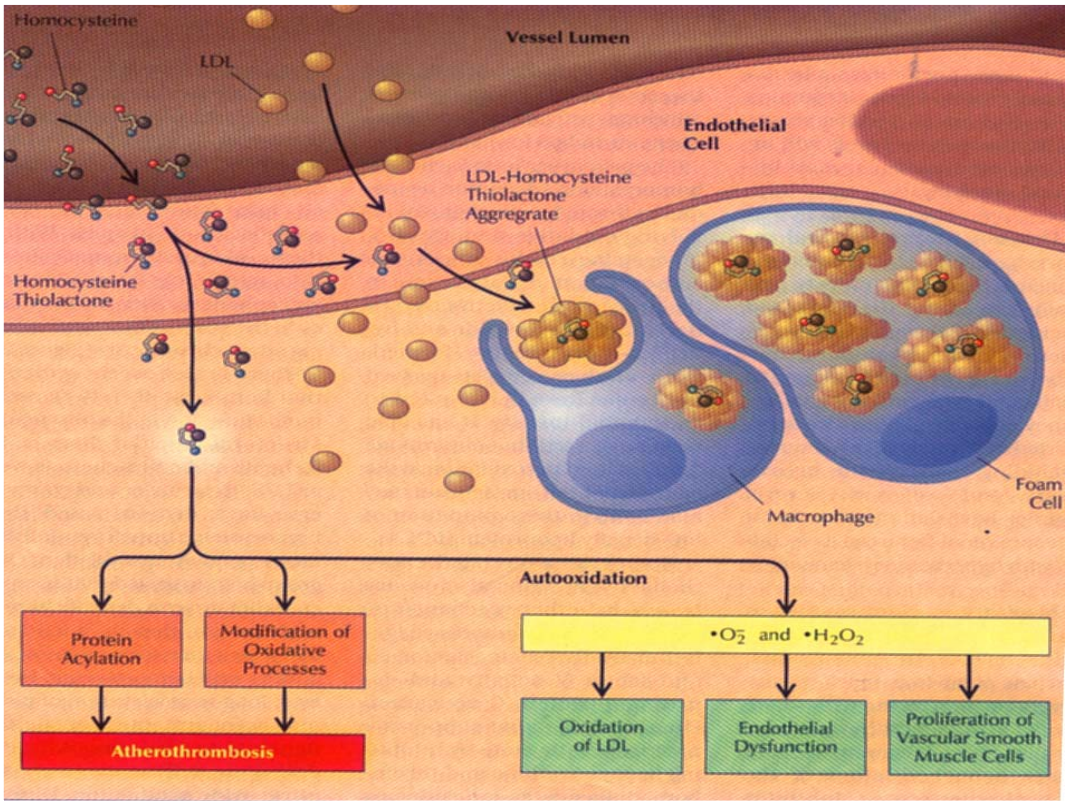

Figure 2-8 Homocysteine-thiolactone reacts with low density lipoprotein (LDL) to from LDL-Homocysteine thiolactone aggregates, then promoting atherosclerosis. In adiition, auto-oxidation of LDL lead to endothelialdysfunction and proliferation of vascular smooth muscle cells[45].

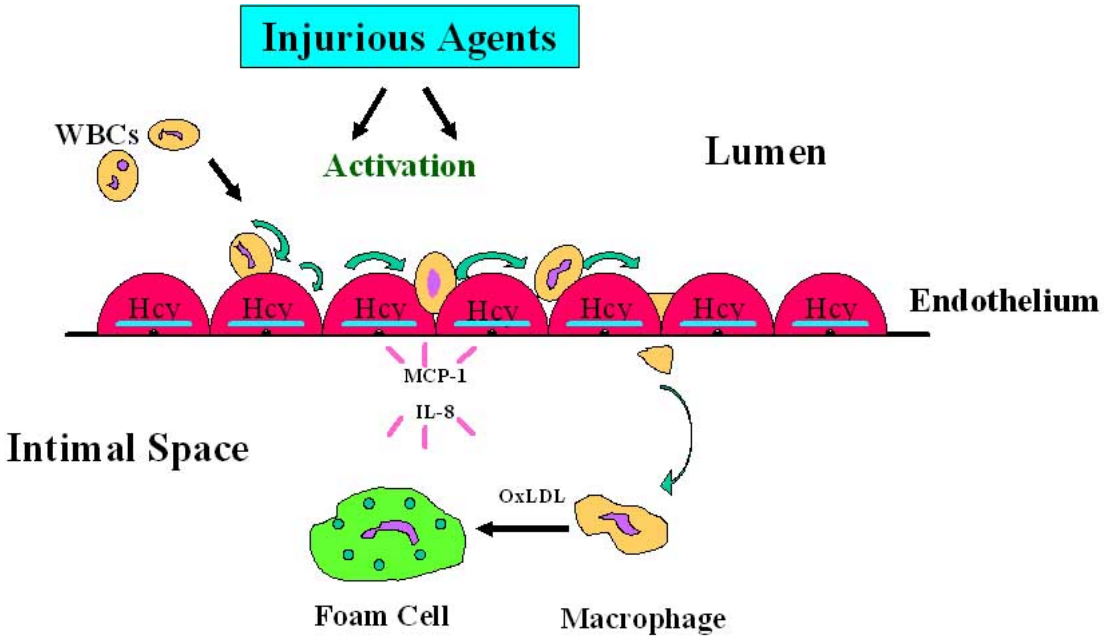

Figure 1-9 Homocysteine may induce the expression and secretion of chemokines such as monocyte chemoattractant protein 1 (MCP-1) and interleukin 8 (IL-8) in vascular endothelial cells then generate foam cell[39].

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8. Treatment of hyperhomocysteinemia

The high level of plasma homocysteine can be reduced by using vitamin supplementation which consist of folic acid, vitamin B_6 and vitamin B_{12}. Many investigation prove that folic acid is the most effective vitamin to use[94-96]. In most patients, small doses of folic acid (1mg to 5 mg per day) may normalize plasma homocysteine concentrations. According to a recent study, both cigareete smoking and coffee consumption were associated with increased homocysteine levels[44]. Smoking and caffeine consumption have previously been shown to be associated with an increased risk of both cardiovascular disease and osteoporosis. The link between coffee consumption and increased homocysteine level is yet controversial.

CHAPTER III

MATERIALS AND METHODS

MATERIALS

1. Enzymes

Mbo II (10,000 U/mL)	Amersham Biosciences
*Hin*f I (10,000 U/mL)	Amersham Pharmacia Biotech
Proteinase K	GibcoBRL
Taq DNA polymerase (2,000 U/mL)	Pacific Science

2. Oligonucleotide primers

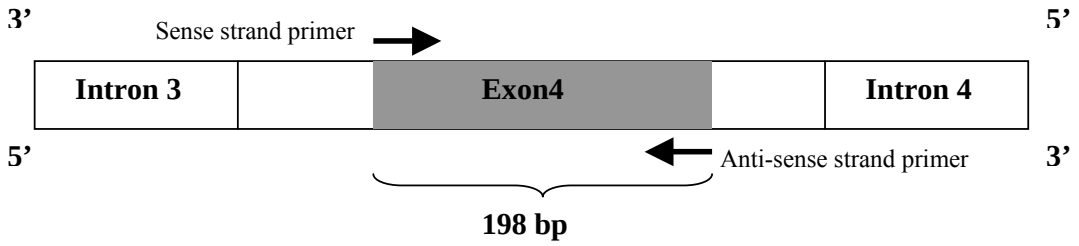
Oligonucleotide primers used to amplify an exon 4 of MTHFR gene were described by Frosst P et al[1] and synthesised by the Bioservice Unit of Mahidol University, Ministry of Science.

Sense strand : 5'-TGA AGG AGA AGG TGT CTG CGG GA-3'
Anti-sense strand : 5'-AGG ACG GTG CGG TGA GAG TG-3'

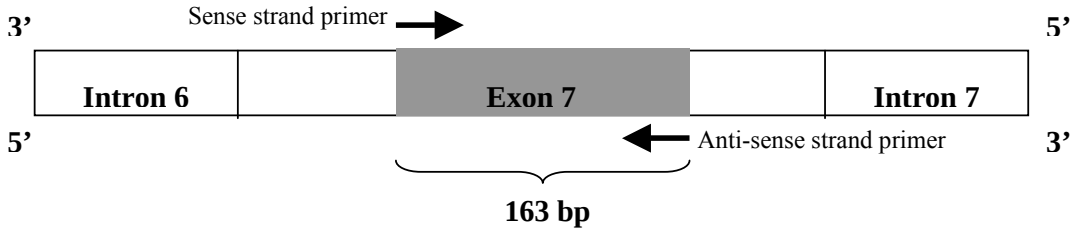
Oligonucleotide primers used to amplify an exon 7 of MTHFR gene were described by Van der Put NM et al[17] and synthesised by Bio Basic Inc.

Sense strand : 5'-CTT TGG GGA GCT GAA GGA CTA CTA C-3'
Anti-sense strand : 5'-CAC TTT GTG ACC ATT CCG GTT TG-3'

a.)



b.)



c.)

1141 CGTACCCAGG AGTGGGACGA GTTCCCTAAC GGCCGCTGGG 1180

1181 GCAATTCCTC TTCCCCTGCC TTTGGGGAGC TGAAGGACTA 1220

1221 CTACCTCTTC TACCTGAAGA GCAAGTCCCC CAAGGAGGAG 1260

1261 CTGCTGAAGA TGTGGGGGGA GGAGCTGACC AGTGAAGAAA 1300

1301 GTGTCTTTGA AGTCTTTGTT CTTTACCTCT CGGGAGAACC 1340

1341 AAACCGGAAT GGTCACAAAG TGACTTGCCT GCCCTGGAAC 1380

Figure 3-1 The diagrammatic view the location of primers used in the PCR that annealing to the genomic DNA (a and b). Partial sequence of the exon 7 of the MTHFR gene (c), the oligonucleotide primers anneal the underline sequence to amplified the 163 bp PCR product.

3. Chemical substances

Chemical substance	Company
Agarose	Sigma
Ammonium chloride (NH_4Cl, MW=63.4)	Sigma
Ammonium persulfate $(NH_4)_2S_2O_8$, MW=228.20)	Merck
Boric acid H_3BO_3, MW=61.83)	Merck
Bromophenol Blue	Sigma
Buffer (10x PCR reaction buffer : 100 mM Tris-HCl , 15 mM $MgCl_2$, 500 mM KCl and 1% Triton X-100)	Pacific Science
Buffer (10x reaction buffer for *Hin* f I enzyme: 10mM Tris-HCl, 7mM $MgCl_2$, 100 mM NaCl and 7 mM 2-mercaptoethanol)	Amersham Pharmacia Biotech
Buffer (10x reaction buffer for *Mbo* IIenzyme: 10mM Tris-HCl, 7mM $MgCl_2$, 10mM KCl, 7mM 2-mercaptoethanol and 0.01%BSA)	Amersham Biosciences
2'-Deoxynucleoside 5'-triphosphate	Pharmacia Biotech
Ethanol (C_2H_5OH , MW=46.0)	Merck
Ethidiumbromide ($C_{21}H_{20}N_3Br$, MW=394.3)	GibcoBRL
Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA, $C_{10}H_{14}N_2Na_2O_8.H_{2O}$, MW=372.24)	Pharmacia Biotech
Glycerol	Sigma
Guanidine-HCl (CH_5N_5.HCl, MW=95.53)	Sigma
Hydrochloric acid (HCl)	Merck
Ladder marker	Invitrogen
Lauryl sulfate (SDS=Sodium dodecyl sulfate , $C_{12}H_{25}O_4SNa$, MW= 288.38)	Sigma
Mineral oil	Pacific science
Sodium hydroxide (NaOH, MW=40.0)	Merck
Trizma base	Pacific science
Tris-HCl	Sigma

Chemical substance	Company
Xylene cyanol	Sigma

4. Instruments

Instruments	Company
Autoclave	Hirayama, Japan
Automatic Pipette	Gilson, France
Bench top centrifuge	Haraeus Sepatech, USA
DNA Thermal cycler	Perkin Elmer, USA
Hotplate Stirrer	Corning, USA
Hot Air Oven	Memmert, Germany
Incubator	Heraeus Sepatech, USA
Microcentrifuge	Heraeus Sepatech, USA
Mini-protean II electrophoresis cell	Biorad, USA
Mini-protean III electrophoresis cell	Biorad, USA
pH meter	Beckman, USA
Polaroid camera	Fotodyne, USA
Refrigerated centrifuge 4237R	ALC product, Italy
Submarine agarose gel electrophoresis set (Gel Mate2000)	Toyobo, Japan
UV-transluminator	Fotodyne, USA
Water bath	Precision pacific Inc,USA

5. Miscellaneous

Plastic wares	Company
Disposable plastic transfer pipette	Elkay, UK
Microcentrifuge tube (0.5,1.5 mL)	Scientific plastics, USA
Pipette tip	Axygen Scientific Inc.,USA
Centrifuge tube (50 mL)	Elkay, UK
Thin wall PCR reaction tube (0.2 mL)	Sorenson BioScience Inc., USA

6. Reagents

6.1 Reagents for DNA extraction from human blood samples

a. 0.5M EDTA pH 8.0 :

Dissolved 93.0 g EDTA (disodium salt) in approximately 400 ml. distilled water. Ten grams of NaOH pellets was added to adjust pH to 8.0. The final volume of this solution was made up to 500 ml and autoclaved.

b. 5% EDTA pH 7.4 :

Dissolved 25 g EDTA (disodium salt) in 400 ml distilled water. The pH of this solution was adjusted to 7.4 with 10 N NaOH, and then final volume was made up to 500 ml before autoclaving to sterile the solution.

c. 7.5 M Guanidine-HCl :

Dissolved 72.0 g guanidine-HCl in 50 ml sterile distilled water and adjusted pH to 7.6. The final volume was made up to 100 ml and filtered with 1 μm filter.

d. Proteinase K solution :

Dissolved 100 mg proteinase K in 10 ml sterile distilled water and mixed well. One milliliter of proteinase K solution was aliquoted into 1.5 ml microcentrifuge tube and stored at -20^0c until use.

e. 10% (W/V) SDS solution :

Dissolved 10 g SDS in 100 ml sterile distilled water.

f. Stock lysis buffer :

118 mM NH_4Cl, 7.6 mM Trizma base, 3.6 mM EDTA pH 7.2

Dissolved 6.35 g NH_4Cl, 1.33 g EDTA (disodium salt) and 0.92 g Trizma in 800 ml distilled water and adjusted pH to 7.2. After adding distilled water up to 1 Liter, the solution was autoclaved and kept at 4^0c.

Working lysis buffer :

The stock lysis buffer was diluted 1:2 before use.

g. TE buffer pH 8.0 :

Mixed 10 ml of 1 M Tris-HCl pH 7.6 and 2 ml of 0.5 M EDTA pH 8.0. Adjusted the final volume to 1 Liter and autoclaved.

h. 1 M Tris-HCl pH 7.6 :

Dissolved 60.6 g Tris-HCl in 400 ml distilled water and added 13 ml of 6 M HCl to adjust pH to 7.6. Adjusted the final volume to 500 ml and autoclaved.

i. 70% ethanol :

Diluted 700 ml of absolute ethanol with 300 ml of sterile distilled water and mixed well.

6.2 Reagents for DNA amplification

a. 10x buffer PCR :

500 mM KCl, 15 mM MgCl2, 100 mM Tris-HCl pH 8.8 and 1% Triton X-100

b. Deoxynucleoside triphosphates :

100 mM each of dATP, dCTP , dGTP, dTTP

c. Taq DNA polymerase

d. Mineral oil

6.3 Reagents for restriction enzyme digestion

a. Enzyme *Hin*f I

b. 10x buffer for enzyme *Hin*f I :

1,000 mM NaCl, 500 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$,

10 mM Dithiothreitol

c. Enzyme *Mbo* II

d. 10x buffer for enzyme *Mbo* II :

100 mM Tris-HCl pH 7.5, 100mM $MgCl_2$, 10mM Dithiothreitol

6.4 Reagents for agarose gel electrophoresis

a. 0.7 %, 2.0 % agarose gel :

Dissolved 0.7 g or 2 .0 g of agarose gel in 1xTBE buffer

b. Ethidium bromide :

Dissolved 1 g ethidium bromide in 100 ml of water.

c. Gel loading buffer :

Dissolved 0.125 g each of bromophenol blue and xylene cyanol in 50 ml DDW containing 15 ml of glycerol.

d. 10x Tris-borate buffer (TBE) :

Dissolved 108 g Trizma base, 55 g boric acid and 9.3 g EDTA (disodium salt) in 1 L distilled water and autoclaved. This stock solution was diluted 10 times before use.

e. DNA ladder marker 250 ng/μl :

Dissolved 5 μ l of 1 μg/μl DNA molecular marker (100 or 25 bp ladder) in 15 μl of sterile distilled water.

METHODS

1. Study subjects

The population was divided into 2 groups. All volunteers were recruited after they provided informed consent. The first group was 98 normal healthy control subjects (65 men in age 48.96±6.23, 26 women in age 41.20±5.68). The healthy control subjects were from blood donor clinic at the Pramongkut hospital. The second group was 199 coronary artery disease (CAD) patients (129 men in age 61.93±9.61, 70 women in age 63.91±8.81). The CAD patients were recruited from Her Majestry's Cardiac Centre, Siriraj Hospital, Mahidol University. The prevalence of CAD was determined by the presence of a pathological coronary angiogram. Severity of CAD was classified according to the number of affected arteries, i.e. as single-vessel disease (≥ 50% luminal diameter stenosis in one coronary artery or its major branches), two-vessel coronary artery disease (≥ 50% stenoses in two coronary arteries, and three-vessel disease (≥ 50% stenoses in three coronary arteries).

2. Determination of homocysteine, folate and vitamin B_{12}

Fasting blood samples were collected into EDTA-containing tubes. The EDTA tube for homocysteine analysis was immediately placed on ice and centrifuged within one hour of the blood draw. The levels of plasma homocysteine concentrations were measured by use of immunoassay method, fluorescence polarization immunoassay or FPIA, by Tropical Laboratory Medicine, Faculty of tropical medicine, Mahidol University (Kits were supplied by Abbott Laboratories LTD). The level of folate and vitamin B_{12} were analyzed using Chemiluminescence technique (Kits were supplied by Boehringer Mannheim) by Clinical Chemistry Unit, Department of Clinical Pathology, Faculty of Medicine Siriraj Hospital, Mahidol University.

3. Determination of cholesterol, triglyceride and HDL-cholesterol

Fasting serum were determined the levels of cholesterol, triglyceride and HDL-cholesterol using spectrophotometry technique (Kits were supplied by Human, Germany).

4. DNA extraction

Genomic DNA was extracted from peripheral leukocytes by Guanidine-HCl method (UCLA, 1993). Five microliters of venous blood was collected from each subject with informed consent into a 50 ml focal tube containing 200 µl of 5% EDTA as a blood anticoagulant. Two volumes of working lysis buffer were added into the tube. The solution was mixed by vortexing then let stand for 10 min at room temperature. After centrifugation at 600 g for 10 minutes at 4ºC (ALC 4237R, Italy), the red supernatant was discarded and this step was repeated twice more. The 40 µl enzyme Proteinase K solution was added into the white blood cell pellet and mixed homogeneously. The solution was added with 800 µl sterile double distill water and 300 µL of 10% SDS, mixed gently to avoid the air-bubble and incubated overnight at 37ºC. Following the addition of Guanidine-HCl, the mixture was incubated at 68-70 ºC for 10 min. The solution was mixed again and incubated at 68-70ºC for another 5 min. The mixture was centrifuged at 9,800xg for 10 min. The supernatant was gently mixed and incubated at 68-70ºC for an additional of 5 min. The mixture was centrifuged as described. DNA from the supernatant was then precipitated with two volumes of cold absolute ethanol followed by washing once with 70% ethanol. Genomic DNA was finally resuspended in TE buffer (10 mM Tris-HCl, 1mM EDTA pH 8.0) and stored at –20ºC until use. Quality of the extracted gDNA was verified by agarose gel electrophoresis.

5. DNA amplification by polymerase chain reaction (PCR)

5.1 Principle of PCR

The polymerase chain reaction (PCR) is a powerful and widely used technique that allows specific DNA sequences to be copied or amplified over a million fold in a simple enzyme reaction[97]. PCR is a rapid and versatile *in vitro* method for selective amplification of a specific target DNA sequences within a heterogeneous source of DNA (e.g. total genomic DNA or a complex cDNA)[98]. To permit such selective amplification, some prior DNA sequence information from the target sequences is required (Figure 3-2). This information is used to design two oligonucleotide primers (amplimers) which are specific for the target sequence and which are often about

15-25 nucleotides long. After the primers are added to denature template DNA, they bind specifically to complementary DNA sequences at the target site. In the presence of a suitably heat stable DNA polymerase and DNA precursors (the four deoxynucleoside triphosphates, dATP, dCTP, dGTP, dTTP), they initiate the synthesis of new DNA strands which are complementary to the individual DNA strands of the target DNA segments, and which overlap each other.

PCR allows the amplification of target DNA sequences through 20-40 repeated cycles of DNA synthesis. Each cycle has three stages carried out at different temperatures: (i) denaturation – the reaction is heated to above 90 ºC (about 93 ºC to 95 ºC for human genomic DNA) to separate the strands of the double helix; (ii) annealing – the reaction is cooled to allow the primers bind to the single- stranded template DNA. Normally, annealing temperature is about 50 ºC to 70 ºC depending on melting temperature (*Tm*) of the expected duplex (the annealing temperature is typically about 5 ºC below the calculated *Tm*; (iii) extension – the reaction is typically about 70 ºC to 75 ºC where the DNA polymerase is most active for DNA synthesis and the target DNA sequence is copied.

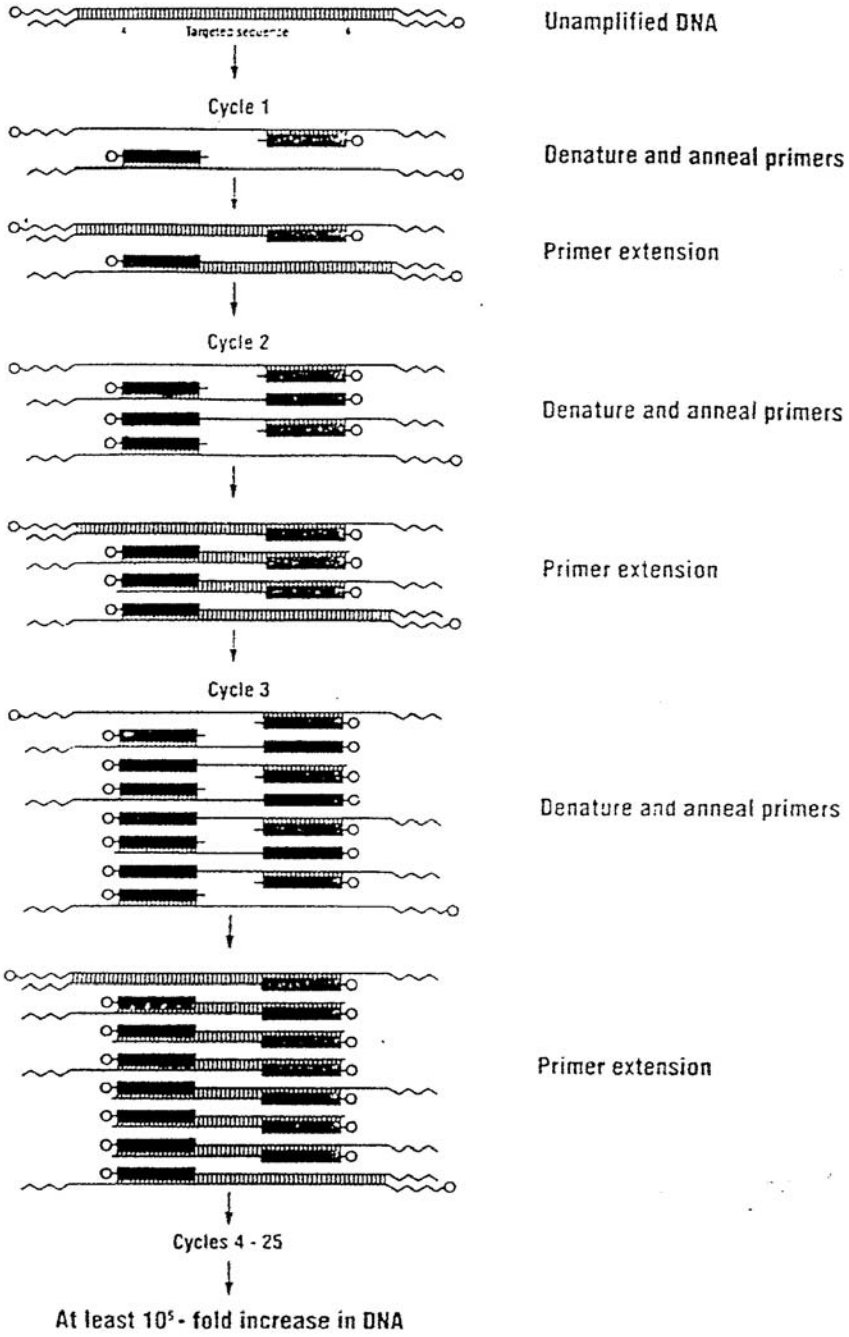


Figure 3-2 The schematic diagram of the principle polymerase chain reaction (PCR)

5.2 Procedure of PCR

The C677T mutation in the MTHFR gene was analyzed by PCR of genomic DNA using the method described by Frosst *et al*. The oligonucleotide primers, sense strand : 5'-TGA AGG AGA AGG TGT CTG CGG GA-3' and anti-sense strand : 5'-AGG ACG GTG CGG TGA GAG TG-3', were used to amplify the exon 4 of MTHFR gene. Nevertheless, the temperature profile was optimized in this study. A 25µl PCR reaction mixture consisted of 5 µl of genomic DNA, 10 mM Tris-HCl pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.04mM of each nucleoside triphosphate, 5 pmol of each primer and 0.5 unit of Taq DNA polymerase. The reaction mixture was covered with a drop of mineral oil. PCR reaction was performed in a 0.6 ml microcentrifuge tube and run in a Perkin-Elmer Cetus Thermal Cycler. Thermocycling condition consisted of 40 cycles of denaturation at 95ºC for one min, annealing at 65ºC for one min and extension at 72ºC for one min, proceeded by an initial denaturation step at 95ºC for five min and followed by a terminal extension of seven min at 72ºC. The second A1298C mutation was also analyzed by PCR using the method described by Van der Put NM et al. The sense strand primer : 5'-CTT TGG GGA GCT GAA GGA CTA CTA C-3' and anti-sense strand primer :5'-CAC TTT GTG ACC ATT CCG GTT TG-3' were used to amplify exon 7 of the MTHFR gene. The 25µl PCR reaction mixture was the same as described for the amplification of C677T mutation. The temperature profiles were : initial denaturation at 92ºC for two min, followed by 40 cycles of denaturation at 92ºC for one min, annealing at 60ºC for one min, extension at 72ºC for 30 sec and a final extension time of seven min at 72ºC. This amplification reaction resulted in synthesis of a 198 bp fragment for exon 4 and 163 bp fragment for exon 7. The PCR-amplified DNA products from subjects of CAD patients and healthy control subjects were used in the next step of restriction enzyme analysis (RFLP).

6. Restriction Fragment Length Polymorphism (RFLP)

6.1 Principle of RFLP

Restriction endonucleases are enzymes that cleave DNA molecule. These are bacterial enzymes, believed to be part of the defence against viral infection. The

enzymes recognize a specific sequence of bases in DNA, and cut the molecule where this sequence occurs. This is known as enzyme digestion. The name of enzymes are from the bacteria which they are isolated such as enzyme *Eco*R1 came from *E. coli* strain R1 and it cuts wherever the sequence GAATTC is found in DNA. Other enzymes cut at different sequence, 4 to 8 bases long. If two organisms differ in the distance between sites of cleavage of a particular restriction endonuclease, the length of the fragments produced will differ when the DNA is digested with an enzyme and these different sized fragments are known as RFLP (restriction fragment length polymorphisms). The detection of different sized fragments can be very important in analyzing certain genetic disorders. Differences between individuals occur because about 90% of human DNA is noncoding, that is, does not contain genes, and variations in base sequence between individuals in this noncoding DNA can occur without apparent harmful effect (except for noncoding regulatory sequences). Thus, different individuals will show variations in the structure of their noncoding DNA, usually in the form of single base changes. Sometimes these changes occur in an existing recognition sequence for a restriction endonucleases and the site is then lost, or the change can create a new recognition site for a restriction endonuclease. When the DNA from an individual showing such a change is digested with the restriction endonuclease that cuts at that site, there will be a change in the size of change in base sequence. The DNA from the two individuals will therefore exhibit different fragment lengths when digested with the relevant restriction enzyme. These differences are RFLP.

6.2 Procedure of RFLP

Two mutations of MTHFR gene on C677T and A1298C in exon 4 and exon 7, respectively, were analysed by PCR-RFLP. The PCR amplified DNA were cut with restriction endonuclease enzymes.

C677T mutation : The C to T substitution at nucleotide 677 in the MTHFR gene introduces a restriction site for the *Hin*f I enzyme. This enzyme was used to distinguish the allele by cleaving the mutant fragment into 175 bp and 23 bp fragments. For the digestion reaction, 5 units of *Hin*f I, 1.5 μl of 10x buffer

recommended by manufacturer and 10 µl of PCR product were added to a final volume of 15 µl. Samples were digested overnight at 37 ºC.

A1298C mutation : This 1298AC mutation in the MTHFR gene abolishes an *Mbo* II restriction site. After restriction enzyme analysis of the 163 bp PCR fragment with *Mbo* II, we expected and observed the following: the 1298CC genotype results in 4 fragments, namely 84, 31, 30 and 18 bp, whereas the 1298AA genotype gives 5 fragments. The 84 bp fragment is cut into a 56 and 28 bp fragments, and thus base pair lengths of 56, 31, 30, 28, 18 can be observed. The reaction mixture (15µl) contained 10 µl of the PCR-amplified DNA, 1.5 µl of 10x buffer, 5 units of *Mbo* II and additional volume of sterile double distilled water up to 15 µl. Sample were digested overnight at 37 ºC.

After digestion, the fragments were identified by 8% and 15% polyacrylamide gel electrophoresis for C677T and A1298C mutations respectively. The gel was stained with ethidium bromide and visualized on a UV transilluminator.

7. Agarose gel Electrophoresis

7.1 Principle of agarose gel electrophoresis

Agarose electrophoresis is a method used in molecular biology to separate DNA strands by size, and to determine the size of the separated strands by comparison to strands of known length. The technique of electrophoresis is based on the fact that DNA molecules have a high negative charge due to its phosphate backbone. For this reason, when DNA is placed on a field with an electric current, these negatively charged DNA molecules migrate toward the positive end of the field. DNA molecule migrated through an agarose gel. The agarose forms a porous lattice in the buffer solution and the DNA must slip through the holes in the lattice in order to move toward the positive pole. This slows the molecule down. The smaller molecules are able to move faster than the larger one, so they make it further down the gel than the larger molecules. The DNA is not normally visible during this process, so the marker dye is added to the DNA to avoid the DNA being run entirely off the gel. The marker dye has a low molecular weight, and migrates faster than the DNA, so as long as the marker has not run past the end of the gel, the DNA will still be in the gel (Figure 3-3).

7.2 Procedure of agarose gel electrophoresis

After PCR, the amplified DNA products were verified by agarose gel electrophoresis. Prior to gel casting, dried agarose is dissolved in 1x TBE buffer by heating. The warm gel solution is poured in to a mold which is fitted with a comb. The percentage of agarose in the gel is varied. The 0.7% agarose gel is used to detect the purified DNA from DNA extraction and 2% agarose is prepared to check the amplified PCR product. The DNA ladder marker is loaded into each gel. Five μl of DNA was mixed with 2 μl of loading dye marker. Agarose gel electrophoresis was run in 1x TBE buffer pH 8.0 for 40 min at 100 volts, then the DNA fragments were observed by staining with ethidium bromide and visualized on a UV transilluminator.

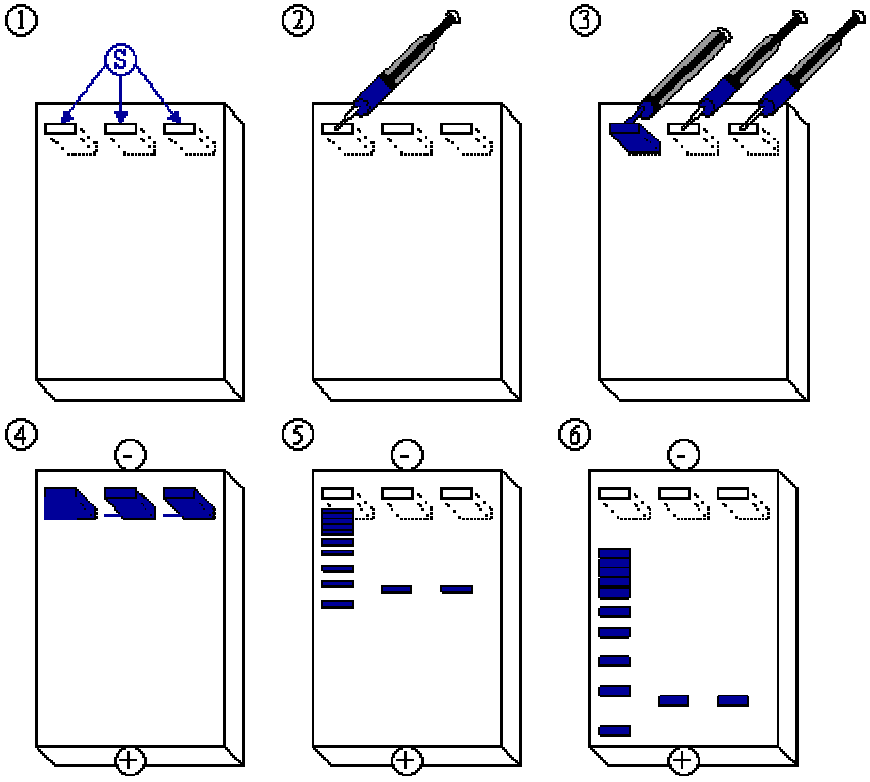


Figure 3-3 The schematic diagram of agarose gel electrophoresis. DNA was mixed with loading dye marker and loaded in each gel and run in TBE buffer at 100 volts. The fragments of DNA migrated with molecular weight.

8. Polyacrylamide gel electrophoresis

8.1 Principle of polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) is often used for high resolution separation of denatured proteins, native proteins and small DNA fragments (Figure 3-4). There are two basic types of polyacrylamide gels. The first is native gels, native polyacrylamide gels contain bis and polyacrylamide (ammoniumpersulfate and TEMED) only, and there are no special additives. Native gels are more convenient to prepare, but have a signification limitation: they cannot be used to separate mixtures of proteins based on their relative sizes. The second is denaturing gels; denaturing polyacrylamide gels contain additives that maintain the molecules to be separated in a denatured state. Proteins are denatured using the detergent SDS. SDS disrupts interactions between side groups and disrupts all of the disulfide bonds that stabilize the higher order structure of proteins. This means that all denatured proteins have roughly the same shape. For this reason the separation in the gel is now purely dependent on the molecular mass of the proteins.

Polyacrylamide forms gels with pores of a much more controlled and uniform size than does agarose. Consequently, polyacrylamide gels can be used to separate molecules that differ in size by as little as 2% of their molecular weight. The formation of pores in polyacrylamide gels is a chemical process, resulting from the combined action of polymerization and chemical cross-linking. The result is a very regular matrix with a much more uniform pore size.

Polyacrylamide gels are composed of two chemicals, monoacrylamide and N,N' methylene bis acryamide. The size of the pores is controlled by varying the ratio of bis to acrylamide (which determines the extent of the cross-linking) and the overall pertage of acrylamide (which determines how tightly packed the polymer chains will be in solution).

8.2 Procedure of polyacrylamide gel electrophoresis

Polymorphisms of MTHFR gene were detected by polyacrylamide gel electrophoresis. The percentage of polyacrylamide depends on size of the DNA fragments. To detect the C677T polymorphism, 8% polyacrylamide gel was used and

15% polyacrylamide gel was used to detect the A1298C polymorphism. Fifteen microliters of 8% polyacrylamide solution, containing 3 ml of 40% acrylamide solution (19:1), 1.5 ml of 10x TBE buffer, 10.5 ml of distilled water, 80 µl of 20% ammonium persulfate (to initiate free radicals which are important for chain reaction) and 8 µl of TEMED were mixed together gently. TEMED accelerated the polymerization of acrylamide and bis-acrylamide by catalyzing the formation of free radicals from ammonium persulfate. The free radicals would drive the polymerization. Fifteen microliters of 15% polyacrylamide solution composed of 5.6 ml of 40% polyacrylamide solution (19.:1), 1.5 ml of 10x TBE buffer, 7.9 ml of distilled water, 80 µl of 20% ammonium persulfate and 8 µl of TEMED. The gel was left to polymerize at room temperature for 40 minutes. After the polymerization was completed, the gel mold was set to the electrophoresis apparatus. 1X TBE was used as a running buffer. The solution of the DNA marker and /or DNA samples were mixed with 2 µl of gel loading buffer and applied into each well and electrophoresed at 100 volts for 60 min to detect the C677T mutation or 150 min to detect the A1298C mutation, respectively at room temperature. When the electrophoresis was finished, the DNA fragments in the gel was stained with ethidium bromide solution then placed on the short wavelength UV transilluminator for visualization.

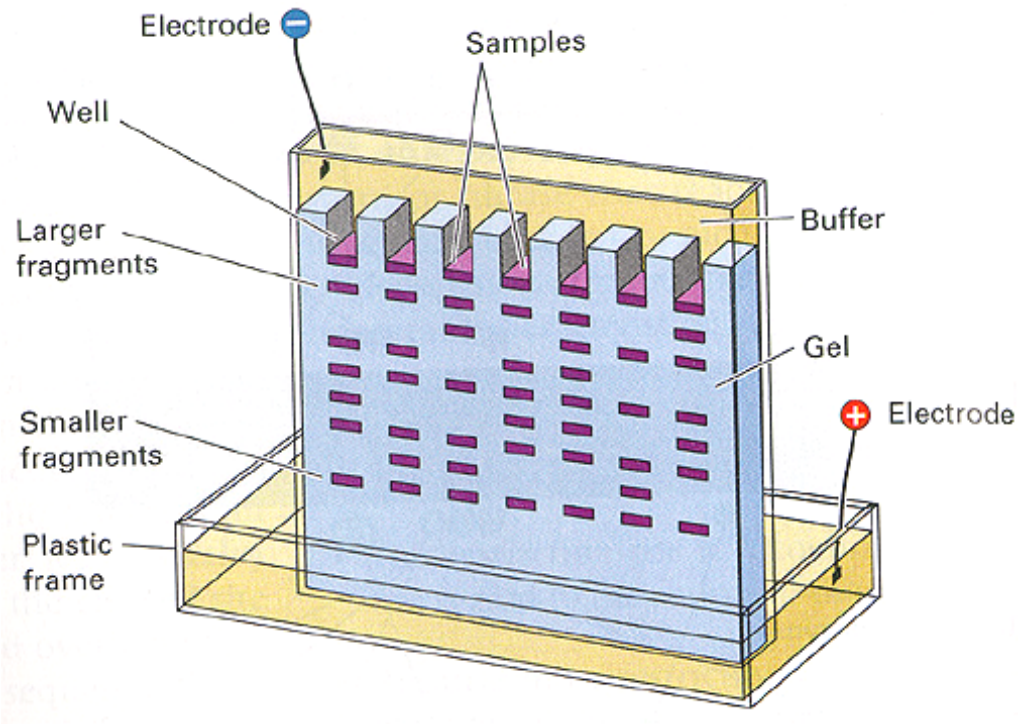


Figure 3-4 The schematic diagram of polyacrylamide gel electrophoresis

9. Statistical analysis

Hardy-Weinberg equilibrium was determined for all of the genotypes using the Chi-square (χ^2) test. Genotype distribution and allele frequencies between the study groups were compared by Chi-square (χ^2) analysis. ANOVA and compared mean t-test were carried out to test the difference of homocysteine concentrations. The relative risk and 95% confidence interval (95% CI) were estimated using exposure Odds ration. The nominal levels of statistical significance for all analyses were < 0.05. All calculations were conducted by SPSS and MedCalC program.

CHAPTER IV

RESULTS

1. Clinical characteristics of the study population

The subjects were divided into 2 groups. The first group was 98 normal healthy control subjects recruited from blood donor clinic at the Phramongkut hospital. The second group was 199 coronary artery disease (CAD) patients, which were divided from Her Majesty's cardiac centre, Siriraj Hospital, Mahidol University. Severity of CAD was classified by the significant plaque burden in different level, as single vessel disease, double vessels disease and triple vessels disease. The baseline data, clinical and biochemistry characteristics of CAD patients were summarized in Table 4-1. Baseline data and biochemistry characteristic of healthy control subjects were not determined.

Table 4-1 Clinical and biochemical characteristics of CAD patients and healthy control subjects.

Parameter	Healthy subjects (n=98)	CAD patients (n=199)	p-value
Age (years)	48.21±5.19 (n=94)	62.63±9.35 (n=190)	<0.0001
Male:Female	3:1	2:1	-
Blood pressure			
systolic (mmHg)	-	151.43±25.84 (n=165)	-
diastolic (mmHg)	-	80.37±17.30 (n=165)	-
Cholesterol (mg/dl)	178.18±25.06 (n=33)	186.52±46.58 (n=192)	0.1055
Triglyride (mg/dl)	163.07±75.86 (n=33)	141.93±83.73 (n=192)	0.1760
HDL-cholesterol (mg/dl)	47.45±3.69 (n=33)	42.73±12.69 (n=191)	0.0353
Homocysteine (μmol/L)	9.26±1.90 (n=98)	13.58±6.07 9 (n=88)	<0.0001
Folate (ng/ml)	-	9.08±9.38 (n=81)	-
B_{12} (pg/ml)	-	772.42±416.26 (n=81)	-

2. DNA extraction

Genomic DNA was extracted from peripheral leukocytes of a healthy control subjects and CAD patients by Guanidine HCl method (UCLA 1993). The quality of the extracted DNA was determined by electrophoresis on 0.7% agarose gel. The gDNA bands were visualized under the UV-transilluminator after ethidium bromide staining.

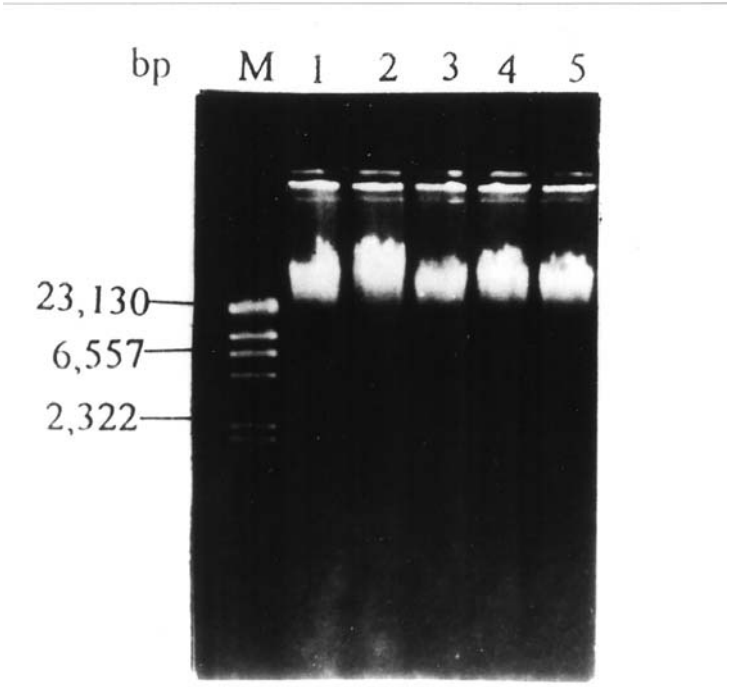


Figure 4-1 Ethidium bromide staining pattern of extracted DNA from peripheral blood leukocytes by Guanidine-HCl extraction. Lane M was *Hin*dIII digested λ DNA marker. Lanes 1-5 were gDNA from different individuals.

3. Analysis of C677T mutation in exon 4 of the MTHFR gene

The C677T mutation was determined by PCR-RFLP. Exon 4 of the MTHFR gene was amplified with specific primers. The generated fragment has a length of 198 bp. The optimal annealing temperature for exon 4 gene amplification was 65°C, 1 min (Figure 4-2). The cytosine to thymine substitution at nucleotide 677 creates a *Hin*f I recognition site. In the presence of the mutation *Hin*f I-digested the 198 bp fragment into a 175 bp and 23 bp fragments (Figure 4-3 and 4-4). The genotypes were analysed by 8% polyacrylamide gel electrophoresis in TBE buffer. The *Hin*f I digested exon 4 was shown in Figure 4-5

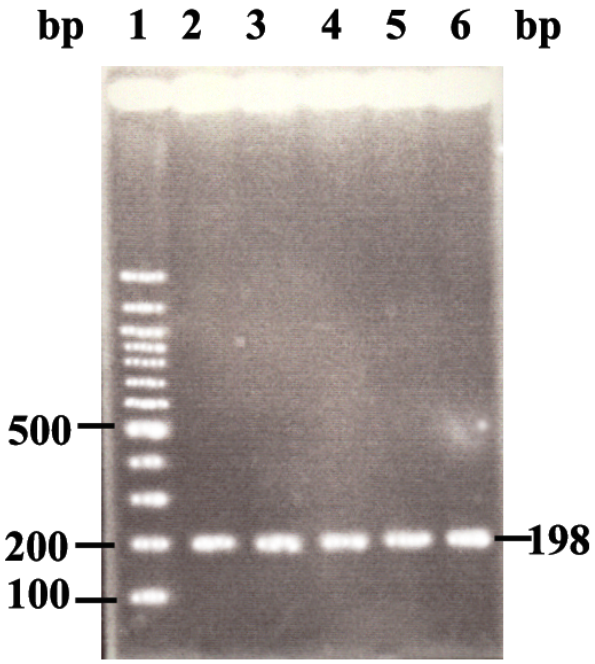


Figure 4-2 2% agarose gel electrophoretic patterns of PCR-amplified product of C677T mutation. Lane M was 100 bp DNA molecular weight marker and lanes 1-4 were PCR-amplified product.

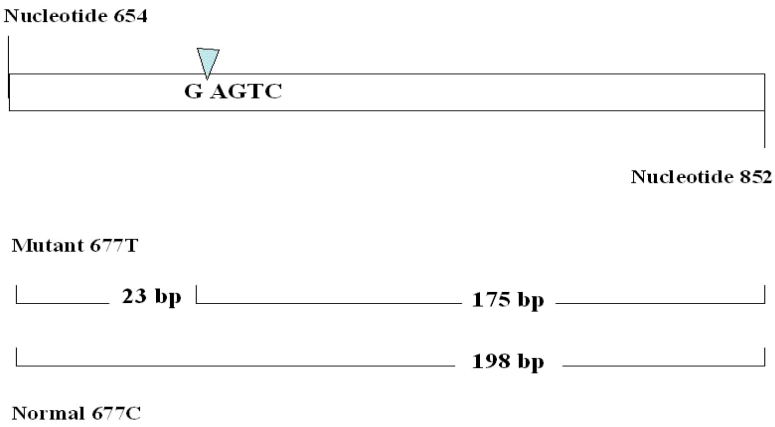


Figure 4-3 For 677CT mutation analysis, genomic DNA was amplified from nucleotide 654 to nucleotide 852 (198 bp size). In the PCR-amplified DNA which had one restriction site cut for restriction enzyme *Hin*f I would digest the PCR-amplified DNA into 2 fragments of 175 and 23 bp in the homozygous mutation (677T) of the MTHFR gene, whereas the PCR-amplified DNA from the homozygous 677C remain undigested.

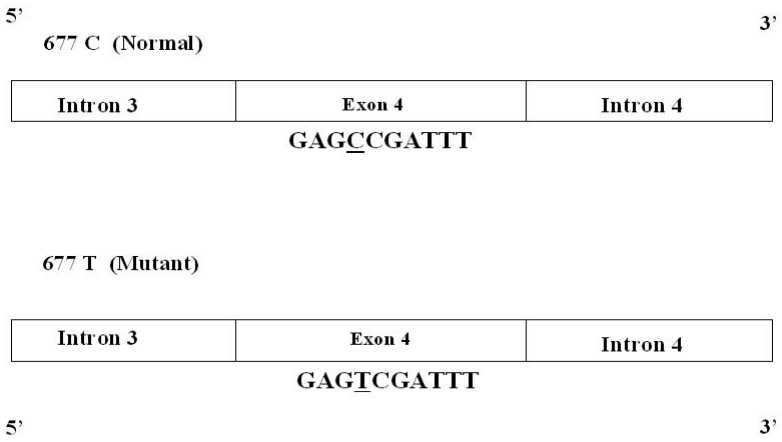


Figure 4-4 Diagrammatic presentation of the base substitution nucleotide 677 of the human MTHFR gene. A genomic DNA fragment on exon 4 of the MTHFR gene was amplified by PCR which produced 198 bp product in the presence of the base substitution (C to T) at nucleotide 677.

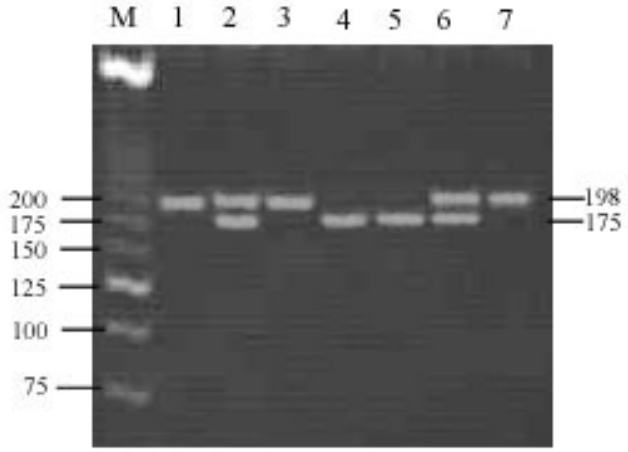


Figure 4-5 8% polyacrylamide gel electrophoretic pattern of the *Hin*f I-digested C677T mutation on exon 4 of the MTHFR gene. Lane M was 25 bp DNA molecular weight marker. Lane 1 was the undigested PCR-amplified DNA. Lanes 2-7 were *Hin*f I-digested DNA. Lanes 3 and 7 were wild type CC (198 bp). Lanes 2 and 6 were heterozygous CT (198, 175 and 23 bp) and lanes 4 and 5 were homozygous TT (175 and 23 bp). The 23 bp was run out of gel.

4. Analysis of A1298C mutation in exon 7 of MTHFR gene

Exon 7 of the MTHFR gene was amplified by PCR using the optimal annealing temperature which was 60°C for 1 min. The amplified-DNA product was 163 bp (Figure 4-6). The A1298C mutation in exon 7 of MTHFR gene was determined by *Mbo* II restriction enzyme digestion. This mutation abolished the *Mbo* II restriction site. After restriction enzyme digested PCR-amplified DNA, the DNA fragments were analyzed by 15% polyacrylamide gel electrophoresis. The mutation pattern in exon 7 of MTHFR gene and the *Mbo* II restriction map were shown in Figures 4-7 and 4-8, respectively. Following *Mbo* II digestion of the homozygous wild type (AA)163 bp fragment resulted in 5 fragments (56, 31, 30, 28 and 18 bp). The heterozygous genotype (AC) resulted in 6 fragments (84, 56, 31, 30, 28 and 18 bp) and the homozygous mutant genotype resulted in 4 fragments (84, 31, 30 and 18 bp). The fragments were identified using 15% polyacrylamide gel electrophoresis (Figure 4-9).

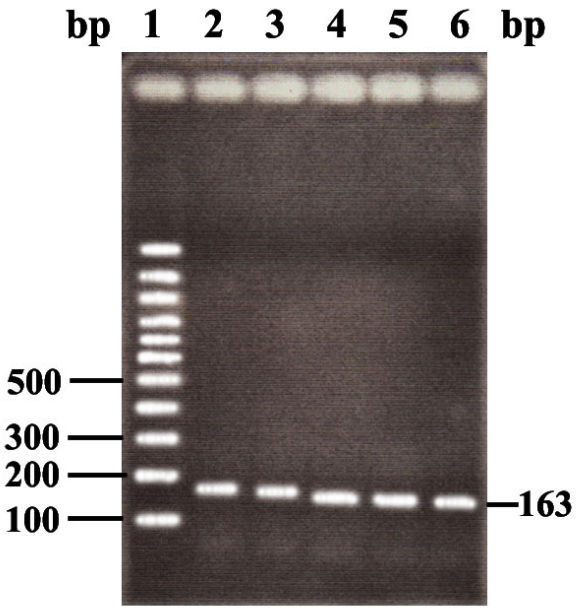


Figure 4-6 2% agarose gel electrophoretic of PCR-amplified product of exon 7 on the MTHFR gene (A1298C). Lane M was 100bp DNA molecular weight marker and lanes 1-4 were PCR-amplified product (163 bp).

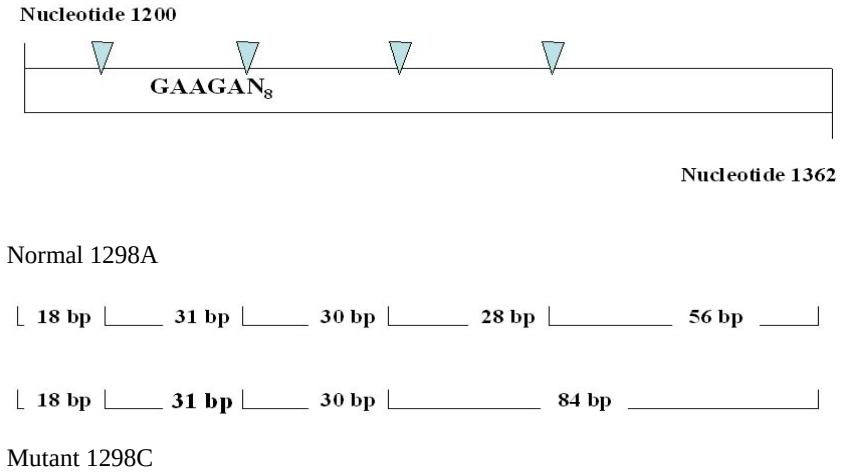


Figure 4-7 For A1298C mutation analysis, genomic DNA was amplified from nucleotide 1200 to 1362 (163 bp size). The substitution of A to C at nucleotide 1298 on exon 7 of MTHFR gene abolished an *Mbo* II restriction site, the 84 bp fragment was not digested into 56 and 28 bp fragments.

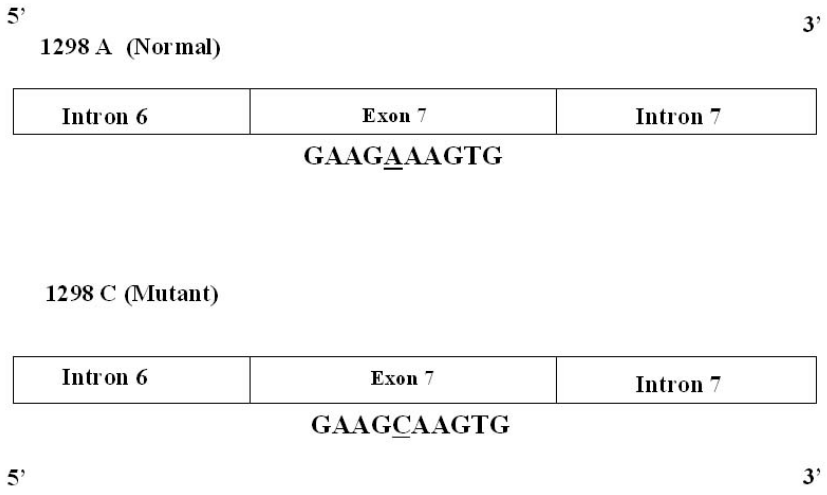


Figure 4-8 Diagrammatic of the A to C substitution at nucleotide 1298 on exon 7 of the human MTHFR gene. The PCR-amplification produced a 163 bp product.

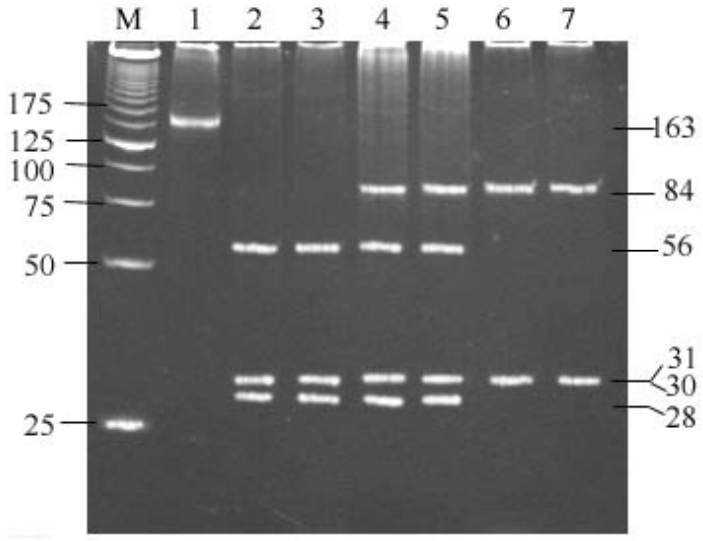


Figure 4-9 15% polyacrylamide gel electrophoretic pattern of the *Mbo* II-digested A1298C mutation on exon 7 of the MTHFR gene. Lane M was 25 bp DNA molecular weight marker. Lane 1 was the undigested PCR-amplified DNA. Lanes 2-7 were *Mbo* II-digested DNA. Lanes 2 and 3 were wild type AA (56, 31, 30, 28 and 18 bp). Lanes 4 and 5 were heterozygous AC (84, 56, 31, 30, 28 and 18 bp). Lanes 6 and 7 were homozygous CC (84, 31, 30 and 18 bp). The 18 bp was run out of gel.

5. The biochemical and clinical characteristics in the MTHFR gene variants

The biochemical and clinical characteristics of 199 CAD patients with CC, CT and TT genotypes of C677T and AA, AC and CC genotypes of A1298C mutations were compared and shown in Table 4-2 and Table 4-3, respectively.

Table 4-2 Biochemical and clinical characteristics from CAD patients in the C677T mutation study.

Parameter	677CC	677CT	677TT	P- value
Age (years)	63.17±9.44 (n=137)	61.33±9.20 (n=58)	64.67±8.39 (n=3)	0.544
Male:Female	2:1	1:1	2:1	-
Blood pressure systolic (mmHg) diastolic (mmHg)	 79.82±15.25 (n=110) 151.72±27.07 (n=110)	 81.63±21.50 (n=52) 150.67±23.99 (n=52)	 154.00±10.58 (n=3) 78.67±1.53 (n=3)	 0.955 0.994
Cholesterol (mg/dl)	187.44±49.11 (n=131)	185.07±41.36 (n=59)	175.33±39.88 (n=3)	0.900
Triglyceride (mg/dl)	138.06±65.96 (n=131)	150.61±113.78(n=59)	140.00±107.70 (n=3)	0.796
HDL-cholesterol (mg/dl)	42.63±11.79 (n=131)	42.55±14.29 (n=59)	50.67±20.84 (n=3)	0.374
Homocysteine (μmol/L)	12.93±4.66 (n=54)	13.56±4.70 (n=33)	50	<0.005
Folate (ng/ml)	7.78±4.20 (n=49)	11.37±14.36 (n=29)	6.4 (n=1)	0.204
B_{12} (pg/ml)	748.24±332.30 (n=50)	810.36±540.88 (n=29)	884 (n=1)	0.350

Table 4-3 Biochemical and clinical characteristics from CAD patients and healthy control subjects in the A1298C mutation study.

Parameter	1298AA (n=98)	1298AC (n=80)	1298CC (n=21)	P- value
Age (years)	62.17±8.95 (n=93)	63.88±9.43 (n=77)	59.95±10.56 (n=20)	0.828
Male:Female	2:1	1:1	4:1	-
Blood pressure systolic (mmHg) diastolic (mmHg)	 80.29±15.24 (n=82) 151.64±24.40(n=82)	 79.03±20.43 (n=66) 148.98±26.09 (n=66)	 85.94±12.42 (n=17) 159.88±31.03 (n=17)	 0.295 0.005
Cholesterol (mg/dl)	185.49±44.81 (n=94)	186.24±46.27 (n=78)	192.19±56.66 (n=21)	0.767
Triglyceride (mg/dl)	145.28±82.34 (n=94)	129.63±52.96 (n=78)	172.62±152.79 (n=21)	0.401
HDL-cholesterol (mg/dl)	42.92±11.75 (n=94)	42.42±13.18 (n=78)	44.38±12.70 (n=21)	0.161
Homocysteine (μmol/L)	14.51±7.68 (n=41)	12.74±3.78 (n=36)	12.89±5.34 (n=11)	0.463
Folate (ng/ml)	8.42±4.13 (n=35)	10.22±13.68 (n=33)	7.63±4.59 (n=11)	0.887
B_{12} (pg/ml)	757.92±481.58 (n=35)	773.76±378.71 (n=34)	814.36±322.98 (n=11)	0.960

6. Analysis of the relationship between C677T and A1298C mutations with plasma homocysteine concentrations.

The effect of C677T and A1298C mutations of the MTHFR gene on plasma homocysteine concentrations were analyzed and compared between CAD patients and healthy control subjects. The levels of homocysteine were classified by genotype distribution (Table 4-4). Homocysteine concentration depends on age, so the ages of all subjects were selected between 40-70 years. For C677T mutation, the homozygous genotype (TT) was not found in healthy control subjects. The levels of homocysteine concentration were significantly higher in CAD patients in both wild type (CC) ($p<0.0001$) and heterozygous mutation (CT) ($p=0.0011$) than the healthy control subjects. In healthy control subjects, homocysteine concentrations were compared between heterozygous (CT) and wild type (CC). It was significantly different ($p=0.0204$).On the contrary, homocysteine between 677CC and 677CT in CAD patients were not significantly different ($p=0.3570$). For A1298 mutation, there were no significant differences in mean homocysteine concentrations in individuals who were either heterozygous (AC) or homozygous (CC) compared with individuals homozygous wild type (AA) ($p>0.05$). However, homocysteine concentrations in CAD patients who were wild type (AA) and heterozygous (AC) were significantly different when compared with healthy control subjects ($p<0.0001$).

Table 4-4 Concentrations of homocysteine according to MTHFR C677T and A1298C genotypes in healthy control subjects and CAD patients

Parameter	Hcy level in Healthy control subjects(μmol/L) (n= 98) [a]	Hcy level in CAD patients (μmol/L) (n=82)[b]	P-value
677			
CC	9.01±1.82 (75)	12.75±4.62 (51)	<0.0001
CT	10.06±2.02 (23)	13.75±4.81 (30)	0.0011
TT	-	50.00 (1)	-
1298			
AA	9.04±2.03 (47)	14.76±7.90 (38)	<0.0001
AC	9.48±1.77 (49)	12.47±3.48 (35)	<0.0001
CC	9.50±2.73 (2)	12.80±5.89 (9)	0.4723

677

CC^a,CT^a p=0.0204 CC^b,CT^b p=0.3570

1298

AA^a,AC^a p=0.2600 AA^a,CC^a p=0.7570 AC^a,CC^a p=0.9877

AA^b,AC^b p=0.4892 AA^b,AC^b p=0.1188 AC^b,CC^b p=0.8285

7. Analysis of the relationship between combined mutation (C677T/A1298C) and plasma homocysteine concentrations.

The effect of the combined mutation between the C677T and A1298C genotypes on homocysteine concentrations was analyzed in the healthy control subjects and CAD patients (Table 4-5). CAD patients with the 677TT/1298AA genotype showed the highest level of homocysteine (50 μmol/L). In addition, the homocysteine concentrations in CAD patients with 677CC/1298AA, 677CC/1298AC, 677CT/1298AA and 677CT/1298AC genotypes were statistical significantly highest than healthy control subjects ($p<0.0001$, $p<0.0001$, $p=0.0253$ and $p=0.0029$, respectively). Furthermore, healthy subjects with combined heterozygosity for the two MTHFR variants (677CT/1298AC genotype) had significantly higher homocysteine concentrations than subjects with the 677CC/1298AA genotypes ($p=0.0156$) and 677CC/1298AC genotypes ($p=0.0051$). In CAD patients, homocysteine concentrations were significantly higher in individuals with 677CT/1298AC genotypes compared with individuals with 677CC/1298AC genotype ($p=0.0064$).

Table 4-5 Concentration of homocysteine according to combined MTHFR C677T/A1298C mutations in healthy control subjects and CAD patients

Parameter	Hcy level in Normal subjects (μmol/L) (n=98)[a]	Hcy level in CAD patients (μmol/L) (n=81)[b]	p-value
677CC/1298AA	8.95±2.10 (36)	13.84±5.39 (21)	<0.0001
677CC/1298AC	9.09±1.50 (37)	11.42±3.02 (24)	<0.0001
677CC/1298CC	9.15±2.73 (2)	14.24±6.25 (6)	0.3256
677CT/1298AA	9.36±1.80 (11)	13.76±5.49 (16)	0.0253
677CT/1298AC	10.70±2.07(12)	14.77±3.46 (11)	0.0029
677CT/1298CC	-	12.35±3.18 (2)	-
677TT/1298AA	-	50.00 (1)	-

677/1298

CC/AA[a],CC/AC[a] p=0.7435 CC/AA[a],CC/CC[a] p=0.8974 CC/AA[a],CT/AA[a] p=0.5620

CC/AA[a],CT/AC[a] p=0.0156 CC/AC[a],CC/CC[a] p=0.9577 CC/AC[a],CT/AA[a] p=0.6189

CC/AC[a],CT/AC[a] p=0.0051 CC/CC[a],CT/AA[a] p=0.8885 CC/CC[a],CT/AC[a] p=0.3601

CT/AA[a], CT/AC[a] p=0.1139

CC/AA[b],CC/AC[b] p=0.0657 CC/AA[b],CC/CC[b] p=0.8780 CC/AA[b],CT/AA[b] p=0.9649

CC/AA[b],CT/AC[b] p=0.6158 CC/AA[b],CT/CC[b] p=0.7081 CC/AC[b],CC/CC[b] p=0.1155

CC/AC[b],CT/AA[b] p=0.0904 CC/AC[b],CT/AC[b] p=0.0064 CC/AC[b],CT/CC[b] p=0.6800

CC/CC[b],CT/AA[b] p=0.8619 CC/CC[b],CT/AC[b] p=0.8228 CC/CC[b],CT/CC[b] p=0.7016

CT/AA[b],CT/AC[b] p=0.5945 CT/AA[b],CT/CC[b] p=0.7311 CT/AC[b],CT/CC[b] p=0.3791

8. Analysis of the relationship between homocysteine concentrations and MTHFR gene mutations in different severity groups of CAD patients

It was observed that, mean homocysteine concentrations were significantly increased with severity of CAD (single, double and triple vessels disease) ($p<0.0001$) (Table 4-6). For C677T mutation, the levels of homocysteine concentrations were significantly different in healthy control subjects and double-vesseled disease ($p=0.0204$ and $p=0.0193$, respectively) when compared with the heterozygous mutant (CT) and wild type (CC). However, A1298C mutation showed no effect on increasing the level of homocysteine in all subjects ($p>0.05$).

Table 4-6 Correlation between MTHFR genotypes, plasma homocysteine levels and severity of CAD patients

Parameter	Hcy level in Normal subjects (μmol/L) (n=98)[a]	Hcy level in 1 vessel CAD patients (μmol/L) (n=22)[b]	Hcy level in 2 vessels CAD patients (μmol/L) (n=25)[c]	Hcy level in 3 vessels CAD patients (μmol/L) (n=35)[d]	p value
Means of Hcy level (μmol/L)	9.25±1.91	11.30±3.90	12.93±5.03	15.46±7.52	<0.0001
677					
CC	9.01±1.82 (75)	12.07±3.75 (13)	11.59±4.38 (18)	14.25±5.11(20)	<0.0001
CT	10.06±2.02(23)	10.19±4.05 (9)	16.37±5.26 (9)	14.72±3.92 (14)	0.0001
TT	-	-	-	50 (1)	-
1298					
AA	9.04±2.03 (47)	10.34±3.44 (12)	13.72±6.73 (8)	16.28±4.62 (17)	<0.05
AC	9.48±1.77 (49)	12.81±4.10 (7)	12.27±2.76 (13)	12.50±3.95 (15)	<0.0001
CC	9.50±2.73 (2)	11.54±5.60 (3)	13.48±7.88 (4)	13.35±4.60 (2)	>0.05

677 CC^{a},CT^{a} p=0.0204 CC^{b},CT^{b} p=0.2762 CC^{c},CT^{c} p=0.0193 CC^{d},CT^{d} p= 0.9351

1298 AA^{a},AC^{a} p=0.2600 AA^{b},AC^{b} p=0.1769 AA^{c},AC^{c} p=0.4949 AA^{d},AC^{d} p=0.0194 AA^{a},CC^{a} p=0.7570 AA^{b},CC^{b} p=0.6374 AA^{c},CC^{c} p=0.9570 AA^{d},CC^{d} p=0.4079

AC^{a},CC^{a} p=0.9877 AC^{b},CC^{b} p=0.6947 AC^{c},CC^{c} p=0.6300 AC^{d},CC^{d} p=0.7814

9. Analysis of the relationship of homocysteine concentrations in different severity groups of CAD patients and combined MTHFR genotype mutations

The combined double heterozygous genotype (677CT/1298AC) was significantly correlated with increase level of homocysteine when compared with combined homozygous wild type (677CC/1298AA) in healthy control subjects ($p=0.0156$). Furthermore, the levels of homocysteine were significantly higher when compared between 677CT/1298AC and 677CC/1298AC in healthy control subjects ($p=0.0051$) and double-vesseled disease ($p=0.0065$) (Table 4-7).

Table 4-7 Correlation between combination MTHFR genotypes, plasma homocysteine levels and severity of CAD patients

Parameter	Hcy level in Normal subjects(μmol/L) (n=98)[a]	Hcy level in 1 vessel CAD patients (μmol/L) (n=22)[b]	Hcy level in 2 vessels CAD patients (μmol/L) (n=25)[c]	Hcy level in 3 vessels CAD patients(μmol/L) (n=35)[d]	P value
677/1298C					
CC/AA	8.95±2.10 (36)	9.43±2.64 (4)	11.69±4.85 (6)	16.63±5.02 (11)	<0.05
CC/AC	9.09±1.50 (37)	12.81±4.10 (8)	11.01±1.30 (9)	10.66±3.27 (8)	<0.05
CC/CC	9.15±2.73 (2)	14.75±0.92 (2)	13.11±9.61 (3)	16.60 (1)	>0.05
CT/AA	9.36±1.80 (11)	10.82±3.83 (9)	19.80±10.04 (2)	15.65±4.14 (6)	<0.05
CT/AC	10.70±2.07(12)	-	15.10±3.26 (4)	14.59±3.81 (7)	>0.05
CT/CC	-	-	14.60 (1)	10.10 (1)	-
TT/AA	-	-	-	50.00 (1)	-

677/1298 CC/AA^a,CC/AC^a p=0.7435 CC/AA^a,CC/CC^a p=0.8974 CC/AA^a,CT/AA^a p=0.5620 CC/AA^a,CT/AC^a p=0.0156 CC/AC^a,CC/CC^a p=0.9577
CC/AC^a,CT/AA^a p=0.6189 CC/AC^a,CT/AC^a p=0.0051 CC/CC^a,CT/AA^a p=0.8885 CC/CC^a,CT/AC^a p=0.3601 CT/AA^a, CT/AC^a p=0.1139
CC/AA^b,CC/AC^b p=0.1690 CC/AA^b,CC/CC^b p=0.0579 CC/AA^b,CT/AA^b p=0.5275 CC/AC^b,CC/CC^b p=0.5416 CC/AC^b,CT/AA^b p=0.3172
CC/CC^b,CT/AA^b p=0.1988
CC/AA^c,CC/AC^c p=0.6912 CC/AA^c,CC/CC^c p=0.7688 CC/AA^c,CT/AA^c p=0.1508 CC/AA^c,CT/AC^c p=0.2565 CC/AC^c,CC/CC^c p=0.4954
CC/AC^c,CT/AA^c p=0.0116 CC/AC^c,CT/AC^c p=0.0065 CC/CC^c,CT/AA^c p=0.5071 CC/CC^c,CT/AC^c p=0.7085 CT/AA^c,CT/AC^c p=0.3994
CC/AA^d,CC/AC^d p=0.0093 CC/AA^d,CT/AA^d p=0.6898 CC/AA^d,CT/AC^d p=0.3730 CC/AC^d,CT/AA^d p=0.0266 CC/AC^d,CT/AC^d p=0.0508
CT/AA^d,CT/AC^d p=0.6401

10. Study on the genotype frequencies of C677T and A1298C mutations of the MTHFR gene

10.1 C677T and A1298 C genotype frequencies

Genotype frequencies of the C677T and A1298C polymorphisms were analyzed in the 98 healthy control subjects and 199 CAD patients (Table 4-8). Frequencies of CC, CT and TT genotypes of the C677T were 76.53, 23.47 and 0% in healthy control subjects and 68.84, 29.65 and 1.51% in CAD patients, respectively. Frequencies of AA, AC and CC genotypes of the A1298C were 47.96, 50.00 and 2.04% in healthy control subjects and 49.25, 40.20 and 10.55% in CAD patients, respectively. Results from a Chi-squares analysis indicated that genotype distributions in both healthy control subjects and CAD patients were not differentially significant for C677T polymorphism ($p=0.23$). On the contrary, they were differentially significant for A1298C polymorphism ($p=0.02$). In addition, 1298CC genotype showed a significantly increase in Odds Ratio (OR=5.663; 95%CI=1.300-24.666; P=0.019).

Table 4-8 Distribution of C677T and A1298C mutations allelic frequencies in healthy control subjects and CAD patient

Parameter	Healthy control (n=98)	CAD patients (n=199)	OR	95% CI	P-value
677					
CC	75 (76.53%)	137 (68.84%)	0.7059	0.404 -1.232	0.2753
CT	23 (23.47%)	59 (29.65%)	1.3742	0.787 -2.400	0.3261
TT	0	3 (1.51%)	-	-	-
1298					
AA	47 (47.96%)	98 (49.25%)	1.0529	0.649-1.708	0.9321
AC	49 (50.00%)	80 (40.20%)	0.6723	0.413-1.094	0.1396
CC	2 (2.04%)	21 (10.55%)	5.6629	1.300- 24.666	0.0188

10.2 C677T and A1298C allelic frequencies

Allelic frequencies of the C677T and A1298C mutations were calculated and shown in Table 4-9. The allelic frequencies of the C677T mutation in healthy control subjects (12%) were less than CAD patients (16%). The allelic frequencies of A1298C in healthy control subjects and CAD patients were 27% and 31%, respectively. The allelic frequencies of 677T between CAD patients and healthy control subjects was not statistically significance ($p=0.17$). The allelic frequencies of 1298C between CAD patients and healthy control subjects was not statistically significance ($p=0.42$).

Table 4-9 Distribution of C677T and A1298C mutations genotype in CAD patients with atherosclerotic plaque in single, double and triple vessels disease

Parameter	Healthy subjects (n=98)	CAD patients (n=199)	OR	95% CI	P-value
677					
C	0.88	0.84	0.6811	0.409-1.134	0.1738
T	0.12	0.16			
1298					
A	0.73	0.69	0.8385	0.573-1.227	0.4166
C	0.27	0.31			

11. Study on the relationship between genotype frequencies and severity of CAD

11.1 Genotype frequencies and severity

This study analyzed the correlation between mutant genotypes (677TT and 1298CC) and severity of CAD. Table 4-10 showed the genetic distributions in healthy control subjects and CAD patients with single-, double- and triple- vesseled disease. The C677T genotype distributions were not statistically significance ($p=0.09$). The frequencies of the 677TT genotypes were found in only triple-vesseled disease. In addition, the A1298C genotype distributions were statistically significance ($p=0.05$). The frequencies of 1298CC genotype showed significantly different in double-vesseled disease compared to normal healthy subjects ($p=0.02$) or triple-vesseled disease compared to normal healthy subjects ($p=0.01$).

Table 4-10 Distribution of C677T and A1298C mutations genotype in CAD patients with atherosclerotic plaque in single, double and triple vessels disease

Parameter	Healthy subjects (n=98)[a]	1vessel CAD (n=53)[b]	2 vessels CAD (n=69)[c]	3 vessels CAD (n=77)[d]	p-value
677					
CC	75 (76.53%)	39 (73.58%)	49 (71.01%)	49 (63.64%)	0.0896
CT	23 (23.47%)	14 (26.42%)	20 (28.98%)	25 (32.47%)	
TT	0	0	0	3 (3.90%)	
1298					
AA	47 (47.96%)	32 (60.38%)	32 (46.38%)	34 (44.16%)	0.0524
AC	49 (50.00%)	18 (33.96%)	29 (42.03%)	33 (42.86%)	
CC	2 (2.04%)	3 (5.66%)	8 (11.59%)	10 (12.99%)	

677 CC^a VS CC^b ; p= 0.8388 CT^a VS CT^b ; p= 0.8388
CC^a VS CC^c ; p= 0.5333 CT^a VS CT^c ; p= 0.5333
CC^a VS CC^d ; p= 0.0899 CT^a VS CT^d ; p= 0.2486
CC^b VS CC^{ac} ; p= 0.91223 CT^b VS CT^c ; p= 0.9123
CC^b VS CC^d ; p= 0.3168 CT^b VS CT^d ; p= 0.5856
CC^c VS CC^d ; p= 0.4407 CT^c VS CT^d ; p= 0.7830

1298 AA^a VS AA^b ; p= 0.1979 AC^a VS AC^b ; p= 0.0851 CC^a VS CC^b ; p= 0.4772
AA^a VS AA^c ; p= 0.9647 AC^a VS AC^c ; p= 0.3903 CC^a VS CC^c ; p= 0.0257
AA^a VS AA^d ; p= 0.7277 AC^a VS AC^d ; p= 0.4311 CC^a VS CC^d ; p= 0.0110
AA^b VS AA^c ; p= 0.1764 AC^bVS AC^c ; p= 0.4716 CC^b VS CC^c ; p= 0.4148
AA^b VS AA^d ; p= 0.1011 AC^b VS AC^c ; p= 0.4021 CC^b VS CC^d ; p= 0.2842
AA^c VS AA^d ; p= 0.9182 AC^c VS AC^d ; p= 0.9469 CC^c VS CC^d ; p= 0.7903

11.2 Study on the allelic frequencies and severity of CAD

The allelic frequencies of the C677T and A1298C mutations in single-, double- and triple-vesseled diseases subjects were shown in Table 4-11. The 677T allelic frequencies were 12, 13, 14 and 16% in normal subjects, single-, double- and triple-vessels diseases, respectively. The 677T allelic distributions were analyzed by Chi-squared analysis (Table 4-11-1). It was shown that the 677T allelic distribution for triple-vesseled disease and normal subjects were differentially significant ($p=0.04$). In addition, the 1298C allelic distributions showed statistically different in triple-vesseled disease compared to single vessel disease ($p=0.05$).

Table 4-11 Distribution of the allelic frequencies of C677T and A1298C mutations of MTHFR gene

Parameter	Normal subjects (n=98) [a]	1V CAD (n=53) [b]	2V CAD (n=69) [c]	3V CAD (n=77) [d]	P value
677					
C	0.88	0.87	0.86	0.80	0.3741
T	0.12	0.13	0.14	0.20	
1298					
A	0.73	0.77	0.67	0.66	0.1446
C	0.27	0.23	0.33	0.34	

Table 4-11-1 Statistical analysis in allelic frequencies from Table 4-11

Comparison between	χ^2	P-value	Odd ratio	95% CI
a* b, 677 1298	0.036 0.488	0.8503 0.4846	0.8737 1.2663	0.4291-1.7786 0.7281-0.2023
a* c, 677 1298	0.331 0.957	0.5652 0.3270	0.7844 0.7660	0.4122-1.4925 0.4761-1.2322
a* d, 677 1298	4.037 1.886	0.0445 0.1697	0.5275 0.7063	0.2933-0.9486 0.4467-1.1167
b* c, 677 1298	0.010 2.466	0.9196 0.1164	-	-
b* d, 677 1298	1.646 3.630	0.1995 0.0568	-	-
c* d, 677 1298	1.237 0.041	0.2660 0.8397	-	-

11.3 Distribution of combined common mutations

The joint effect of the two polymorphisms (C677T in exon 4 and A1298C in exon 7 on MTHFR gene) were also studied (Table 4-12). It was found that 677CC/1298CC individuals were 4.197-fold increased risk for CAD (OR=4.197; 95%CI=0.945-18.633; p=0.07). Other combined genotypes were not statistically significance.

Table 4-12 Distribution of the combination of 677CT and 1298AC mutations

Parameter	Normal subjects (n=98)	CAD patients (n=199)	OR	95% CI	P-value
677CC/1298AA	36 (36.73%)	62 (31.16%)	0.779	0.469-1.296	0.40
677CC/1298AC	39 (39.80%)	59 (29.65%)	0.638	0.384-1.058	0.11
677CC/1298CC	2 (2.04%)	16 (8.04%)	4.197	0.945-18.633	0.07
677CT/1298AA	11 (11.22%)	34 (17.08%)	1.630	0.787- 3.374	0.25
677CT/1298AC	10 (10.20%)	20 (10.05%)	1.067	0.478-2.379	0.87
677CT/1298CC	-	5 (2.51%)	-	-	-
677TT/1298AA	-	3 (1.51%)	-	-	-

11.4 Distribution of combined two common mutation on severity of CAD

Only seven of nine combined genotypes of C677T and A1298C mutations were ascertained (Table 4-13). It was found that 677CC/1298CC was significantly different in triple-vesseled disease compared with the normal subjects (p=0.04).

Table 4-13 Distribution of the combination of 677CT and 1298AC mutations in different severity of CAD patients

Parameter	Normal subjects (n=98) [a]	1 vessel CAD (n=53) [b]	2 vessels CAD (n=69) [c]	3 vessels CAD (n=77) [d]
677CC/1298AA	36 (36.73%)	21 (39.62%)	20 (28.98%)	21 (27.27%)
677CC/1298AC	39(39.80%)	16 (30.19%)	23 (33.33%)	20 (25.77%)
677CC/1298CC	2 (2.04%)	2 (3.77%)	6 (8.70%)	8 (10.39%)
677CT/1298AA	11(11.22%)	12 (22.64%)	12 (17.39%)	10 (12.99%)
677CT/1298AC	10 (10.20%)	1 (1.89%)	6 (8.70%)	13 (16.88%)
677CT/1298CC	0	1 (1.89%)	2 (2.90%)	2 (2.60%)
677TT/1298AA	0	0	0	3 (3.90%)

677CC/1298AA a* b ; p= 0.8622 , a*c ; p= 0.3799 , a*d ; p= 0.2447
677CC/1298AC a* b ; p= 0.3203 , a*c ; p= 0.4911 , a*d ; p= 0.0786
677CC/1298CC a* b ; p= 0.9188 , a*c ; p= 0.1063 , a*d ; p= 0.0420
677CT/1298AA a* b ; p= 0.1039 , a*c ; p= 0.3625 , a*d ; p= 0.9030
677CT/1298AC a* b ; p= 0.1214 , a*c ; p= 0.9528 , a*d ; p= 0.2834

12. Hardy-Weinberg equilibrium

Genotype frequencies of MTHFR polymorphisms (C677T and A1298C) were used to calculated the Chi-square values to test the deviation of each genotype frequencies of MTHFR polymorphisms in 297 Thai population (199 CAD patients and 98 normal healthy control subjects) from the criteria of Hardy-Weinberg equilibrium (Table 4-14). From χ^2 method, it was shown that the distribution of each genotype from the two these polymorphisms (677CC/677CT/677TT and 1298AA/1298AC/1298CC) was in Hardy-Weinberg equilibrium (χ^2=1.349; p=0.509; df=2 and χ^2=0.357; p=0.836; df=2, respectively).

Table 4-14 The genotype frequencies distribution of MTHFR C677T and A1298C polymorphisms.

MTHFR C677T polymorphism			
Genotype	677CC	677CT	677TT
Observation	212	82	3
Hardy-Weinberg expectation	216	75	6
$\chi^2 = 1.349$; p = 0.509; df = 2			
MTHFR A1298C polymorphism			
Genotype	1298AA	1298AC	1298CC
Observation	145	129	23
Hardy-Weinberg expectation	148	123	26
$\chi^2 = 0.357$; p = 0.836; df = 2			

13. Linkage disequilibrium

Genotype association of MTHFR C677T and A1298C are presented in Table 4-15. Chi-square test was used to determine linkage disequilibrium that indicates the association among these two polymorphisms in this study. No disequilibrium between MTHFR C677T and MTHFR A1298C ($\chi^2 = 5.71$; $p = 0.22$; $df = 4$).

Table 4-15 Genotype association of MTHFR C677T and A1298C in Thai population

MTHFR C677T polymorphism					
Genotype	677CC	677CT	677TT	All	χ^2
1298AA	98	45	3	146	
1298AC	98	30	0	128	5.710
1298CC	18	5	0	23	(p=0.2219)
All	204	80	3	297	df=4

CHAPTER V
DISCUSSION

Coronary artery disease or CAD may develop from many factors such as lipoprotein (a), cholesterol, platelet/fibrinogen, high density lipoprotein (HDL), blood sugar and hyperhomocysteinemia. Homocysteine is an amino acid produces as a normal by product of the breakdown of methionine which is an essential amino acid acquired mostly from eating meat. High homocysteine levels have been linked to damage of the arteries which may increase the risk of heart attack, stroke or other cardiovascular problems. A moderate increase of plasma homocysteine has been recognized as an independent predictor of CAD[46,93,99-100]. Whether high levels of homocysteine are a causal risk factor for CAD is not yet clear. Multiple factors and conditions lead to increases in the plasma concentrations of homocysteine[44,101-103]. In 1969, McCully[20] found that children who died from homocystinuria showed signs of premature arteriosclerosis. The methylation of homocysteine to methionine, in the remethylation pathway, is catalyzed by the enzyme methylenetetrahydrofolate reductase (MTHFR). Genetic polymorphisms of MTHFR are one cause leading to increased homocysteine levels. MTHFR gene C677T and A1298C polymorphisms are common. In the present study, we analyzed the two common MTHFR polymorphisms, C677T and A1298C in 199 Thai CAD patients with coronary angiography compared to 98 healthy control subjects. Furthermore, we investigated the link between MTHFR C677T and A1298C genotypes on plasma homocysteine levels.

MTHFR C677T polymorphism

The MTHFR C677T, thymine in place of cytosine at nucleotide 677 position in exon 4 of the MTHFR gene, replacing alanine (A) by valine (V) in the N-terminal catalytic domain of MTHFR[104]. This thermolabile variant has been consistently associated with elevations in homocysteine concentrations[101]. Previous studies[40,105] investigested the relationship between MTHFR C677T genotype and homocysteine

concentrations. Kolling K *et al.*[106], Hanson NQ *et al.*[107] and Rothenbacher D *et al.*[108] reported that there was a graded increase in the plasma homocysteine concentrations from CC to TT genotypes in both healthy control subjects and CAD patients.

Homocysteine concentrations depend on age[106-108]. Kolling K *et al.*[106], Hanson NQ *et al.*[107] and Rothenbacher D *et al.*[108] suspected that impaired kidney function may contribute to the higher levels in older people. Hence, healthy control subjects and CAD patients were selected between 40-70 years between genotypes and homocysteine concentrations (Table 4-4). From regression analysis, indicated that no correlation between age and homocysteine concentrations in our subjects. The present study showed that the C677T mutation was significantly associated with elevated homocysteine concentrations in all subjects ($p<0.005$) (Table 4-2).

In this study, plasma homocysteine levels in CAD patients were not significantly increased by the C677T. However, there was a trend to show higher homocysteine levels in homozygous 677TT individuals. The homozygous (TT) was strongly associated to increased homocysteine concentration up to 50 μmol/L in CAD patients (Table 4-4). Our results confirmed the previous study[109] that TT homozygosity was associated with reduced activity of enzyme MTHFR and leading to the defect of stabilization of plasma homocysteine level by reversing homocysteine to methionine in the remethylation pathway.

The heterozygous (CT) elevated the levels of homocysteine in both healthy control subjects and CAD patients. The mean difference increasing of plasma homocysteine concentrations between CT and CC genotype were 11.65% in healthy control subject and 4.87% in CAD patients. However, these concentrations were not statistically different in CAD patients. On the contrary, the mean plasma homocysteine concentrations in healthy control subjects were significant different ($p=0.02$). This results were correlated with previous studies[15,108-110] that the heterozygous (CT) mutation affected to increase the level of homocysteine concentration but nonsignificant may be due to: heterozygous (CT) genotype has only one allele mutant, which may lead to mild hyperhomocysteinemia.

Our study showed that CAD patients had higher levels of homocysteine than the healthy control subjects. Although, CAD patients were a significantly higher than

healthy control subjects but it was accepted that CAD patients had a high concentrations of homocysteine than healthy control. Moreover, plasma homocysteine levels progressively increased with more severe coronary atherosclerosis (Table 4-6) ($p<0.0001$). Barrett S *et al.*[124] found a linear relationship between homocysteine levels and severity of the coronary blockage. For every 10% elevation of homocysteine, there was nearly the same rise in the risk of developing severe coronary heart disease[124]. This results confirmed the observation of Kolling *et al.*[106]

The frequency of subjects homozygous for the 677TT genotype was 1.51% in CAD patients. The TT genotype was not found in healthy control subjects. The prevalence of homozygosity for the C677T mutation in this study was much lower than other observations (Table 5-1). It is well established that the proportion of an allele varies considerably from place to place, but usually there is little difference between neighboring populations so that the greatest variation is observed at large distances. We found that the distribution of C677T genotype in healthy control subjects and CAD patients were not significant (Table 4-8) ($p=0.2312$). Many of the previous studies demonstrated that homozygous (TT) genotype was not associated with the presence of CAD[106,108,113-115]. On the other hand, some other studies have shown an association of homozygous (TT) genotype with CAD[13,81,111-112].

The frequency of 677T allele was not significant in patients with CAD compared with the healthy control subjects (0.16 and 0.12, respectively, $p=0.1738$). Our results were confirmed with Abu-Amero *et al.*[115] who found that there was no significant difference in T allele frequency between CAD patients (0.20) and healthy control subjects (0.15) in Arabs. In addition, Angeline *et al.*[117] found that there was no significant of T allele frequency in CAD patients (0.12) and healthy control subjects (0.08).

Table 5-1 Genotype prevalence of the MTHFR C677T polymorphism in different populations

Study area	Total No.		Genotype prevalence (%)		P value	References
	CAD	Healthy	CAD	Healthy		
Thailand	199	98	1.51	0	-	Present
Thailand	401	108	3	0.9	0.04	121
Arabs	545	625	3.7	2	0.86	115
Japan	362	778	16	10	0.0067	81
Japan	230	199	17	11	<0.05	111
Netherlands	60	111	15	5	0.05	12
Israel	169	313	28	14	0.0002	80
Canada	152	121	15	11	>0.05	99
India	52	20	1.92	0	-	122
America	772	329	10.9	12.5	>0.05	107
UK	739	-	9.74	-	-	15
Germany	312	479	9.9	10.4	0.295	108
Germany	2121	-	11.9	-	-	106
Italy	302	168	15.23	17.86	0.542	118
France	-	66	-	18	-	110
Poland	161	211	10.56	8.53	>0.05	83

Table 5-2 Allelic frequencies of the MTHFR C677T polymorphism in different populations

Study area	Total No.		Allelic frequencies		P value	References
	CAD	Healthy	CAD	Healthy		
Thailand	199	98	0.16	0.12	0.17	Present
Thailand	401	108	0.16	0.10	0.03	121
Arabs	545	625	0.20	0.15	>0.05	115
Japan	362	778	0.42	0.33	<0.0001	81
Japan	-	199	-	0.34	-	111
European	-	735	-	0.32	-	123
India	52	20	0.115	0.075	-	122
Italy	302	168	0.25	0.45	0.3189	118
France	-	66	-	0.37	-	110
Poland	161	211	0.23	0.26	>0.05	83

MTHFR A1298C polymorphism

The substitution of alanine (A) to cytosine (C) at nucleotide 1298 produces a glutamate to alanine (E429A). The A1298C mutation affects the C-terminal regulatory domain of the enzyme MTHFR which is the binding site of S-adenosylmethionine (SAM), an allosteric inhibitor.

van der Put *et al*.[17] and Weisberg *et al*.[77] indicated that the A1298C mutation was associated with decrease enzyme activity which was more pronounced in the homozygous than heterozygous state. In this study, the results indicated that the heterozygous (1298AC) and homozygous (1298CC) mutations of the MTHFR gene did not affect the homocysteine concentrations. This study was not correlated with van der Put[17] and Weisberg[77]. However, many studies reported similar results[15,83,106-108,118-119]. There was no additional effect of A1298C on plasma homocysteine. Agreement with our results, Yamada *et al.*[120] found that purified homozygous 1298CC showed no difference from wild type (1298AA) either in its kinetic or stabilizing properties. Sometimes, the absence of a biochemical phenotype *in vitro* does not necessarily rule out the possible importance of the A1298C polymorphism *in vivo*.

The frequency of the 1298CC homozygote was 7.41% in Thai population and the 1298CC genotype prevalence in our study was statistically significant between CAD patients (10.55%) and healthy control subjects (2.04%) (p=0.0188). This was correlated with the study of Szczeklik *et al.*[83] who suggested that the relationship of homozygous 1298CC genotype with CAD and found that they were statistically associated (p=0.0103). Furthermore, the Odds ratio showed the 5.663-fold increased risk in CAD patients.

In these studies, the 1298C allelic frequency was 0.31 in CAD patients which was lower than other studies. This frequency showed no statistically with healthy control subjects (p=0.4166). Similarly, Abu-Amero *et al*.[115] also found that there was no significant difference in 1298C allelic frequency between CAD patients and healthy control subjects in Arabs (p=0.65). However, Szczeklik *et al.*[83] indicated that

the frequency of allele 1298C was significantly higher in CAD than healthy control subjects (p=0.001).

Table 5-3 Genotype prevalence of the MTHFR A1298C polymorphism in different populations

Study area	Total No.		Genotype prevalence (%)		P value	References
	CAD	Healthy	CAD	Healthy		
Thailand	199	98	10.55	2.04	0.02	Present
Arabs	540	625	7.4	9.1	0.21	115
India	52	20	17.30	2.0	-	122
America	772	329	11.7	7.9	>0.05	107
UK	739	-	7.44	-	-	15
Germany	312	479	9.7	13.8	0.346	108
Milan & Naples	-	180	-	6.1	-	119
Germany	2121	-	11.4	-	-	106
Italy	302	168	9.3	7.1	0.5352	118
France	-	66	-	12.5	-	110
Poland	161	211	11.18	3.79	0.003	83

Table 5-4 Allelic frequencies of the MTHFR A1298C polymorphism in different populations

Study area	Total No.		Allelic frequencies		P value	References
	CAD	Healthy	CAD	Healthy		
Thailand	199	98	0.31	0.27	0.42	Present
India	52	20	0.404	0.350	-	122
Italy	302	168	0.33	0.33	0.9615	118
France	-	66	-	0.32	-	110
Poland	161	211	0.30	0.20	0.001	83

CombinedC677T and A1298C of MTHFR polymorphisms

It is interesting that to date, various researchers reported that the combined C677T and A1298C variants was associated with reduced MTHFR specific activity and higher homocysteine concentrations[17-18,77]. Chango *et al.* studies the MTHFR activity in combined variants of the MTHFR genotype in 66 normal healthy French subjects[110]. The enzyme activity of 677CC/1298AC, 677CC/1298CC variants were 60%, 52% and 36%, respectively when compared with the MTHFR activity of 677CC/1298AA. Only compound heterozygote for both polymorphisms had higher homocysteine concentrations[17-18,77].

In the present study, the distribution of these C677T and A1298C MTHFR variants followed the Hardy-Weinberg equilibrium. In addition, we found that only seven of nine possible combined genotypes of C677T and A1298C polymorphisms were present in our population.

The combined heterozygote of both polymorphisms (677CT/1298AC) showed higher significantly homocysteine concentrations when compared with wild type (677CC/1298AA), in healthy control subjects (p=0.0156). On the contrary, such polymorphisms were not significantly in CAD patients (p=0.6158). The level of homocysteine concentrations in 677CT/1298AC MTHFR variant were significantly higher than 677CC/1298AC in healthy control subjects and CAD patients (p=0.0051 and p=0.0064, respectively). It was reported by van der Put *et al.*[17 that combined heterozygosity for the C677T and A1298C MTHFR variants predisposes individuals to increased homocysteine concentrations. Our study showed similar results that there was a tendency to increase homocysteine concentrations in individuals with the 677CT/1298AC genotype compared with individuals with the 677CC/1298AA genotype.

CHAPTER VI
CONCLUSIONS

1. In this investigation, the prevalence of homozygous 677TT genotype of MTHFR gene is 1.51% in Thai CAD patients, whereas, in healthy control subjects is not observed. The 677T allele frequency is 0.16 in Thai CAD patients that no significant difference from healthy control subjects (0.12).

2. The prevalence of homozygous 1298CC genotype is 10.55% in Thai CAD patients and 2.04% in healthy control subjects (p=0.02). The allele frequency of 1298C is 0.31 in Thai CAD patients and 0.27 in healthy control subjects.

3. The 677T allele has a trend to increased the homocysteine concentration, whereas, the 1298C allele does not effected.

4. The combined heterozygous mutation C677T and A1298C of the MTHFR gene are correlated with elevation the level of homocysteine (p=0.0156).

5. MTHFR C677T and A1298C genotypes, alone or combined, are not major effect in developing CAD.

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APPENDIX

Biochemical and clinical data of coronary artery disease patients

No.	Sex	Age	C677T	A1298C	CAD type	Hcy (µmol/L)	Vitamin B_{12} (pg/ml)	Folate (ng/ml)	BP_1 (mmHg)	BP_2 (mmHg)	Chol (mg/dl)	Trig (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
6	M	70	CT	AA	3V	21.7			123	73	99	134	32	82
8	M	76	CC	AA	3V				155	90	113	178	39	116
9	F	61	CC	AA	3V	21.7					83	203	51	135
10	M	83	CC	AC	3V				117	75	105	38	26	71
11	M	64	CC	AC	3V	14.9	1080	6.8	148	89	101	209	45	144
12	M	47	CC	AA	3V	14.2	787	10.1	180	120	137	197	47	123
15	M	58	CC	AA	1V	7.93	493	8.3			84	158	55	86
17	M	50	CC	AC	3V	12.1	667	2.8			271	105	28	222
20	F	59	CC	AA	1V						118	207	31	152
22	M	67	CC	CC	3V				150	78	80	180	41	124
23	M	59	CT	AC	3V	18.2	351	1.5	116	71	232	192	23	123
24	M	67	CC	AA	2V				200	88	99	211	36	155
29	M	59	CC	AA	3V	26.3	315	4.8			128	187	27	134
30	M	73	CT	AC	3V				193	100	152	222	36	156
31			CC	AA	3V									
32	M	62	CC	AA	3V	17.4	549	3.7	154	63	98	158	50	88
34	M	75	CC	AA	2V				145	95	197	209	42	113
37	F	53	CC	AA	3V	18.4	529	6.2	139	88	278	312	29	187
39	M	69	TT	AA	3V				158	79	218	258	28	138
40	M	65	CC	AC	2V				159	66	167	109	56	89
41	M	64	CT	AA	1V	15.9	552	5.8	132	62	196	102	39	137
42	F	76	CC	AA	3V				115	70	308	150	40	238
43	M	64	CT	AA	2V	26.9	2000	4.1	140	78	227	268	25	148
44	M	54	CC	AC	3V	6.77	563	5.26	140	75	174	87	36	121
45	M	74	CC	AC	2V						278	105	46	211
48	M	63	CT	AA	3V	16.4			132	70	208	109	10	146

Biochemical and clinical data of coronary artery disease patients (continued)

No.	Sex	Age	C677T	A1298C	CAD type	Hcy (µmol/L)	Vitamin B_{12} (pg/ml)	Folate (ng/ml)	BP_1 (mmHg)	BP_2 (mmHg)	Chol (mg/dl)	Trig (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
49	F	88	CC	AC	3V						188	103	37	130
55	M	61	CC	CC	3V						267	193	29	199
59	M	83	CC	AC	2V				131	48	127	66	38	76
60	F	59	CC	AC	2V				118	64	266	114	43	200
62	M	78	CC	AC	1V	21.9	505	3.6	144	70	219	105	31	167
63	M	54	CC	AA	2V	16.5	575	5.3	157	96	220	235	31	142
64	F	70	TT	AA	3V				162	80	169	115	55	91
65	M	72	CC	AA	3V				163	96	190	119	50	116
67	F	66	CT	AC	2V						204	92	41	145
68	M	63	CC	AA	3V	17.9	1250	9.8	185	70	211	265	30	128
77	F	52	CC	AA	2V	13.4	313	2.7	190	110	202	182	44	127
78	F	56	CC	AC	3V	10.7	941	10	167	94	215	42	47	160
79	M	69	CC	AC	3V				181	83	175	89	51	206
80	M	61	CT	AA	2V				152	82	155	135	38	90
82	F	63	CC	AA	2V	10.4	1191	5.5	178	94	247	168	44	169
84	M	63	CC	AA	3V				141	88	114	77	34	65
88	M	65	CC	AA	2V				123	69	201	80	33	152
95	F	81	CC	AA	3V						201	423	26	90
102	M	67	CT	CC	2V	14.6	1486	3.6			185	101	3.1	134
104	M	57	CC	CC	3V				139	88	219	166	33	153
107	M	63	CT	CC	3V				178	93	312	804	34	117
109	M	76	CC	AA	1V				181	64	207	56	33	163
112	F	56	CT	CC	3V	10.1	564	6.7	205	105	177	221	36	97
120	F	72	CT	AA	2V				166	78	162	114	49	90
122	M	67	CC	AC	1V	20	275	2.7	121	59	145	84	30	98
123	M	50	CT	AA	3V	10.8	522	7.2	146	89	185	106	39	125
125	F	52	CC	AC	1V	11.4	929	9.4	182	87	236	170	65	137

Biochemical and clinical data of coronary artery disease patients (continued)

No.	Sex	Age	C677T	A1298C	CAD type	Hcy (µmol/L)	Vitamin B_{12} (pg/ml)	Folate (ng/ml)	BP_1 (mmHg)	BP_2 (mmHg)	Chol (mg/dl)	Trig (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
126	M		CT	AA	2V				152	82	161	128	43	92
128			CC	AC	2V				168	86	175	145	26	120
130	M	48	CC	AA	3V	10.4	1038	6.4	124	69	183	130	33	124
131	M	69	CC	AC	3V				166	75	164	86	32	115
132	M	69	CT	AA	2V				144	69	252	116	43	186
136	F	62	CC	AC	2V	11.8	789	4.2	130	77	235	164	45	157
137	M	49	CC	AC	1V	11.4	908	5.6			225	163	35	157
142	M	67	CC	AC	2V	13.9	764	5.2			189	106	21	147
143	M	68	CC	AC	3V				140	90	206	117	52	131
144	M	57	CT	AA	3V						210	181	35	139
148	M	62	CC	AC	2V	10.2	1201	6.5	143	61	162	68	40	108
150	M	43	CC	AA	1V									
152	M	64	CC	AA	2V	16.9	1114	7.7	154	83	172	90	33	124
157	M	71	CC	AC	3V				172	73	240	186	35	168
161	M	55	CC	AC	2V				130	63	177	107	55	101
166	M	41	CC	CC	2V	7.83	1055	7.5	134	81	141	98	42	79
168	M	59	CT	AA	2V				130	75	166	87	28	121
174	M	64	CC	AC	1V				105	97	168	91	44	106
175	M	64	CC	CC	1V	15.4	471	5.5			159	141	54	77
176	M	64	CC	AA	3V				191	101	169	136	27	115
179	M	55	CC	AA	2V				140	81	237	120	44	169
181	F	46	CC	CC	3V						169	117	40	106
184	M	44	CT	AC	3V	14.1	754	5.4	156	100	243	238	42	153
185	M	55	TT	AA	3V	50	884	6.4	142	77	139	47	69	
187	M	50	CT	AA	1V	8.57	983	7.3	124	76	153	77	59	79
189	M	49	CC	AC	2V	9.97	549	13.4	120	63	196	95	35	142
191	M	71	CC	AA	1V				131	79	178	174	59	84

Biochemical and clinical data of coronary artery disease patients (continued)

No.	Sex	Age	C677T	A1298C	CAD type	Hcy (µmol/L)	Vitamin B_{12} (pg/ml)	Folate (ng/ml)	BP_1 (mmHg)	BP_2 (mmHg)	Chol (mg/dl)	Trig (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
192	F	70	CT	AC	3V	16.2	125	81	190	199	39	111		
194	M	58	CC	AA	3V	11.3	771	6.1	221	125	195	66	51	128
195	M	64	CC	AC	2V						141	250	21	70
196	M	74	CC	AA	2V						244	61	78	154
197	M		CC	CC	1V	14.1	1077	6.9	154	88	156	237	42	67
201	M	69	CC	AA	1V						244	61	78	154
202	M	66	CT	AA	2V				134	84	214	109	51	141
203	M	61	CT	AA	3V				200	90	202	276	48	99
204	F	70	CT	AC	3V	16.5	331	7.23			210	184	45	104
212	M	47	CC	AC	2V	9.63	784	5.6	129	73	184	109	32	130
213			CC	AC	1V									
214	F	49	CT	AA	1V	8.66	922	7.7	132	70	229	451	30	113
217	M	50	CT	AA	1V	9.8	784	10.7	157	66	183	167	41	109
219	M	44	CT	AC	2V	13.6	757	6.7	190	88	248	134	34	187
221	M	74	CC	CC	3V				162	80	200	112	54	124
223	F	60	CC	AC	2V	10.5	1006		167	84	201	104	43	137
224	M	51	CC	AC	3V	7.56	872	10.7	154	90	129	46	42	81
230	F	77	CC	AC	3V				186	90	307	125	47	235
231	M	67	CC	AC	1V	11.5	1258	4.54	117	55	168	187	35	96
232	F	66	CC	AC	3V				122	75	115	83	21	77
236	F	58	CC	AA	2V	8.56	350	10	156	67	179	43	53	117
237	F	70	CC	AC	3V				171	60	267	104	79	167
238	M	48	CT	AC	3V						176	61	64	100
240	M	67	CC	CC	3V				105	80	301	140	65	208
243	F	70	CC	CC	3V				198	81	220	93	76	125
245	F	67	CT	AC	3V	18	375	5.49	163	113	213	136	40	146
249	M	48	CT	AA	2V	12.7	504	12	132	84	183	87	61	104

Biochemical and clinical data of coronary artery disease patients (continued)

No.	Sex	Age	C677T	A1298C	CAD type	Hcy (µmol/L)	Vitamin B_{12} (pg/ml)	Folate (ng/ml)	BP_1 (mmHg)	BP_2 (mmHg)	Chol (mg/dl)	Trig (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
250	M	67	CC	AC	3V				179	98	257	204	43	173
253	M	79	CC	AC	1V				192	101	117	174	37	45
254	F	67	CT	AA	1V				184	79	210	12	70	116
256	F	58	CT	AC	2V	18.8	1887	20	160	73	214	96	81	114
257	M	67	CC	AA	3V				125	69	228	159	40	156
259	M	70	CC	CC	2V				155	72	186	211	32	112
260	M	44	CT	AA	3V	12.4	738	2.41	117	75	202	99	49	133
263	M	71	CC	CC	2V	11.2	613	5.46	160	90	137	95	36	82
264	M	55	CC	AC	2V				112	77	181	105	26	134
265	M	54	CT	AA	3V				181	90	154	98	27	107
267	F	62	CT	AA	3V	18.9	315	7.25	151	51	140	136	43	69
268	M	47	CT	AC	3V				110	70	248	188	42	168
269	M	50	CC	CC	2V	24.2	665	20	203	93	195	137	41	127
270	M	65	CC	AC	2V				120	55	197	49	57	130
272	F	70	CT	AA	3V	13.7	530	20	119	73	165	95	53	93
274	M	52	CC	CC	3V	16.6	632	4.96	156	103	214	62	64	138
275	M	63	CC	AC	1V						156	208	25	89
280	M	70	CT	AA	2V						147	64	51	83
284	F	69	CT	AC	1V				124	84	179	158	53	94
289	M	75	CC	AC	2V				140	89	125	101	42	63
290	M	50	CC	AC	3V				134	60	164	66	52	99
291	M	74	CC	AA	1V				200	110	200	215	32	125
298	M	70	CC	AC	3V	9.05	966	20	210	94	174	142	41	109
299	M	47	CT	AA	1V	9.69			147	72	175	69	45	
302			CC	AA	1V									
305	F	73	CC	AC	1V				147	69	147	72	64	69
309	F	61	CC	AC	3V				191	101	171	156	32	108

Biochemical and clinical data of coronary artery disease patients (continued)

No.	Sex	Age	C677T	A1298C	CAD type	Hcy (µmol/L)	Vitamin B_{12} (pg/ml)	Folate (ng/ml)	BP_1 (mmHg)	BP_2 (mmHg)	Chol (mg/dl)	Trig (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
311	F	65	CC	AC	3V	8.83	802	20			175	152	45	100
313	M		CC	AA	2V				140	78	199	209	35	122
315	M	48	CT	AA	1V	17.6	824	20	173	111	184	237	24	113
317	F	67	CC	AC	1V	10.1	342	5.2	155	75	165	134	47	9
319	M	75	CC	AA	1V	14.5	367	6.22	202	75	153	123	35	93
323	M	64	CC	CC	2V				212	109	244	190	58	148
327	F	65	CT	AA	1V	9.72	2000	14.3			207	289	35	114
333	F	66	CT	AA	1V				153	93	145	123	43	77
335	F	66	CC	AA	3V	9.95	1013	8.2	145	82	212	322	42	106
336	F	64	CT	AC	3V				171	99	152	63	53	86
341	F	64	CT	AC	2V				150	70	222	136	84	111
346	F	61	CC	AC	3V	15.5			181	79	198	194	35	127
348	F	73	CC	AC	2V				156	74	150	144	31	94
351	M	61	CC	AA	1V				128	73	188	338	30	25
352	M	63	CC	AC	1V				160	76	190	175	35	175
353	M	76	CT	AC	3V				129	67	145	138	23	99
354	M	58	CC	AA	3V	19.6	310	5.71	155	94	253	133	43	194
356			CC	AC	2V									
365	M	66	CC	AA	1V				152	68	194	213	49	126
366	M	48	CC	CC	2V	7.3	452	11.6	122	70	116	89	39	60
367	M	40	CT	CC	1V	5.12	909	6.7	161	87	176	93	45	115
370	F	49	CC	AA	1V	8.95	725	10.8	164	97	202	119	50	132
373	F	60	CT	AC	3V	7.91	1339	19.1	195	93	138	150	52	61
374	M	64	CC	AA	2V				139	61	134	65	47	74
377	M	62	CT	AA	1V				143	92	208	88	49	135
382			CC	AA	1V									

Biochemical and clinical data of coronary artery disease patients (continued)

No.	Sex	Age	C677T	A1298C	CAD type	Hcy (µmol/L)	Vitamin B_{12} (pg/ml)	Folate (ng/ml)	BP_1 (mmHg)	BP_2 (mmHg)	Chol (mg/dl)	Trig (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
385	F	68	CT	AC	3V	11.2	872	10.7	120	60	150	128	36	91
387	M	62	CC	AA	2V				144	80	178	132	35	118
389	F	70	CT	AC	2V	16.6	224	6.24	149	66	176	124	46	107
393	M	58	CT	AC	3V				125	83	200	119	41	135
396	M	51	CC	AA	3V				144	91	217	82	43	162
397	M	73	CC	AA	3V				106	45	159	47	48	102
398	M	64	CC	AC	2V				146	65	152	140	37	91
401	M	58	CC	AA	1V						247	105	55	176
409	F	60	CT	AC	2V	11.4	1462	7.5	141	75	189	88	64	107
411	F	75	CC	AC	2V	10.4	817	7.2	146	63	177	159	49	97
412	F	48	CC	AA	2V	4.4	2000	15.7	144	79	199	121	58	119
413	F	81	CC	AA	2V				151	71	182	73	44	117
416	F	65	CC	AA	1V	7.55			115	63	242	169	50	147
417	F	64	CC	AC	2V	11.3			153	60	160	118	35	95
418	F	67	CT	AA	1V	6.64			158	80	143	72	42	79
435	M	55	CC	AC	1V				132	83	217	70	59	129
439	F	42	CC	AA	3V	15.8	588	7.4	185	99	168	179	37	95
450	F	54	CC	AC	1V	16.9	530	5.46	132	73	157	105	36	102
452	M	71	CT	AA	2V	11.1	339	8	140	67	148	96	39	91
453	M	74	CT	AA	1V	8.55	17.3	10.2	151	57	233	93	53	162
457	M	62	CC	AA	1V				174	106	142	147	43	78
469	M	67	CC	AA	2V				154	71	190	87	39	123
476	F	65	CC	AC	2V	11.4	784	4.4	159	82	162	306	33	65
483	F	72	CC	AC	2V						243	238	47	120
487	F	71	CT	CC	2V	15.3	1034	4.97	124	63	182	145	40	93
490	F	60	CC	AC	1V	8.38	499	9.3	158	79	230	132	53	139
504	M	62	CC	AA	2V				131	84	188	134	40	100

Biochemical and clinical data of coronary artery disease patients (continued)

No.	Sex	Age	C677T	A1298C	CAD type	Hcy (µmol/L)	Vitamin B_{12} (pg/ml)	Folate (ng/ml)	BP_1 (mmHg)	BP_2 (mmHg)	Chol (mg/dl)	Trig (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
509	M	73	CC	AC	1V				119	72	177	77	45	109
512	M	55	CC	AC	1V				101	58	239	170	41	140
513	F	55	CC	AA	1V						242	91	55	141
514	M	60	CC	AA	2V				124	73	266	64	46	173
516	M	65	CC	AA	1V				104	51	143	80	33	68
522	M	64	CC	AA	1V				136	72	131	81	44	80
524	F	69	CT	AA	2V				155	82	152	324	34	66
526	F	50	CC	AA	2V				189	105	232	98	58	126
550	M	72	CT	AA	3V				163	80	175	95	40	94
563	F	63	CC	AA	2V				185	88	279	150	55	163
578	F	69	CC	AA	1V	13.3	335	10.9	129	71	148	80	59	63
582	F	64	CT	AA	2V				154	56	184	127	42	124

Classifications of clinical and biochemical data according to severity of CAD patients

Genotype	Biochemical	1V CAD	2V CAD	3V CAD	P value
677CC					
	Age (years)	60.11 ±6.7	59.17± 7.1	59.82± 7.8	0.867
	Hcy (μmol/L)	11.90± 3.9	11.66 ±4.5	14.25 ±5.1	0.190
	B_{12} (pg/ml)	615 ±305.4	849.5 ±417.5	759.61 ±261.9	0.214
	Folate (ng/ml)	7.06 ±2.8	8.35 ±4.9	8.27 ±4.8	0.722
	BP_1 (mmHg)	136.94± 24.7	150.28± 26.4	160.03± 27.4	0.015
	BP_2 (mmHg)	74.61 ±15.1	77.77 ±14.0	86.00 ±15.3	0.018
	Chol (mg/ml)	183.85± 45.5	191.19 ±40.9	186.84 ±56.6	0.833
	Trig (mg/ml)	141.5 ±61.1	131.30± 59.3	143.13 ±67.2	0.688
	HDL (mg/ml)	45.81 ±12.6	41.13 ±10.1	42.45 ±13.0	0.303
	LDL (mg/ml)	110.84 ±43.0	124.19± 32.7	135.08 ±38.6	0.046
677CT					
	Age (years)	57.23 ±10.0	62.44 ±7.5	59.32 ±8.5	0.267
	Hcy (μmol/L)	10.19 ±4.0	16.37 ±5.26	14.72 ±3.9	0.017
	B_{12} (pg/ml)	996.29 ±464.2	1188.57± 694.59	568.00 ±324.0	0.030
	Folate (ng/ml)	10.36 ±5.2	8.59 ±5.7	14.50 ±21.7	0.701
	BP_1 (mmHg)	149.00 ±19.0	148.78± 15.0	152.53 ±32.3	0.888
	BP_2 (mmHg)	81.00 ±13.0	76.00 ±8.7	89.32 ±31.0	0.225
	Chol (mg/ml)	183.69 ±26.3	192.88 ±32.64	184.32 ±56.0	0.788
	Trig (mg/ml)	149.08 ±117.2	130.33 ±66.47	174.04 ±151.2	0.329
	HDL (mg/ml)	44.23 ±11.9	45.24 ±19.8	40.19 ±11.7	0.551
	LDL (mg/ml)	106.75 ±20.4	121.18± 33.1	113.05 ±28.8	0.404
677TT	Age (years)	-	-	64.67 ±8.4	-
	Hcy (μmol/L)	-	-	50	-
	B_{12} (pg/ml)	-	-	884	-
	Folate (ng/ml)	-	-	6.4	-
	BP_1 (mmHg)	-	-	154.00 ±10.6	-
	BP_2 (mmHg)	-	-	78.76 ±1.5	-
	Chol (mg/ml)	-	-	175.33 ±39.9	-
	Trig (mg/ml)	-	-	140.00 ±107.7	-
	HDL (mg/ml)	-	-	50.67 ±20.8	-
	LDL (mg/ml)		-	114.5 ±32.2	-

BIOGRAPHY

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