ความแปรผันของยีนและประสิทธิภาพในการทำงานของเมทิลีนเททระไฮโดรโฟเลตรีดักเทสในผู้ป่วยเด็กที่เป็นมะเร็งเม็ดเลือดขาวแบบเฉียบพลัน

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GENETIC POLYMORPHISM AND METHYLENETETRAHYDROFOLATE REDUCTASE ACTIVITY IN CHILDREN WITH ACUTE LYMPHOBLASTIC LEUKEMIA

Miss Tiparat Oungsakul

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เอนไซม์เมทิลินเททระไฮโดรโฟเลตรีดักเทส (MTHFR) เป็นเอนไซม์สำคัญในกระบวนการเมทิเลชันและสร้างดีเอ็นเอ เอนไซม์นี้มีการแปรผันของยีนที่พบได้บ่อย 2 ตำแหน่ง คือ ที่นิวคลีโอไทด์ตำแหน่ง 677(C⟶T) และ 1298 (A⟶C) ทำให้สมรรถนะของเอนไซม์ลดลง การรีเมทิเลชันของโฮโมซีสทีอีนไปเป็นเมไธโอนีนเสียไป ส่งผลให้ระดับของโฮโมซีสทีอีนในเลือดสูงขึ้น การรีเมทิเลชันของโฮโมซีสทีอีนไปเป็นเมไธโอนีนและเมทิเลชันของดีเอ็นเอเปลี่ยนไปได้เช่นกันจากการใช้ยาเมธโทเทรกเสท(MTX) ผลของการให้ MTX ร่วมกับการลดการทำงานของ MTHFR เนื่องจากการแปรผันของยีน อาจเป็นผลทำให้ระดับของโฮโมซีสทีอีนในเลือด (tHcy) สูงขึ้น การวิจัยครั้งนี้ทำการศึกษาความสัมพันธ์ระหว่างการแปรผันของ*MTHFR* ยีนกับการทำงานของ MTHFR ในผู้ป่วยเด็กที่เป็นโรคมะเร็งเม็ดเลือดขาวแบบเฉียบพลันซึ่งได้รับยาMTX ในขนาดสูง (HDMTX) ตรวจวัดการแปรผันของยีนโดยใช้เทคนิค polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP)และตรวจวัดการทำงานของเอนไซม์ MTHFR จากระดับโฮโมซีสทีอีนโดยใช้เทคนิค high performance liquid chromatography (HPLC) จากการทดลองพบว่าหลังการได้รับยา MTX ระดับของ tHcy สูงขึ้นอย่างมีนัยสำคัญเมื่อเทียบกับก่อนได้รับยา ไม่พบ homozygous ของยีนที่เปลี่ยนไปทั้งสองตำแหน่ง (677TT/1298CC) และ homozygous ร่วมกับ heterozygous ของยีนที่เปลี่ยนไป (677TT/1298AC และ 677CT/1298CC) ในคนไทยที่ทำการศึกษา การแปรผันของ *MTHFR* ยีนอาจมีผลกับระดับของโฮโมซีสทีอีน หลังการให้ MTX (tHcy PMT) โดยเฉพาะในคนที่มีการแปรผันของยีนแบบ heterozygous ทั้งสองตำแหน่ง (677CT/1298AC) อาจใช้ tHcy PMT เป็นตัวชี้วัดการเกิดพิษจาก MTX ได้ในเด็กที่เป็นมะเร็งเม็ดเลือดขาวแบบเฉียบพลันที่ได้รับการรักษาด้วย MTX ในขนาดสูง

ภาควิชา เภสัชวิทยา ลายมือชื่อนิสิต.....

สาขาวิชา เภสัชวิทยา ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา 2546 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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5,10-Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme involved in DNA methylation and synthesis. *MTHFR* has two common polymorphisms (C677T and A1298C), both reduce enzyme activity and impaire remethylation of homocysteine (Hcy) to methionine resulting in hyperhomocysteinemia. Remethylation of Hcy to methionine and DNA methylation are also affected by methotrexate (MTX) treatmant. A combined effect of MTX and reduced MTHFR activity by polymorphisms may lead to the elevation of total Hcy (tHcy). The objective of this study was to examine the correlation between the *MTHFR* genotype and phynotype in children with acute lymphoblastic leukemia (ALL) receiving high dose MTX (HDMTX). Genotyping of *MTHFR* were detected by polymerase chain reaction/ restriction fragment length polymorphism (PCR/RFLP) method. Phynotyping of MTHFR, Hcy, was detected by high performance liquid chromatography (HPLC) technique. Our data indicated that after patients treated with HDMTX, tHcy was significantly higher than basal line. The homozygous of both mutant alleles (677TT/1298CC) and one homozygous combined with heterozygous of mutant alleles (677TT/1298AC and 677CT/1298CC) were undetected in Thai population studied. The *MTHFR* polymorphisms may affect the tHcy after MTX treatment (tHcy PMT) especially in the conbined heterozygosity (677/1298AC).The tHcy PMT may be a marker for MTX cytotoxicity in ALL children treated with HDMTX.

Department Pharmacology Student's signature……..

Field of study Pharmacology Advisor's signature…….....

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LIST OF ABBREVIATIONS

%	**=** percent
°C	= degree celcius
μl	= microlitter
μM	= micromolar
10-formylTHF	= 10-formyltetrahydrofolate
5,10-MTHF	= 5,10-methylenetetrahydrofolate
5-formylTHF	= 5-formyltetrahydrofolate
5-MeTHF	= 5-methyltetrahydrofalate
A	= adenine
AICAR	= aminoimidazole carboxamide ribonucleotide
ALL	= acute lymphoblastic leukemia
BMT	= before methotrexate treating
C	= cytosine
Cbl	= cobalamin
CBS	= cystathionine β-synthase
CO_2	= carbondioxide
CSA	= cysteine sulfinic acid
DHF	= dihydrofolate
DHFR	= dihydrofolate reductase
DNA	= deoxyribonucleic acid
dTMP	= deoxythymidine monophosphate
dUMP	= deoxyuridine monophosphate

LIST OF ABBREVIATIONS (CONTINUE)

EAAs = excitatory amino acids

Fig = Figure

FiGlu = formiminoglutamate

G = guanine

GAR = glycinamide ribonucleotide

HCA = homocysteic acid

Hcy = homocysteine

HDMTX = high dose methotrexate

hFR = human folate receptor

HPLC = high performance liquid chromatography

hr = hour

L = Liter

M = molar (mole/liter)

MDR = multi drug resistance

Mg = milligram

Min = minute

ml = milliliter

mol = mole

MS = methionine synthase

MTHFD = methylenetetrahydrofolate dehydrogenase

MTHFR = methylenetetrahydrofolate reductase

MTX = methotrexate

NMDA = N-methyl-D-aspartate

PCR	= polymerase chain reaction
pM	= picomolar
PMT	= post methotrexate treating
RFLP	= restriction fragment length polymorphism
RNA	= ribonucleic acid
SAH	= S-adenosylhomocysteine
SAM	= S-adenosylmethionine
Sec	= second
SHMT	= serine-hydroxy-methyl transferase
T	= thymine
tHcy	= total homocysteine
THF	= tetrahydrofolate
TS	= thymidylate synthase
U	= unit

CHAPTER I

Introduction

Methotrexate (MTX) is an antifolate chemotherapeutic drug. It plays a central role in the treatment of acute lymphoblastic leukemia (ALL), and is used in the treatment of other neoplastic and non-neoplastic diseases, including psoriasis, rheumatoid arthritis and systemic lupus erythematosus. The prognosis of ALL has improved with intensive chemotherapy. High dose of MTX (>1g/m^2) is required for the effective treatment in ALL patients (Refsum et al, 1991). Its principal pharmacological mechanism is the inhibition of enzymes involved in folate homeostasis resulting in cellular depletion of reduced folates. The 5,10-methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in the generation of bioactive folate compounds(Quinn, 1996; Kishi *et al*, 2003). A simplified diagram of MTX targeted enzymes is illustrated in Fig.1

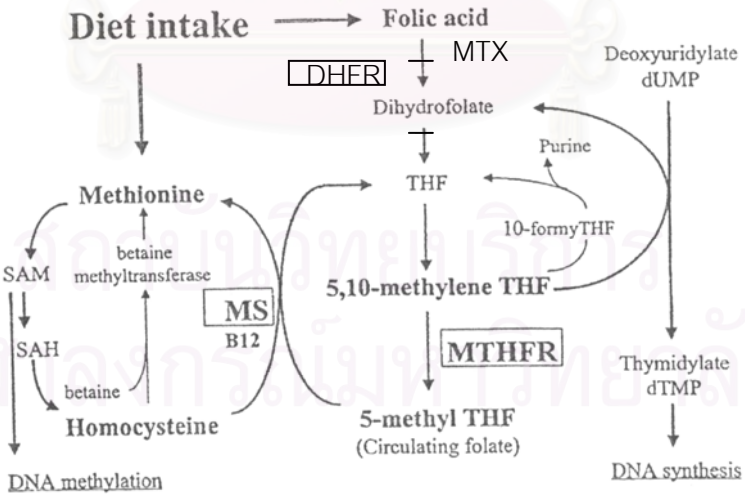

Figure 1. A simplified diagram of MTX targeted enzymes. DHFR, dihydrofolate reductase; THF, tetrahydrofolate; 5,10- methylene THF, 5,10-methylenetetrahydro-folate; 5-methylTHF, 5-methyltetrahydrofolate; MTHFR, methylenetetrahydrofolate reductase; SAM, S-adenosylmethionine; and SAH, S-adenosylhomocysteine (Ma *et al*, 1999).

Despite MTX clinical success, the major factor limiting its use is its toxicity. The prolonged administration of MTX can lead to several toxicities such as gastrointestinal symptom (nausea, vomiting, abdominal pain or diarrhea), hepatitis with raised transaminases, alopecia, hypersensitivity pneumonitis and a serious complication of neurotoxicity (dementia, seizures, confusion, visual deficits or hemiparesis) which is thought to be affected by the elevation of plasma homocysteine level (hyperhomocysteine or hyperhomocysteinemia) (Quinn, 1996; McKendry,1997; Toffoli *et al*,2003; Kishi *et al*, 2003). The pathogenesis of neurotoxicity is remains unclear. It has been suggested that hyperhomocysteine may be responsible for the neurotoxicity associated with MTX (Quinn, 1996; Kishi *et al*, 2003). It is possible that polymorphic enzymes involved in folate metabolism may be related to these different effects especially MTHFR. Recently, a gene mutation is discovered in one of these metabolic routes that may predispose to the development of side effects. MTHFR received much interest after the first described polymorphism of MTHFR gene in the mid-1990s. MTHFR catalyzes the reduction of 5,10-methylenetetrahydrofolate (5,10-MTHF) to 5-methyltetrahydrofolate (5-MeTHF) which is the predominant circulatory form of folate and carbon donor for the remethylation of homocysteine to methionine.

The common point mutations have been found in MTHFR gene. The first identified polymorphism of the MTHFR gene found by Frosst *et al*, is a cytosine (C) to thymine (T) substitution at nucleotide 677 which converts an alanine to a valine (677C→T). Reduction in activity of MTHFR is associated with the heterozygous or homozygous variant 677T genotype. The variant TT genotype has about 30% of wild type (677CC) activity and presents in about 10% of white and Asian populations. Heterozygote (677CT) has about 60% activity and constitutes approximately 40% of the population (Ulrich, 2001; Ranganathan *et al*, 2003). Homozygotes of the 677T allele are predisposed to hyperhomocyeteine increased by the decrease in MTHFR activity, particularly in the context of suboptimal folate status.

Recently, a second common polymorphism in the MTHFR gene has been reported. It is a substitution of A to C at 1298 nucleotide results in a glutamate to an alanine conversion (1298 A→C). This polymorphism also associated with decreased enzymatic activity (van der Put *et al*, 1998) elevated homocysteine (Hcy) concentration, and decreased folate concentration in plasma (Weisberg *et al*, 1998).

Both polymorphisms reduced MTHFR activity resulting in the depletion of 5-MeTHF for Hcy remethylation, the accumulation of 5,10-MTHF which is precursor for thymidylate and purine synthesis, and subsequently increased plasma homocysteine level (Ranganathan *et al*, 2003).

Plasma homocysteine concentration, a representative marker of intracellular folate status (Surtees *et al*, 1998; Toffoli *et al*, 2002) is associated with the TT *MTHFR* 677genotype and is also increased after MTX therapy. Therefore it could be hypothesized that patients with C677T or A1298C or C677T plus A1298C polymorphisms are predisposed to hyperhomocysteine receiving high dose MTX (HDMTX).There are some studies reported the association between C677T polymorphism of *MTHFR* and toxicity of MTX (Haagsma *et al*, 1999; Urano *et al*, 2002; Ranganathan *et al*, 2003) with a few studies describing the relationship of both C677T and A1298C polymorphisms with MTX toxicity (Kishi *et al*, 2003).

Hypothesis

There is a correlation between MTHFR genetic polymorphism and homocysteine concentration in ALL children treated with MTX.

Objective

This study aims to evaluate that polymorphisms are related to MTX toxicity and/or associated with the elevation of plasma homocysteine concentration in ALL children.

Expected outcome

1. The characteristics of genetic control of MTHFR activity in ALL children

2. The application of (1) for MTX dosage adjustment to suit each individual of ALL children

CHAPTER II

Literature Review

Pharmacogenomics is the study of how genetic inheritance influences response to drugs. A greater understanding of the genetic determinants of drug response has the potential to revolutionize the use of many medications, with the goal of selecting the optimal drug therapy and dosage for each patient, especially important in the challenging field of oncology. By increasing our ability to prospectively identify patients at risk for severe toxicity will be great benefit to a particular treatment.

Human genomic DNA contains a variety of known polymorphisms, such as single-nucleotide substitutions, insertion/deletions, and copy number variations in nucleotide repeated motifs. A single-nucleotide substitution is the most common form of polymorphism and is called a SNP. SNPs are not only important as markers for constructing dense genetic maps but also have potential as direct functional polymorphic variants involved in common and genetically complex diseases as well as drug response. SNPs within the coding regions (cSNPs) of functional genes introduce biological variation directly into the gene products through the creation of missense substitutions or premature termination codon. SNPs present in noncoding regions may affect gene expression especially regulatory elements and some intronic SNPs activate cryptic splice site, leading to alternative splicing. Recently developed technologies using high-throughput genotyping methods have accelerated SNP discovery by identifying the specific location in a gene at a frequency of > 1% across a population (Goto *et al*, 2001).

It is necessary to build a comprehensive catalogue of SNPs in human candidate genes for better understanding of its role in common diseases. Recently, several studies characterizing SNPs on a large scale have been reported for this purpose (Goto *et al*, 2001). In addition, the exploitation of all relevant SNP variation will improve the diagnosis and treatment of disease as shown in cancer patients using gene-based information for better treatment of chemotherapeutic drugs.

A central goal of cancer pharmacogenomics is the prediction of drug response based on a patient's genetic profile. Ideally, a widely available and inexpensive genetic test performed on an easily accessible patient biological sample (i.e., primary tumor or peripheral blood) could be used to predict drug efficacy or toxicity. Every gene has some level of polymorphism, and determining which polymorphisms are relevant for predicting patient response to chemotherapy represents a major challenge. Since the mechanism of action of many chemotherapeutic agents is known, polymorphisms in candidate genes that likely to influence drug response should be identified. The prediction of cancer treatment outcome based on gene polymorphisms is becoming possible for many classes of chemotherapeutic agents.

Methylenetetrahydrofolate reductase

The enzyme methylenetetrahydrofolate reductase, MTHFR, [5-methyltetrahydrofolate: (acceptor) oxidoreductase, EC 1.7.99.5] is one of key enzymes in the folate metabolism and catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate (5,10-MTHF) to 5-methyltetrahydrofolate (5-MeTHF). 5-MeTHF is the predominant circulatory form of folate, serves as a methyl donor (carbon donor) for the remethylation of homocysteine (Hcy) to methionine. This reaction is important in one carbon metabolism because methionine, along with other functions, is the precursor of S-adenosylmethionine (SAM), the universal methyl donor of biological methylation reactions. Included among these essential methylation reactions is DNA methylation. The 5,10-MTHF, a substrate for MTHFR, is an intracellular form of folate required for de novo synthesis of thymidylate and is therefore vital for DNA synthesis (Fig. 2).

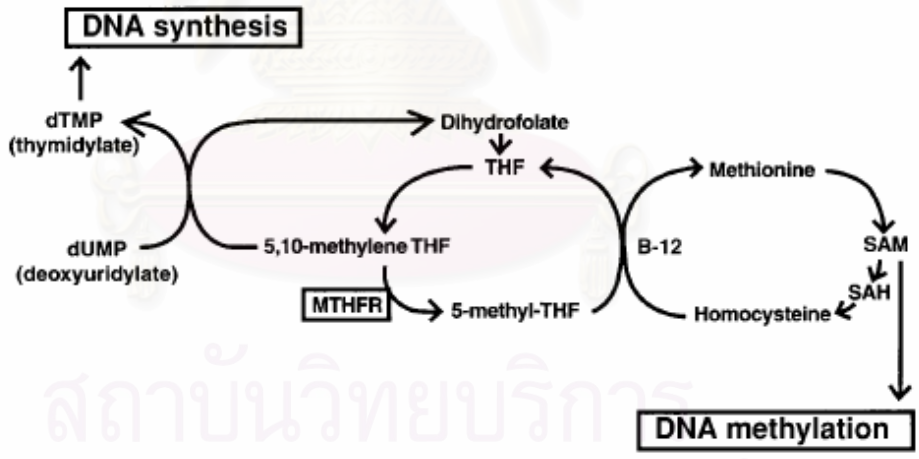

Figure 2. Role of MTHFR in DNA synthesis and methylation. dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; B-12, vitamin B-12 (James *et al*, 1999).

Mutation in methylenetetrahydrofolate reductase MTHFR gene decreases the activity of MTHFR and the synthesis of 5-methyltetrahydrofolate (5-methyl-THF), which is used for the remethylation of homocysteine to methionine. Insufficient synthesis of 5-methyl-THF and methionine results in a decrease in methionine and in S-

adenosylmethionine (SAM) and an accumulation of homocysteine and S-adenosylhomocysteine (SAH). A reduction in SAM:SAH reduces the efficiency of DNA (cytosine-5-)-methyltransferase and is associated with DNA hypomethylation.

The gene encoding *MTHFR* has been mapped to chromosomal region 1p36.3. The *MTHFR* gene product consists of 656 amino acid residue and represents a key enzyme in the folate cycle. It reduces 5,10-MTHF to 5-MeTHF, thus catalyzing the only reaction in the cell that ultimately generates 5-MeTHF, the biologically active folate derivative.

Two polymorphisms exist in *MTHFR* that are located at nucleotide position 677 (MTHFR 677C→T) and position 1298 (MTHFR 1298A→C). First polymorphism of *MTHFR*, a missense mutation in exon 4 of the *MTHFR* gene, (Fig. 3), is a cytosine (C) to thymine (T) transition at nucleotide 677(677C→T), which converts an alanine (ala) to valine (val) amino acid substitution. For *MTHFR* 677C→T, there are three genetic polymorphisms: variant homozygous (677TT, val /val), variant heterozygous (677CT, val /ala), and wide type homozygous (677CC, ala /ala). *MTHFR* 677C→T mutation is associated with reduced enzyme activity, decreased formation of 5-MeTHF and an accumulation of formylated tetrahydrofolate polyglutamates in erythrocytes.

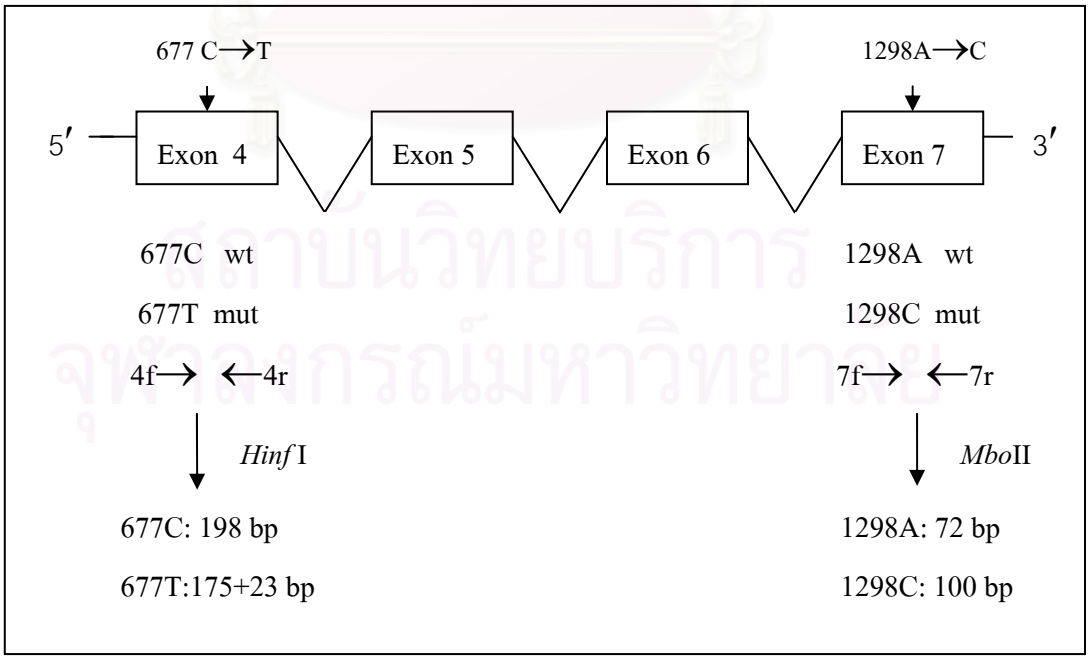

Figure 3. Schematic genomic structure of the MTHFR gene in the region of exon 4 to exon 7 (Urano *et al*, 2002).

Furthermore, *MTHFR* 677TT is frequently associated with higher plasma tHcy levels as compared to heterozygotes or people without mutation. This polymorphism in the catalytic domain of MTHFR results in a 50-60% decrease in the specific activity of MTHFR when it is present in the homozygous state. The gene frequency for 677C→T is observed in all countries (Sunder-Plassmann; 2003) and varies among ethnic groups with T allele having a frequency of around 30% in Europeans and Japan and only around 11% in African Americans (Carmel *et al*, 2003).

Second polymorphism has been discovered in the *MTHFR* gene (van der Put *et al*, 1998; Weisberg *et al*, 1998). It is located in exon 7 of the *MTHFR* gene (Fig. 3), which is associated with decreased enzyme activity in vitro. This genetic variant consists of an adenine (A) to cytosine (C) transition at nucleotide 1298(1298A→C), which converts a glutamate (glu) to alanine (ala) amino acid substitution. For *MTHFR* 1298A→C, there are three genetic polymorphisms: variant homozygous (1298CC, ala /ala), variant heterozygous (1298AC, glu /ala), and wide type homozygous (1298AA, glu /glu). *MTHFR* 1298A→C alone influences neither folate status nor tHcy concentrations, and it is associated with an approximately 35% decrease in MTHFR activity, but not with thermolability. The 1298C allele frequency is about 18% among Asian population, and about 30% in Western Europe (Carmel *et al*, 2003).

By contrast, both polymorphisms are associated with decreased folate plasma concentration and higher plasma tHcy concentrations (Sunder-Plassmann; 2003). Studies on a relatively small number of individuals have shown that ~10% of individuals are homozygous for the 1298C allele and roughly 20% of individuals are heterozygous carriers of both the C677T and A1298C polymorphisms. Although it has been shown that the A1298C polymorphism, in either heterozygosity or homozygosity, is not associated with higher plasma tHcy concentrations in patient with neural tube defects and geneticthei parents (van der Put *et al*, 1998; Weisberg *et al*, 1998), it has been reported that combined heterozygosity for the C677T and A1298C variants is associated with reduced MTHFR specific activity (Table 1) and higher Hcy concentration when compared with heterozygosity of each variants (van der Put *et al*, 1998).

Table 1. Relationship of *MTHFR* combined genotype. The specific enzyme activity and residual activity between C677T and A1298C genotype (van der Put *et al*, 1998).

Genotype	**677CC**	**677CT**	**677TT**
Specific *MTHFR* activity			
1298AA	26.2 (± 6.7)	17.5 (± 5.3)	6.5 (± 2.6)
1298AC	21.8 (± 5.1)	12.5 (± 3.7)	NO
1298CC	16.0 (± 4.2)	NO	NO
Residual *MTHFR* activity			
1298AA	66.0 (± 8.2)	56.1 (± 10.5)	17.6 (± 14.4)
1298AC	65.8 (± 8.8)	51.9 (± 9.4)	NO
1298CC	61.0 (± 8.5)	NO	NO

NO = not observed

Methotrexate

The modern era of antimetabolite cancer chemotherapy began in the late 1940s with the demonstration by *Farber* and colleagues that the antifolate aminopterin (2,4-diamino, 4-deoxyfolic acid) could induce remissions in pediatric acute leukemia. Today, the most widely used antifolate in medical oncology is methotrexate (Fig.4), the N^{10}-methyl derivative of aminopterin, which has documented activity in acute leukemia, non-Hodgkin's lymphoma, breast cancer, head and neck cancer, choriocarcinoma, osteogenic sarcoma and bladder cancer. Antifolates are also widely used in the treatment of nonmalignant diseases such as rheumatoid arthritis, psoriasis, bacterial and plasmodial infections and in the opportunistic infections associated with AIDS (takamoto, 1996).

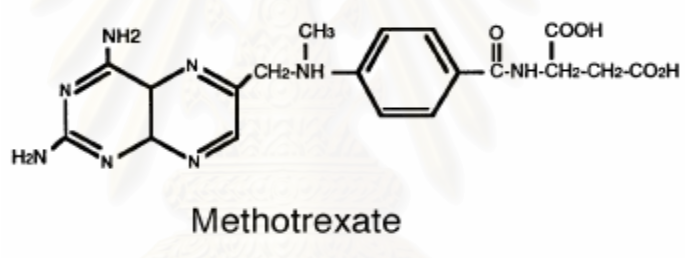

Figure 4. Chemical structure of methotrexate. Chemical name is 4-amino-4-deoxy-10-methylpteroyl -L- glutamic acid. Its molecular formular is $C_{20}H_{22}N_8O_5$ with the molecular weight of 454.4 (Takimoto, 1996).

Mechanism of action

Methotrexate, MTX, analogue of folic acid, is an antimetabolite whose primary mechanism of action is the inhibition of enzyme dihydrofolate reductase (DHFR). This enzyme is required to maintain the cellular pool of tetrahydrofolate (THF) during thymidylate synthesis. Specifically, deoxythymidylate is synthesized from deoxyuridyrate by methylation in a reaction catalyzed by thymidylate synthase. The methyl donor is 5,10-methylenetetrahydrofolate (MTHF), which is oxidized

during the reaction, producing dihydrofolate (DHF). DHF is then reduced to THF in an NADPH-dependent reaction catalyzed by DHFR (Fig.1). MTHF is regenerated in a one carbon transfer reaction, with serine usually supplying the carbon group. MTX, or more likely its polyglutamate metabolites, can interfere with other enzymes involved in folate homeostasis as well.

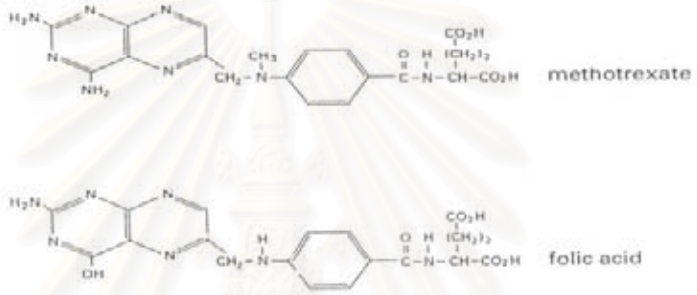

Figure 5. The chemical structure of methotrexate and folic acid (Rasmussen *et al*, 2000).

MTX is designed to mimic a folate molecule (Fig.5), to bind and block at the active site of enzyme (Goodsell, 2002). MTX differs from the essential vitamin, folic acid, by the substitution of an amino group for a hydroxyl at the 4-position of the pteridine ring. This minor structural alteration changes the normal substrate into a tight binding inhibitor of DHFR, the enzyme principally responsible for the maintenance of the intracellular reduced folate pool. Reduced forms of folic acid, called tetrahydrofolates, are essential cofactors which serve as single carbon atom donors in the enzymatic synthesis of thymidylate and purine nucleotide. Tetrahydrofolates are only biologically active in their fully reduced forms. One reduced folate, 10-formylterahydrofolate, (Fig. 6) is responsible for donating single carbon groups in the *de novo* biosynthesis of purine nucleotides in the reactions catalyzed by the enzymes glycinamide ribonucleotide (GAR) and aminoimidazole carboxamide

ribonucleotide (AICAR) transformylase (Fig.6). Another reduced folate, 5,10-MTHF, donates a methyl group to deoxyuridylate (dUMP) during the biosynthesis of thymidylate (dTMP) in the reaction catalyzed by thymidylate synthase (TS). During this process, 5,10-MTHF is oxidized to DHF and must be converted back to its THF form by DHFR in order to maintain the reduced folate pool. In the presence of ongoing thymidylate synthesis, the inhibition of DHFR leads to the partial depletion of intracellular reduced folates, which can ultimately contribute to the impaired production of essential nucleotide precursors for DNA synthesis.

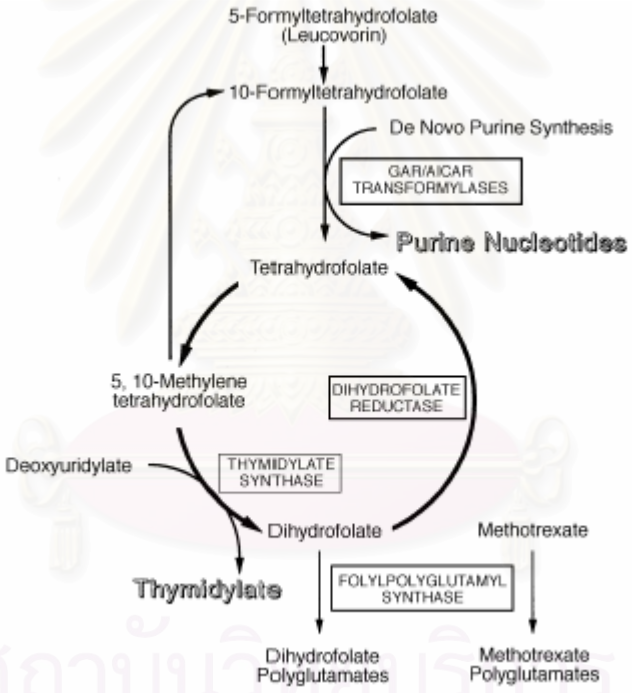

Figure 6. Enzymatic reactions of folate metabolism. The inhibition of dihydrofolate reductase by methotrexate and its polyglutamated derivatives leads to a partial depletion of the intracellular reduced folate pool and to the accumulation of dihydrofolate. Dihydrofate polyglutamates and methotrexate polyglutamates can inhiblt thymidylate synthase, and glycinamide ribonucleotide (GAR) and aminoimidazole carboxamide ribonucleotide (AICAR) transformylases. These multiple drug effects ultimately impair the synthesis of thymidylate and purine nucleotide, which are essential precursors of DNA synthesis (Takimoto, 1996).

In mammalian cells, the enzyme folylolyglutamyl synthase can attach up to six glutamate molecules to the pteridine ring of naturally occurring folates and antifolates, such as MTX. This polyglutamation reaction has several biologically important consequences. First, by increasing the size and ionization state of the MTX molecule, it decreases cellular efflux and traps the drug within the cell, thereby prolonging drug action. Furthermore, the increased propensity of malignant cells to polyglutamate methotrexate compared to normal tissues may also account for some of the drug's selective cytotoxicity. Methotrexate polyglutamates are direct inhibitors of DHFR and, compared to monoglutamated drugs, have increased potency as inhibitors of other folate dependent enzymes such as TS, GAR and AICAR transformylases. Polyglutamation of the natural enzyme substrate, DHF, which accumulates as a consequence of DHFR inhibition, is also an important component of MTX action. Dihydrofolate polyglutamates are, by themselves, potent inhibitors of TS and GAR and AICAR transformylases, they further contribute to the impaired production of thymidylate and purine nucleotide precursors. Thus, the inhibition of DNA synthesis by MTX is a multifactorial process which results from both the partial depletion of intracellular reduced folates and from the direct inhibition of folate depentdent enzymes by MTX and dihydrofolate polyglutamates. The relative contributions from each of these mechanisms toward the generation of MTX induced cytotoxicity can vary in different cancer cell lines. Decreased polyglutamation due to the loss of folylpolyglutamyl synthase (FPGS) activity has been identified as a potentially important mechanism of clinical MTX resistance in human soft tissue sarcomas. Furthermore, normal tissues with high FPGS activity, such as the liver, accumulate and retain methotrexate polyglutamates for prolonged periods of time which may be a process responsible for the occasional hepatotoxicity seen during chronic MTX administration.

Although the depletion of thymidylate and purine nucleotides can inhibit DNA synthesis, the precise events leading to MTX induced cell death are still under investigation. Cytotoxic DNA damage may result from the loss of DNA precursors, which leads to ineffective DNA repair and strand breakage. Another consequence of the inhibition of TS is the intracellular accumulation of dUMP, which can be converted into the deoxytriphosphate nucleotide, dUTP, and misincorporated into DNA. The DNA excision repair enzyme, uracil-DNA-glycosylase, recognizes and removes these uracil residues in DNA leading to further fragmentation and potentially cytotoxic DNA damage. However, the addition of

thymidylate alone to methotraxate treated cells does not prevent cytotoxicity unless a purine source, such as hypoxanthine, is also provided. This further emphasizes the importance of both purine and thymidylate effects in the generation of MTX cytotoxicity.

Membrane transport

Folate transport has been extensively studied because of its importance as a mechanism of antifolate drug resistance. Cellular uptake of MTX occurs via the same active transport pathways responsible for the influx of normal physiologic folates. At least two distinct carrier mediated active transport systems are responsible for the uptake of MTX into mammalian cells. One system, called the reduced folate carrier, is a relatively low affinity transporter of both MTX and reduced folates with affinity constants in the micromolar range. A second system utilizes a high affinity, membrane associated, folate binding protein, called the human folate receptor (hFR) which has affinity constants for reduced folates and folic acid in the nanomolar range. Some tumors, such as ovarian cancers, express high levels of hFR on the cell surface. The relative contribution of these two distinct transport pathways to the uptake of MTX in clinical cancer chemotherapy is an area of active research. However, *in vitro* resistance to MTX resulting from the decreased transport activity of one or both of these carrier systems has been reported. Newer, more lipophilic antifolates, such as trimetrexate or piritrexim, are not substrates for either folate transport system and enter cells by energy independent mechanisms such as passive diffusion. Cell lines resistant to MTX because of decreased MTX transport generally retain their sensitivity to these newer lipophilic antifolates. Efflux of MTX from the cell is also mediated by several different transport systems, some of which are distinct from the folate uptake pathways. As previously mentioned, drug efflux is greatly influenced by the degree of MTX polyglutamation. MTX efflux is not associated with the P-glycoprotein multidrug resistance (MDR) phenotype which confers cross resistance to numerous other anticancer agents.

Despite its clinical success, MTX is associated with a number of gastrointestinal, haematopoietic and neurologic toxicities. The neurotoxicity is frequently the most unexpected and worrisome. MTX related neurotoxicity can be classified as occurring in three forms with relation to time from therapy.

1. An acute syndrome, usually within one day of treatment, which may include nausea, emesis, headache, somnolence, lethargy or seizures.

2. A subacute syndrome, occurring one to two weeks after exposure, characterized variably by seizures, affective disturbances or focal neurologic deficits, usually transient, including paresis, aphasia, anesthesia, blurred vision and pseudobulbar palsy.

3. A delayed leukoencephalopathy of variable severity, occurring weeks to months following therapy, which may be progressive and is characterized by disturbances of higher cognitive functions.

The pathogenesis of MTX induced neurotoxicity is not well understood despite its use for nearly 50 years. MTX neurotoxicity may arise from the polymorphisms of methylenetetrahydrofolate reductase (MTHFR) and MTHFR gene polymorphisms may cause elevation of plasma homocysteine level.

Folate

Folates are involved in several important metabolic routes including 5,10-methyltetrahydrofolate/10-formyltetrahydrofolate, 5,10-methylenetetrahydrofolate and 5-methyl- tetrahydrofolate which deliver one carbon units for synthesis of purine, pyrimidine and methionine respectively.

Folate structure and function

Folic acid is a water soluble B vitamin. The name folic acid is derived from the Latin word folium, or leaf. It was first isolated from spinach leaves in 1941 and was synthesized in 1946. Its chemical name is pteroylmonoglutamic acid. The term folic acid is used for its synthetic form presents in multivitamins, folic acid tablets, and fortified foods. Synthetic folic acid is relatively stable and exists as monoglutamate, a form that is rapidly absorbed without being processed.

The natural form is referred to as folate (falacin), which occurs naturally as polyglutamate derivatives with the glutamate moieties linked via γ-carboxyl peptide bonds. Several dietary sources rich in natural folate include a wide variety of fruits and vegetables, particularly green leafy vegetables such as spinach, brussel sprouts and turnip greens. Other foods rich in folate include potatoes, orange, beans, yeast, and organ foods such as liver. Natural folate mainly consists of 5-methyltetrahydrofalate (5-MeTHF) and 10-formyltetrahydrofolate (10-formylTHF) in their polyglutamate derivatives. Both compounds are readily oxidized and the rates vary directly with oxygen concentration, temperature, alkalinity, exposure to light and concentration of cupric and ferric ions. Therefore, a considerable amount of folate can be destroyed in cooking, processing and storage (van der Put *et al*, 2001).

The generic term folate includes both naturally occurring polyglutamates and the synthetic form, folic acid. The common feature of all folates is the p-aminobenzoic acid part of the molecule attached to the apterin-ring on the NH_2 end, and one or more glutamic acid in a γ-amide linkage at the carboxyl end (Fig. 7). The active center of folate is between the N^5 and the N^{10} sites. Folate derivatives are formed by different one-carbon subgroups, which are added to the active site. Essentially, tetrahydrofolate (THF) is carrying a one-carbon unit present

as formate in its most oxidized form and as methyl in its most reduced form; all of these one-carbon subgroups can be converted into each other.

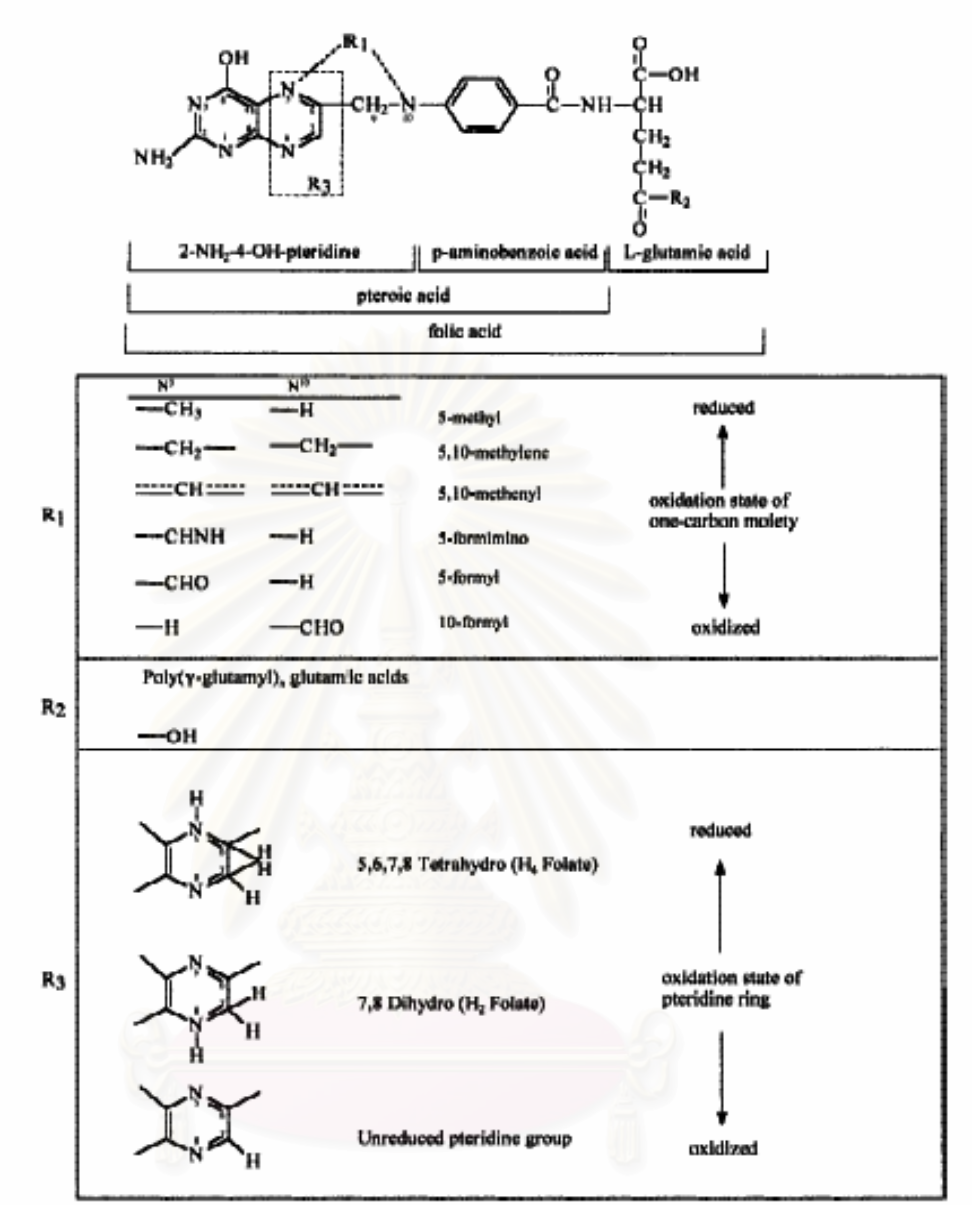

Figure 7. Folic acid structure and its derivatives (van der Put *et al*, 2001)

In mammalian tissues, folate functions as substrate in series of interconnected metabolic cycles involving thymidilate and purine biosynthesis (adenosine and guanine), methionine synthesis via homocysteine (Hcy) remethylation, serine and glycine interconversion and the metabolism of histidine and formate. Folate is also indirectly a methyl donor in many methylation reactions via S-adenosylmethionine (SAM), for example, in the regulation of gene expression. Thus, folate is directly or indirectly essential for cell function, division and differentiation. Acquisition of folate, therefore, is critically important to

the viability of proliferating cells. The shutdown of DNA synthesis and SAM synthesis arising from folate deficiency perturbs the cell cycle and could lead to premature cell death. Eukaryotic cells are unable to synthesize folate *de novo*, so folate is an essential nutrient. Thus, mammals are dependent on transport systems for uptake of folate compounds from the environment.

Folate one-carbon metabolism

Vital cellular processes depend on folate-mediated one-carbon metabolism, i.e., the transfer of a carbon group. The major metabolic folate pathways are shown schematically in Fig. 8 and Fig. 9. Folate acts as donor and acceptor of one-carbon units in a variety of critical enzymatic reactions involved in one-carbon metabolism. The one-carbon units are principally derived from the β-carbon of serine, but also from glycine, methyl- and dimethylglycine, formate and histidine. One-carbon units are thus obtained from THF-mediated reactions, which are required for several major cellular processes like nucleic acid biosynthesis, protein biosynthesis, amino acid metabolism, methyl-group biogenesis and vitamin metabolism.

One carbon units derived from the third carbon of serine are transferred to THF in a reaction catalyzed by serine hydroxyl methyl transferase (SHMT), generating 5,10-methylenetetrahydrofolate (5,10-MTHF) and glycine (Fig. 8, reaction 1). SHMT is the major provider of one-carbon units in the cell, particularly in replicating cells. Serine can enter the mitochondria, where it is converted to formate, which reenters the cytoplasm and acts as a primary carbon donor. The glycine-derive one carbon units are generated from the second carbon of glycine by the glycine cleavage system, which results in the formation of 5,10-MTHF (Fig. 8, reaction 2). Formate-derived one-carbon units are formed by the ATP-dependent enzyme formyl synthetase, which activates formyl to 10-formylTHF (Fig 8, reaction 3). One-carbon units thus enter the active pool of one-carbon at the level of 5,10-MTHF and 10-formylTHF. 10-FormylTHF and 5,10-methenylTHF are substrates for purine biosynthesis, while 5,10-MTHF is involved in thymidine biosynthesis. Apart from this role in the biosynthesis of purines and thymidine and, thus, DNA and RNA, the folates provide a source of methyl-groups for over 100 methyltransferase-catalyzed reactions. This is done by converting 5,10-MTHF to 5-MeTHF, which is used to methylate Hcy to

methionine by the vitamin B_{12}-dependent enzyme methionine synthase (MS) (Fig. 8, reaction 4 and 5). Methionine can be activated by ATP to SAM, the ultimate methyl-donor in the body.

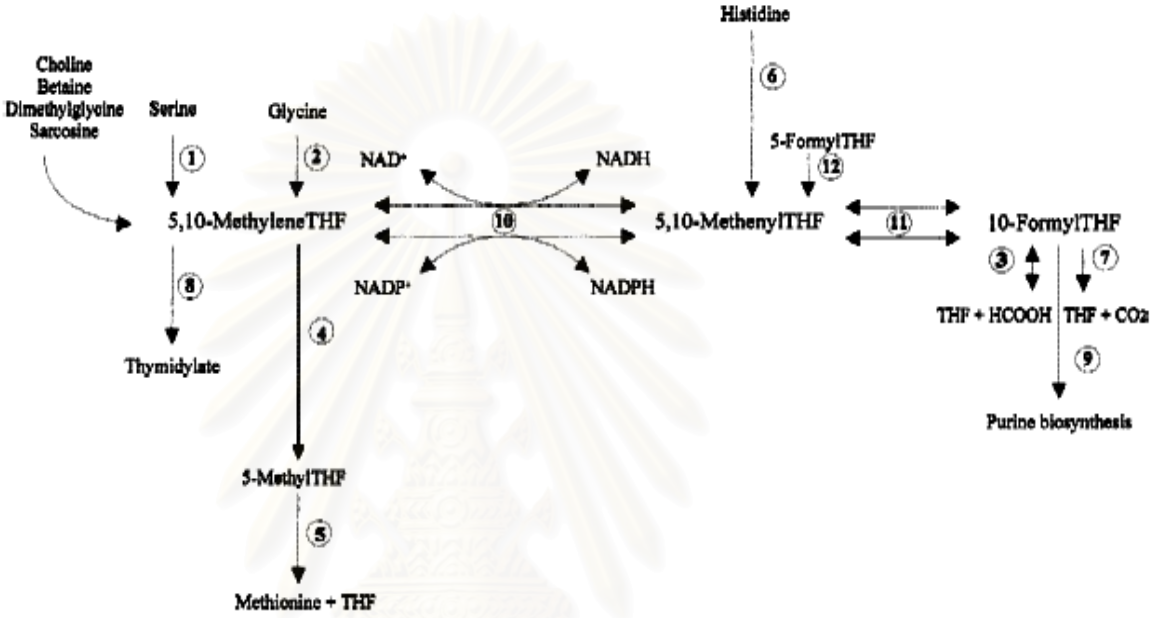

Figure 8. Simplified folate metabolism indicating its one carbon donors and acceptors involved in methyl group biogenesis, thymidylate sunthesis and purine synthesis. 1,serine hydroxymethyltransferase; 2, glycine cleavage pathway; 3, 10-formyltetrahydrofolate synthase; 4, methylenetetrahydrofolate reductase; 5, methionine synthase; 6, glutamate formiminotransferase; 7, 10-formyltetrahydrofolate dehydrogenase; 8, thymidylate synthase; 9, 5-amino-4-imidazolecarboxamide ribonucleotide transformylase (AICAR) and glycineamide ribonucleotide transformylase (GAR); 10,methylenetetrahydrofolate dehydrogenase; 11, methenyltetrahydro- folate cyclohydrolase; 12, methenyltetrahydrofolate synthetase. THF, tetrahydrofolate (van der Put *et al*, 2001).

An alternative one-carbon source is formiminoglutamate (FiGlu). During the catabolism of histidine, a formimino-group is transferred to THF followed by the release of ammonia and by the generation of 5,10-methylTHF by the two enzyme activities, glutamate formiminotransferase

and formiminoTHF cyclodeaminase (Fig. 8, reaction 6 and Fig. 9). This pathway represents only a minor source of one-carbon and may exist only in liver and kidney. The enzymes seem to be absent in fibroblasts and blood cells. Excess one-carbon units are removed from the one-carbon pool by their oxidation to carbondioxide (CO_2) by formylTHF dehydrogenase (Fig. 8, reaction 7). This enzyme also catalyze the hydrolysis of 10-formylTHF to THF and formate. Activity of this bifunctional protein is restricted to the liver. The function of the hydrolase activity is yet unclear, it may represent an additional mechanism for regeneration of the unsubstituted THF under conditions in which utilization of substituted folate for biosynthetic purpose is impaired.

Thymidine biosynthesis

Folate is required for the synthesis of thymidylate. This reaction is catalyzed by thymidylate synthase (TS) and involves the addition of formaldehyde group to the 5-position of deoxyuridylate. In this reaction the one-carbon group of 5,10-MTHF is transferred to deoxyuridine monophosphate (dUMP), resulting in the formation of deoxythymidine monophosphate (dTMP) and dihydrofolate (DHF) (Fig. 8, reaction 8). Synthesis of deoxynucleotides, which is mediated by thymidylate synthase and ribonucleotide reductase, is considered to be the rate-limiting step in DNA synthesis. DHF formed in the thymidylate synthase reaction has to be reduced to THF before it can participate in one-carbon transfer reactions (Fig.9). This reduction is also catalyzed by dihydrofolate reductase (DHFR), an enzyme that catalyzes the reduction of folic acid to DHF. The major role of DHFR appears to be the reduction of DHF formed in the thymidylate synthase reaction. The thymidylate synthase expression level is related to the replication state of a cell. A multi-enzyme complex called replitase is formed during the S-phase of the cell cycle. This replitase contains thymidylate synthase, DHFR, DNA polymerase, thymidine kinase, deoxycytidine monophosphate kinase, nucleotide diphosphate kinase and ribonucleotide reductase.

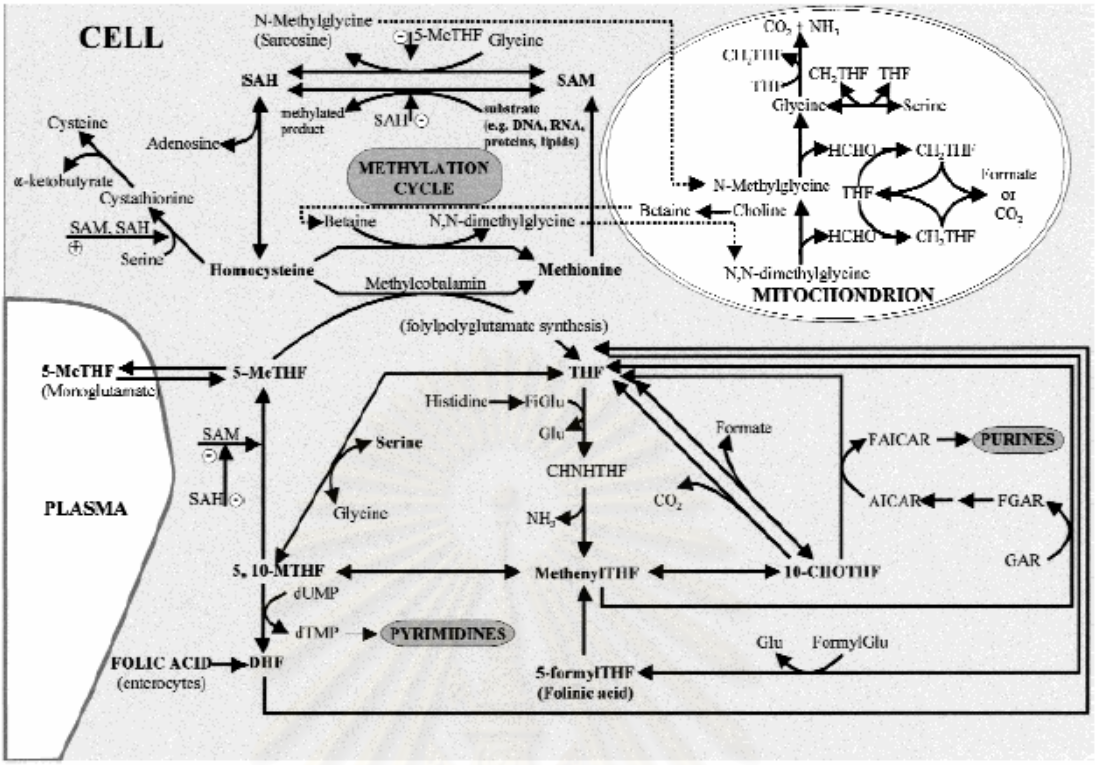

Figure 9. Extended folate metabolism, including compartmentation. MTHFR, methylenetetrahydrofolate reductase; SHMT, serine hydroxymethyltransferase; BHMT, betaine homocysteine methyltransferase, MAT, methionine adenosyltransferase; SAH-hydrolase, S-adenosylhomocysteine hydrolase; MT, methyltransferase; CBC, cystathionineβ-synthase; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; THF, tetrahydrofolate; and 5-MeTHF, 5-methyltetrahydrofolate (van der Put *et al*, 2001).

Purine biosynthesis.

One-carbon units at the oxidation level of formate are utilized in the de novo purine biosynthesis. The C_8 and C_2 position in the purine ring are derived from the folate one-carbon pool from 5,10-methenylTHF in a reaction catalyzed by glycinamide ribonucleotide (GAR) transformylase to FGAR and from 10-formylTHF by formylTHF 5-amino-4-imidazole carboamide ribonucleotide (AICAR) transformylase to FAICAR, respectively (Fig. 8, reaction 9 and Fig. 9). The 10-formyl need for purine biosynthesis can be either directly derived from the 10-formylTHF synthetase-catalyzed reaction (Fig. 8, reaction 3), or can be derived from the oxidation of 5,10-MTHF dehydrogenase and methenylTHF cyclohydrolase (Fig. 8, reaction 10 and 11). Any 5-formylTHF

(leucovorin) is converted to 5,10-methenylTHF by methenylTHF synthetase (Fig. 8, reaction 12). In mammalian tissues the dehydrogenase, cyclohydrolase and synthetase activities are catalyzed by a single trifunctional protein, methylenetetrahydrofolate dehydrogenase (MTHFD).

Methyl group synthesis

An important part of one-carbon utilization involves the reduction of 5,10-MTHF to 5-MeTHF by the enzyme MTHFR (Fig. 8, reaction 4). This reaction is physiologically irreversible. The methyl group of 5-MeTHF is donated to Hcy resulting in methionine synthesis, which is catalyzed by MS (Fig. 8, reaction 5 and Fig. 9). This is the sole enzyme that is able to metabolize the methyl-group of 5-MeTHF. MS plays a major role in methyl-group metabolism, as it allows the reutilization of the Hcy backbone as a carrier of methyl-groups. This reaction channels the one-carbon units derived from formate and amino acid such as serine, histidine and glycine into the methylation cycle, providing a methyl-group for the synthesis of SAM via methionine and sequentially a methyl-group that is used by many essential methyltransferase enzymes.

Cycling of intracellular folates

The folate one-carbon metabolism is, as pointed out earlier, connected to the Hcy cycle by the synthesis of 5-MeTHF by MTHFR and the use of 5-MeTHF as methyl donor for Hcy remethylation. 5-MeTHF donate its methyl-group via catalysis by the cobalamin (Cbl) containing MS to Hcy, whereby methionine and THF are formed. MS converts the circulating form of folate, 5-MeTHF, to THF, which can then support a variety of cellular reactions. MS is the only enzyme that is able to demethylate 5-MeTHF and thus occupies a strategic position in the control of folate metabolism. MS provides methyl-groups for the methylation cycle; in doing so, it recycles 5-MeTHF back to THF. After uptake from blood, demethylation of 5-MeTHF via MS is needed before it can be conjugated into a polyglutamate and retained by the cell. Intracellular remethylation of THF can occur by donation of one-carbon group from serine to form 5,10-MTHF, which can subsequently be

reduced to 5-MeTHF by MTHFR (Fig. 10). Thus, cycling of folates is dependent on the MS and the MTHFR enzyme (van der Put *et al*, 2001).

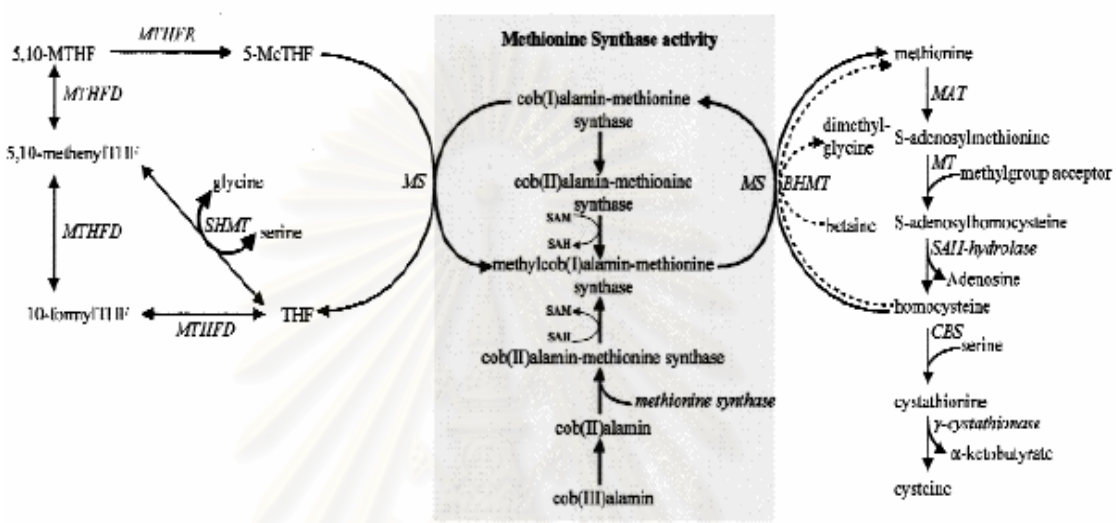

Figure 10. Key enzymes of homocysteine metabolism. Enzymes are presented in italics. MTHFR, methylenetetrahydrofolate reductase; SHMT, serine hydroxymethyltransferase; BHMT, betaine homocysteine methyltransferase, MAT, methionine adenosyltransferase; SAH-hydrolase, S-adenosylhomocysteine hydrolase; MT, methyltransferase; CBC, cystathionineβ-synthase; SAM, S-adenosylmethionine; SAH, S-adenosyl- homocysteine; THF, tetrahydrofolate; and 5-MeTHF, 5-methyltetrahydrofolate (van der Put *et al*, 2001).

Homocysteine

Biochemical aspects

Homocysteine (Hcy) is an amino acid with a free thiol (sulphydryl, -SH) group. It readily oxidizes to form various disulfides at physiological pH because the pKa of the thiol group is 8.9. Hcy is not found in the diet but is an intermediate formed during the metabolism of methionine to cysteine. Methionine is an essential amino acid and the only source of Hcy in the body. The essential role of methionine derives from its utilization in the synthesis of proteins and S-adenosylmethionine (SAM). A small portion of the latter compound provides the polyamine moiety necessary for the formation of spermine amd spermidine. The bulk of SAM, however, is the methyl donor in a wide range of transmethylation reactions yielding vitally important methylated compounds such as creatine and methylated DNA. The transfer of the methyl group results in the formation of S-adenosylhomocysteine (SAH). This compound is toxic and rapidly loses its adenosine to become Hcy (Rasmussen *et al*, 2000).

Hcy is a sulfur-containing, nonproteinogenetic amino acid biosynthesized from methionine that takes a key place between folate and activated methyl cycles, and which has three main metabolic fates (Fig. 11): to be remethylated to methionine, to enter the cysteine biosynthetic pathway, or to be released into the extracellular medium. Clearly, this third metabolic fate of Hcy is the direct cause of increased concentration of total homocysteine (tHcy) in extracellular fluids like urine and plasma. Another pathway of Hcy metabolism that involving its deamination, has been shown to be of no significant physiological importance in man and will not be discussed (Medina *et al*, 2001).

Homocysteine in plasma

Studies with isolated cells show that Hcy export into extracellular media reflects an imbalance between Hcy production and metabolism, either at low or high Hcy concentrations. At low Hcy concentrations, Hcy export rates are higher because methionine synthase activity is lower; for this reason, plasma total Hcy is markedly increased with folate or vitamin B_{12} deficiency. On the other hand, at high Hcy concentrations, Hcy export increase with decreased CBS activity. In spite of the relevance of

increased export in explaining increased homocysteinemia, to date there is no comprehensive study of Hcy transport across the plasma membrane, or identification of the carriers involved, although the involvement of a general, nonspecific transporter for neutral amino acid efflux (such as the L system) may be suspected.

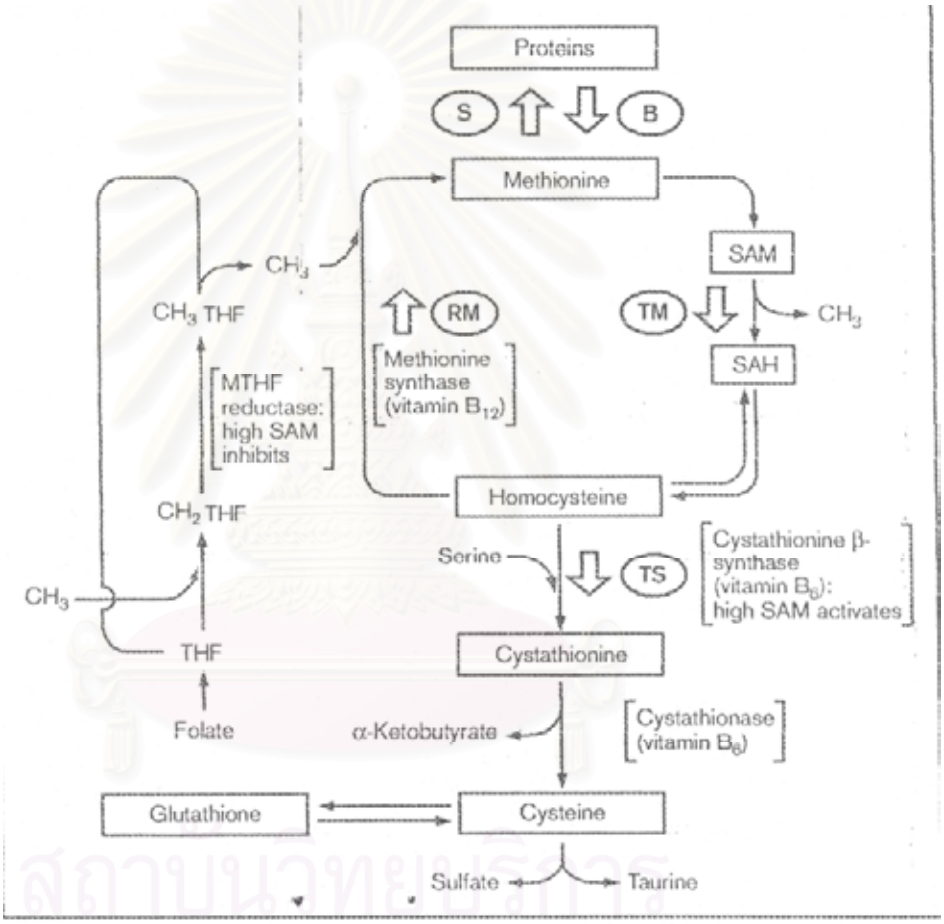

Figure 11. Sulfur amino acid metabolism. Open arrows indicate metabolic rates that can measured using tracer methods. Square brackets surround the names of enzyme.CH_3, methyl; CH_2THF, methylenetetrahydrofolate; CH_3THF, methyltetrahydrofolate; RM, remethylation pathway; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate; TM, transmehtylation pathway; TS, transsulfuration pathway (Hoffer, 2002).

Human plasma contains both reduced and oxidized species of Hcy. The thiol group of Hcy allows it to form a disulfur bond with other Hcy molecules, with free cysteine, or with thiol groups of plasma proteins, such as albumin (Fig. 12). It is remarkable that the oxidized forms are overwhelmingly in the majority (up to 99%) and reduced Hcy represents not more than 1% of total Hcy. The sum of all forms of Hcy existing in plasma is usually called total homocysteine (tHcy).

Protein-bound Hcy represents up to 75-80% of total Hcy. Both experimental and clinical studies demonstrate the presence in plasma of binding site for aminothiols, which interact preferentially with Hcy. A rapid equilibrium exists between free and protein-bound Hcy fractions in vivo. Thus, transitorily increased free Hcy, induced by an increase in Hcy export or by methionine loading, becomes progressively bound to plasma protein in a redistribution which takes place in less than 24 hours.

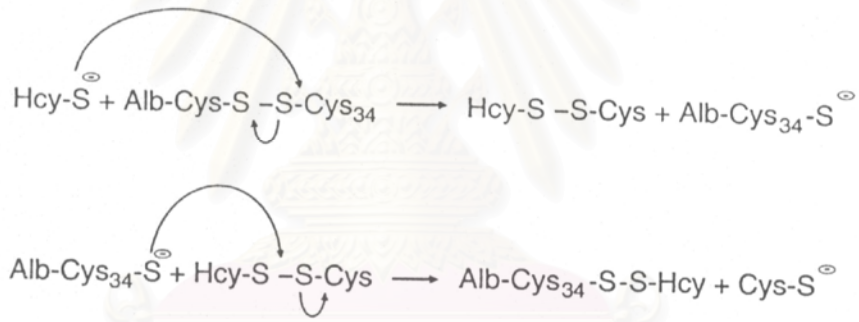

Figure 12. Mechanism of albumin bound homocysteine. Homocysteime first displaces the cysteine residue present in position 34 of albumin. The favored reactions are as follows: the homocyateine sulfydryl group reacts with the sulfur of the cysteine residue, forming the homocysteine-cysteine mixed disulfide and free albumin thiolate anion. Subsequently, albumin thiolate anion attacks the homocysteine-cysteine mixed disulfide on the homocysteine sulfur to form albumin-bound homocyeteine and cysteine thiolate anion (Perna *et al*, 2003).

Factors influencing plasma Hcy levels include demographic, genetic and physiological factors, as well as acquired determinants including habits, nutrition, diseases, transplants and medication. Normal concentrations of total Hcy in plasma, in adult, are in the range of 5-16 μM, although perhaps 10 μM should be considered the desirable upper limit, because it is possible to achieve this level by optimal nutrition with

respect to folic acid and both vitamin B_6 and B_{12}. Over 16 μM, three ranges of hyperhomocysteinemia are defined:moderate (16-30 μM), medium (30-100 μM) and severe (> 100μM) hyperhomocysteinemia, reaching values as high as 500 μM in patients with homocystinuria (Medina *et al*, 2001).

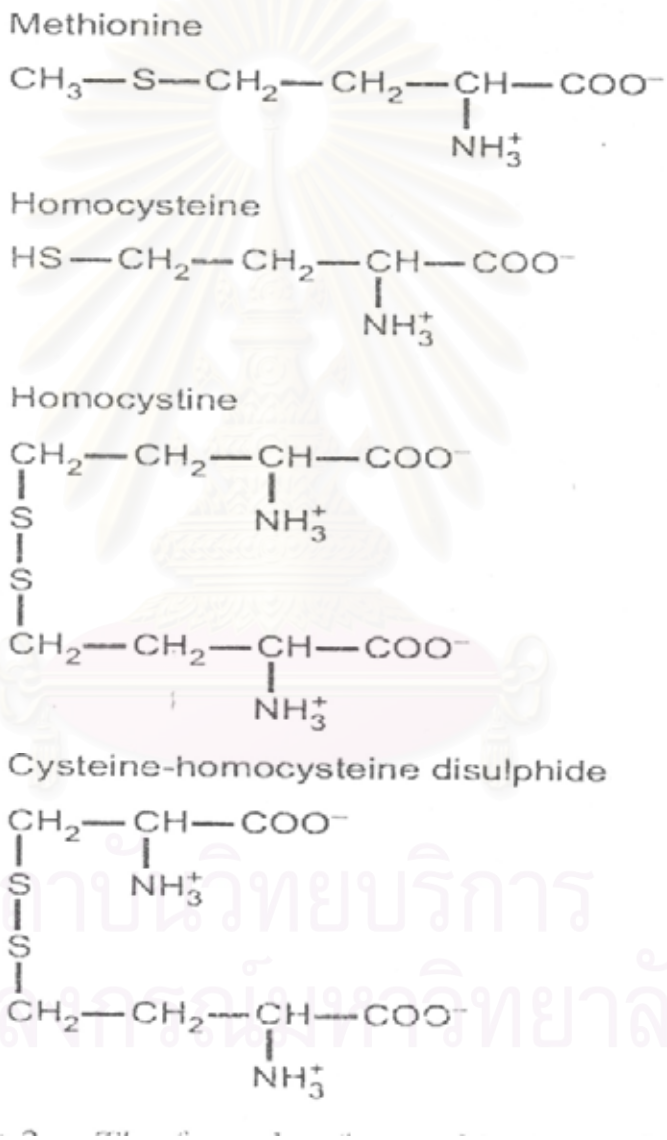

Figure 13 The formulation of methionine and circulating homocysteine species. Most of homocysteine in plasma is in the oxidized form as disulfides, the majority forming disulfides with the free sulphydrylgroups of cysteine incorporated in proteins, probably mainly albumin (Rasmussen *et al*, 2000).

The source and fate of homocysteine in plasma

The origin of Hcy in plasma is uncertain, but in vitro studies point to the liver and proliferating cells as the important sources. Only about 6 μmol/day, or less than 0.05% of total cellular Hcy production, is excreted in the urine. Thus, plasma Hcy must be taken up by cells and then metabolized (Rasmussen *et al*, 2000).

Circulating species of homocysteine in plasma

Hcy occurs in plasma as the free thiol, its symmetrical disulfide (homocystine), an asymmetrical mixed disulfide homocysteine-cysteine or conjugated with protein through disulfide bonding (Fig. 13) (Rasmussen *et al*, 2000).

The concentration of free Hcy in plasma is very low and accounts for less than 2% of plasma Hcy in normal subject. The concentration of Hcy and homocysteine-cysteine represent approximately 10-15% and protein bound Hcy accounts for over 80% of the measured total Hcy in normal plasma (Fig.14). Binding of Hcy to plasma proteins seems to be saturable, with a maximal capacity of about 140 μmol/L. Proteins containing reactive cysteine residue that carry Hcy in the circulation have not been identified with certainly, although there is some evidence suggesting that albumin may be the major carrier (Hoffer, 2002)(Rasmussen *et al*, 2000).The following terminology was recently recommended: homocysteine in general is refered to as ‘Hcy’; total homocysteine as ‘tHcy’; protein bound homocysteine as ‘bHcy’; non protein bound (‘free’) homocysteine as ‘fHcy’; homocysteine-cysteine mixed disulfide as ‘Hcy-Cys’; homocystine as ‘Hcy-Hcy’; homocysteine-disulfieds generally as ‘Hcy-SR’; and reduced homocysteine as ‘HcyH’ (Hoffer, 2002).

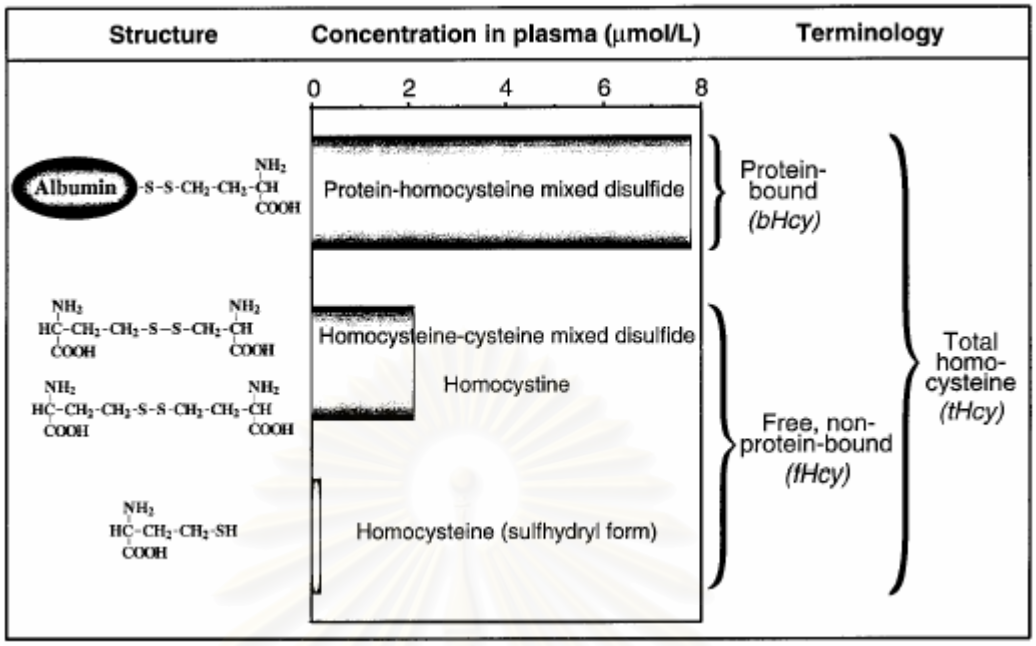

Figure 14. Homocysteine and the major related disulfides in normal human plasma (Friedman *et al*, 2001).

Homocysteine metabolism

Hcy is involved as an essential intermediate in the transfer of activated methyl groups from tetrahydrofolate to SAM in the so called activated methyl cycle. Hcy is also an intermediate in the lineal pathway of synthesis of cysteine from methionine, known as the transulfuration pathway. A third metabolic role for Hcy is its activation to homocysteinyl-tRNA, with the potential production of highly reactive derivative Hcy thiolactone. Hcy thiolactone is presented in mammalian cell cultures but has been reported to be absent in human plasma (Rasmussen *et al*, 2000), possibly because non-specific esterases, present in plasma as well as on the surface of endothelial cells, rapidly hydrolyse the thiolactone to Hcy. A metabolic scheme is presented in Fig. 15 and the main metabolic roles of Hcy are further explained below.

The activated methyl cycle or remethylation pathway

In many biosynthetic reactions, a transfer of methyl groups is required. Tetrahydrofolate can behave as a carrier and donor of methyl

groups, but its transfer potential is not high enough for most biosynthetic methylations. The universal activated methyl donor is SAM, which is synthesized by the transfer of an adenosyl group from ATP to the sulfur atom of methionine in a nonequilibrium reaction. When SAM transfers its methyl group to an acceptor, SAH is formed. The hydrolysis of SAH acted on by S-adenosylhomocysteine hydrolase leads to the formation of Hcy and adenosine (Medina *et al*, 2001).

In the activated methyl cycle, Hcy is remethylated to methionine by transfer of a methyl group from *N*-5-methyltetrahydrofolate, catalyzed by methionine synthase or *N*-5-methyltetrahydrofolate-hmocysteine methyltransferase, an enzyme that requires vitamin B_{12} as a cofactor. This is the last point of the activated methyl cycle and the point shared with the folate cycle. Tetrahydrofolate can accept one carbon groups in either *N*-5 and/or *N*-10 nitrogen atoms. For instance, the transfer of a one carbon group from serine produces glycine and *N*-5,*N*-10-methylenetetrahydrofolate. This folate cycle is closed with the reaction catalyzed by MTHFR, to produce *N*-5-methyltetrahydrofolate.

In the liver and kidney of rats, a substantial proportion of Hcy is remethylated by an alternative route in which betaine serves as a methyl donor for the reaction catalyzed by betaine-homocysteine methyltransferase. In humans, some evidence indicates that a significant amount of dietary choline can be utilized for betaine dependent methyl transfer.

Recently, an alternative pathway for the conversion of Hcy into methionine has been proposed. This biochemical pathway involves Hcy thiolactone formation. It seems that Hcy thiolactone formation is a sign of inadequate methylation of Hcy-tRNA to methionine-tRNA. Because in many cases N-terminal methionine is released from the polypeptide after chain maturation, Hcy converted by this pathway can enter the cellular methionine pool.

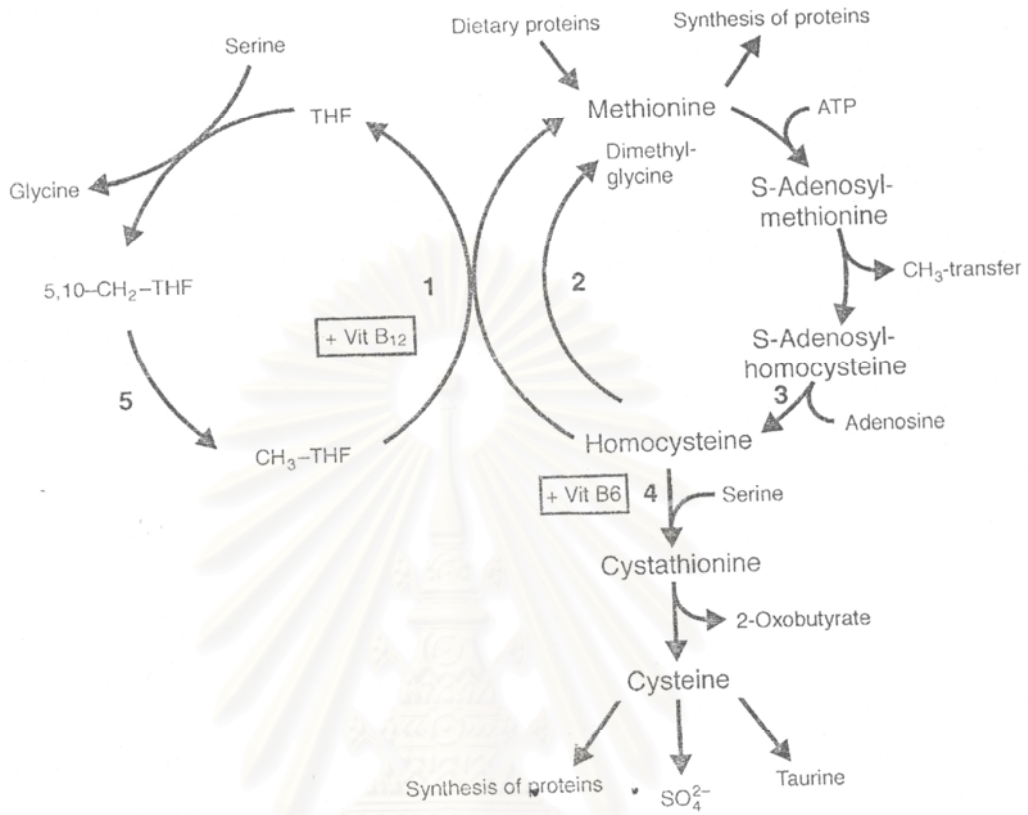

Figure 15. The metabolism of homocysteine. The enzymec **(1)** 5-methyltetrahydrofolate:homoctsteine methyltransferase (EC 2.1.1.13), which uses cobalamin (vitamin B12) as a coenzyme, transfers a methyl group from 5-methyltetrahydrofolate (5-MeTHF) to homocysteine to form methionine. 5-MeTHF is made by reduction of 5,10-methylenetetrahydrofolate (5,10-MTHF), the compound of central importance in folate metabolism, by the enzyme **(5)** 5,10-methylenetetrahydrofolate reductase (EC 1.7.99.5).An alternative pathway for the methylation of homocysteine to methionine is mediated by the enzyme**(2)** betaine:homocysteine methyl transferase (EC 2.1.1.5) using betaine as methyl donor. S-adenosylmethionine (SAM) is the methyl donor in a wide range of transmethylation reactions. The loss of the methyl group results in the formation of S-adenosyl-homocysteine (SAH), which issubsequently converted to homocysteine by the enzyme **(3)** S-adenosylhomocysteine hydrolase (EC 3.3.1.1). In the transsulfuration pathway, homocysteine is condensed with serine to form cystathionine by the pyridoxal phosphate (vitamin B6) dependent enzyme **(4)** cystathionine-β-synthase (EC 4.2.1.22) (Rasmussen *et al*, 2000).

The transulfuration pathway

Cysteine is biosynthesized from methionine through the so called transulfuration pathway. The first three steps of this pathway are shared with the activated methyl cycle and lead to formation of Hcy from methionine. In the transulfuration pathway, Hcy is the substrate of the vitamin B_6-dependent enzyme CBS, which catalyzes its condensation with serine to form cystathionine. This is the critical step in the pathway because it is irreversible under physiological conditions; from this point on, Hcy is committed to follow this pathway. In the last step of the transulfuration pathway, cystathionine is cleaved by γ-cystathionase, another vitamin B_6-dependent enzyme, to form 2-oxoglutarate and cysteine. Excess cysteine is oxidized to taurine and eventually to inorganic sulfates. Thus, in addition to the synthesis of cysteine, this pathway can catabolize effectively potentially toxic excess Hcy that is not required for methyl transfer (Medina *et al*, 2001).

Impairment of homocysteine metabolism

1. Determinants of homocysteine metabolism impairment

There is a tight regulation of Hcy metabolism based upon the very different affinities of methionine synthase and CBS for Hcy: the first enzyme shows low Km values for Hcy (below 0.1 mM), and the second one has high Km values for Hcy (over 1 mM). Thus, at low Hcy concentrations, methionine conservation is favored; and at high Hcy concentrations, immediate and long term drainage of Hcy via the transulfuration pathway is ensured.

As stated above, abnormal elevations of Hcy in plasma and urine are the result of increased levels of Hcy export, and this reflects an imbalance between Hcy production and metabolism. Several congenital and nutritional disorders, as well as renal failure can induce this situation.

Congenital disorders involve polymorphism in the genes coding for MTHFR, methionine synthase and CBS. The most common genetic defect associated with mild hyperhomocysteinemia is a point mutation, namely, a C to T substitution at nucleotide 677 (C677→T) in the open

reading frame of the gene for methylenetetrahydrofolate reductase. This point mutation causes a substitution of valine for alanine in the functional enzyme, giving rise to a thermolabile variant of the enzyme with decreased total activity. This is an autosomal recessive mutation, and the frequency of the C677→T polymorphism varies among racial and ethnic groups, with 10-13% of T/T homozygous and 50% C/T heterozygous among Caucasion and Asian populations and very low incidence among African-Americans. The widely documented elevations in plasma total Hcy levels associated with the homozygous T/T genotype might lead to a higher expected incidence of cardiovascular disease in the T/T population. However, little or no evidence has been found so far linking the T/T genotype with increased rates of cardiovascular disease, although some reports seem to link the T/T genotype with increased incidence of certain forms of vascular disease in selected populations. In this context, it has been suggested that an elevated plasma Hcy level may not necessarily be deleterious, but it could promote vascular blockage under conditions predisposing to vascular disease.

Nutritional disorders that potentially lead to an impairment of Hcy metabolism include deficiencies of vitamin B_{12}, folate and vitamin B_6, as the de novo synthesis of methionine groups requires both vitamin B_{12} and folate cofactors whereas the synthesis of cystathionine requires pyridoxal 5'-phosphate (vitamin B_6). Although it has been shown that deficiencies of vitamin B_{12} and folate are related to increased plasma concentrations of Hcy, the relationship of Hcy levels to vitamin B_6 status is less clear.

A study in rats has identified the kidney as a major site for the removal and metabolism of Hcy. It seems that a metabolic channeling occurs leading this Hcy, removed from the blood by the kidney, to be metabolized primarily through the transulfuration pathway. Renal impairment commonly causes hyperhomocysteinemia, reflecting the key role of kidney in Hcy clearance from plasma; this fact may contribute to the high incidence of vascular complications in patients with chronic renal failure (Medina *et al*, 2001).

2. Role of S-adenosylmethionine in the control of homocysteine metabolism

Medina et al. (2001) have proposed a central role for SAM in the coordinated control of Hcy metabolism. SAM is an allosteric inhibitor of

methylenetetrahydrofolate reductase, an in vitro inhibitor of betaine-homocysteine methyltransferase and an activator of CBS. The ability of SAM to act as an enzymatic effector of Hcy metabolism provides a means by which remethylation and transulfuration pathways can be coordinated. When cellular SAM concentration is low, the synthesis of 5-methyltetrahydrofolate will proceed uninhibited whereas cystathionine synthesis will be suppressed, resulting in the conversion of Hcy for methionine synthesis. Conversely, when SAM concentration is high, Hcy is derived through the transulfuration pathway because of inhibition of 5-methyltetrahydrofolate synthesis and stimulation of cystathionine synthesis. Thus, although the primary effect of this coordinated control is the regulation of cellular SAM concentrations, it also contributes to the maintenance of a Hcy concentration compatible with the need for de novo methyl groups.

3. Hypomethylation

SAM is the main methyl group donor. Cellular methylation, which is involved in the synthesis of phospholipids, nucleic acids, amines and other neurotransmitters, regulates gene expression and modifies protein function, cannot occur without the proper function of methyltransferases. In the presence of adenosine, Hcy is efficiently converted to SAH, a potent inhibitor of methyltransferase reactions. In fact, SAH is the product of methyltransferase reactions, which use SAM as the substrate. Most methyltransferase bind SAH with higher affinity than SAM and, consequently, there are subject to potent product inhibition. Thus, one of the basic biochemical mechanisms of hyperhomocysteinemia toxicity is a hypomethylation through SAH accumulation.

The SAM/SAH ratio is a good and important indicator of cellular methylation status. It has recently been reported that physiologically relevant concentrations of Hcy (but not other thiols) in the presence of adenosine inhibited the growth of vascular endothelial cells by a mechanism involving a decreased carboxymethylation of $p21^{ras}$. This effect of Hcy is not observed in smooth muscle cells, a difference that might be explained by the ability of Hcy to dramatically increase levels of SAH in vascular endothelial cells but not vascular smooth muscle cells.

Mature human erythrocytes are highly differentiated cells that have lost the ability to biosynthesize proteins *de novo*. During cell aging in

circulation, erythrocyte proteins undergo spontaneous post-translational modifications, regarded as protein fatigue damage. New molecules cannot replace these damaged proteins, but they can be repaired to a significant extent through an enzymatic transmethylation reaction. Membrane protein methylation levels are consistently reduced in erythrocytes of both chronic renal failure and hemodialysis patients, two population groups will higher mean plasma Hcy levels (and, thus, increased hypomethylation) than healthy subjects. Very recently, it has been shown that peripheral arterial occlusive disease patient present disturbed erythrocyte and plasma SAM/SAH ratios, suggesting that methylation may be impaired in these patients.

In the case where the efficiency of methylatransferase reactions must be preserved, an efficient removal of SAH is required. This is effectively accomplished by SAH hydrolase, an enzyme that appears to act in close proximity to the methtltransferase, at least in the nucleus. Interestingly, the polypeptide folding pattern at the catalytic domain of SAH hydrolase is almost identical to that reported for the DNA methyltransferases and suggests that SAH can travel easily between the catalytic pockets of the two enzymes. This binding similarity further supports an important role for excess SAH in the regulation of methylation reactions. Very recently, it has been shown for the first time that moderate elevation in plasma total Hcy concentration is positively associated with parallel increases in plasma SAH concentrations and lymphocyte DNA hypomethylation. These data support an indirect mechanism for Hcy pathogenicity secondary to inhibition of DNA methyltransferase: the disruption of the heritable methylation patterns in DNA can lead to alterations in chromatin structure and alteration in gene expression that can promote chronic diseases (Medina *et al*, 2001).

Hyperhomocysteinemia has been associated with both folate and cobalamine deficiencies, and also with pregnancy complications, neural tube defects, mental disorders, cognitive impairment in the elderly, psoriasis and some tumors. Furthermore, moderately increased concentrations of tHcy have been associated with an increased risk of cardiovascular disease, including atherosclerosis and thrombosis. All this explains the increased interest of basic and clinical biomedical researchers in Hcy, as shown by the explosion of articles containing the keyword Hcy during the past few years.

To understand clearly the basis of hyperhomocysteinemia pathogenicity, its metabolic roles should be revisited. In fact, new

metabolic roles are being described for Hcy that could help to understand the pathological consequences of increased levels of Hcy. They include its interactions with key modulator molecules, its own ability to modulate certain enzyme activities, or its involvement in protein modification (Medina *et al*, 2001).

Hyperhomocysteine related methotrexate neurotoxicity

Elevated plasma homocysteine has also been documented in leukemic children receiving a high dose of MTX (Quinn, 1996). MTX readily interferes with homocysteine metabolism. MTX impairs the regeneration of tetrahydrofolate from dihydrofolate by inhibition of DHFR, involving other folic metabolism enzyme, thereby decreasing cellular concentrations of reduced folates. Consequently, depletion of 5-methyltetrahydrofolate will inhibit the remethylation of homocysteine. Cellular homocysteine elevates and results in a rise in plasma homocysteine concentration. What are the consequences of hyperhomocysteinemia? Stricking effects are seen in patients with classic homocystinuria caused by CBS deficiency, including seizures and premature vascular disease resulting in stroke, myocardial infarction and venous thromboembolism.

Focal neurologic deficits, mineralizing microangiopathy and radiographic ischemic white matter changes are reported as consequences of MTX therapy. This suggests that occlusive vascular disease and resultant ischemia may be implicated in the pathogenesis of MTX neurotoxicity. Homocysteine,in light of current evidence of its role in vascular damage, may be in part as the mediator of this toxicity. Moreover, certain individuals may be at high risk for MTX induced hyperhomocysteinemia, including those with polymorphisms of important enzyme in homocysteine metabolism such as the thermolabile variant of MTHFR which has only 35% to 40% of normal activity. Individuals with this allele manifest intermediate hyperhomocysteinemia.

There is another related aspect of homocysteinemetabolism that may prove to be important in the pathogenesis of MTX neurotoxicity. Patients with classic homocystiuria are known to have unusual sulfur containing amino acids in their plasma and urine that are not found in the plasma or urine of normal individuals. One of these amino acids has been identified as homocysteic acid (HCA), an oxidized metabolite of

homocysteine. Cysteine sulfinic acid (CSA) is another sulfur containing amino acid that may accumulate in hyperhomocysteimic states resulting from folate deficiency caused by the shunting of homocysteine through the transulfuration pathway yielding cysteine. The oxidative conversion of cysteine to CSA is well characterized. Both HCA and CSA belong to a class of putative neurotransmitters known as excitatory amino acids (EAAs). Both HCA and CSA are known as endogenous agonists of the N-methyl-D-aspartate (NMDA) receptor, a subtype of glutamate receptor widely distributed throughout the central nervous system. These receptors mediate a majority of the excitatory synaptic in the CNS. Excitatory amino acids likely play a role in the pathogenesis of seizures, and NMDA receptor antagonists have been shown to be the effective anticonvulsive agents (Quinn and Kamen, 1996; Quinn et al, 1997). It has been proposed that HCA, produced in excess in patients with hyperhomocysteinemia and homocystinuria, is the cause of the seizures frequently occurring in this disorder. It is plausible that these EAAs, which can accumulate in hyperhomocysteinemic state, may be responsible in part for the seizures resulting from MTX administration. Furthermore, folate, cobalamin and pyridoxine deficiencies conditions characterized by Hcy accumulatation are also associated with neuropsychiatric symptomatology. It has been postulated that this neuropsychiatric toxicity is also mediate by the effects of EAAs, which could accumulate due to the hyperhomocysteinemia or hyperhomocysteine. Likewise, it is plausible that EAAs are excitotoxicity. For example, neuronal injury consequent to the actions of excessive EAAs activity manifest clinically symptom such as MTX related leukoencephalopathy (Quinn and Kamen, 1996).

CHAPTER III

Materials and Methods

1. Subjects

29 children with acute lymphoblastic leukemia (ALL) treated with methotrexate are participated in this study.

Inclusion criteria

All children with ALL receive methotrexate given as high dose by intravenous injection according to their body mass index (BMI).

Exclusion criteria

- Patients receiving drugs that interfere with folate metabolic pathways such as antiepileptic drug, nitrous oxide, theophylline, D-penicillamine and sulfazalazine.

- Patients diagnosed with renal or hepatic impairments.

2. Sample preparations

ALL patients received high dose methotrexate (HDMTX) with 5 g/m^2, 2 g/m^2 or 1.5 g/m^2 for patient assigned to the high risk, standard risk or low risk, respectively. Blood was collected in EDTA tube before HDMTX infusion and after stopped HDMTX immediately in each case.

Blood sampling

Two milliliters of whole blood was collected by venipuncture into a Vacutainer Tube containing EDTA, cooled on ice, and centrifuged at 3000g for 10 min. After centrifugation, plasma was separated and kept frozen at -20°C until analysis.

3. Isolation of Genomic DNA

DNA was extracted from whole blood. Genomic DNA was isolated using the DNA Blood Mini Kit.

4. PCR Amplification and MTHFR polymorphism detection

To determine MTHFR genotype, we use the polymerase chain reaction (PCR)/restriction fragment length polymorphism (RFLP) method.

5. Agarose Gel Electrophoresis

The restriction digest products are separated on 4% agarose gel and stained by ethidium bromide. The running condition is 100 volts for 1 hour. The gel is then visualized under UV visible.

Principle of Polymerase Chain Reaction

Basic aspects of polymerase chain reaction

The polymerase chain reaction (PCR) is an *in vitro* DNA amplification method that involves a repeated cycling process of a number of defined stages. Reagents required for the PCR include a DNA polymerase, each of the four nucleotide dNTP building blocks of DNA in equimolar amounts, and a source of template DNA, such as genomic DNA or cDNA containing the target sequence. The technique also demands the availability of two oligonucleotide primers designed to complement DNA sequences flanking the region of interest. The primers may be of variable length but are usually in the region of 15-30 base pair (bp) and although for some specific purpose may vary or be degenerate in their sequence, they are usually directly complementary. This demands that, in general, sequence information be available for part of the DNA sequence that is to be amplified.

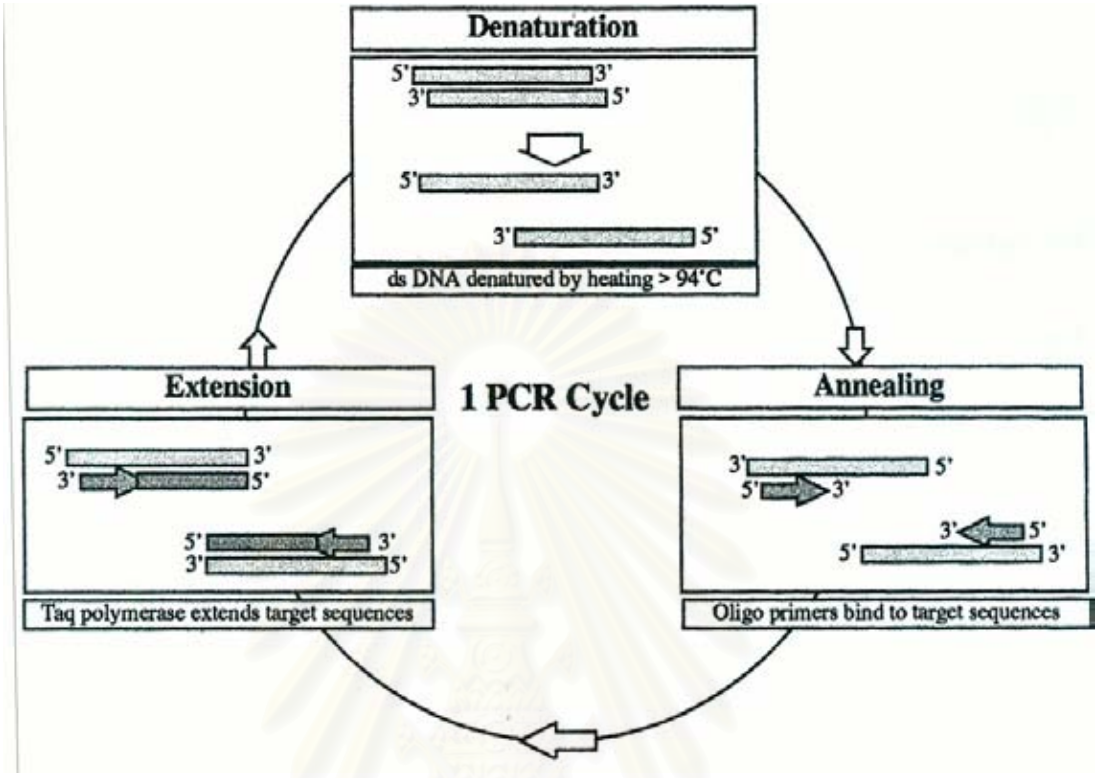

Figure 16. Three stages of one PCR cycle (Rapley, 1998).

Amplification takes place in repeated cycles made up of the defined stages, termed denaturation, annealing and extension (Fig.16). In the first stage of denaturation the template DNA is heated in excess of 90°C for at least 60second to separate the double stranded DNA and produce two single strands. This is followed by a second stage, known as annealing, where the temperature is reduced to 35- 55°C for a time interval of between 60 and 120 second to allow the oligonucleotide primers to bind to their complementary DNA sequences on the single strands produced in the previous stage. In the final stage of the cycle an enzymatic primer extension reaction is carried out producing complementary copies of the initial single strands from the primers bound to the DNA. This step usually takes place at 72°C for 60-180 second by a DNA polymerase that is able to withstand the high denaturation temperature. This heat stable enzyme is the key to the PCR and was initially isolated from the bacterium *Thermus aquaticus* found in hot

springs and termed Taq DNA polymerase. The three separate stages are usually repeated between 25 and 40 times. In this way the double stranded products of the previous cycle become new templates for the next cycle such that in each round the amount of the specific target DNA flanked by the primers essentially doubles (Fig.17). This results in the near exponential accumulation of the specific target DNA sequence of up to a million fold in 3-4 hours. Linear amplification of the initial strands also takes place and contributes to the PCR not being 100% efficient. Because of the repeated nature of the cycles, the PCR may be automated and numerous automatic thermal cycles have been designed and produced specifically for the PCR.

In general, lengthy template sample preparations are not required for the PCR to work efficiently and because of the sensitivity of the technique relatively crude DNA samples may be used as templates. It is also possible to analyze DNA samples of poor condition by PCR as only relatively short intact sequences are required. Thus, paraffin embedded material or even ancient samples may be analyzed. This now makes previously difficult retrospective studies quite straightforward to perform (Rapley, 1998).

Reaction components of PCR

The components of a polymerase chain reaction require the following: DNA polymerase, deoxynucleoside triphosphates, Tris buffer, non-ionic detergent, magnesium chloride, gelatin or bovin serum albumin (BSA), primers and target DNA (Rasmussen, 2000).

1. DNA polymerase

The most commonly used DNA polymerase is *Taq* polymerase isolated from *T. aquaticus.* Its advantages of heat stability and high temperature optimum make an ideal choice, although other heat stable DNA polymerase could be used. The activity of *Taq* polymerase roughly doubles from 65°C to 72 °C, the temperature of the assay is also important. *Taq* polymerase activity is optimal over a fairly broad pH range from 8.2-9.0 in 10 mM Tris (measured at 25 °C), but declines at higher or lower pH.

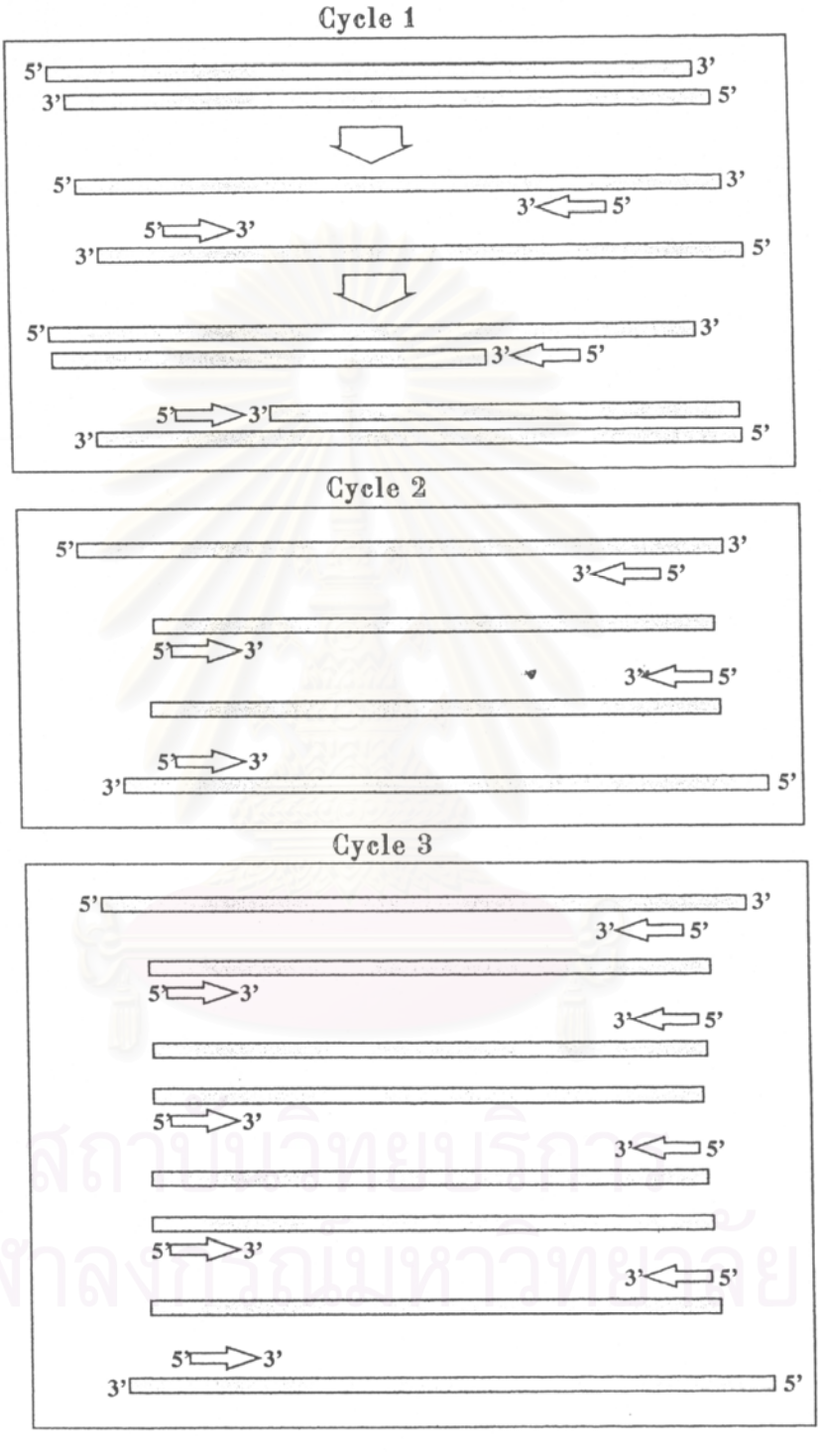

Figure 17. First few cycles in the PCR (Rapley, 1998).

2. Deoxynucleoside triphosphates (dNTPs)

dNTPs is a premixed solution containing the sodium salts of dATP, dCTP, dGTP and dTTP, each at a concentration of 10 mM in water. The total concentration of nucleotides, therefore, is 40 mM (pH 7.5). Addition of 1 μl of dNTPs to a 50 μl reaction will give a final concentration of 200μM for each dNTP. Precursor dNTPs can be obtained by either freeze-dried or as neutralized aqueous solutions. They are stable at -20°C for some month and the freeze dried reagents may require neutralization by KOH before use. dNTPs can be stored at -20°C for several months.

3. Reaction buffer

This contains 10 mM Tris-HCl (pH 9.0 at 25°C), 50 mM KCL, 1.5 mM MgCl2, and 0.1% Triton®X-100. Store at -20°C.

4. Primers

Oligonucleotide primers are generally synthesized in the range 18-30 bases, though it is possible to amplify low complexity DNA (e.g. plasmid or previously amplified DNA) with shorter primers.

Reaction conditions of PCR

The selection of times, temperatures, and number of cycles depends on the DNA being amplified and the primers chosen. Reaction volumes of between 10 to 100 μl are generally used. Small volumes are an advantage for batch screening large numbers of samples, because of the saving in reagent costs, but if only a few samples are being processed it may be technically easier to work to a slightly larger reaction volume of 25-50 μl. Concentrations of primers are generally 25- 100 pmol of each primer for a 50μl reaction. Incubation times should be kept as short as possible, to reduce the overall cycling time and to minimize the risk of nonspecific amplification. Denaturing and annealing times of 30 second should be adequate and extension times which allow 1 min per kilobase of target followed by a final extended incubation time (2 min per kilobase) are more than enough. The number of cycles required depends on the abundance of target and efficiency of the PCR.

Confirmation of PCR product

Confirmation of the correctly sized amplified PCR product is usually carried out by agarose gel electrophoresis. It may also be possible to confirm the PCR product by analysis with restriction endonucleases. This depends on the presence of a suitable restriction site within the amplified sequence. Further confirmation that the amplified product is the expected sequence may be derived from hybridization studies, such as Southern blotting, with a labeled probe that anneals to a position internal to the amplified sequence.

The PCR has found many applications and may be used in the diagnosis of disease states or species identification. It is capable of detecting many sources of DNA with great specificity and therefore can be used to detect viral or bacterial infection. Multiplex PCR may be undertaken to analyze more than one product in the same reaction. This involves adding more than one set of specific oligonucleotide primer pairs to the reaction mix. If the primers produce amplified products of different size their detection is greatly facilitated, requiring only simple electrophoresis and staining. This gives a considerable saving in sample DNA, time and cost.

Principle of Restriction Fragment Length polymorphisms

Restriction endonucleases

Restriction endonucleases are bacterial enzyme that cut double stranded DNA molecules in a precise and reproducible manner. Three different classes of restriction endonucleases have been recognized, each with a slightly different mode of action. Type II restriction endonecleases are the most important and frequently used cutting enzymes. Such enzymes are named usually by a three letter abbreviation that identifies their bacterial origin. To distinguish between enzymes with the same origin, Roman numerals are added, for example *Hpa*I is derived from *Haemophilus parainfluenza* and *Eco* RI from *Escherichia coli* RY 13 (Table 2) (Rapley, 1998).

Table 2. Recognition sequence of frequently used restriction endonucleases (Rapley, 1998).

Enzyme	Organism	Recognition sequence	Cut sequence
Alu I	*Arthobacter luteus*	AG/CT	-AGCT-
		TC/GA	-TCGA-
Bam HI	*Bacillus amyloliquefaciens*	G/GATCC	-GGATCC-
		CCTAG/G	-CCCTAGG-
Bgl II	*Bacillus globigii*	A/GATCT	-AGATCC-
		TATAG/A	-TGTAGG-
Eco RI	*Escherichia coli*	G/AATTC	-GAATTC-
		CTTAA/G	-CTTAAG-
Hae III	*Haemophilus aegyptius*	GG/CC	-GGCC-
		CC/GG	-CCGG-
Hind III	*Haemophilus influenza Rd*	A/AGCTT	-AAGCTT-
		TTCGA/A	-TTCGAA-
Kpn I	*Klebsiella pneumonia*	GGTAC/C	-GTGGTACC-
		C/CATGG	-CCATGG-
Pst I	*Porvidencia stuartti*	CTGC/C	-CTGCG-
		G/ACGC	-GACGC-
Sma I	*Serratia marcescens*	CCC/GGG	-CCCGGG-
		GGG/CCC	-GGGCCC-

Restriction enzymes recognize specific sequence in double stranded DNA and cleave the DNA, usually within the recognition site, to yield fragments of defined length. The specific recognition sequence of double stranded DNA for the vast majority of type II restriction enzymes is usually between 4 and 6 base pairs in length and generally palindromic; in other words, a sequence of bases is the same on both strands when read in a 5’ to 3’ direction. Restriction enzymes cut the DNA at every point at which the target sequence occurs. Many restriction endonucleases cut in the middle of the recognition sequence to produce a “blunt end”, whereas others cut at different nucleotides usually 2 or 4 bases apart resulting in DNA fragments with short single stranded overhangs at each end known as “sticky or cohesive ends” (Fig.18). Using restriction endonucleaes to digest DNA, a physical map of the molecule can be obtained, identifying the sites of cleavage, separated by actual distance along the strand. This is known as a restriction map. Such a map can obtain for any DNA sequence regardless of the presence of mutations or if its function is unknown.

Detection and analysis of the reaction product

The product of a PCR should be a fragment or fragments of DNA of defined length. The simplest way to check, is to load a fraction of the reaction product and appropriate molecular weight markers onto an agarose gel of 0.8-4% containing ethidium bromide. The product should be readily visible under ultraviolet transillumination.

I. Blunt end production

cleavage point in recognition sequence

↓

5’--------AGCT----------3’ Alu I 5’-----AG CT-----3’

3’--------TCGA----------5’ → 3’-----TC GA-----3’

↑

II. Cohesive end production

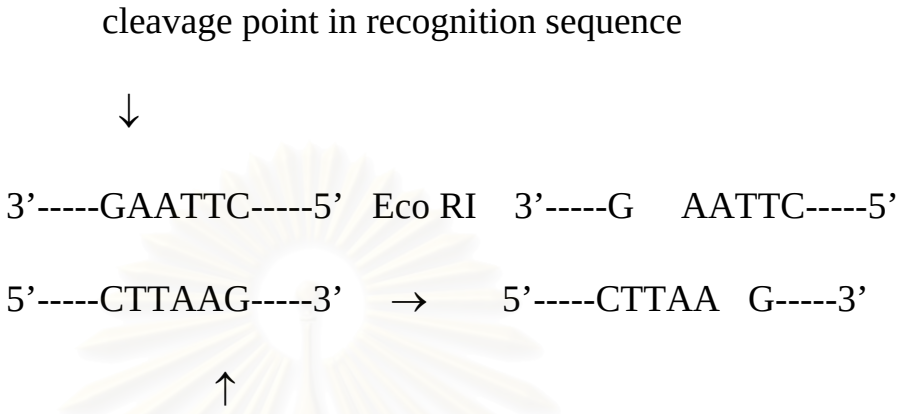

Figure 18. Restriction endonucleases generate either blunt ended or cohesive ended fragments during cleavage of DNA (Rapley, 1998).

Principle of Gel electrophoresis of DNA

The ability to separate and visualize DNA strands from as small as 5 bp to as large as 5,000,000 bp forms. The wide size range of DNA molecules that can be handled effectively derived from the application of three essentially similar to a large extent overlapping techniques of gel electrophoresis, namely polyacrylamide gel electrophoresis (Table 3). In each case, DNA molecules are moved through a gel matrix by the application of an electric field. The gel matrix consists of pores through which the DNA molecule must pass. In both polyacrylamide and agarose gel electrophoresis, a voltage applied at the ends of the gel produces an electric field with a strength determined by both the length of the gel and the potential difference at the ends. Owing to the presence of negatively charged phosphate groups along the backbone of the DNA molecule, the DNA chain will migrate toward the anode at the application of an electric field. Because the charge to mass ratio of DNA molecules is constant, the rate of migration in the absence of the gel would also be constant. However, in the gel matrix, it is frictional drag through the gel that essentially governs the rate of migration. Larger molecules move more slowly because of greater frictional drag and because they worm their way through the pores of the gel less efficiently than smaller molecules (Rapley, 1998).

Table 3. Resolution of DNA gels (Rapley, 1998).

Gel matrix	%	Range of separation	Comments
Polyacrylamide	20	5-100 bp	Xylene cyanol migrates at around 50 bp
	15	20-150 bp	
	12	50-200 bp	
	8	60-400 bp	Standard concentration for DNA sequencing gels
	6	100-600 bp	
	3.5	1 kb- 2 kb	Xylene cyanol migrates at around 450 bp
Agarose	3	0.1-1 kb	Can separate small fragments differing from each other by a small amount. Must be poured rapidly
	2	0.2-1.5 kb	Do not allow to cool to 50 °C before pouring
	1.5	0.3- 3 kb	As for 0.8%, bromophenol blue runs at about
	1	0.5- 5 kb	500bp
	0.8	1- 71 kb	As for 0.8% General purpose gel; separation not greatly affected by choice of running buffer; bromophenol blue runs at about 1kb
	0.6	3- 10 kb	Gel mechanically weak but with care can use low melting point agarose
	0.4	5- 30 kb	As above
	0.3	5- 40 kb	Gel very weak mechanically; separation in20-40 kb range improved by using high ionic strength; use only high melting point agarose

Pulse field		Up to 2000 kb Up to 5000 kb	FIGE (Field Inversion Gel Electrophoresis) CHEF (Contour clamped homogenous field electrophoresis)

Agarose Gel Electrophoresis

Agarose is a linear polymer that is extracted from seaweed and is sold as a white powder. The powder is melted in buffer and allowed to cool, whereby the agarose forms a gel by hydrogen bonding. The hardened matrix contains pores, the size of which depends on the concentration of agarose. The concentration of agarose is referred to as a percentage of agarose to a volume of buffer (W/V), and agarose gels are normally in the range of 0-3%. The resolving range of the gel is determined by the concentration of the agarose.

Gel electrophoresis is the standard method for separating DNA molecules of different lengths. It has many applications in size analysis of restriction fragments and restriction mapping, in purification of individual fragments to be cloned or sequenced, in examination of PCR products and during DNA sequencing. Electrophoresis is the movement of charged molecules in an electric field, negative charged molecules migrating towards the positive electrode and positively charged ones towards the negative electrode. The technique was originally carried out in aqueous solution, but this is not particularly useful for DNA separations because the predominant factors influencing migration rate in solution phase are the shape of a molecule and its electric charge. Most DNA molecules are in the same shape (linear) and although the charge of a DNA molecule is dependent on its length, the differences in charge are not sufficient to result in effective separation. The situation is different when electrophoresis is carried out in a gel, because now shape and charge are less important and molecular length is the critical determinant of migration rate. This is because the gel is a network of pores through which the DNA molecules have to travel to reach the positive electrode. Shorter molecules are less impeded by the pores than longer molecules and so move through the gel more quickly. Molecule of different lengths therefore form bands in the gel.

Two types of gel are used in molecular biology: agarose gels and polyacrylamide gels. Agarose is a polysaccharide that forms gels with pores ranging from 100-300 nm in diameter, the size depending on the concentration of agarose in the gel. Gel concentration therefore determines the range of DNA fragments that can be separated, a 0.3% gel, for example, being used for molecules between 5 and 50 kb, and a 5% gel being used for 100-500 bp molecules. The separation range is also affected by the electroendosmosis value (EEO) of the agarose, this being a measure of the amount of bound sulfate and pyruvate anions. The greater the EEO, the slower the migration rate for a negative charged molecule such as DNA. An agarose gel is prepared by mixing the appropriate amount of agarose powder in a buffer, heating to dissolve the agarose, and then pouring the molten gel on to a perspex plate with tape around the sides to prevent spillage. A comb is placed in the gel to form wells for the samples. The gel is allowed to set and the electrophoresis then carried out with the gel submerged under buffer. One or two dyes, of known migration rates, are added to the DNA samples before loading so the progress of the electrophoresis can be followed, and the bands of DNA are visualized by soaking the gel in ethidium bromide solution, this compound intercalating between DNA base pairs and fluorescing when activated with ultraviolet radiation(Rasmussen, 2000).

Procedure

The MTHFR 677C→T mutation alters an alanine into a valine residue. The MTHFR 677C →T mutation was analyzed according to *Fross et al; 1995*. The primers for PCR amplification were (677forword) 5′-TGAAGGAGAAGGTGTCTGCGGGA-3′ and (677 reverse) 5′-AGGACGGTG CGGTGAGAGTG-3′. The PCR reactions were conducted in a 50 μL reaction mixture containing 10 mM Tris-HCl, pH8.3, 50 mM KCl, 0.1% TritonX-100, 1.5 mM $MgCl_2$, 0.2 mM of dNTP, 100 ng genomic DNA, 10 pmol of each primer, and 0.2 units Taq DNA polymerase(Promega, Wisconsin, USA). The reaction mixture was incubated at 94 °C for 4 min for denaturation step, followed by 35 cycles of 94 °C for 30 sec, 64 °C for 30 sec and 72 °C for 45 sec, with a final extension for 7 min at 72 °C in the GeneAmp PCR system. The PCR products were digested with the restriction enzyme *Hinf* I**.** Ten μL purified PCR fragment were digested with 1μL (10 U/mL) *Hinf* I at 37 °C and incubated overnight in order to completely digest the DNA. The restriction digested fragments were then separated on 4% agarose gel

electrophoresis. The wide type homozygous (677 CC, ala/ala) genotype was characterized by a 198 base-pair (bp) fragments (bands), the heterozygous (677 CT, ala/val) genotype is characterized by both 198 and 175 bp fragments and the mutant homozygous (677 TT, val/ val) genotype is characterized by 175 and 23 bp fragments.

The MTHFR 1298A →C mutation alters a glutamate into an alanine residue, abolishing a *Mbo*II site. The MTHFR 1298A →C mutation was analyzed according to *Weisberg et al, 1998*.The primers for PCR were (1298 forword) 5'-CTTTGGGGAGCTGAAGGACTACTAC-3' and (1298 reverse) 5'-CACTTTGTGACCATTCCGGTTTG-3'. The PCR reactions were conducted in a 50 μL reaction mixture containing 10 mM Tris-HCl, pH8.3, 50 mM KCl, 0.1% TritonX-100, 2.0 mM $MgCl_2$, 0.2 mM of dNTP, 100 ng genomic DNA, 10 pmol of each primer, and 0.2 units Taq DNA polymerase (Promega®, Wisconsin, USA). The reaction mixture was incubated at 95 °C for 5 min for denaturation step, followed by 35 cycles of 95 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min, with a final extension for 7 min at 72 °C in the GeneAmp PCR system. The PCR products were digested with the restriction enzyme *Mbo*II**.** Ten μL purified PCR fragment were digested with 1μL (10 U/mL) *Mbo*II at 37 °C and incubated overnight in order to completely digest the DNA. The restriction digested fragments were then separated on 4% agarose gel electrophoresis. The wide type homozygous (1298AA, glu/ glu) genotype is characterized by a 72 bp fragments (bands), the heterozygous (1298 AC, glu/ ala) genotype is characterized by both 72 and 100 bp fragments and the mutant homozygous (1298 CC, ala/ ala) genotype is characterized by 100 bp fragments.

6. Homocysteine Assay

Homocysteine was measured by a high performance liquid chromatography (HPLC) method and detected by fluorescence detector

Procedure

Plasma total homocysteine (tHcy) was determined by High Performance Liquid Chromatography (HPLC). A mixture of 50 μL of plasma or homocysteine calibrator (final concentrations as homocysteine = 5,10,20,40,60 μM), 25 μL of cystamine dihydrochloride (2 μM) and 25 μL of phosphate-buffered saline (PBS, pH7.4) was incubated with 10 μL of 100 g/L tris (2-carboxyethyl) phosphine (TCEP) for 30 min at room temperature to reduce and release protein-bound thiols, after which 100 μL of 100 g/L trichloroacetic acid containing 1 mmol/L EDTA was added for deproteinization. After the sample was centrifuged for 10 min at 12000g, 50 μL of the supernatant was added to an autosampler vial containing 10 μL of 1.55 mol/L NaOH; 125 μL of 0.125 mol/L borate buffer containing 4 mM EDTA, pH 9.5 and 50 μL of 1 g/L SBD-F the borate buffer. The sample was then incubated for 60 min at 60°C. Separation of the SBD- derivatized plasma thiols was eluted at room temperature with an adsorbosphere C_{18} analytical column, 150 ×3.2 mm, 5 μm particle size and 3 cm guard column, using a 10 μL injection volume. The mobile phase was 0.1 M potassium phosphate buffer (KH_2PO_4), pH2.0 (pH adjusted by *O*-phosphoric acid), containing 60 ml/L acetonitrile at a flow rate of 0.7 ml/min. L-Homocysteine calibrators (0-60μM free thiol) were prepared in 0.1M HCl, and in pooled EDTA plasma. The internal standard was cystamine dihydrochloride, which was added to all samples to achieve a final concentration of 2 μM free thiol. Calibration was performed daily in PBS and in plasma (standard addition).

Fluorescence signals (385 nm excitation, 515 nm emission) were measured using Shimadzu RF-10AXL (Shimadzu, Kyoto, Japan) detector.

7. Method validation of homocysteine assay

Analytical method validation includes all of procedures recommended to demonstrate that a particular method for the quantitative measurement of an analyst in homocysteine is reliable and reproducible. The parameters essential to the validation include selectivity, linearity, recovery, within-run and between-run precision and accuracy.

Procedure

1. Selectivity

Selectivity includes the ability to separate the homocysteine from plasma sample, and other sulfur amino acid. Blank plasma samples were evaluated to determine the presence of any interference across the retention windows of homocysteine.

2. Linearity

The linearity of an analytical method is its ability to elicit test results that are directly, or by a well defined mathematical transformation, proportional to the concentration of analyst in samples within a given range. Linearity can be expressed as a calibration curve which is the relationship between instrument response and known concentration of the analyst. A calibration curve should be prepared in the same biological matrix as the samples in the intended by spiking with known concentrations of the analyst.

The stock solution of homocysteine was prepared and appropriate volumes added to a 50 µl aliquots of plasma. The standard mixture of homocysteine ranged 5-60 µM. Plasma standards were prepared and analyzed as described above. Peak area and concentrations of each analyst was plotted and the relationship between these variables was explained by regression analysis.

3. Precision

The precision of a bioanalytical method is a measure of the random error and is defined as the agreement between replicate measurements of the same sample. Precision can be considered as having a within assay batch component or repeatability which defines the ability to repeat the same methodology with the same analyst, using the same reagents in a short interval of time, e.g. within a day. This is also known as intra-assay precision. The ability to repeat the same methodology under different conditions, e.g. change of analyst, reagents or equipment; or on subsequent occasions, e.g. loss several days or weeks, is covered by the between batch precision or reproducibility, also known as inter-assay precision.

- *Intra-assay*

Plasma samples spiked with honocysteine at 10, 20 and 40 μM were prepared and analyzed as described above.

- *Inter-assay*

The inter-assay was evaluated over three days with five replicates of plasma samples being prepared in the same manner as those described in intra-assay.

The precision is expressed as the percentage relative standard deviation (%RSD) of the replicate measurements. The %RSD value should be within ± 15%.

$$\% \text{ RSD} = (\text{standard deviation/mean}) \times 100$$

4. Absolute Recovery

The recovery of an analyst in an assay is the detector response obtained from an amount of the analyst added to and extracted from plasma, compared to the detector response obtained for the true concentration of the pure standard. Recovery relates to the extraction efficiency of an analytical method within the limits of variability. Values

for absolute recovery of method not less than 80% have all been used as numerical acceptance limits.

$$\%\text{Absolute recovery} = \frac{\text{response of analyst spiked into matrix (processed)}}{\text{response of analyst of pure standard (processed)}} \times 100$$

7. Reagents and Chemicals

1. L-Homocystine (Sigma ,USA)
2. Tris (2-carboxyethyl) phosphine ,TCEP (Sigma ,USA)
3. Cystamine dihydrochloride (Sigma ,USA)
4. Trichloroacetic acid
5. Phosphate-buffered saline (PBS), pH7.4
6. EDTA (Sigma ,USA)
7. NaOH (Sigma ,USA)
8. Borate buffer (Sigma ,USA)
9. Methanol (HPLC grade)
10. *O*-phosphoric acid (Merck)
11. potassium phosphate buffer (Sigma ,USA)
12. Acetonitrile, HPLC grade
13. Ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate,SBD-F (Sigma ,USA)
14. Synthetic oligonucleotide →MTHFR677-F', MTHFR677-R'
15. Synthetic oligonucleotide →MTHFR1298-F', MTHFR1298-R'
16. Enzyme Taq DNA polymerase (Promega, Wisconsin, USA)
17. dNTP Mix (Promega, Wisconsin, USA)

18. Restriction enzyme for MTHF R677→*Hinf* I (Promega, Wisconsin, USA)

19. Restriction enzyme for MTHF R1298→ *Mbo* II (Promega, Wisconsin, USA)

20. TAE Buffer (Promega, Wisconsin, USA)

21. Agarose gel (Promega, Wisconsin, USA)

22. DNA Ladder (marker) (Promega, Wisconsin, USA)

23. Ethidium Bromide solution (Promega, Wisconsin, USA)

8. Equipment

1. High Performance Liquid Chromatography (HPLC); Shimadzu LC-10AD, Kyoto, Japan

2. Fluoresence detector; Shimadzu RF-10AXL, Kyoto, Japan

3. Analytical column (Alltech®), 150 ×3.2 mm, 5 μm with an adsorbosphere C_{18} and 3 cm guard column

4. Refrigerated ultracentrifuge (ALC® 4237R)

5. Blood DNA mini kit (Promega, Wisconsin, USA)

6. Polymerase chain reaction GeneAmp ,PCR (Eppendoef®)

7. A power supply

8. Gel electrophoresis chamber (Bio-Rad®)

9. A casting plate

10. A well forming comb
11. Digital balance (Mettler® AJ 180)
12. pH meter (Beckman®,USA)
13. Sonicator (Elma®, Germany)
14. Vortex mixture (Clay adam®, USA)
15. Shaker bath (Heto®)
16. Hot air oven (Memmert®)
17. Automatic pipette size 10, 20,100, 200, 1000 ul (Pipetman Gilson®)
18. 0.2 ml PCR tube
19. 0.5 ml Microtrifuge tube
20. 1.5 ml Microtrifuge tube
21. Blue Multi Pipette Tips 100-1000 ul/ rack
22. Yellow Multi Pipette Tips 5-200 ul/ rack
23. White Multi Pipette Tips 0.5-10 ul/ rack
24. Rack for 1.5 ml Microtrifuge tube
25. PCR rack
26. Clear microtube storage box

Statistical analysis

Plasma tHcy valued presented as mean ± standard deviation. A paired t-test evaluated the difference in plasma total homocysteine between before and after treated with MTX. The t-test for the C667T and A1298C mutant genotype compared with each wild type genotype was used to determine whether there were any significant differences in tHcy concentration. The correlation between genotype and tHcy concentration were tested by one-way ANOVA. $P < 0.05$ was considered statistically different. Statistics were computed with SPSS for Windows.

CHAPTER IV

Results

Result of validation of the HPLC method

I. Selectivity

1. Fig.19 showed peaks of blank plasma without adding standard homocystine and internal standard (cysteamine dihydrochloride). The chromatogram showed the HPLC pattern of sulfur amino acids in plasma. The 4 peaks were identified as:1 = cysteine, retention time was 2.90 min; 2 = cysteamine, retention time was 3.44 min; 3 = cysteinylglycine, retention time was 4.00 min; 4 = homocysteine, retention time was 4.56 min. From chromatogram illustrated that HPLC technique could detect the sulfur amino acids in plasma.

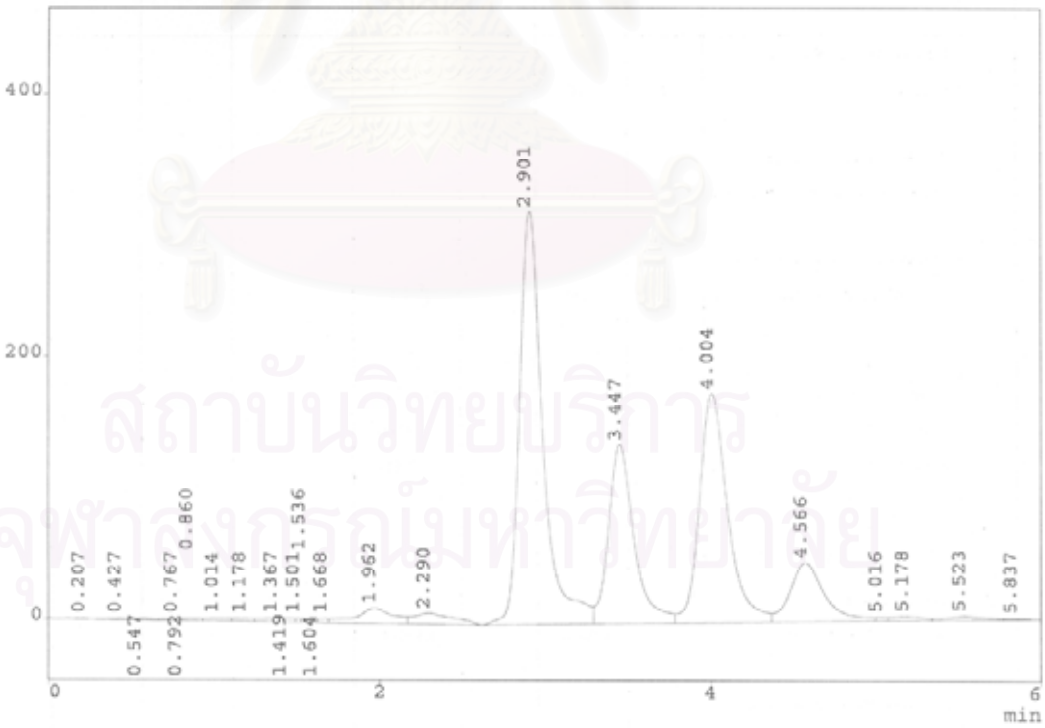

Figure 19.Representative chromatogram of standard condition of Hcy in blank plasma without adding standard homocystine and internal standard (cysteamine dihydrochloride).

2. Fig.20 showed peaks of blank plasma with adding internal standard (cysteamine dihydrochloride) 2 μM. The chromatogram showed 4 peaks of sulfur amino acids :1 = cysteine, retention time was 2.86 min; 2 = cysteamine, retention time was 3.41 min; 3 = cysteinylglycine, retention time was 3.95 min; 4 = homocysteine, retention time was 4.49 min. The chromatogram illustrated the second peak was higher than the second peak of Fig. 19. Other 3 peaks (cysteine, cysteinylglycine, homocysteine) were as high as Fig.19. It meant that the second peak was internal standard.

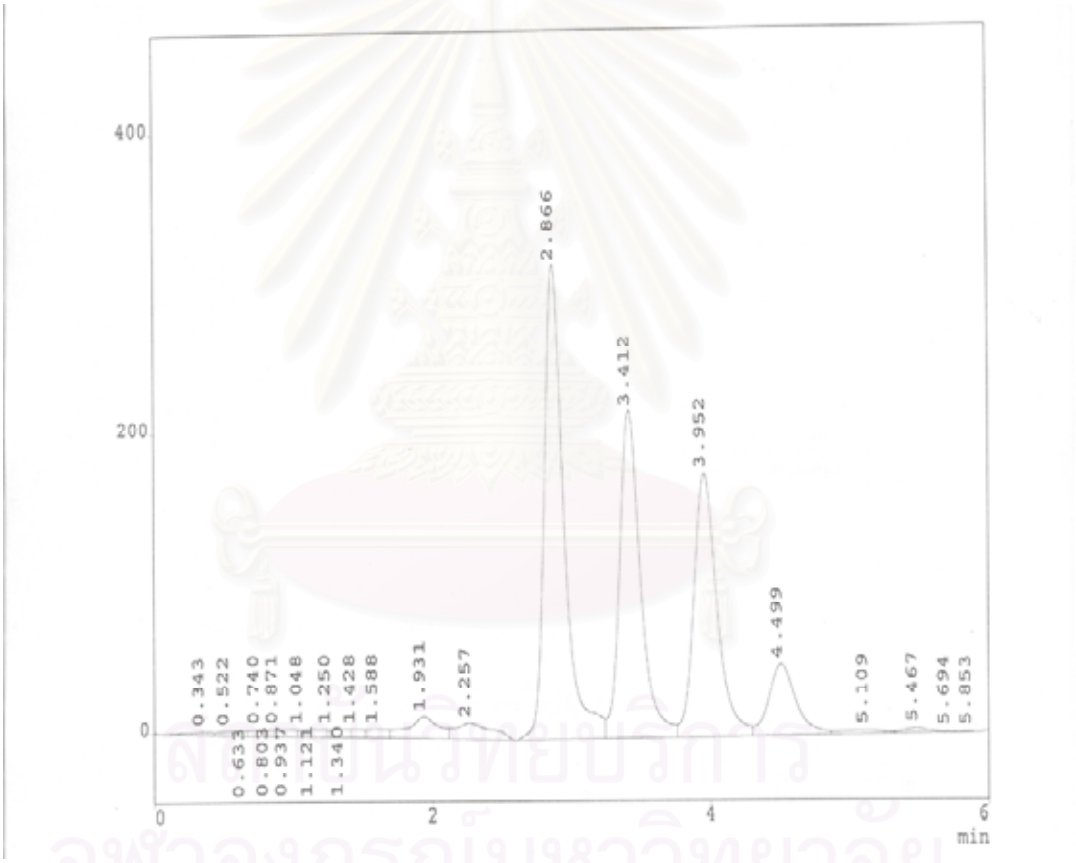

Figure 20. Representative chromatogram of standard condition of Hcy in blank plasma with adding internal standard 2 μM (without standard homocysteine).

3. A representative of blank plasma with adding standard homocystine 10 μM and internal standard (cysteamine dihydrochloride) 2 μM. The chromatogram (Fig. 21) showed 4 peaks of sulfur amino acids :1 = cysteine, retention time was 2.85 min; 2 = cysteamine, retention time was 3.38 min; 3 = cysteinylglycine, retention time was 3.91 min; 4 = homocysteine, retention time was 4.45 min. The chromatogram illustrated the second peak was as high as the second peak of Fig.20 and the fourth peak was higher than the fourth peak of Fig.20. Other 2 peaks (cysteine, cysteinylglycine) were as high as Fig.20. It meant that the second peak was the added internal standard and the fourth peak was the added homocysteine.

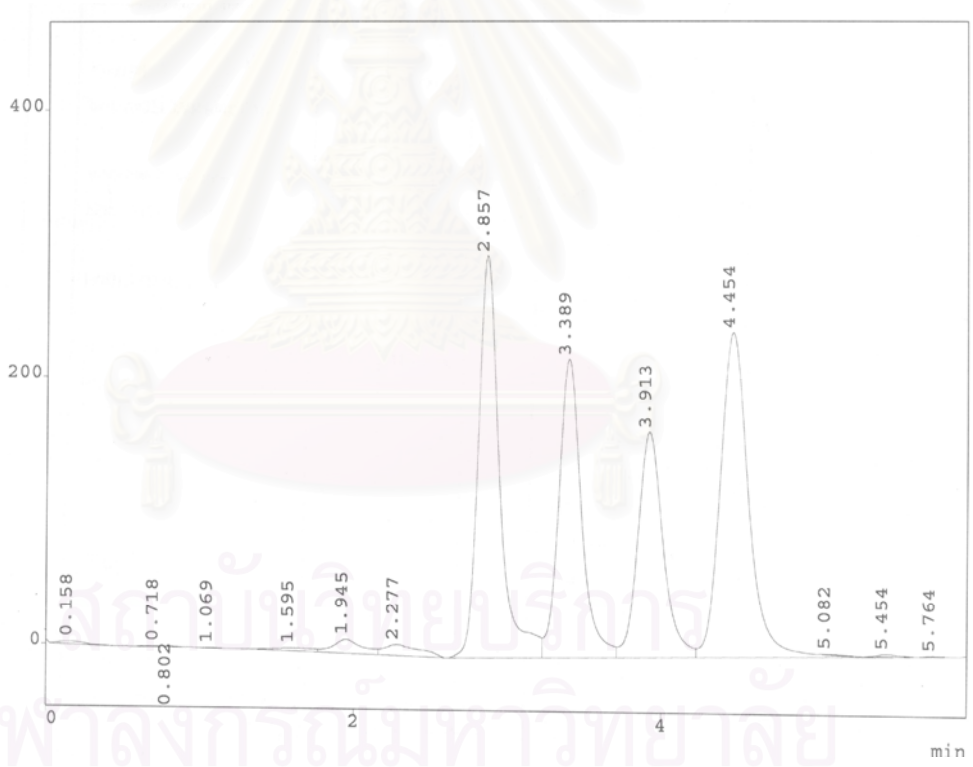

Figure 21. Representative chromatogram of standard condition of Hcy in blank plasma with adding internal standard 2 μM (cysteamine dihydrochloride) and standard homocystine 10 μM.

4. A representative of plasma sample with adding internal standard (cysteamine dihydrochloride) 2 μM. The chromatogram (Fig. 22) showed 4 peaks of sulfur amino acids :1 = cysteine, retention time was 2.84 min; 2 = cysteamine, retention time was 3.36 min; 3 = cysteinylglycine, retention time was 3.91 min; 4 = homocysteine, retention time was 4.57 min.

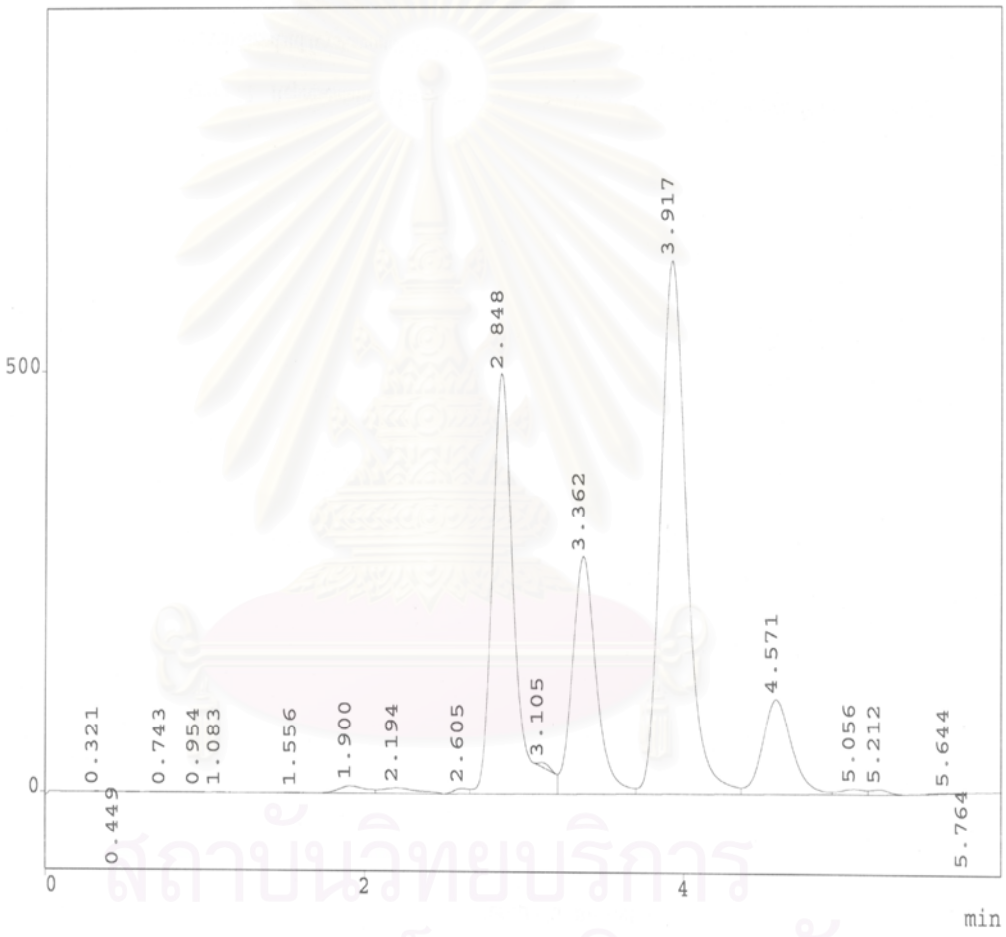

Figure 22. Representative chromatogram of standard condition of Hcy in plasma sample with adding internal standard 2 μM (cysteamine dihydrochloride).

II. Linearity

The calibration curve of Hcy concentration varied at range 5-60 μM. Correlation coefficient of the curve was 0.9965

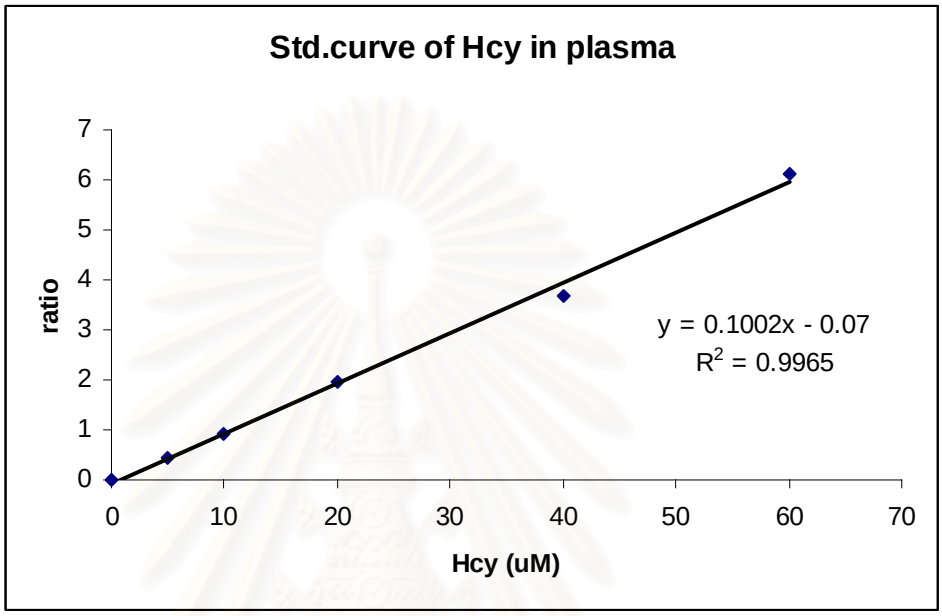

Figure 23. Standard curve of homocysteine in plasma (concentration range 5-60μM).

III. Precision

Six replicates of blank plasma with adding standard homocysteine concentration range 10-40 μM were detected by HPLC method. % RSD (precision) of this method was presented in Table 4.

Table 4. The value of precision

Concentration of Hcy (μM)	% RSD	
	Intra-assay	Inter-assay
10	1.84	2.78
20	2.14	2.48
40	2.63	3.83

IV. Absolute Recovery

Blank plasma with adding standard homocysteine concentration range 10-40 μM was detected by HPLC method. % Absolute Recovery of this method was presented in Table 5.

Table 5. The value of Absolute Recovery

Concentration of Hcy (μM)	% Absolute Recovery
10	92.74
20	93.56
40	90.35

Result of detection the C677T and A1298C genotype

C677T alleles

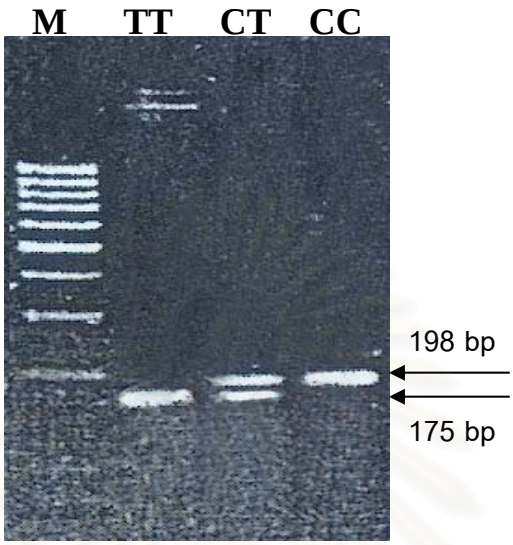

Figure 24. After *Hinf*I digestion, the 198 base-pair (bp) PCR amplification product of the 677CC genotype remains undigested, whereas the 677TT genotype results in 23 and 175 bp fragments. The 677CC genotype is defined by the presence of a single 198 bp band, the 677TT genotype is defined by a single 175 bp band, and 677CT genotype defined by the presence of both 175 and 198 bp bands. Summary of informative bands : 677CC (wild type):single 198 bp band; 677CT (heterozygous):198 and 175 bp bands; 677TT (homozygous variant):175 bp band. Lane1 (M): the molecular weight markers.

A1298C alleles

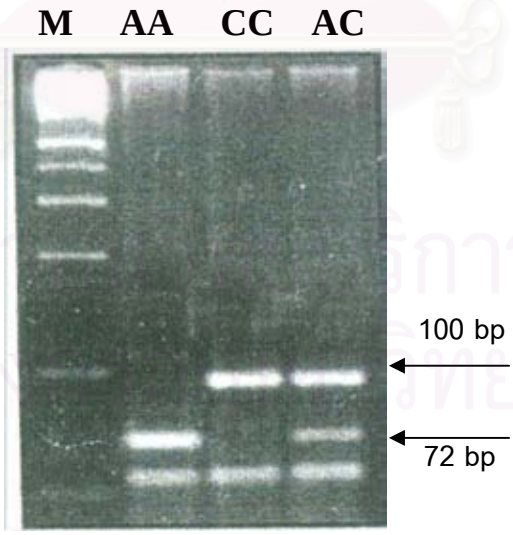

Figure 25. After MboII digestion, the 1298AA genotype is defined by the presence of a single 72 base-pair (bp) band, 1298CC genotype defined by the presence of a single 100 bp band, and the 1298AC genotype defined by the presence of both 72 and 100 bp bands. Summary of informative bamds: 1298AA (wild type): single 72 bp band; 1298AC (heterozygous): 72 and 100 bp bands; 1298CC (homozygous variant):100 bp band. Lane1 (M): the molecular weight markers.

Result of tHcy concentration and *MTHFR* polymorphisms

We determined the polymorphisms of MTHFR genotype and plasma tHcy concentration before methotrexate treating (BMT) and immediately after methotrexate treating (AMT) in 29 ALL children who receiving high dose methotrexate (HDMTX) therapy

The mean tHcy before methotrexate treating (BMT) and the mean post methotrexate treating (PMT) increased in the combined population of 29 individuals classified according to the Dosage of MTX (Table 7),C677T (Table 8) ,A1298C (Table 9) and combined genotypes (Table 10) , the frequency of each genotype was also shown in tables.

Table 6. Characteristics of children with ALL

Characteristic	No (%)
All of the subjects	29 (100)
Sex	
Male	17 (58.6)
Female	12 (41.4)
Age (mean ± SD)	8.08 ± 3.78

From 29 ALL children, 17 were male (58.6%), 12 were female (41.4%). The mean (± SD) age was 8.08(± 3.78) years (range 2 -14 years) (Table 6).

Table 7. The dosage of MTX used and tHcy both BMT and PMT in 29 patients

Dose of MTX	**n (%)**	**tHcy BMT μM (± S.D)**	**tHcy PMT μ M(± S.D)**
1.5 g/m^2	14 (48.3)	4.37 (± 1.69)	8.11 (± 2.87) [a]
2.0 g/m^2	12 (41.4)	5.11 (± 1.60)	10.19 (± 4.18) [a]
5.0 g/m^2	3 (10.3)	4.21 (± 0.64)	8.92 (± 0.24) [a]

[a] significant difference ($p < 0.05$, paired t-test) for tHcy BMT versus tHcy PMT from the same MTX dose

BMT = before methotrexate treating; PMT = post methotrexate treating

Fourteen patients (48.3%) received 1.5 g/m^2, 12 (41.4%) received 2.0 g/m^2 and 3 (10.3%) received 5.0 g/m^2 of MTX. There was a significant increase in plasma tHcy PMT at 1.5, 2.0, and 5.0 g/m^2 when compared with BMT from the same dose ($P < 0.05$, paired t-test) with no significant difference of tHcy between this dosage range (1.5-5.0 g/m^2)(Table 7).

Of the 29 individuals, 20 (69%) were homozygous for the 677C allele (wild type), 8 (27.6%) were heterozygous and 1 (3.4%) was homozygous for the 677T allele. There was significantly increased plasma tHcy PMT compared with BMT from the same genotype in 677 CC but not the 677TT. Individual with the 677TT was omitted from the statistical analysis of both BMT and PMT because of the small size group. Only one patient in the study had 677TT genotype, the small number of patients with genotype limited our statistical power. There were no significant differences of tHcy compared between genotype (Table 8).

Table 8. *MTHFR* C677T genotype distribution and tHcy both BMT and PMT in ALL children

***MTHFR* genotype**	**n (%)**	**tHcy BMT μM (± S.D)**	**tHcy PMT μ M(± S.D)**
677CC (wild type)	20 (69)	4.70(± 1.43)	8.45 (± 3.16)[a]
677CT	8 (27.6)	4.79 (± 1.98)	10.40 (± 4.04) [a]
677TT	1 (3.4)	2.68 (± 0.0)	6.39 (± 0.0)

[a] significant difference ($p < 0.05$, paired t-test) for tHcy BMT versus tHcy PMT from the same genotype

With respect to the A1298C polymorphism, Table10 Showed that 17 individuals were 1298AA (wild type genotype), 9 were 1298AC (heterozygous genotype) and 3 were 1298CC (variant homozygous genotype). There was significantly increased plasma tHcy PMT compared with BMT from the same genotype ($P < 0.05$, paired t-test). There were no significant differences of tHcy compared between genotype (Table 9).

Table 9. *MTHFR* A1298C genotype distribution and tHcy both BMT and PMT in ALL children

***MTHFR* genotype**	**n (%)**	**tHcy BMT μM (± S.D)**	**tHcy PMT μM (± S.D)**
1298AA(wild type)	17 (58.6)	4.24 (± 1.09)	8.33 (± 3.02) [a]
1298AC	9 (31.0)	5.65 (± 2.10)	10.82 (± 3.97) [a]
1298CC	3 (10.3)	4.07 (± 1.31)	8.92 (± 3.45) [a]

[a] significant ($p < 0.05$, paired t-test) for tHcy BMT versus tHcy PMT from the same genotype

Regarding the two common MTHFR polymorphisms, we can detect six of nine combined genotypes. No individuals were homozygous for both the 677T and 1298C alleles (677TT/1298CC genotype), and 677CT/1298CC and 677TT/1298AC. There was significantly increased plasma tHcy PMT compared with BMT from the same genotype ($P < 0.05$, paired t-test).There were no significant differences of tHcy compared between these genotypes (Table 10).

Table 10. *MTHFR* C677T and A1298C genotype distribution and tHcy both BMT and PMT in ALL children

***MTHFR* genotype**	**n (%)**	**tHcy BMT μ M(± S.D)**	**tHcy PMT μM (± S.D)**
677CC/1298AA	11 (37.9)	4.62 (± 1.06)	7.93 (± 2.06) [a]
677CC/1298AC	6 (20.7)	5.17 (± 2.08)	10.38 (± 4.59) [a]
677CC/1298CC	3 (10.3)	4.07 (± 1.31)	6.48 (± 1.64) [a]
677CT/1298AA	5 (17.2)	3.71 (± 0.75)	9.62 (± 4.72) [a]
677CT/1298AC	3 (10.3)	6.59 (± 2.20)	11.71 (± 2.92) [a]
677CT/1298CC	0 (0)	-	-
677TT/1298AA	1 (3.4)	2.68 (± 0.00)	6.39 (± 0.00)
677TT/1298AC	0 (0)	-	-
677TT/1298CC	0 (0)	-	-

[a] significant difference ($p < 0.05$, paired t-test) for tHcy BMT versus tHcy PMT from the same genotype

In this study, there was no occurrence of seizure among patients with MTHFR C677T and A1298C polymorphisms and there were no significant differences in the prevalence of different genotypes associated with the elevation of plasma tHcy concentration when receiving HDMTX.

CHAPTER V

Discussion and Conclusions

We found that in ALL patients, after MTX infusion, plasma Hcy levels were markedly increased and significantly higher than basal levels same as previous reported by Kishi et al. (2003). It is reasonable to hypothesize that elevated Hcy caused by MTX could be a marker in ALL children received MTX for MTX cytotoxicity, which may be affected by MTHFR activity.

Two MTHFR polymorphisms are studied in the persent investigation. The first C677T polymorphism consists of 3 genotypes, the 677CC (homozygous, wild type), 677 CT (heterozygous) and 677TT (homozygous) with the frequency of mutant alleles around 30% similar to the report found in Japanese and Europeans(Carmel *et al*,2003). The 677TT is found only in one patient. The tHcy after treated with MTX do not differ among the above genotype, like previous reported by Hanson *et al* in 2001 in that the C677T polymorphism may not affect the tHcy after MTX treatment.

The second studied MTHFR polymorphism is the A1298C. The frequency of 1298AA (homozygous, wild type) is around 60% together with 40% of the mutant alleles of 1298AC (heterozygous) and 1298CC (homozygous) which seems to be higher than those reported by Carmel *et al* (2003).

Several studies regarding the A1298C polymorphism have shown that presence of the mutant 1298C allele is not a significant risk factor for neural tube defects (van der Put *et al*,1998; Weisberg *et al*, 1998), for coronary artery disease(CAD) or deep vein thrombosis (DVT)(Hanson *et al*, 2001). In this study, we show a represent of patients who documented ALL and received HDMTX that there are no significant difference in the prevalence of the 1298AA, 1298AC and 1298 CC genotype in individuals after treated with MTX. Our study also confirms the results from other studies (van der Put *et al*, 1998; Weisberg *et al*, 1998; Hanson *et al*, 2001) in that for MTX treating the heterozygosity or homozygosity of the A1298C polymorphism is not associated with tHcy concentration.

With respect to these two common polymorphisms (C677T and A1298C) in the MTHFR gene, we found six of the nine combined genotypes are presented in our study. Some studies report that combined heterozygosity of the C677T and A1298C MTHFR variants predisposes individuals to the increased tHcy (van der Put *et al*, 1998;) or there is a tendency toward the increase in tHcy concentration in individuals with the 677CT/1298AC genotype compared with individuals with the 677CC/1298AA genotype, although the difference did not reach statistical significance (Hanson *et al*, 2001). In this study also showed same results as Hanson *et al* (2001). We found that there is no significant difference in tHcy in individuals with the 677CT/ 1298AC genotype compared to wild type, 677CC/1298AA genotype. But the combined heterozygosity (677CT/ 1298AC) showed the highest tHcy PMT among all combined genotypes.

Various studies have examined the haplotype distribution of the two MTHFR polymorphisms. van der Put *et al* (1998) reported that an individual with a 677TT genotype always has a 1298AA genotype as also found in our study and the 677T and 1298C alleles are always in trans configuration. Other studies such as Friedman et al (1999), Shen *et al* (2001), and Hanson *et al* (2001) and one present study detected no homozygous for both polymorphisms (677TT/1298CC) Weisberg *et al* (1998) observed 1 individual with a 677TT/1298AC genotype and Hanson *et al* (2001) found 2 individuals with the 677CT/1298CC genotype, 3 individuals with the 677TT/1298AC genotype which have not been found in this study.

In conclusions, tHcy PMT may be the marker for MTX cytotoxicity in ALL children treated with HDMTX. The MTHFR polymorphisms may affect the tHcy concentration especially in combined heterozygosity (677CT/1298AC). The homozygous of both MTHFR polymorphisms (677TT/1298CC) and one homozygous combined with heterozygous of mutant alleles (677TT/1298AC and 677CT/1298CC) are undetected Thai population studies. Number of patients should be increased to confirm the association of MTHFR polymorphisms and tHcy level in HDMTX treatment.

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APPENDIX

ACUTE LYMPHOBLASTIC LEUKEMIA (ALL)

Q: what is ALL?

A: ALL is the commonest childhood cancer. The peak age is between 2-6 years. Boys are affected more frequently as compared to girls.

Q: How do you diagnose ALL?

A: On clinical suspicion, a variety of tests are done:-blood tests, bone marrow aspiration & biopsy, monoclonal antibody tests, immunophenotyping etc.

However, primary diagnosis is made by bone marrow examination. By definition, the presence of 30% or more blasts in the marrow is diagnostic of acute leukemia.

The distinction between ALL and AML is made primary by morphology of blasts in the marrow and the characteristic pattern of staining with cytochemical stains. Morphologically, the lymphoblasts are smaller than the myeloblasts and do not stain with cytochemical stains except with PAS.

Q; what are the prognostic features of ALL?

A: The various clinical and laboratory factors that determine prognosis are as follows:-

1. Age at diagnosis

   Infants with ALL (especially young infants of less than 6 months have a high risk of treatment failure. The poor outcome for infants with ALL is strongly associated with the presence of t (4; 11) translocation involving the MLL gene.

   Young children (1-9 years) have a favourable outcome as compared to older children or infants.

2. WBC count at diagnosis

   Patients with high WBC counts at diagnosis (> 50,000/cu mm) have a poorer prognosis.

3. Gender

   Girls have a slightly better prognosis. One reason for better prognosis in girls is the occurrence if testicular relapses among boys. Also boys are at higher risk for bone marrow relapse.

4. Cellular morphology

   In the past, ALL was classified into 3 types –L_1, L_2 and L_3 using the FAB criteria for prognosis. However this classification is no longer applied. However molecular and biology characteristics are used for determining the outcome.

a) Immunophenotype:-

B-cell precursor ALL - Represent 80-85% of childhood ALL. Approximately 80% of B cell precursor ALL express the cALLa, CD 10 antigen. The lack of cALLA is associated with a worse prognosis.

Stage of B cell maturation – Patients with early pre B phenotype have the best prognosis, pre B phenotype have an intermediate prognosis and B-cell type have the worst prognosis.

T-cell ALL – Approximately 15% of children with ALL have a T-cell phenotype. In patients with T-cell ALL, CD2 has a favourable prognosis, whereas CD7+, CD2- and CD5- immunophenotype has a worse prognosis.

b) Chromosome number

Hyperdiploidy (> 50 chromosomes per cell or DNA index > 1.16) have a favourable prognosis. Hyperdiploid leukemic cells are susceptible to undergoing apoptosis.

Trisomy 4 and 10 are associated with favorable prognosis. Hyperdiploidy (< 45 chromosomes per cell) have a high risk of treatment failure.

c) Chromosomal translocations t (8; 14), t (9; 22), t (4; 11) and t (1; 19) are associated with unfavorable prognosis, t (12; 21) has a favourable prognosis.

5. Rapidity of leukemic cytoreduction following onset of treatment

   Patients who have a rapid reduction in the leukemic cell in the bone marrow within 7-14 days following multiagent chemotherapy have a better prognosis.

6. CNS disease at presentation

   Patients with CNS manifestations to onset have an unfavourable prognosis

7. Mediastinal mass

   Patients with a mediastinal mass at onset have a favourable prognosis.

   CCG divided patients into “standard risk” or “high risk” based on age and WBC criteria. Standard risk patients are patients between 1-9 years and those who have WBC count < 50,000 / cu mm at diagnosis. The remaining patients are classified as high risk ALL. The “very high risk” category includes –presence of t (9; 22); M_3 marrow on day 29 or M_2 or M_3 marrow on day 43; or hypodiploidy (DNA index < 0.95). Infants with ALL are considered “high risk” and have special chemotherapy protocols.

Q: How do you work up a patient of ALL?

A: The essential laboratory work up is

- Hb, total and differential WBC count
- Bone marrow aspirate

- Chest-X ray (mediastinal mass)
- Uric acid electrolyte: Na, K, Ca, PO_4
- LDN/ Renal function tests/ Liver function tests
- Diagnostic lumbar puncture

Q: what is the treatment of ALL?

A: Successful treatment of children with ALL requires the control of systemic disease (marrow, liver, spleen, lymph nodes etc) as well as the treatment (or prevention) of extramedullary disease in sanctuary sites like the CNS and the testicular region.

Only 3% of patients have detectable CNS involvement at diagnosis (>, 5 WBC/ mm2 with presence of lymphoblasts), however, unless specific therapy is directed toward the CNS, 50% or more children will eventually develop overt CNS leukemia. Hence, all children with ALL should receive form of CNS prophylaxis

Treatment of ALL is divided into stages:-

- Remission induction
- Consolidation or intensification
- Maintenance therapy

CNS sanctuary therapy is given at each stage.

Induction chemotherapy

Three drug induction therapy using vincristine. Prednisolone/ dexamethasone, L-asparaginase in conjunction with intrathecal therapy results in complete remission rate of greater than 95%. For patients with high risk, an anthracyclin (daunorubicin) may be included. The aim of induction phase is to induce remission. In general, patients will achieve complete remission within the first 4 weeks. Patients who require more than 4 weeks to achieve remission o those who demonstrate more than 25% blasts in the bone marrow or have persistent blasts in the peripheral blood after 1 week of intensive induction therapy have a poor prognosis. (A bone marrow is done at the end of induction to establish remission status).

CNS prophylaxis:- Intrathecal (IT)

Chemotherapy with IT methotrexate with or without systemic methotrexate is used for CNS prophylaxis. For patients at high risk of CNS relapse (e.g. age > 10 years, presence of hyperleukocytosis or T cell ALL), use of cranial irradiation is controversial.

Consolidation/ Intensification

It is given to consolidate the remission achieved during induction phase. It may involve the use of intermediate or high dose methotrexate, same drugs as used in induction , other drug combinations, extended use of high dose L-asparaginase or combinations of above.

Maintenance

Most protocols include daily oral mercaptopurine and weekly oral methotrexate. If the patients has not had cranial irradiation , intrathecal chemotherapy for CNS prophylaxis is continued in the maintenance phase.

Monthly pulses of vincristine and prednisolone/ dexamethasone are often added to the standard maintenance regimen. Maintenance chemotherapy is generally given for 2 to 3 years to achieve complete remission.

Q: How do you monitor a patient during maintenance?

A: During this period, it is important to monitor and maintain the WBC count (ANC) between 1,000 to 1,500 or as mentioned in the protocol sheet. It is also important to monitor for clinical signs of relapse like persistent unexplained fever, hepatosplenomegaly, lymphadenophathy, testicular enlargement or new CNS deficits as well as suspicious peripheral blood values.

Q: How to manage a relapse?

A: Relapse may occur during or after completion of therapy and may occur in the bone marrow, testes or CNS. Allogenic bone marrow transplant should be considered in early relapse in therapy or within 6 months of termination of therapy or late marrow relapse with high tumor load as indicated by a peripheral blast count of 10,000/ ul or more. For patients with late relapse (> 36 months in remission), a primary chemotherapy approach should be considered with bone marrow transplantation reserved for a subsequent marrow relapse.

Q: What is radiation therapy?

A: Radiation therapy is treatment with high energy x-rays. High levels of radiation can kill cells and keep them from growing and diving. There is no pain or discomfort during the treatment. It is like having an ordinary x-ray taken except that the child needs to be held still for a few minutes.

The area to be irradiated is marked with a dye. This dye should not be washed off for the duration of therapy as it serves as a guide for aiming the radiation. While radiation therapy is being received, soap or lotion should not be used on these lines or within the radiation field, where the skin becomes tender. The area should also be kept dry.

There are various side effects of radiation therapy

- **Skin damage** - The skin in the treated area may be sensitive and therefore should be protected against sunlight and irritation. Sun blocking lotion containing PABA should be used to prevent burning. If the head is affected, soft hats and scarves may be worth. Body power, cornstarch, topical antibiotics or steroids may relieve itching, pain and speed healing.

- **Sore throat**

- **Hair loss** - is usually temporary with hair growth beginning about 3 months after completion of treatment.

- **Nausea, vomiting and headaches** – These last for about 4-5 hours and are relieved by anti-emetics

- **Diarrhea** – It is usually after radiation to the abdomen or pelvic area. It usually responds to simple measures.

- **Late effects** – Following irradiation to the brain/ CNS, some children may be drowsy. This symptom may begin at various times and may be seen as late as 5-7 weeks after therapy has been completed. It usually lasts about 5 to 10 days.

- **Long term effects** – Radiation therapy to the head may affect intelligence/ coordination. Also, growth may be affected. There is increased possibility of developing a secondary tumor at the radiation site.

Q: What are the precautions advised to patients?

A: The following precautions are advised to the patients while on therapy:

◆ **Immunization**

To avoid live viral vaccines.

◆ **Mouth care**

Teeth should be brushed after each meal using soft toothbrush. To prevent the severe tooth decay that can result when saliva flow is reduced from radiation to the head and neck, fluoride mouthwash may be recommended. Infants and toddlers may be given mouth care by wrapping a soft cloth around the patient's finger and gently wiping the teeth and gums with a solution of mouth rinse. When a patient has low blood counts, mouth care should be gentle.

◆ **Bleeding**

Contact sports should be avoided. To control bleeding, apply pressure with a clean cloth till bleeding stops.

◆ **Danger signs**

If the child shows any of the following symptoms, the parents should immediately notify the child's physician.

- A fever or other sign of infection, a just "not looking well".
- Exposure to a contagious infection especially chicken pox or measles, unless the child is immune from prior exposure.
- Persistent headaches pain or discomfort anywhere in the body.

- Difficulty in walking or bending.
- Pain during urination or bowel movements.
- Reddened or swollen areas.
- Vomiting unless post chemo/ radiotherapy.
- Problems with eyesight such as blurred or double vision.
- Bleeding or multiple bruises.
- Marked depression or a sudden change in behavior.

VITAE

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