RESEARCH ARTICLES

Hyperhomocysteinemia and Genetic Polymorphisms of Methylenetetrahydrofolate Reductase in Acute Lymphoblastic Leukemia Children Treated with High Dose Methotrexate

Tiparat Oungsakul[1], Pornpen Pramyothin[1*], Suradej Hong-ing[2]

[1]*Department of Pharmacology, Faculty of Pharmaceutical Sciences, Chulalongkorn University*
[2]*Department of Pediatrics, Bone Marrow Transplant Program, Ramathibodi Hospital, Mahidol University*

Abstract

5,10-Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme involved in DNA methylation and synthesis. *MTHFR* has two common polymorphisms (C677T and A1298C), both with reduced enzyme activity and may impair remethylation of homocysteine (Hcy) to methionine resulting in hyperhomocysteinemia. Remethylation of Hcy to methionine and DNA methylation are also affected by methotrexate (MTX) treatment. A combined effect of MTX and reduced *MTHFR* activity by genetic 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 tHcy in children with acute lymphoblastic leukemia (ALL) receiving high dose MTX (HDMTX). Genotyping of *MTHFR* was detected by polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) method. tHcy was detected by high performance liquid chromatography (HPLC) technique. Our data indicated that after ALL patients treated with HDMTX, tHcy was significantly higher than basal line. The combination of both homozygous mutant alleles (677T/T + 1298C/C) and homozygous plus heterozygous mutant alleles (677T/T + 1298A/C and 677C/T + 1298C/C) were undetected in Thai population studied. The *MTHFR* polymorphisms may affect the tHcy after MTX treatment (tHcy PMT) especially in the combined heterozygosity (677C/T + 1298A/C) which presented the highest value of tHcy PMT. Therefore the tHcy PMT may be used as a marker for the detection of MTX cytotoxicity in ALL children treated with HDMTX.

Key words : hyperhomocysteinemia, methylenetetrahydrofolate reductase, methotrexate, acute lymphoblastic leukemia

* Address correspondence and print requests to: Pornpen Pramyothin, Department of Pharmacology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand.

การเพิ่มขึ้นของระดับโฮโมซีสเทอินในเลือด และความแปรผันของยีนเมทิลีนเททระไฮโดรโฟเลท รีดักเทส ในเด็กป่วยเป็นมะเร็งเม็ดเลือดขาวแบบเฉียบพลัน ที่ได้รับการรักษาด้วยเมโทเทรกเซสในขนาดสูง

ทิพย์รัตน์ อึ้งสกุล[1], พรเพ็ญ เปรมโยธิน[1*], สุรเดช หงษ์อิง[2]

[1]*ภาควิชาเภสัชวิทยา คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย*
[2]*ภาควิชากุมารเวชศาสตร์ และ โครงการปลูกถ่ายไขกระดูก โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล*

บทคัดย่อ

5, 10 เมทิลีนเททระไฮโดรโฟเลท รีดักเทส (MTHFR) เป็นเอนไซม์สำคัญเกี่ยวกับเมทิเลชั่นและการสร้างดีเอ็นเอ MTHFR มีการแปรผันของยีนที่พบบ่อยอยู่สองแห่ง (C677T และ A1298C) ซึ่งเมื่อเกิดขึ้นจะทำให้การทำงานของเอนไซม์ลดลง และอาจทำให้รีเมทิเลชั่นของโฮโมซีสเทอิน (Hcy) ไปเป็นเมไทโอนินเสียไป เป็นผลให้ระดับของโฮโมซีสเทอินในเลือดเพิ่มขึ้น รีเมทิเลชั่นของโฮโมซีสเทอินไปเป็นเมไทโอนินและดีเอ็นเอเมทิเลชั่นมีการเปลี่ยนแปลงได้เช่นกันจากการใช้ยาเมโทเทรกเซส (methotrexate, MTX) ผลรวมของการใช้ยา MTX และการลดการทำงานของเอนไซม์ MTHFR จากการแปรผันของยีน อาจนำไปสู่การเพิ่มขึ้นของระดับโฮโมซีสเทอินรวม (tHcy) วัตถุประสงค์ของการวิจัยครั้งนี้คือการหาความสัมพันธ์ระหว่าง MTHFR genotype และ tHcy ในเด็กป่วยเป็นมะเร็งเม็ดเลือดขาวแบบเฉียบพลัน (ALL) ที่ได้รับการรักษาด้วยเมโทเทรกเซสในขนาดสูง (HDMTX) การตรวจหา MTHFR genotype ใช้วิธีของ PCR/RFLP การวัดค่า tHcy ใช้เทคนิค HPLC ผลการวิจัยพบว่าหลังจากที่เด็ก ALL ได้รับ HDMTX ระดับของ tHcy เพิ่มสูงขึ้นจากเดิม ไม่พบคนที่เป็นโฮโมไซกัสของการแปรผันยีนทั้งสองตำแหน่ง (677T/T+1298C/C) และคนที่เป็นโฮโมไซกัสของยีนหนึ่งตำแหน่งร่วมกับเฮเทอโรไซกัสของยีนอีกหนึ่งตำแหน่งที่เหลือ (677T/T + 1298A/C และ 677C/T+1298 C/C) ในคนไข้เด็กไทยที่ทำการศึกษา การแปรผันของยีน MTHFR อาจมีผลต่อระดับ tHcy หลังจากได้รับการรักษาด้วยเมโทเทรกเซส (tHcy PMT) โดยเฉพาะในเฮเทอโรไซกัสของยีนทั้งสองตำแหน่ง (677C/T + 1298 A/C) ซึ่งวัดค่า tHcy PMT ได้สูงสุด ดังนั้นค่า tHcy PMT อาจนำมาใช้เป็นตัวบ่งชี้การเกิดพิษของ MTX ในเด็ก ALL ที่ได้รับ HDMTX

คำสำคัญ: การเพิ่มขึ้นของระดับโฮโมซีสเทอินในเลือด, เมทิลีนเททระไฮโดรโฟเลท รีดักเทส, เมโทเทรกเซส, มะเร็งเม็ดเลือดขาวแบบเฉียบพลัน

Introduction

Methotrexate (MTX) is an antifolate chemotherapeutic drug. It plays a central role in the treatment of acute lymphoblastic leukemia (ALL). The prognosis of ALL has improved with intensive chemotherapy. High dose of MTX (>1 g/m^2) is required for the effective treatment in ALL patients [1]. Its principal pharmacological mechanism is the inhibition of enzymes involved in folate homeostasis resulting in cellular depletion of reduced folates. The 5,10-methylene-tetrahydrofolate reductase (MTHFR) is a key enzyme in the generation of bioactive folate compounds[2-3]. 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 symptoms, hepatitis, alopecia, hypersensitivity, pneumonitis and a serious complication of neurotoxicity which is thought to cause by the elevation of serum homocysteine or hyperhomocysteinemia[2-5]. The pathogenesis of neurotoxicity remains unclear. It is possible that polymorphic enzymes involved in folate metabolism may be related to these neurotoxic effects especially MTHFR. MTHFR catalyzes the reduction of 5,10-methylenetetrahydrofolate (5,10-MTHF) to 5-methyltetrahydrofolate (5-MeTHF) which is the predominant circulating form of folate and carbon donor for the remethylation of homocysteine to methionine.

The common point mutation firstly identified polymorphism of the MTHFR gene found by Frosst *et al*, 1995[13] is a 677C→T which converts an alanine to a valine. The variant T/T genotype has about 30% of wild type (677C/C) activity and presents in about 10% of white and Asian populations. Heterozygote 677C/T has about 60% activity and constitutes approximately 40% of the population [6,7]. Homozygotes (677T/T) are predisposed to hyperhomocysteinemia by the decrease in MTHFR activity, particularly in the context of suboptimal folate status. The second common polymorphism in the MTHFR gene is a 1298 A→C resulting in a glutamate to an alanine conversion. This polymorphism is associated with decreased enzymatic activity[8] elevated homocysteine (Hcy) concentration, and decreased folate concentration in plasma [9].

Plasma homocysteine concentration, a representative marker of intracellular folate status [5,10] may be associated with the MTHFR polymorphisms. There are some studies reported the association between C677T polymorphism and toxicity of MTX [7,11,12] with a few describing the relationship of both C677T and A1298C polymorphisms with MTX toxicity [3].

This study aimed to evaluate the relationship between MTHFR polymorphisms and the elevation of plasma homocysteine concentration in ALL Thai children receiving high dose of MTX.

Patients and Methods

Subjects

Twenty nine children with ALL treated with MTX are participated in this study.

Inclusion criteria

All children with ALL received MTX 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 drugs, nitrous oxide, theophylline, D-penicillamine and sulfazalazine.
- Patients diagnosed with renal or hepatic impairments.

Sample preparations

ALL patients received high dose methotrexate (HDMTX) at 5 g/m^2 or 2 g/m^2 or 1.5 g/m^2 according to the high risk or standard risk or low risk groups. Blood was collected in EDTA tube before HDMTX infusion and immediately after stopped HDMTX 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 3000 g for 10 min. After centrifugation, plasma was separated and kept frozen at -20°C until analysis.

Isolation of genomic DNA

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

PCR amplification and MTHFR polymorphism detection

The polymerase chain reaction/restriction fragment length polymorphism (PCR/ RFLP) method was used for the determination of MTHFR genotype. The MTHFR 677C→T mutation was analyzed according to the method of Frosst *et al*, 1995 [13]

Agarose Gel Electrophoresis

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

Homocysteine Assay

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

Statistical analysis

Plasma tHcy values were presented as mean ± standard deviation. A paired *t*-test was used to evaluate the difference in plasma tHcy between before and after MTX treatment. The *t*-test for the C677T 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.

Results

tHcy concentration and *MTHFR* polymorphisms

From 29 ALL children, 17 were male (58.6%), 12 were female (41.4%). The mean age was 8.08 years ranging from 2 to14 years (Table 1).

Table 1. Characteristics of ALL children.

Characteristic	No (%)
All subjects	29 (100)
Sex	
Male	17 (58.6)
Female	12 (41.4)
Age (mean ± S.D.)	8.08 ± 3.78

Table 2. Doses of MTX used and tHcy both in BMT and PMT of 29 patients.

Dose of MTX	No (%)	tHcy BMT μM (± S.D.)	tHcy PMT μM (± 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 treatment; PMT = post methotrexate treatment

Table 3. *MTHFR* C677T genotype distribution and tHcy both in BMT and PMT of ALL children.

MTHFR genotype	No (%)	tHcy BMT μM (± S.D.)	tHcy PMT μM (± S.D.)
677C/C	20 (69)	4.70 (± 1.43)	8.45 (± 3.16)[a]
677C/T	8 (27.6)	4.79 (± 1.98)	10.40 (± 4.04)[a]
677T/T	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

Table 4. *MTHFR* A1298C genotype distribution and tHcy both in BMT and PMT of ALL children.

MTHFR genotype	No (%)	tHcy BMT μM (± S.D.)	tHcy PMT μM (± S.D.)
1298A/A	17 (58.6)	4.24 (± 1.09)	8.33 (± 3.02)[a]
1298A/C	9 (31.0)	5.65 (± 2.10)	10.82 (± 3.97)[a]
1298C/C	3 (10.3)	4.07 (± 1.31)	8.92 (± 3.45)[a]

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

Table 5. *MTHFR* C677T plus A1298C genotype distribution and tHcy both in BMT and PMT of ALL children.

MTHFR genotype	No (%)	tHcy BMT μ M(± S.D.)	tHcy PMT μM (± S.D.)
677C/C + 1298A/A	11 (37.9)	4.62 (± 1.06)	7.93 (± 2.06)[a]
677C/C + 1298A/C	6 (20.7)	5.17 (± 2.08)	10.38 (± 4.59)[a]
677C/C + 1298C/C	3 (10.3)	4.07 (± 1.31)	6.48 (± 1.64)[a]
677C/T + 1298A/A	5 (17.2)	3.71 (± 0.75)	9.62 (± 4.72)[a]
677C/T + 1298A/C	3 (10.3)	6.59 (± 2.20)	11.71 (± 2.92)[a]
677C/T + 1298C/C	0 (0)	-	-
677T/T + 1298A/A	1 (3.4)	2.68 (± 0.00)	6.39 (± 0.00)
677T/T + 1298A/C	0 (0)	-	-
677T/T + 1298C/C	0 (0)	-	-

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

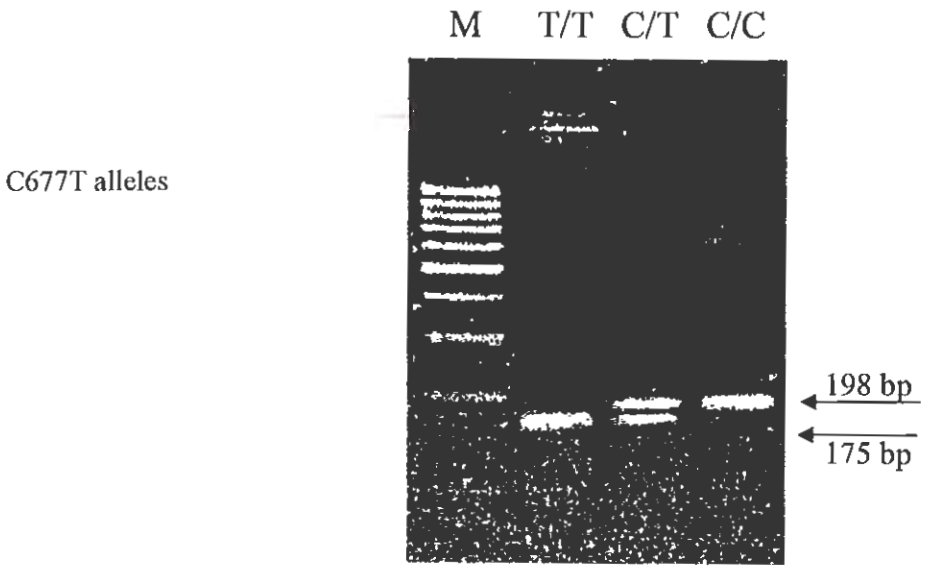

Figure 1. After *Hinf*I digestion, the 198 base-pair (bp) PCR amplification product of the 677C/C genotype remains undigested, whereas the 677T/T genotype results in 23 and 175 bp fragments. The 677C/C genotype is defined by the presence of a single 198 bp band, the 677T/T genotype is defined by a single 175 bp band, and 677C/T genotype defined by the presence of both 175 and 198 bp bands. Summary of informative bands: 677C/C (wild type): single 198 bp band; 677C/T (heterozygous): 198 and 175 bp bands; 677T/T (homozygous variant): 175 bp band. Lane1 (M): the molecular weight markers.

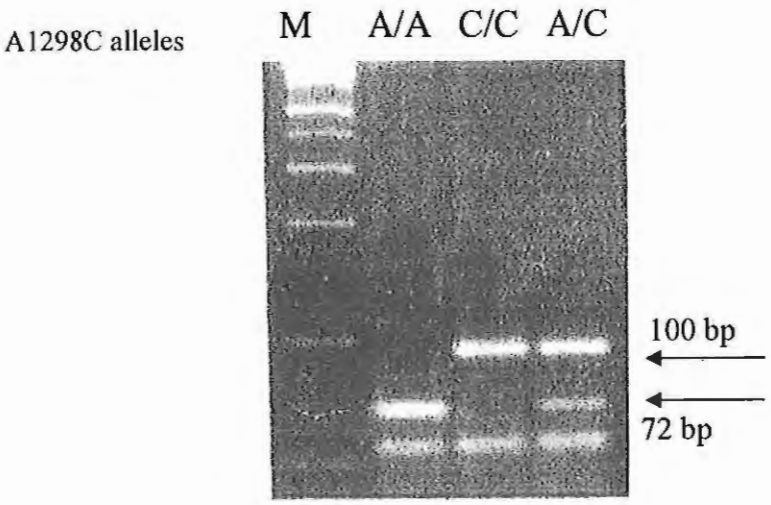

Figure 2. After MboII digestion, the 1298A/A genotype is defined by the presence of a single 72 base-pair (bp) band, 1298C/C genotype defined by the presence of a single 100 bp band, and the 1298A/C genotype defined by the presence of both 72 and 100 bp bands. Summary of informative bands: 1298A/A (wild type): single 72 bp band; 1298A/C (heterozygous): 72 and 100 bp bands; 1298C/C (homozygous variant):100 bp band. Lane1 (M): the molecular weight markers.

The polymorphisms of MTHFR genotype and plasma tHcy concentration before methotrexate treatment (BMT) and immediately post methotrexate treatment (PMT) in 29 ALL children who received HDMTX therapy were determined. The mean tHcy BMT and the mean PMT increased in the combined population of 29 individuals classified according to the dosage of MTX (Table 2), C677T (Table 3), A1298C (Table 4) and combined genotypes (Table 5), the frequency of each genotype is also shown in Tables.

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 the dose of 1.5, 2.0, and 5.0 g/m^2 when compared with BMT from the same doses ($p< 0.05$) with no significant difference of tHcy BMT among this dosage range (1.5-5.0 g/m^2) (Table 2). Of the 29 individuals, 20 (69%) were homozygous 677C/C (wild type), 8 (27.6%) were heterozygous 677C/T and 1 (3.4%) was homozygous 677T/T alleles. There was a significant increased plasma tHcy PMT compared with BMT from the same genotype. Individual with the 677T/T was omitted from the statistical analysis of both BMT and PMT due to small sample size of only one patient. There were no significant differences of tHcy BMT compared between genotype (Table 3).

With respect to the A1298C polymorphism, 17 individuals were 1298A/A (wild type), 9 were 1298A/C (heterozygous genotype) and 3 were 1298C/C (variant homozygous genotype). There was a significant increased plasma tHcy PMT compared with BMT from the same genotype ($p<0.05$) with no significant differences of tHcy BMT compared between genotype (Table 4). Regarding the two common MTHFR polymorphisms, we can detect six from nine combined genotypes. There were no individuals with combination of 677T/T plus 1298C/C, 677C/T plus 1298C/C and

677T/T plus 1298A/C genotypes. There was a significantly increased plasma tHcy PMT compared with BMT from the same genotype ($p < 0.05$) with no significant differences of tHcy BMT compared between these genotypes (Table 5).

In this study, there was no occurrence of seizures 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. Figures 1 and 2 showed the PCR products of C677T and A1298C genotype polymorphisms, respectively.

Discussion and Conclusion

We found that in ALL patients, after MTX infusion, plasma Hcy levels were markedly increased and significantly higher than basal levels same as previously reported by Kishi *et al*,2003[3]. It is reasonable to hypothesize that elevated Hcy caused by MTX could be a marker in ALL children for MTX cytotoxicity, which may be affected by MTHFR polymorphisms.

Two MTHFR polymorphisms were studied in the present investigation. The first C677T polymorphism consisted of 3 genotypes, the 677C/C (wild type), 677C/T (heterozygous) and 677T/T (homozygous) with the frequency of mutant alleles around 30% similar to the report found in Japanese and European Caucasians [14]. The 677T/T was found only in one patient. Since the tHcy after MTX treatment did not differ in this patient, like previously reported by Hanson *et al*, 2001 [15], the C677T polymorphism may not affect the tHcy concentration.

The second studied MTHFR polymorphism was the A1298C. The frequency of 1298A/A (wild type) was around 60% together with 40% of the mutant alleles of 1298A/C (heterozygous) and 1298C/C (homozygous) which were higher than those reported by Carmel *et al*, 2003 [14]. The presence of this mutant allele may not associate with tHcy in patient treated with HDMTX as shown by no differences in tHcy PMT among the above genotypes. Several studies reported that this mutant allele is not a significant risk factor for neural tube defects [8,9] coronary artery disease (CAD) and deep vein thrombosis (DVT) [15].

With respect to C677T and A1298C polymorphisms, we found six of the nine combined genotypes presented in our study. Three missing combinations were 677T/T plus 1298C/C genotype which has also been undetected by other studies[15,16,17] 677T/T plus 1298A/C genotype (found only 1 by Weisberg *et al*, 1998[9]) and 677C/T plus 1298C/C genotype (found only 2 by Hanson *et al*, 2001[15]). Although, there is no significant difference in tHcy in individuals with the 677C/T plus 1298A/C genotype compared to wild type (677C/C plus 1298A/A genotype), this combined heterozygosity showed the highest tHcy PMT among all combined genotypes.

In conclusions, tHcy PMT may be used as a marker for MTX cytotoxicity in ALL children treated with HDMTX. The MTHFR polymorphisms may affect the tHcy PMT especially in combined heterozygosity (677C/T plus 1298A/C). The homozygous of both MTHFR polymorphisms (677T/T plus 1298C/C) and one homozygous combined with heterozygous of mutant alleles (677T/T plus 1298A/C ; 677C/T plus 1298C/C) are undetected in Thai population by our study. Number of patients should be increased to confirm the association of MTHFR polymorphisms with tHcy level in HDMTX treatment.

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