Methylenetetrahydrofolate reductase (MTHFR) C677T and thymidylate synthase promoter (TSER) polymorphisms in Indonesian children with and without leukemia.
Methylenetetrahydrofolate reductase (MTHFR) C677T and thymidylate synthase promoter (TSER) polymorphisms in Indonesian children with and without leukemia

Elisa Giovannetti\textsuperscript{a}, Dewa G. Ugrasena\textsuperscript{c}, Eddy Supriyadi\textsuperscript{d}, Laura Vroling\textsuperscript{a}, Antonino Azzarello\textsuperscript{a}, Desiree de Lange\textsuperscript{b}, Godefridus J. Peters\textsuperscript{a}, Anjo J.P. Veerman\textsuperscript{b}, Jacqueline Cloos\textsuperscript{b}

\textsuperscript{a}Department of Medical Oncology, VU Institute for Cancer and Immunology, VU University Medical Center, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands
\textsuperscript{b}Department of Paediatric Oncology/Hematology, VU Institute for Cancer and Immunology, VU University Medical Center, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands
\textsuperscript{c}Department of Child Health, Airlangga University Surabaya, Indonesia
\textsuperscript{d}Department of Paediatric Hemato-oncology, Gadjah Mada University, Jogjakarta, Indonesia

Leukemia Research 32 (2008) 19–24
Studies investigating ethnic differences in polymorphisms in enzymes that are related to sensitivity (antileukemic and toxic side effects) to MTX are highly relevant, especially since there is much debate about the best treatment schedule with either high or low dose MTX.
ABSTRACT

Genetic variations in the polymorphic tandem repeat sequence of the enhancer region of the thymidylate synthase promoter (TSER), as well as in methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism, influence methotrexate sensitivity. We studied these polymorphisms in children with acute lymphoblastic leukaemia (ALL) and in subjects without malignancy in Indonesia and Holland.

The frequencies of TT and CT genotypes were two-fold higher in Dutch children. The TSER 3R/3R repeat was three-fold more frequent in the Indonesian children, while the 2R/2R repeat was only 1% compared to 21% in the Dutch children. No differences of these polymorphisms were found between ALL cells and normal blood cells, indicating an ethnic rather than leukemic origin. These results may have implications for treatment of Indonesian children with ALL.

Keywords: Acute lymphoblastoid leukemia; Paediatric patients; Methotrexate; MTHFR C677T; TSER 2R/3R; Indonesia
1. Introduction

Acute lymphoblastic leukemia (ALL) is the most common malignancy that affects children, representing nearly one-third of all paediatric cancers. Annual incidence of ALL is about 30 cases per million people, with a peak incidence in patients aged 2–5 years [1]. The optimisation of the use of antileukemic agents that were developed from the 1950s through the 1980s, has resulted in a substantial reduction in death rates from ALL, particularly in children [2]. An important drug in the treatment of ALL is methotrexate (MTX), which inhibits several enzymes involved in folate homeostasis. MTX is an inhibitor of dihydrofolate reductase and decreases intracellular levels of reduced folates such as 5,10-methylenetetrahydrofolate (CH2-THF), which is required for DNA synthesis and for maintaining the balance of the deoxynucleotide pool [3]. Methylenetetrahydrofolate reductase (MTHFR) catalyzes the reduction of 5,10-CH2-THF to 5-CH3-THF, the predominant circulatory form of folate and carbon donor for the remethylation of homocysteine to methionine (Fig. 1).

![Diagram of the human folate metabolic pathway](image)

**Figure 1.** Overview of the human folate metabolic pathway. S-adenosylmethionine (SAM); S-adenosylhomocysteine (SAH); dihydrofolate (DHF), dihydrofolate reductase (DHFR), tetrahydrofolate (THF), serine hydroxymethyltransferase (SHMT); 5,10-methylenetetrahydrofolate (5,10-methyleneTHF); 5,10-methylenetetrahydrofolate reductase (MTHFR); 5-methyltetrahydrofolate (5-methylTHF); 10-formyltetrahydrofolate (10-formylTHF); methionine synthase (MS); thymidylate synthase (TS); deoxythymidine monophosphate (dTMP); deoxyuridine monophosphate (dUMP); fluoro-dUMP (FdUMP).
MTHFR is located on the short (p) arm of chromosome 1, at position 36.3 (1p36.3). Up to 12–15% of Caucasian individuals are homozygous for a C→T polymorphism located at nucleotide 677 (referred to as the TT genotype). The resulting substitution of alanine to valine increases thermolability and reduces the activity of MTHFR [4]. Accumulation of 5,10-CH2-THF resulting from the MTHFR C677T polymorphism may have an effect on the response to MTX, and recent studies have suggested that the TT genotype may be associated with an increased toxicity of MTX in leukemia patients [5,6]. The risk of cardiovascular disease, neural tube defects and several cancers, including ALL, may also be increased, although the evidence to support these relationships is controversial [4,7,8].

Thymidylate synthase (TS) is a key-enzyme in de novo DNA synthesis, which catalyses the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), and is another critical target for MTX [3]. Inhibition of this enzyme results in deoxythymidine triphosphate (dTTP) depletion, chromosome breaks and cell death [9]. The TYMS gene is located on chromosome 18p11.32 and has a unique tandem repeat sequence in the enhancer region (TSER) that has been shown to be polymorphic [10], containing either two (2R) or three (3R) 28-bp repeats. The presence of the 3R versus 2R was shown to influence gene expression in vitro and in vivo [10], and in childhood ALL, homozygosity for the 3R, was reported to be associated with poorer outcome compared to the presence of at least one 2R allele [10].

The prevalence of the MTHFR C677T and TSER polymorphisms may be different among various populations and may affect the efficacy of MTX, a chemotherapeutic agent that is extensively used for the treatment of paediatric ALL. Therefore, identification of these polymorphisms may be an important pharmacogenetic determinant to predict response or toxicity to chemotherapy in children affected by leukaemia. Ethnic variations in the frequency of both the C677T MTHFR and the TSER polymorphic tandem repeat sequences have been described earlier [11,12]. However, to our knowledge, identification of the MTHFR C677T and TSER polymorphisms in Indonesian ALL patients has not been performed before. ALL treatment protocols in Indonesia are analogous to the Dutch protocols (DCLSG ALL-6 and ALL-9), which are based on tolerability and efficacy in this Caucasian population. Since ethnic differences may exist between Dutch and Indonesian children, which may affect drug sensitivity, we determined the ethnic variations in TSER and MTHFR polymorphisms in Dutch and Indonesian children with ALL, in Dutch adult controls, and in Indonesian children without malignancies.
2. Materials and methods

2.1. Patient and control samples

The patients of Indonesian origin included in this study were 71 children diagnosed with ALL treated at Dr. Sarjito Hospital, Yogyakarta, Indonesia and Dr. Soetomo Hospital, Surabaya, Indonesia with the Wijaya Kusuma ALL 2000 protocol. Control samples were from 44 patients without any malignancy selected within the range of 3–8 years of age, while the mean age of the children affected by ALL was 6.1 years (Table 1). For DNA isolation we used isolated mononuclear cells frozen on DMSO and stored in liquid nitrogen (n = 24) and cells from bone marrow slides (n = 91). Furthermore, we used as a comparison the results obtained in 157 samples of ALL children of Caucasian origin [13].

2.2. Isolation of mononuclear cells from fresh bone marrow or blood

The sample was diluted 1:1 with wash medium (Phosphate Buffered Saline, pH 7.4 containing 1% Fetal Calf Serum) and put on a ficoll-gradient with ratio 2:1. The interphase was collected and the cells were washed two times with wash medium. The mononuclear cell pellet was dissolved in 500 µl wash medium. Cell count ranged from $10.0 \times 10^6$ to $16.7 \times 10^6$ total cells.

Table 1. Demographics of 115 Indonesian children

<table>
<thead>
<tr>
<th></th>
<th>% sex (number)</th>
<th>% age (years) (number)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Paediatric leukaemia (71)</td>
<td>51.4 (36)</td>
<td>48.6 (34)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Paediatric control (44)</td>
<td>59.1 (26)</td>
<td>40.9 (18)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Of one child the gender data was not available.
<sup>b</sup> No exact data on age of the control samples is available but control patients were selected within the range of 3–8 years of age, while the mean age of the children affected by ALL was 6.1 years

2.3. DNA isolation

Genomic DNA was extracted from mononuclear cells from vials and bone marrow slides using two different methods.

1. DNA extracted from cells stored in liquid nitrogen was obtained using Qiagen DNA Tissue Kit (Qiagen, Hilden, Germany). The DNA isolation was performed
according the protocol included in the Kit. Because cells were used instead of tissue the incubation step with proteinase K was reduced from overnight to 1 h. The DNA was finally suspended in 200 µl double distilled water (DDH) and stored at −20°C.

2. Before DNA was isolated from bone marrow slides an extra step with Hank’s Balanced Salt Solution and shock buffer was used for a better separation and purification between the mononuclear cells and erythrocytes. An overnight proteinase K treatment was followed by phenol-chloroform-isoamylalcohol (25:24:1) extraction. DNA was precipitated with 3 M sodium acetate (1/10 of total volume) and 2.5 volumes of 100% ethanol. Glycogen (4 ng) was added because we expected a small amount of DNA. This pellet was diluted in 20 µl DDH and stored at −20°C.

In order to have internal controls for the assays we isolated DNA from cell lines, previously characterized as heterozygous for polymorphisms of *TSER* and *MTHFR* (LS174T and HCT116 colon cancer cells, respectively). Pellets of about 5 × 10⁶ cells were frozen until DNA was extracted by using the extraction QIAamp DNA mini Kit (Qiagen). Genomic DNA obtained from these cells was used as control in each experiment.

**2.4. *TSER* genotyping**

*TSER* polymorphisms were analyzed by Polymerase Chain Reaction (PCR) using forward: 5’-GCT CCG AGC CGG CCA CAG GCA TGG CGC GG-3’ and reverse primers: 5’-GTG GCT CCT GCG TTT CCC CC-3’ as previously described [13].

The amplified DNA fragments were visualized on a 2% agarose gel (RESPONSETM Research PCR agarose, 335010 Biozym) with ethidium bromide. Homozygotes for the double repeat (2R/2R) produced a singlet 210-bp band. Heterozygotes (2R/3R) produced 210-bp and 238-bp fragments and homozygotes for the triple repeat (3R/3R) produced a 238-bp fragment.

PCR products were also checked with the Genescan (ABI prism® 3100 - Avant, Applied Biosystems, Foster City, CA). PCR products (5 µl) were combined with 12.5 µl loading mixture consisting of 12 µl Hi-Di formamide and 0.5 µl Gene ScanTM-500 ROX TM Size standard (Applied Biosystems). The samples were run on the ABI prism® 3100 sequencer and analyzed using the version 1.2 Genescan Analysis software (Applied Biosystems).
2.5. **MTHFR C677T genotyping**

The **MTHFR C677T** polymorphisms were studied with a Taqman® probes-based assay using the ABI PRISM 7500 Sequence Detection System instrument equipped with the SDS version 1.3.0 software (Applied Biosystems). Forward and reverse primers and probes were produced by Applied Biosystems (C_1202883_20), on the basis of gene sequence, i.e. 5’-GAA AAG CTG CGT GAT GAT GAA ATC G[G/A]C TCC CGC AGA CAC CTT CTC CTT CAA-3’ for the VIC- and FAM-probes, respectively. The PCR reactions were performed using about 50 ng of genomic DNA diluted in 11.875 µl DNase-RNase free water, 12.5 µl of TaqMan Universal PCR Master Mix, with AmpliTaq Gold®, and 0.625 µl of the assay mix (forward and reverse specific primers and the specific probes), in a total volume of 25 µl. After thermal cycling the 7500 instrument determined the allelic content of each sample in the plate by reading the fluorescence generated. A substantial increase in VIC fluorescence only indicated homozygosity for wild type allele, in FAM fluorescence only homozygosity for mutant allele, while both fluorescence signals indicated heterozygosity.

2.6. **Statistical analysis**

Frequencies of TSER and **MTHFR C677T** polymorphisms between the Caucasian and Indonesian ALL samples and differences were compared using the chi-square test. Statistics were performed on SPSS software, version 9.0 (Chicago, USA).

**Table 2.** TSER polymorphisms in several patient and population groups

<table>
<thead>
<tr>
<th>Origin</th>
<th>Ethnicity</th>
<th>Number</th>
<th>% Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>2R/2R</td>
</tr>
<tr>
<td>Paediatric control</td>
<td>Indonesian</td>
<td>44</td>
<td>0.0 (0)</td>
</tr>
<tr>
<td>Paediatric leukaemia</td>
<td>Indonesian</td>
<td>71</td>
<td>1.4 (1)</td>
</tr>
<tr>
<td></td>
<td>Dutch [13]</td>
<td>157</td>
<td>21.0 (33)</td>
</tr>
<tr>
<td>Adult control</td>
<td>Caucasian [12]</td>
<td>96</td>
<td>19.0 (18)</td>
</tr>
<tr>
<td></td>
<td>Chinese [12]</td>
<td>96</td>
<td>2.0 (2)</td>
</tr>
</tbody>
</table>
3. Results

We analyzed the *TSER* genotype in 71 samples of Indonesian origin from leukemia cells and compared the results to the 157 samples of Caucasian origin that were published earlier [13]. In addition, blood samples of 44 Indonesian paediatric children without any malignancies were analysed to evaluate whether the difference in genotype would be due to ethnicity and not to a characteristic of the leukaemia cells. This has for instance been described for colon cancer where occasionally only one allele is found due to loss of heterozygosity [14].

Data analyzed with Genescan showed PCR fragments of 208 and 236 bp precisely, representing the two-and-three-repeat sequences of *TSER*, respectively. In Table 2 the allele frequencies are listed and compared to figures that others and we had found previously.

The frequency of *TSER* genotypes in the Indonesian ALL samples was not significantly different from the control children, although a relatively small number of controls was analyzed, while a significant difference was found between Caucasian and Indonesian ALL samples. The *TSER* triple tandem repeat frequency was 33.1% in the Caucasian children samples whereas in the Indonesian samples, we observed a significantly higher triple repeat frequency of 76.1% (p < 0.001). The TSER 2R/2R genotype was present in 1.4% of the Indonesian samples and 21.0% in the Caucasian samples and the 2R/3R repeat variant in 22.5% of the Indonesian samples and 45.9% in the Caucasians. Therefore, the 3R allele was more frequently present in Indonesian children.

We also analyzed the *MTHFR C677T* polymorphism in 65 samples of leukaemia cells from Indonesian children and 32 blood samples of Indonesian paediatric children without any malignancies, while 6 ALL samples and 12 control samples, in which we previously analyzed *TSER* genotype, were not suitable for this analysis. The genotype distribution in the Indonesian ALL patients was not significantly different
from the controls. However, as shown in Table 3, the frequency of this polymorphism was significantly different in Indonesian and Caucasian Dutch ALL (p = 0.003) and control samples (p < 0.001).

Finally, the evaluation of the distribution of \textit{TSER} and \textit{MTHFR} polymorphisms demonstrated that 63.6\% of Indonesian children (59.3\%, ALL and 72.4\%, control) had a \textit{TSER} 3R/3R and \textit{MTHFR} CC genotype (Table 4), while the other children had either a \textit{TSER} 2R/3R and a \textit{MTHFR} 677 CC or CT genotype.

Table 4. Distribution of \textit{TSER} 2R/2R and \textit{MTHFR} C677T polymorphisms in 97 Indonesian children

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Polymorphism</th>
</tr>
</thead>
<tbody>
<tr>
<td>2R/2R</td>
<td>1.1 (1)</td>
</tr>
<tr>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td>2R/3R</td>
<td>14.8 (14)</td>
</tr>
<tr>
<td></td>
<td>2.3 (2)</td>
</tr>
<tr>
<td>3R/3R</td>
<td>63.6 (63)</td>
</tr>
<tr>
<td></td>
<td>14.8 (14)</td>
</tr>
<tr>
<td></td>
<td>2.3 (2)</td>
</tr>
</tbody>
</table>

4. Discussion

In this paper we demonstrate that large differences exist between Indonesian and Dutch children, both in polymorphisms of \textit{TSER} and \textit{MTHFR}. In Indonesian children a much higher proportion of the 3R allele, and specifically of the \textit{TSER} 3R/3R group, was present, while for \textit{MTHFR}, the frequency of TT genotype was very low. There is no difference for \textit{TSER} between Caucasian childhood ALL [13] and adult controls [12], while the genotype distribution in the Indonesian ALL samples was slightly different from the control children, but that may be due to the relatively small numbers. The allele frequencies that we have found in the Indonesian children are best comparable to those reported for the Chinese population in contrast to other Southwest Asian countries [12].

The frequencies of \textit{MTHFR} TT and CT genotypes are two-fold higher in Dutch children in comparison with Indonesian [13]. These results are in agreement with previous data in both adult Indonesian Javanese population [15] and in Indonesian mothers and newborns [16], compared to Caucasian populations [12,13]. All these studies demonstrate that the frequency of the T allele widely differs among ethnic population, ranging from 0.06, as in Indonesian Javanese individuals [15], to 0.59, as in the Mexican population [17], and seems closely related to folic acid food content. For instance, insufficient intake of folate in the pregnant women, which is a common social problem in developing countries, including Indonesia, is considered to be a survival disadvantage for fetuses homozygous for the T allele [18]. Finally,
since the $C677T$ polymorphism has been associated with elevated homocysteine levels, which is associated with increased risk for vascular disease, it has also been suggested that it may play a role in the pathogenesis of pre-eclampsia. However, there are conflicting results regarding an increased frequency of $TT$ homozygous or $CT$ heterozygous among mothers with pre-eclampsia $[16,19]$, as well as in the role of $MTHFR$ polymorphisms to modulate the risk of cardiovascular diseases, neurological defects and several cancers $[4]$. These discrepancies could be partly explained by studies in larger sample sizes, revealing the heterogeneity in the frequency of $C677T$ among areas and ethnic population.

Because of the different prevalence of the $MTHFR C677T$ as well as of the $TSER 2R/3R$ genotypes, and the fact that MTX is a widely used chemotherapeutic agent for the treatment of ALL, one of the most common paediatric cancers globally, the study of these polymorphisms may be an important pharmacogenetic determinant to enable physicians to provide an effective tailored chemotherapy to leukaemia patients.

Since the Indonesian children from the centers in this study are being treated according to protocols derived from Dutch clinical practice, the question arises whether these polymorphisms have consequences for the treatment of ALL with MTX. Although $TSER$ genotype was related to ALL prognosis $[7,10]$, no clear relation between $TSER$ genotype and MTX sensitivity was found in our previous study in 157 Caucasian children $[14]$. Indeed, there are indications that a $G \rightarrow C$ transition in a $3R$ allele may also be important for $TYMS$ expression $[20]$. Similarly, for MTX based treatment outcome other genes involved in folate homeostasis such as methionine synthase reductase ($MTRR$) and serine-hydroxymethyltransferase ($SHMT$), can play an additional role $[13,21]$. These studies provide evidence that more genes need to be considered when evaluating MTX sensitivity and subsequent ALL treatment outcome. However, $TSER$ polymorphisms may also play a role in dosing of MTX. A higher dose of the widely used drug MTX had to be used in Japanese rheumatoid arthritis patients with the $TSER 3R/3R$ $[22]$, while, a recent study by Robien et al. $[5]$, suggested that the incidence of mucositis was higher in patients with a $TSER 2R$ and $TYMS 1494del6$ combined genotype. The lower incidence of toxicity in $TYMS 3R/3R$ may be related to the relation between $TYMS$ polymorphisms and TS activity, which exists in normal tissues but not in cancerous specimens $[23]$. This means that normal cells with a $TYMS 3R/3R$ have a higher activity of $TS$ and are less sensitive to MTX. Hence, patients with a triple repeat may have been undertreated in the past. For this specific group of Indonesian patients, and possibly other Asian countries as well, dosing according to Western protocols might represent under-dosing. The findings here support the individualising of MTX prescription, as is advocated during
the maintenance phase of ALL treatment. MTX dosage should be varied according to the White Blood Cell Count. This is stated in many ALL protocols, but seems often to be forgotten in clinical practice.

Several studies reported that not only the variable number of TSER tandem repeats, but also MTHFR C677T polymorphisms, may play a role in toxic side effects in leukaemia patients [5,8], although several studies did not support a relationship between this polymorphism and the toxicity of MTX treatment [24,25]. Similarly, while Taub et al. [26] reported that MTHFR C677T affects the MTX chemosensitivity of leukaemia blasts from six patients, our previous study showed no relationship between MTHFR C677T polymorphism and ex vivo MTX sensitivity of lymphoblasts obtained from 157 ALL paediatric patients [14]. However, MTHFR C667T variant allele was associated with an increased risk of relapse in 520 paediatric patients treated with MTX [26], while the combination of TSER 3R and MTHFR T677A1298 haplo-type significantly reduced the event free survival after MTX treatment in 201 children with ALL [21].

Therefore, studies investigating ethnic differences in polymorphisms in enzymes that are related to sensitivity (antileukaemia and toxic side effects) to MTX are highly relevant, especially since there is much debate about the best treatment schedule with either high or low dose MTX. Individualized treatment based on pharmacogenetic differences might increase survival of these children. Although a low dose is more cost effective (more children can be treated), it might represent an undertreatment for Indonesian children, who are more frequently homozygous for the TSER 3R/3R polymorphism. In addition, because of the lower frequency of the MTHFR 677T allele, they may experience less toxicity with respect to Caucasian children. Therefore future research should focus on characterization of both side-effects and clinical outcome in these patients in relation to genetic polymorphisms. Finally, since the use of the required higher dose of MTX in paediatric patients of Indonesia might be limited by lack of financial resources, alternative, less expensive treatments, such as with aminopterin [27], might be considered.

Conflicts of interest
All the authors do not have any commercial or other associations that might pose a conflict of interest.

Acknowledgement
This study was supported by a grant (KWF-IN-2006-22) from the Dutch Cancer Society.
Contributions. E. Giovannetti contributed to the analysis and interpretation of
data, writing the article. D.G. Ugrasena and E. Supriyadi contributed to the study conception and analysis of data. L. Vroling, A. Azzarello and D. de Lange contributed to the analysis and interpretation of data. G.J. Peters contributed to the study conception, interpretation of data, writing and revising the article. A.J.P. Veerman contributed to the study conception, interpretation of data, writing and approving the final version of the article. J. Cloos contributed to the study conception, interpretation of data, writing and approving the final version of the article.
Methylenetetrahydrofolate reductase (MTHFR) C677T and thymidylate synthase promoter (TSER) polymorphisms in Indonesian children with and without leukemia

References


