

High frequency of the 3R/3R polymorphism in the thymidylate synthase enhancer region in Indonesian childhood acute lymphoblastic leukemia

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ABSTRACT

Background Deoxyuridylate monophosphate (dTMP) is necessary for DNA synthesis and thymidylate synthase (TS) is an important target of cancer chemotherapy. Ethnic variations of the polymorphic tandem repeat sequence in the enhancer region of the TS promoter has previously been described to influence the outcome of acute lymphoblastic leukemia (ALL). A triple repeat is associated with a higher TS gene expression than a double repeat, resulting in poorer outcome of ALL patients treated with anti-folate methotrexate (MTX).

Objective In this study, we determined the incidences of TS and methylenetetrahydrofolate reductase (MTHFR) polymorphism and ethnic variations between Indonesian and Caucasian ALL cell samples obtained at diagnosis. Furthermore, we determined the involvement of TS polymorphisms in MTX sensitivity using a thymidylate synthase inhibition assay (TSIA).

Methods ALL cell samples were obtained at diagnosis from 101 Indonesian and 157 Caucasian children treated with MTX prospectively. Genotyping for TS and MTHFR was analyzed by Genescan and Lightcycler. TS polymorphism was determined by PCR assay and MTHFR polymorphism and was analyzed by melting curve analyses on lightcycler.

Results Homozygous TS triple repeats were more than twice as common in Indonesian samples (76.3%) than in Caucasian samples (33.1%). Heterozygotes of the MTHFR mutations were seen in 15% of the screened Indonesian samples.

Conclusion There are significant ethnic variations in TS gene regulatory elements of leukemic cells. A difference was found between the MTX sensitivity and a double or triple repeat in the Caucasian ALL group. The samples with a triple repeat show a shift in their distribution towards hypersensitivity to MTX. Further investigation on Indonesian samples may give insight in the role of polymorphisms in MTX sensitivity [Paediatr Indones 2006;46:103-112].

Keywords: polymorphism, acute lymphoblastic leukemia, thymidylate synthase, methotrexate, children

A major problem in the treatment of acute lymphoblastic leukemia (ALL) is the resistance to cytostatic agents. An important cytostatic agent used in the treatment of ALL is anti-folate methotrexate (MTX) which inhibits DNA synthesis. The polyglutamylated form of MTX acts as an inhibitor of enzyme thymidylate synthase (TS)¹ and is an important enzyme in folate metabolism. There are several mechanisms responsible for resistance towards MTX. One of them is intrinsic resistance by genetic changes in enzymes involved in folate-metabolism. Modulation of TS or other folate enzymes such as methylenetetrahydrofolate reductase (MTHFR) may result in resistance.

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TS is an important enzyme, its gene is located in chromosome 18 and catalyses the conversion of deoxyuridylylate monophosphate (dUMP) to deoxythymidylate monophosphate (dTMP) (Figure 1). TS is the key enzyme in *de novo* DNA synthesis. Inhibition of this enzyme results in deoxythymidine triphosphate depletion and thereby chromosome breaks and cell death occurs.² Impairments of the TS enzyme have been associated with chromosome damage and fragile site induction.^{3,4} TS has a unique tandem repeat sequence in the 5' untranslated region (UTR) immediately upstream of the ATG codon ini-

tiation start site that has been shown to be polymorphic, containing either two or three 28-bp repeats (Figure 2).⁵ In colorectal cancer, the presence of the triple versus double 28-bp repeat was shown to enhance gene expression in *in vitro* and *in vivo* studies⁶⁻⁸ and is associated with poor prognosis.⁹

Marsh *et al*¹⁰⁻¹¹ in 1999 was the first to describe ethnic variations between different populations in the TS enhancer region. A TS triple repeat in childhood ALL homozygosity was reported to be associated with a poorer outcome than in those with at least one double-repeat allele.¹³ However, in adult ALL, poly-

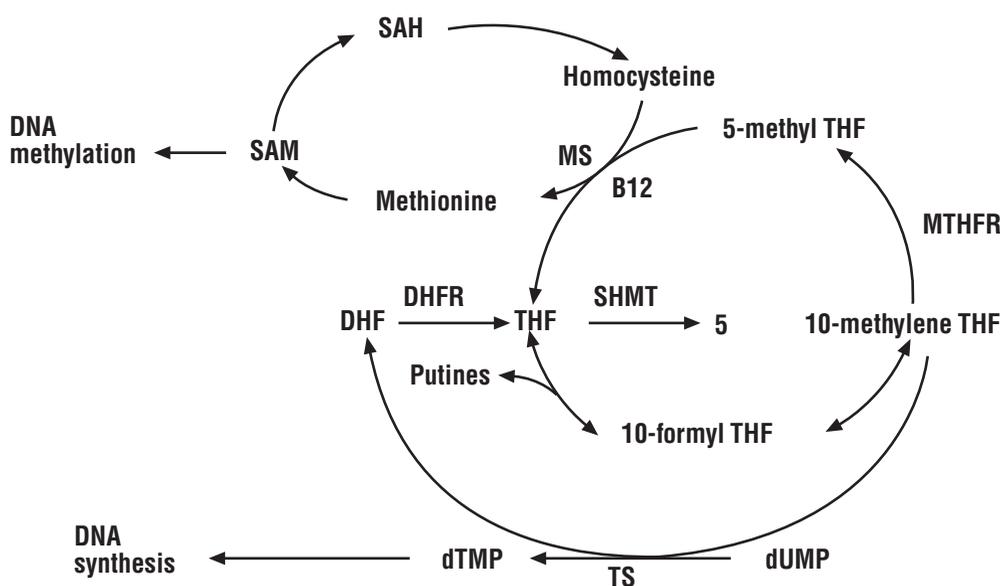


FIGURE 1. OVERVIEW OF THE HUMAN FOLATE METABOLIC PATHWAY.² TS BINDS WITH METHYLENETETRAHYDROFOLATE (METHYLENETHF) WHICH SERVES AS A HYDROXYMETHYL DONOR IN THE CONVERSION OF DUMP TO DTMP IN THE DNA SYNTHESIS PATHWAY.

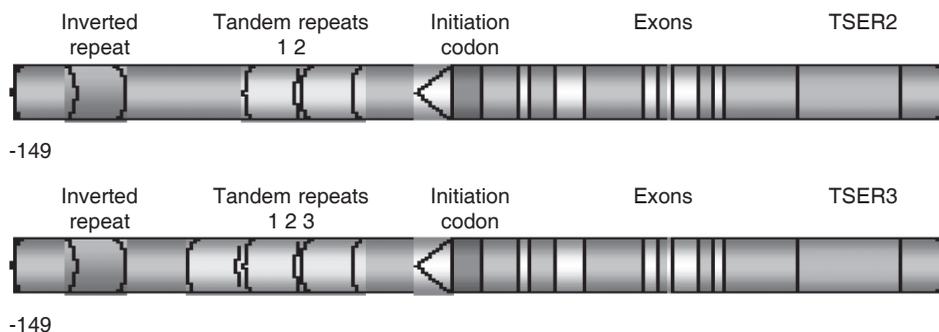


FIGURE 2. SCHEMATIC STRUCTURE OF TS ENHANCER REGION.⁵ CONTAINING EITHER DOUBLE OR TRIPLE 28-BP REPEAT IN THE 5' UNTRANSLATED REGION (UTR).

morphisms of the TS triple repeat seem to result in a higher level of protection of ALL risks than a double repeat.^{14,15}

In addition to polymorphisms of the repeat region, other mutations have also been described in MTX sensitivity. The second most important enzyme in MTX sensitivity is MTHFR which catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) into 5-methyltetrahydrofolate (5-methylTHF) in intracellular folate homeostasis.^{16,17} The substrate 5,10-methyleneTHF is required for DNA synthesis and maintains the balance of the nucleotide pools, whereas 5-methylTHF is required for methylation reactions.^{16,18}

The MTHFR gene is located in chromosome 1p and is subject to several polymorphisms.¹⁹ Among them a mutation C^AT at nucleotide position 677 (exon 4), which results in an alanine to valine substitution.^{16,18} This mutation is associated with lower enzyme activity and increases the frequency of the mutated 677 TT genotype around 10-15% in Caucasians, causes thermolability of MTHFR which leads to lower levels of 5-methylTHF, accumulates 5,10-methyleneTHF, and increases homocysteine levels. This increases the risk of congenital neural tube defect and colorectal cancer.²⁰

The accumulation of 5,10-methyleneTHF results from the MTHFR C677T polymorphism may affect the response of cancer cells towards MTX since activity of the drug is dependent on competitive interactions with folate metabolism.²¹ The 5,10-methyleneTHF is a substrate for the TS enzyme. Since the prevalence of TS tandem repeat and MTHFR C677T polymorphisms and the fact that MTX is commonly used for treating ALL in children, identification of these polymorphisms may be an important pharmacogenetic determinant of predicting response towards MTX. Identification of TS and MTHFR polymorphisms in Indonesian ALL subjects has yet been performed. We also compared ethnic variations of TS and MTHFR between Caucasians and Indonesians, and their involvement in MTX sensitivity.

Methods

Identification of polymorphisms in leukemic cells was performed on individuals with different ethnic

background diagnosed as ALL. Samples were obtained from cells of fresh bone marrow or peripheral blood, frozen in liquid nitrogen vials, put on bone marrow slides or cytospins.

Subjects were children diagnosed with ALL treated at Sardjito Hospital, Yogyakarta, or Soetomo Hospital, Surabaya, with the Wijaya Kusuma ALL 2000 protocol. Vials and bone marrow samples were taken from 101 (42 female and 53 male) children, 6 had missing data. Ages ranged from birth to 14 years. The ALL subtypes were morphologically classified according to the French-American-British (FAB) criteria. From 101 Indonesian samples, 79 had FAB type L1; 11 had L2; 2 had L3, and 9 were not available.

Whole blood was diluted 1:1 with wash medium phosphate buffered saline (PBS), pH 7.4 and 1% fetal calf serum (FCS) and put on a ficoll-gradient with 2:1 ratio. The interphase was collected and the cells were washed two times with wash medium. The mononuclear cells were dissolved in 500 μ l wash medium. Cell count ranged from 10.0-16.7x10⁶.

Vials stored in the nitrogen tank were thawed in a 37°C water bath, transferred in a tube and diluted in 5 ml PBS+0.1% bovine serum albumin (BSA). After centrifugation and washing with wash medium the pellet was suspended in 200 μ l PBS+0.1% BSA.

Genomic DNA was extracted from samples. DNA isolation was done in two ways, from vials and bone marrow slides. First, DNA was extracted from cells using Qiagen DNA Tissue Kit (Qiagen, Hilden, Germany) and stored in liquid nitrogen. Isolation was performed according to protocol included in the kit. Proteinase K incubation time was reduced from overnight to 1 hour since cells were used instead of tissue. DNA was suspended in 200 μ l of double distilled water (DDH) and stored at -20°C. Hank's balanced salt solution (HBSS) and shock buffer was added for better separation and purification between mononuclear cells and erythrocytes before conducting DNA isolation from bone marrow slides. An overnight treatment was followed by phenolchloroform-isoamylalcohol (FCI, 25:24:1) extraction. DNA was precipitated with 3M Sodium Acetate (1/10 of total volume) and 2.5 volumes of 100% ethanol. Glycogen (4 μ g) was used because of the small amount of DNA. This pellet was suspended in 20 μ l DDH and stored at -20°C.

TS and MTHFR genetic polymorphisms were analyzed by Genescan and Lightcycler, respectively.

All isolated DNA samples from Indonesia (n=101) were screened for TS polymorphism while 15 samples were screened for MTHFR polymorphisms. Data of polymorphisms and MTX sensitivity from Caucasian patients were available from a previous study.

For TS polymorphisms, forward and reverse primers were 5'- GCT CCG AGC CGG CCA CAG GCA TGG CGC GG-3' and 5'- GTG GCT CCT GCG TTT CCC CC-3', respectively. Eurogentec synthesized primers used for PCR products were visualized on gel. Primers for gene scan were labeled with fluorescein for measurement. The reverse primer was labeled with FAM and synthesized by Isogen Bioscience BV from Maarssen, Netherlands. Presence of the tandem repeat sequence in the 5'- terminal of the regulatory region of the TS gene, a fragment which contains the 28-bp repeats, was amplified and detected using a protocol described by Horie *et al* and Marsh *et al*.

PCR was conducted on a Peltier Thermal Cycler-200 in a 50 μ l final volume containing 100 ng of genomic DNA, 1 mM MgCl₂, 5.0 μ l of Mg-free buffer 10x (Amersham Biosciences), 0.2 mM dNTPs, 0.2 μ M of each primer, 10% DMSO, and 2.5 units of Taq DNA Polymerase (5U/ μ l; Amersham Biosciences). The reactants were added and mixed on ice and transferred to a preheated 94°C block for amplification of 35 cycles. Each cycle of amplification consisted of denaturation for 1 minute at 94°C, annealing for 1 minute at 60°C and extension for 2 minutes at 72°C followed by 5 minutes at 72°C after the last cycle. The amplified DNA fragments were visualized on a 2% agarose gel 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. As a positive control a heterozygous cell line LS 174T was used.

Parts of the PCR products were visualized using gel electrophoresis and checked with the Genescan (ABI prism® 3100 - Avant, Applied Biosystems). Since the latter method was more sensitive, all further samples were only visualized on the Genescan. The genomic PCR assay was performed again on all the isolated Indonesian samples, except that the reverse primer was labeled at the 5'-end with fluorescein-FAM (Isogen Bioscience BV). PCR products (5 μ l) were combined with 12.5 μ l loading mixture consisted of 12 μ l Hi-Di formamide and 0.5 μ l Gene

Scan™-500 ROX™ size standard (Applied Biosystems, Foster City, California). The samples were run on the ABI PRISM sequencer (3100 genetic Analyzer; Applied Biosystems) and analyzed using Genescan Analysis software (version 1.2, Applied Biosystems). For the identification of single nucleotide polymorphisms in the MTHFR gene of the primer pair, the fluorescent detection probe and anchor probe for determination were provided by Genes-4U (MTHFR C677T ToolSet™ for LightCycler™).

In determining MTHFR polymorphisms, the C677T (Ala→Val) mutations were analyzed using the melting curve analyses on Lightcycler (Roche Diagnostics) and the MTHFR C677T ToolSet™ for LightCycler™ (Genes 4U, licensed by Roche Diagnostics) based on the protocol and recommended by the manufacturers. The 3' end of one probe was labeled with the fluorescent donor dye fluorescein (FLU), whereas the 5' end of an adjacent probe was labeled with an acceptor dye, LC-Red640 as the anchor probe. After reaching the annealing temperature, hybridization probes are allowed to bind to their specific target region. As the temperature increases, the probes melt at their own specific melting temperature and at that moment the donor dye comes into close proximity to the acceptor dye and fluorescence resonance energy transfer (FRET) occurs which results in a fluorescence signal. A sensor probe with a mismatch (containing the mutation) will melt at a lower temperature than a perfectly matched probe (containing the wild type gene).

The Lightcycler PCR amplification was conducted in 20 μ l final volume of reaction mix (16 μ l) containing 4 μ l Faststart DNA Master Hybridization Probes plus 5x (LC FastStart DNA masterplus hybridisation probes, 0351567 Roche Diagnostic, France) 2,8 μ l Oligo Tool MTHFR 677, 9,2 μ l solvent MTHFR 677 (Genes 4U, licensed by Roche Diagnostics GmbH) and 4 μ l genomic DNA (3 ng). After DNA denaturation and enzyme activation (10 minutes at 95°C), DNA was amplified for 50 cycles (1 second at 95°C, 15 seconds at 57°C and 5 seconds at 72°C). After amplification, melting curves of the DNA probes synthesis complexes were measured by gradually increasing the temperature (0.1°C/second) up to 95°C. The mutated form of MTHFR C677T is visualized based on the fact that the melting temperature of the DNA/probe complex is lower in the case of

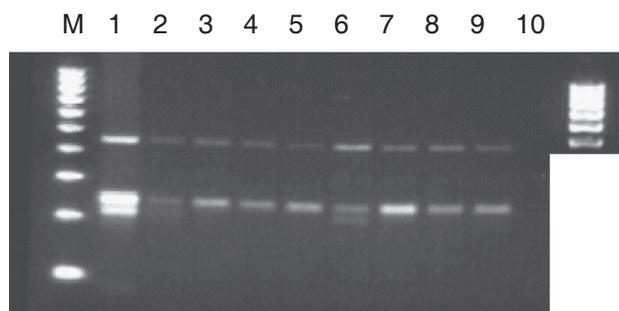


FIGURE 3. PCR ANALYSIS OF TSER POLYMORPHISMS. LANE M SHOWS A 100-BP DNA LADDER LANE 1, POSITIVE CONTROL (LS 174T SELLINE); MOST UPPER BAND (ABOUT 450 BP), ASPECIFIC BINDING; LANE 2-9 PATIENT SAMPLES; LANE 2 AND 6 CONTAINING 2R/3R; LANE 3,4,5,7,8,9 CONTAINING 3R/3R. LANE 10 WATER CONTROL.

DNA-probe T/C mismatch at nucleotide 677 compared with the homozygous wild type (C/C) melting temperature. Monitoring of the C667T genotype was visualized at 640 nm (by Light Cycler software version 4.0, Roche) and the melting curve showed a single peak at 62°C for C/C (homozygous wild type) samples, a single peak at 52.4°C for T/T (homozygous mutant types) samples, and two peaks for the heterozygous samples (consisting one T/T and one C/C allele) at 52.4°C and 62°C, respectively. The positive control was used consisted of lyophilized heterozygous DNA provided by manufacturers.

Frequencies of TSER polymorphisms between Caucasian and Indonesian ALL samples and differences in clinical and hematological features were com-

pared using the chi-square test. Oneway Anova was used for differences in age distribution and incidence of TSER polymorphisms. MTX IC50 was defined as the concentration causing 50% TS inhibition. Correlations between TSER repeats and IC50 were tested by chi-square test. Statistics were performed on SPSS software, version 9.0 (Chicago).

Results

We analyzed the TS genotype of 157 samples of Caucasian ALL patients and 101 samples of Indonesian origin using PCR and Genescan as described above. With gelelectrophoresis we obtained PCR fragments with estimated lengths of 210 and 240 bp, which represent the two- and three-repeats sequences, respectively. From all samples extra PCR fragments longer than 240 bp were observed. These samples may contain TS repeats longer than three repeats or may be an aspecific binding of the primers. These fragments were not relevant with this study, therefore they were excluded from further analysis (Figure 3). The difference in quantity of the two bands may be a result of loss of heterozygosity which is a phenomenon also known in colorectal cancer.^{21,22}

Data analyzed with Genescan showed PCR fragments of 208 and 236 bp precisely, representing the two- and three-repeat sequences (Figure 5). The extra peak in front of the main peak may be a result of hesitation of the tandem repeats depending on the Taq polymerase used for PCR. The frequency of each

TABLE 1. TSER-P (POLYMORPHISMS)

TSER-P	n	Genotype			
		2R/2R	2R/3R	3R/3R	NA
Caucasian	157	32 (20.4%)	72 (40.9%)	52 (33.1%)	0
Indonesian	101	1 (1.1%)	21 (22.6%)	71 (76.3%)	7

TSER-P	MTX sensitivity		
	Sensitive	Intermediate	Resistant
3R/3R	13 (52.0%)	8 (32.0%)	4 (16.0%)
*R/3R	13 (23.0%)	41 (73.2%)	2 (3.6%)

A. Frequencies of TSER polymorphisms between Caucasian and Indonesian ALL

B. MTX sensitivity associated with TSER genotype in Caucasian ALL between genotypes *R/3R and 3R/3R.

genotype in 157 Caucasian and 100 Indonesian samples are shown in **Table 1**. In contrast, the frequency in TSER genotype was significantly different in Caucasian and Indonesian samples. The TS triple tandem repeat frequency was 33.1% in the Caucasian samples which corresponds with the 38% previously reported.¹¹ However, in the Indonesian samples, we observed a significantly higher triple repeat frequency of 76.3% ($P < 0.001$). The TS 2R/2R genotype was present in 1.1% of Indonesian samples and 20.4% in Caucasian samples and the 3R/3R repeat variant in 22.6% of the Indonesian samples and 45.9% in Caucasians. In 7 cases, the fluorescence intensity was extremely low for detection.

The distribution of the C677T genotype was analyzed in 15 samples of Indonesian origin. The analysis of MTHFR genotype in Indonesia showed a frequency of 13.3% ($n=2$) for the heterozygous type (C/T) and 86.7% ($n=13$) for C667 homozygote (wild type). No complete homozygous mutation (T/T) was observed in these samples (**Figure 4**). No further analysis was performed due to a small sample group.

We analyzed the available data known from the Caucasian ALL samples to determine whether there was a relation between TS polymorphisms and MTX sensitivity. TS Inhibition Assay (TSIA) data was available from 81 out of 157 samples. Inhibition of TS was

determined in whole cells by measuring the TS-catalyzed conversion of 3H-dUMP to dTMP and 3H₂O, as previously described.^{23,24} Briefly, 0.1×10^6 cells were incubated in 150 μ l culture medium with/without MTX (provided by Pharmachemie, Haarlem, The Netherlands). After 4 hours, [5-3H]-2'-deoxycytidine (final concentration 1 μ M, 2.5 Ci/mmol) was added as precursor for 3H-dUMP. Data are expressed as TSI50 values, representing the concentration of MTX necessary to inhibit 50% of the TS activity either after 3 hours of MTX exposure followed by an 18-hour drug free period (TSI50, short) or after 21 hours of MTX exposure (TSI50, cont.).²⁴

In this study, data from continuous long-term exposure to MTX was used to determine MTX sensitivity. This data provided insight of only the polyglutamylated-MTX and thereby the TS inhibition. Because the sensitivity for MTX below 0.156 μ M and above 40 μ M concentrations was undetectable, the measurements were divided in 3 categories; hypersensitive, intermediate and insensitive. To determine the effect of a triple repeat on MTX sensitivity the samples were divided in a group consisting at least one double repeat and a group with a triple repeat only (**Table 1**). In addition, a difference was found between the MTX sensitivity and the presence of a double or a triple repeat in Caucasian ALL population. Concern-

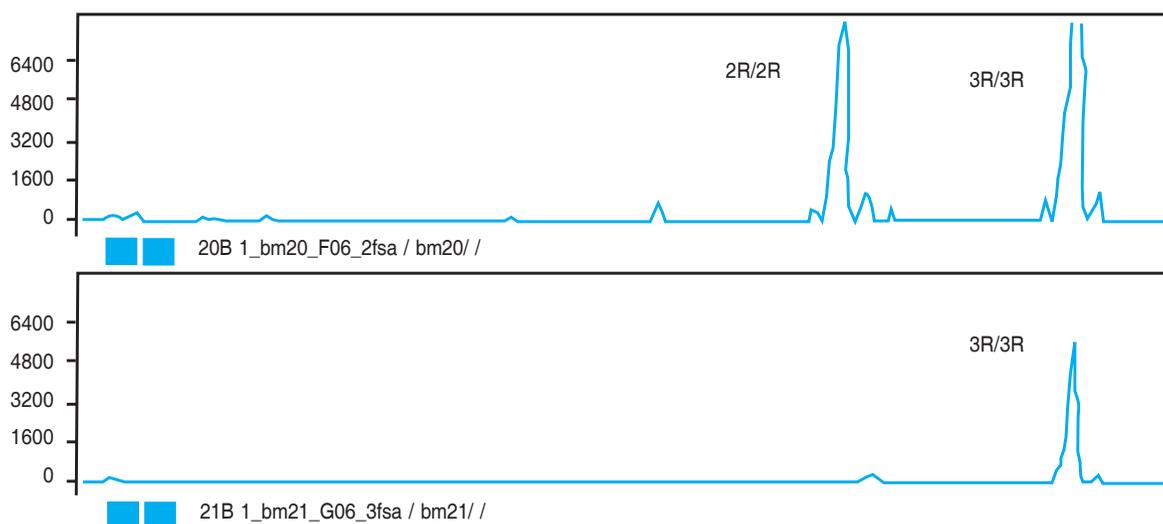


FIGURE 4. GENESCAN ANALYSIS OF TSER POLYMORPHISMS.

THE PCR FRAGMENTS WITH FAM-LABELLED REVERSE PRIMER, WAS VISUALIZED WITH A DNA SEQUENCER.

(A) REPRESENTS A SAMPLE WITH A HETEROZYGOUS GENOTYPE, CONSISTS OF A DOUBLE AND TRIPLE REPEAT.

(B) REPRESENTS A SAMPLE WITH ONLY A TRIPLE REPEAT.

ing the triple repeat there seems to be a shift in the distribution to hypersensitivity to MTX. However, four of the samples (16.0 %) did show resistance to MTX. This could suggest an additional role for other enzymes involved in the folate metabolism, such as MTHFR.

Discussion

Acute lymphoblastic leukemia is the most frequent malignancy that affects children. One of the drugs used in the treatment of this type of leukemia is MTX, which by synthesis of MTX-polyglutamates acts as an inhibitor of TS.¹ Ethnic variations of the polymorphic tandem repeat sequences in the enhancer region of the TS promoter has previously been described to influence the outcome of ALL.^{10,12}

Mutations in the MTHFR gene, which regulates the intracellular folate homeostasis, may also have an effect on the response of cancer cells to MTX. Since polymorphisms can define the sensitivity of a therapy, identification of polymorphisms among different populations is important. This strengthens the need to examine the impact of cancer-treatment-related gene polymorphisms. In this study, we compared the eth-

nic variations of polymorphisms in TS and MTHFR genotype in ALL cells obtained at diagnosis from 157 Caucasian and 101 Indonesian children, which were about to be treated with MTX.

Furthermore, we determined the involvement of TS polymorphisms in MTX sensitivity using a TS inhibition assay. In the present study, we found that the homozygous triple repeats were more than twice as common in Indonesian ALL samples (76.3 %) than in Caucasian ALL samples (33.1%). The Caucasian ALL data corresponded completely with the genotype frequencies previously reported in normal cells of healthy Caucasians.^{11,12} This suggests that the genotype found in Indonesian ALL samples may also represent their normal cells. However, to substantiate this hypothesis we will include normal cells of an Indonesian cohort in future studies. When the normal cells show a different distribution, it may be indicative of a different etiology of ALL in Indonesia. In any way, these ALL data of our study could be of great relevance in the treatment of ALL since the triple repeat may contribute to increased MTX sensitivity.

The difference in genotype was not associated with clinical and hematological features of the patient. There was no difference in sex and age distribution between patients consisting at least one double re-

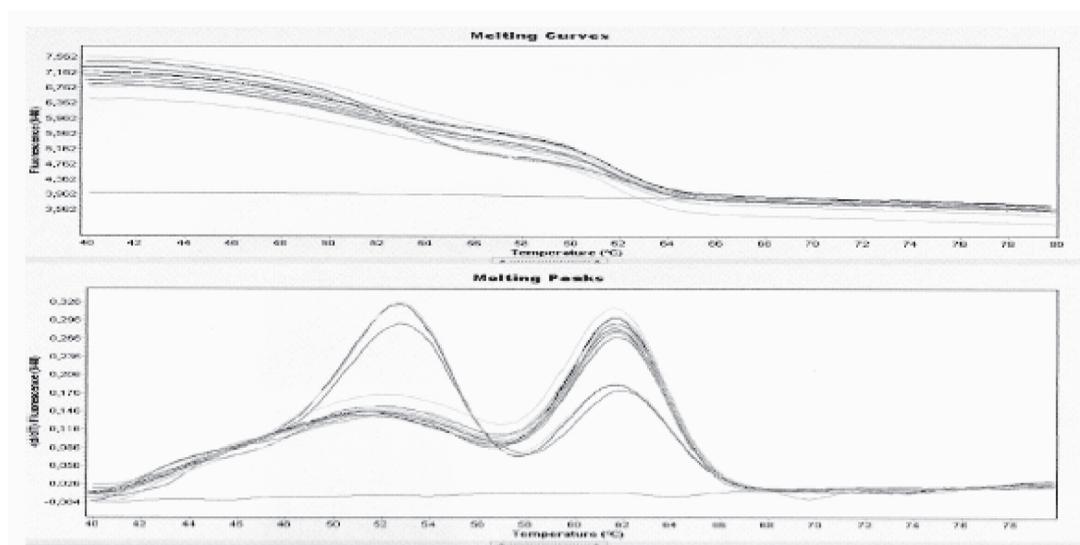


FIGURE 5. TYPICAL EXAMPLE OF MELTING CURVES AND MELTING PEAKS USED TO GENOTYPE C677T MTHFR GENE MUTATION. THE PROBE AND POSITIVE CONTROL THAT WAS USED PROVIDED BY THE MANUFACTURES. MELTING PEAK TEMPERATURES OBTAINED FROM A DERIVATIVE OF THE MELTING CURVES ARE 62,0 °C FOR HOMOZYGOUS WT (C/C), 52,4 °C AND 62,0 °C FOR HETEROZYGOUS (C/T). NO HOMOZYGOUS MUTANT TYPE (T/T) WAS OBSERVED.

peat or consisting a triple repeat only. No relation was found either in WBC and FAB subtype distribution between the polymorphisms. According to several studies on TS polymorphisms, the triple tandem repeat is associated with gene expression and prognosis but giving rather contrasting results. An *in vitro* study showed a stepwise increase in TS gene expression with increasing number of tandem repeats: the presence of a triple repeat results in 2.6 fold greater TS expression than a double repeat.⁶ A retrospective study reported that triple repeat homozygous exhibit 3.6 fold higher TS mRNA levels as compared to double repeat homozygous.⁸ Kawakami *et al*⁷ in 1999 had suggested that mRNA expression levels was not responsible for the genotype dependent differences in TS expression, but the mRNA translation efficiency. Etienne *et al* in 2002 had first described that there was no link between TS activity and TS polymorphisms²⁵ yet other data²⁶ showed that TS activity was significantly higher in 2R/3R heterozygous human cell lines (head and neck, breast, digestive tract).

One of the major findings of the present study was that TS enzymatic activity is significantly influenced by the 5'TS genotype. Interestingly, according to the data in the present study found in Caucasian ALL samples, the triple repeat seems to be associated with hypersensitivity to MTX, which was unexpected. This suggests a good treatment response and thereby a good prognosis. This is in contrast with the results of Krajinovic *et al*¹² which reported a relation between TS polymorphisms and the prognosis of the patient. In 205 childhood ALL cases homozygosity for the TS triple repeat was reported to be associated with poorer outcome than in those MTHFR thermolability, changed intracellular folate distribution, accelerated cellular growth rate, and increased TS activity. The MTHFR 677T mutation increased chemosensitivity with treatment of 5-fluorouracil (5-FU) but a decrease in chemosensitivity was seen in the treatment of MTX.³¹

Several studies reported that MTHFR 677T and 3R/3R TS variants were found to influence susceptibility to ALL.^{15,32,33} Higher 5,10-methyleneTHF and TS levels associated with these variants were suggested to limit DNA damage by reducing uracil incorporation into DNA, thus explaining the protective role of these variants against leukemia. Krajinovic *et al*³³ showed that polymorphisms in the MTHFR in combination with

polymorphisms of the triple repeat of the TS gene resulted in a highly significant reduction of event free survival (EFS).³⁴ These studies provide evidence of the critical role played by the enzymes TS and MTHFR in the folate pathway and their possible gene-gene interactions in MTX sensitivity, and thereby ALL outcome. In our study, heterozygotes of the MTHFR mutations were seen in 13.3% of the screened Indonesian samples (n=15), which is lower than 52.6% in Caucasians previously reported.²⁶ Since polymorphism studies require large population groups, the latter result did not allow further analyses concerning enzyme interactions with TS to be performed. For this purpose the MTHFR polymorphisms will also have to be determined in the complete Indonesian cohort (n=105).

In conclusion, this study shows ethnic variations in the regulatory element of the TS gene in pediatric patients diagnosed with ALL. Furthermore, within the Caucasian ALL samples containing a triple repeat, hypersensitivity towards MTX was observed. Although previous studies show conflicting results they were all associated with more resistance to MTX. The current study shows the opposite. Mutations in the TS enhancer region with or without other enzymes involved may influence gene expression, gene transcription and prognosis of the patient. Therefore it is important that further investigation will unravel the underlying mechanisms of different polymorphisms within the folate pathway, as well as their possible gene-gene interactions and their role as potential predictors of MTX responsiveness and/or toxicity.

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