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# Folate-related Genes Polymorphisms Associated with the Risk of Childhood Leukaemia in the Malaysian Population

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## ABSTRACT

**The folate and methionine metabolism play essential roles in DNA synthesis and DNA methylation, hence their metabolic pathways might affect the susceptibility to diseases such as leukaemia. In this study, we examined the frequencies of several folate-related genes polymorphisms such as methylenetetrahydrofolate reductase MTHFR C677T and A1298C, methylenetetrahydrofolate dehydrogenase1 MTHFD1 G1958A, methionine synthase MTR A2756G and reduced folate carrier RFC1 G80A in 131 childhood leukaemia patients using PCR and PCR-RFLP techniques and determined their association with the risk of developing childhood leukaemia. An increased occurrence of childhood leukaemia was associated with MTHFR 677 CT (OR = 7.22; 95 % CI, 2.98-17.50), MTHFR 1298 AC (OR: 27.92 95% CI: 9.89-78.83) and MTHFR 1298 CC (OR: 13.25 95% CI: 4.42-39.73), RFC1 80 GA (OR: 3.95 95% CI: 1.58-9.83), RFC1 80 AA (OR: 3.83, 95% CI: 1.4-10.26), and SHMT1 1420 CT variant (OR: 5.89, 95% CI: 2.7-12.81) ($p < 0.05$). On the other hand, MTHFD1 1958 GA (OR = 0.42; 95 % CI, 0.20-0.90; $p = 0.03$) and MTRR 66 GG variant (OR = 0.05; 95 % CI, 0.01-0.29; $p < 0.001$) were associated with reduced risk of developing childhood leukaemia. There were no significant association of TS 3R variant and MTR 2576 A > G with the risk of childhood leukaemia variant among the 131 childhood leukaemia patients.**

## INTRODUCTION

Childhood leukaemia represents 4.7 % of the childhood cancers in the Malaysian population (1). Many factors are involved in leukaemogenesis such as genes and environment interaction (2), maternal consumption of folate (3), or changes in DNA that could lead to uncontrolled cellular growth (4). Folate metabolism may become deranged by inadequate nutrition, altered cellular transport, and polymorphisms in folate-related genes (5). Folate is crucial in DNA synthesis and repair where folate deficiency may influence the risk of cancer (6) through mis-incorporation of uracil leading to double strand breaks, chromosomal damage and DNA hypomethylation or dysmethylation (7).

Polymorphisms in folate-related genes listed in Figure 1 have been described and reported to play an essential role in leukaemia susceptibility (5). Reduced folate carrier (RFC) transport mutations have been identified that alter or disrupt RFC expression, resulting in a major loss of antifolate uptake. In ALL, low levels of human RFC have been shown to be associated with poor prognosis (9).

Methylene tetrahydrofolate reductase (MTHFR) polymorphism 677 C > T and 1298 A > C alters normal intracellular folate distribution. Lower activity of MTHFR could lead to increase plasma levels of homocysteine and decrease the formation of methyl-THF that could lead to the pooling of methylene-THF, the methyl group donor for dUMP to dTMP conversion. Therefore, lower activity of MTHFR might favor optimal DNA synthesis by reducing uracil and mis-incoporation rate during uracil excision repairing processes (7, 8). Both MTHFR 1298 and MTHFR 677 polymorphisms were reported to decrease the susceptibility towards lymphoid leukaemia in children and adults (2, 6, 8, 10, 11, 12, 13). As an enzyme that catalyzes the re-methylation of homocysteine to methionine via cobalamin and folate dependent reaction, methionine synthase reductase (MTRR) plays a crucial role in maintaining the active form of cobalamin. The 66 A to G MTRR polymorphisms have a significant influence on the total circulating homocysteine concentration (14). Methylene tetrahydrofolate dehydrogenase1 (MTHFD1) plays an important role in the metabolism of folate and homocysteine, and polymorphisms of MTHFD1 may result

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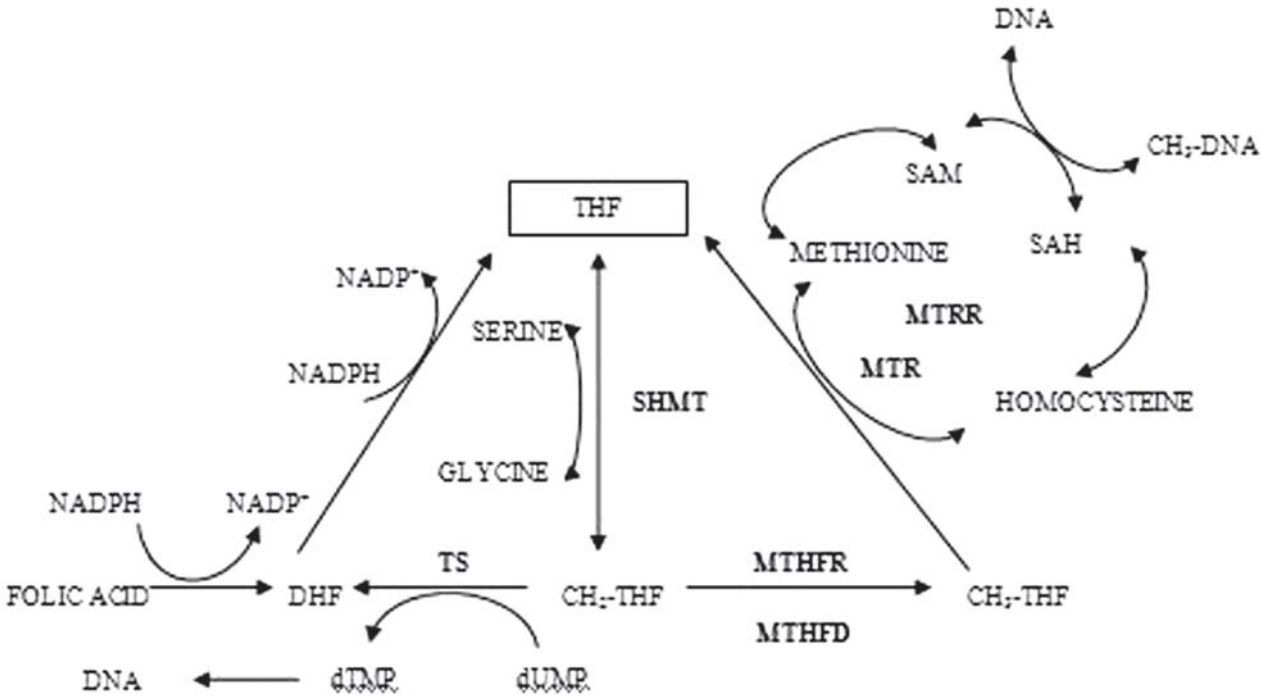


Figure 1: Intracellular metabolism of folate. For clarity, the reaction catalysed by methylenetetrahydrofolate dehydrogenase/ methenyltetrahydrofolate cyclohydrolase/ formyltetrahydrofolate synthetase (MTHFD1) is not shown. MTHFD1 converts CH2-THF in 3 steps and provide formyl-THF (CHO-THF) for purine synthesis. THF indicates tetrahydrofolate; DHF, dihydrofolate; CH2-THF, methylene-THF; CH3-THF; methyl-THF; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; dTMP, deoxy thymidine monophosphate; dUMP, deoxy uridine monophosphate; TS, thymidylate synthase; MTHFR, methylenetetrahydrofolate reductase; MS, methionine synthase; MTRR, methionine synthase reductase; SHMT, serine-hydroxymethyltransferase; DHFR, dihyrdofolate reductase; RFC, reduced folate carrier; MTX-PG, methotrexate-polyglutamate; and NADP, nicotinamide adenine dinucleuotide

in disturbance of the folate-mediated homocysteine pathway. In 2006, De Marco et al reported that heterozygosity and homozygosity of MTHFD1 1958 gene polymorphism as one of the contributing genetic factors for neural tube defect in the Italian population. However, the role of MTHFD1 gene and other folate related genes and in childhood leukaemia susceptibility still remains unclear (15).

Patients with serine hydroxymethyl transferase (SHMT1 1420T allele) polymorphisms in combination with thymidilate synthase gene (TS) repeat (3R) polymorphisms appeared to have a lower risk of ALL (16, 17). Methionine synthase (MTR 2756G) has been reported to be a risk allele for acute lymphoblastic leukaemia (ALL) on its own or in combination with the MTHFR 677 T allele in adults (2). However, it reduces the risk of ALL in adults when combined with the SHMT1 1420 CT/TT variant (16). The aim of this study was to investigate the frequency of folate-related genes polymorphisms in a population of Malaysian childhood leukaemia patients and the interaction of these genes with the risk of childhood leukaemia. We hypothesised that the derangements of folate status caused by the polymorphisms involving folate-related genes and their interactions would influence childhood leukaemia susceptibility.

## MATERIALS AND METHODS

### SUBJECT RECRUITMENT

A total of 131 patients with childhood leukaemia were recruited. Ninety-three of them were patients treated at the Universiti Kebangsaan Malaysia Medical Centre (UKMMC) and another 38 patients were from the Paediatric Institute of the Hospital Kuala Lumpur (HKL). All these patients underwent treatment between the year 2000 to 2008. A total of 150 controls were selected from students attending form two (14 years of age) at the Sekolah Menengah Kebangsaan Wangsa Maju and Sekolah Menengah Kebangsaan Taman Melati Setapak during the year 2005. Informed consent was obtained from the legal guardian for each subject and control. This study was approved by the ethic committee of all participating institutions. Peripheral blood samples were taken from the cases and buccal swabs were taken from the controls. Samples were stored at -80°C before DNA extraction and analysis. The age range of the leukaemia patients were 9 month to 19 years old. There were 114 patients with acute lymphoblastic leukaemia (ALL), 5 with acute myeloid leukaemia (AML), and 12 with relapsed ALL. The controls were 14 years old when their blood samples were taken (2005).

### DNA EXTRACTION

DNA from the peripheral blood samples taken from cases was extracted using the salt saturation method. DNA from buccal swabs of the controls was extracted using the *BuccalAmp™ DNA Extraction Kit QuickExtract™ DNA Extraction Solution 1.0 Catch-All™ Sample Collection Swabs*.

### GENOTYPE ANALYSIS OF FOLATE-RELATED GENE POLYMORPHISMS

Polymerase chain reaction (PCR) amplifications of the genetic variant of each folate-related gene were carried out using forward and reverse primers listed in Table 1.

The polymorphisms for each gene were determined by PCR-restriction fragment length polymorphisms (RFLP) using *Hinf I* restriction enzyme for MTHFR C677T polymorphisms, *Mbo II* for MTHFR A1298C polymorphisms, *Hha I* for RFC A80G polymorphisms, *Ear I* for SHMT C1420T polymorphisms, *Hae III* for MTR A2756G polymorphisms, *Nde I* for MTRR A66G polymorphisms and *Msp I* for MTHFD G1958A polymorphisms. As for the 28-bp double (2R2R) or triple tandem repeats (3R3R) in the promoter region of thymidylate synthase (TS), the resulting bands were directly visualized on agarose gels after PCR (18).

Table 1. List of oligonucleotide forward and reverse primer for each gene in the polymerase chain reaction (PCR)

| Gene | Forward and reverse primer |
|---|---|
| MTHFR (C677T) | Forward primer: 5'-TGAAGGAGAAGGTGTCTGCGGGA-3'<br>Reverse primer: 5'-AGGACGGTGCGGTGAGAGTG-3' |
| MTHFR (A1298C) | Forward primer: 5'-CTTTGGGGAGCTGAAGGACTACTAC-3'<br>Reverse primer: 5'-CACTTTGTGACCATTCCGGTTTG-3' |
| RFC (G80A) | Forward primer: 5'-AGTGTCACCTTCGTCCCCTC-3'<br>Reverse primer: 5'-CTCCCGCGTGAAGTTCTT-3' |
| TS (3R) | Forward primer: 5'- GTGGCTCCTGCGTTTCCCCC -3'<br>Reverse primer: 5'CCAAGCTTGGCTCCGAGCCGGCCACAGGCATGG-3' |
| SHMT1 (C1420T) | Forward primer: 5'-CAGAGCCACCCTGAAAGAGTTC-3'<br>Reverse primer: 5'-GTGGGCCCGCTCCTTTA-3' |
| MTR (A2756G) | Forward primer: 5'-TGTTCCAGACAGTTAGATGAAAATC-3'<br>Reverse primer: 5'-GATCCAAAGCCTTTTACACTCCTC-3' |
| MTRR (A66G) | Forward primer: 5'- GCAAAGGCCATCGCAGAAGACAT-3'<br>Reverse primer: 5'-TGGTGGTATTAGTGTCCTTTTG-3' |
| MTHFD1 (G1958A) | Forward primer: 5'-CCCACTTTGAAGCAGGATTG-3'<br>Reverse primer: 5'-CACCATCCCAATTCCCCTGATG-3' |

DNA sequencing was used to validate the polymorphisms for each folate-related gene. Sequence of each polymorphism was determined using the 3130 × l Applied Biosystem (ABI) Genetic Analyzer with BigDye®Terminator v3.1 Cycle Sequencing Kit.

### STATISTICAL ANALYSIS

Power calculation was performed using the BRIDGES power and sample size calculation program (19). Logistic regression analysis was performed to obtain odd ratios (ORs) and 95% confidence intervals (CI) for the estimation of the relative risks for each polymorphism. Gene-gene interactions within the folate-related genes polymorphism were also explored. All analyses with dominant genetic model were carried out using the SPSS version 16.0 to calculate ORs and CIs.

## RESULTS

Of the the 131 patients, 54.2% were female, and 45.8% were male with an ethnicity distribution of 75.6% Malays, 15.3% Chinese and 9.7% Indians (Table 2) with the majority of patients were ALL (88.5%), followed by relapsed ALL (6.1%) and acute myeloid leukaemia (AML) (3.8%).

Table 3 shows the distribution between folate-related gene polymorphisms in childhood leukaemia patients and controls. The MTHFR 677 CT variant was significantly more common in the childhood leukaemia group (39.7%) compared with the control group, indicating that the MTHFR 677 CT variant is related to increased risk of childhood leukaemia (OR = 7.22; 95 % CI, 2.98-17.50; $p < 0.00$) in our population.

The frequency of MTHFR 1298 AA homozygous dominant genotype was lower in patients (6.1%) compared with the control group (50.7%). Significant increased risk was also observed for MTHFR 1298 AC (OR = 27.92; 95% CI, 9.89-78.83; $p < 0.00$), and MTHFR 1298 CC variant (OR

Table 2. Demographic data for 131 childhood leukaemia patients and 150 childhood control

| | Race | | | Gender | |
|---|---|---|---|---|---|
| | Chinese (%) | Malay (%) | Indian (%) | Male (%) | Female (%) |
| Control | 2 (1.3) | 134 (89.3) | 14 (9.3) | 37 (24.7) | 113 (75.3) |
| Childhood leukaemia | 20 (15.3) | 99 (75.6) | 12 (9.7) | 60 (45.8) | 71 (54.2) |
| Total | 22 (7.8) | 233 (82.9) | 26 (9.3) | 97 (34.5) | 184 (65.5) |

Table 3. Distribution of folate related genes in childhood leukaemia patients and control

| Genotype | | Childhood Leukaemia n = 131 (%) | Control n = 150 (%) | OR (95% CI) | *p* value |
|---|---|---|---|---|---|
| MTHFR 677 | CC | 74 (56.5) | 120 (80) | 1 | |
| | CT | 52 (39.7) | 21 (14.0) | 7.22 (2.98-17.50) | 0.00 |
| | TT | 5 (3.8) | 9 (6.0) | 1.63 (0.33-3.31) | 0.55 |
| MTHFR 1298 | AA | 8 (6.1) | 76 (50.7) | 1 | |
| | AC | 93 (71.0) | 40 (26.7) | 27.92 (9.89-78.83) | 0.00 |
| | CC | 30 (22.9) | 34 (22.7) | 13.25 (4.42-39.73) | 0.00 |
| RFC1 80 | GG | 18 (13.7) | 49 (32.7) | 1 | |
| | GA | 62 (47.3) | 61 (40.7) | 3.95 (1.58-9.83) | 0.00 |
| | AA | 51 (38.9) | 40 (26.6) | 3.83 (1.43-10.26) | 0.01 |
| TS | 3R | 91 (69.5) | 86 (57.3) | 1 | |
| | 3R2R | 32 (24.4) | 51 (34.0) | 0.49 (0.22-1.09) | 0.08 |
| | 2R | 8 (6.1) | 13 (8.7) | 1.26 (0.34-4.36) | 0.73 |
| SHMT1 1420 | CC | 54 (41.2) | 85 (56.7) | 1 | |
| | CT | 74 (56.5) | 38 (25.3) | 5.89 (2.7-12.81) | 0.00 |
| | TT | 3 (2.3) | 27 (18.0) | 0.43 (0.10-1.78) | 0.24 |
| MTHFD1 1958 | GG | 68 (51.9) | 45 (30.0) | 1 | |
| | GA | 44 (33.6) | 85 (56.7) | 0.42 (0.20-0.90) | 0.03 |
| | AA | 19 (14.5) | 20 (13.3) | 0.43 (0.15-1.27) | 0.13 |
| MTR 2756 | AA | 98 (74.8) | 98 (65.3) | 1 | |
| | AG | 32 (24.4) | 47 (31.3) | 0.85 (0.39-1.85) | 0.68 |
| | GG | 1 (0.8) | 5 (3.3) | 0.24 (0.02-3.31) | 0.28 |
| MTRR 66 | AA | 30 (22.9) | 17 (11.3) | 1 | |
| | AG | 98 (74.8) | 115 (76.7) | 0.38 (0.14-1.02) | 0.06 |
| | GG | 3 (2.3) | 18 (12.0) | 0.05 (0.01-0.29) | 0.00 |

= 13.25, 95% CI, 4.42-39.73, p < 0.00) in childhood leukaemia patients compared to the control group.

The frequencies of the RFC1 80 GA variant and RFC 80 AA variant in childhood leukaemia patients were higher (47.3% and 38.9%) compared with the control group (40.7% and 26.7%), indicating that RFC1 80 GA variant (OR = 3.95; 95% CI, 1.58-9.83; p < 0.00) and RFC1 80 AA variant (OR = 3.83; 95% CI, 1.43-10.26; p < 0.00) could be related to an increased risk of leukaemia. There is also an increased risk of leukaemia for patients having the SHMT1 1420 CT variant (OR = 5.89; 95% CI, 2.7-12.81; p < 0.00).

The MTHFD1 1958 GA (OR = 0.42; 95% CI, 0.20-0.90; p = 0.03) and MTRR 66 GG variants (OR = 0.05; 95% CI, 0.01-0.29; p < 0.00) were associated with a decreased risk of leukaemia in the population.

We also analysed the gene-gene interaction between the MTHFR 677 CT (Table 4), MTHFR 1298 AC (Table 5) and other folate-related gene polymorphisms. In order to look for allele interaction, we combined the frequencies of homozygous mutants and heterozygous variants in the analysis. We observed that the interaction between MTHFR 677 CC variant with MTHFR 1298 AC variant (OR = 11.9; 95% CI, 4.8-29.3), MTHFR 1298 CC variant (OR = 4.2; 95% CI, 1.6-11.2), MTHFD 1958 GA (OR = 2.82; 95% CI, 2.09-7.3), RFC1 80 GA variant (OR = 2.9; 95% CI, 1.2-7.3) and RFC1 80 AA variant (OR = 5.14; 95% CI, 2.01-13.2) all suggested an increased risk of leukaemia (Table 4). There is also an interaction between MTHFR 677 CT and TT variant with RFC1 80 GG variant (OR = 4.8; 95% CI, 1.7-14.2) indicating an increased leukaemia risk.

The interaction between MTHFR 1298 AC and CC variant with MTRR 66 AA (OR = 30.7; 95% CI, 3.9-239), MTRR 66 AG (OR = 11.4; 95% CI, 1.9-66.5) and SHMT 1420 CT (OR = 15.1; 95% CI, 3.1-72.7) also suggested an increased leukaemia risk as shown in Table 4.

Table 4. Interaction between MTHFR 677 polymorphisms and other polymorphisms in folate related genes in control and childhood leukemia

| Genotype | Control (n) | Childhood patients (n) | OR (CI) | *p* value |
|---|---|---|---|---|
| MTHFR677 CC v/s | | | | |
| MTHFR1298 AA | 55 | 7 | 1 | 0 |
| AC | 33 | 50 | 11.9 (4.8-29.3) | 0* |
| CC | 32 | 17 | 4.2 (1.6-11.2) | 0.04* |
| MTHFD 1958 GG | 34 | 43 | 1 | 0.1 |
| GA | 7 | 25 | 2.82 (2.09-7.3) | 0.03* |
| AA | 4 | 0 | | N/A |
| MTR 2756 AA | 81 | 53 | 1 | 0.77 |
| AG | 36 | 20 | 0.85 (0.45-1.62) | 0.62 |
| GG | 3 | 1 | 0.51 (0.52-5.03) | 0.56 |
| MTRR 66 AA | 12 | 16 | 1 | 0.14 |
| AG | 92 | 56 | 0.46 (0.20-1.04) | 0.61 |
| GG | 16 | 2 | 0.09 (0.02-0.49) | 0.00* |
| RFC GG | 34 | 7 | 1 | 0.00 |
| GA | 52 | 31 | 2.9 (1.2-7.3) | 0.02* |
| AA | 34 | 36 | 5.14 (2.01-13.2) | 0.00* |
| SHMT 1420 CC | 67 | 17 | 1 | |
| CT | 30 | 55 | 7.23 (3.6-14.5) | 0* |
| TT | 23 | 2 | 0.34 (0.1-1.5) | 0.17 |
| TS 3R | 68 | 44 | | 0.85 |
| 3R2R | 42 | 23 | 0.85 (0.45-0.6) | 0.61 |
| 2R | 10 | 7 | 1.08 (0.4-3.1) | 0.88 |
| MTHFR 677 CT+TT v/s | | | | |
| MTHFR1298 AA | 21 | 1 | 0.95 (0.2-5.1) | 0* |
| AC | 7 | 43 | 0.007 (0.001-0.09) | 0* |
| CC | 2 | 13 | 1 | 0.95 |
| MTHFD 1958 GG | 11 | 25 | 2.02 (0.62-6.6) | 0.3 |
| GA | 11 | 23 | 1.9 (0.56-6.1) | 0.3 |
| AA | 8 | 9 | 1 | 0.5 |
| MTR 2756 AA | 17 | 45 | | |
| AG | 11 | 12 | | |
| GG | 2 | 0 | N/A | |
| MTRR 66 AA | 5 | 14 | 5.6 (0.4-76.05) | 0.2 |
| AG | 23 | 42 | 3.7 (0.3-42.5) | 0.3 |
| GG | 2 | 1 | 1 | 0.41 |
| RFC GG | 15 | 22 | 4.8 (1.7-14.2) | 0.04* |
| GA | 9 | 32 | 3.4 (1.0-11.6) | 0.05 |
| AA | 6 | 15 | 1 | 0.12 |
| SHMT 1420 CC | 18 | 37 | 8.2 (0.86-79) | 0.07 |
| CT | 8 | 19 | 9.5 (0.9-98.8) | 0.06 |
| TT | 4 | 1 | 1 | 0.16 |
| TS 3R | 18 | 47 | 7.8 (0.76-80.3) | 0.08 |
| 3R2R | 9 | 9 | 3 (0.26-34.6) | 0.38 |
| 2R | 3 | 1 | 1 | 0.07 |

* $p < 0.05$

Table 5. Interaction between MTHFR 1298 polymoprhisms and other folate related genes in control and childhood leukemia

| Genotype | Childhood control (n) | Childhood leukemia (n) | OR (CI) | *p* value |
|---|---|---|---|---|
| MTHFR 1298 AA v/s | | | | |
| MTHFR677 CC | 55 | 7 | | 0.87 |
| CT | 14 | 1 | 0.56 (0.06-4.9) | 0.6 |
| TT | 7 | 0 | | N/A |
| MTHFD 1958 GG | 22 | 5 | | 0.27 |
| GA | 46 | 3 | 0.29 (0.06-1.3) | 0.11 |
| AA | 8 | 0 | | N/A |
| MTR 2756 AA | 48 | 7 | | 0.52 |
| AG | 24 | 1 | 0.29 (0.03-2.5) | 0.25 |
| GG | 4 | 0 | | N/A |
| MTRR 66 AA | 12 | 2 | | 0.9 |
| AG | 57 | 5 | 0.65 (0.05-8.2) | 0.7 |
| GG | 7 | 1 | 0.88 (0.03-25.6) | 0.9 |
| RFC GG | 24 | 2 | | 0.53 |
| GA | 34 | 5 | 0.4 (0.03-5.6) | 0.5 |
| AA | 18 | 1 | 0.2 (0.01-4.2) | 0.3 |
| SHMT 1420 CC | 40 | 4 | | 0.3 |
| CT | 20 | 5 | 3.3 (0.7-15.4) | 0.12 |
| TT | 16 | 0 | N/A | |
| TS 3R | 41 | 5 | | 0.22 |
| 3R2R | 29 | 1 | 0.28 (0.03-2.6) | 0.26 |
| 2R | 6 | 2 | 2.7 (0.4-17.4) | 0.29 |
| MTHFR 1298 AC+CC v/s | | | | |
| MTHFR 677 CC | 65 | 68 | 0.23 (0.03-1.6) | 0.14 |
| CT | 7 | 51 | 5.1 (0.6-42.8) | 0.13 |
| TT | 2 | 5 | | 0 |
| MTHFD 1958 GG | 23 | 63 | 2.4 (0.7-8.1) | 0.2 |
| GA | 39 | 41 | 0.91 (0.3-3) | 0.9 |
| AA | 12 | 19 | | 0.09 |
| MTR 2756 AA | 50 | 91 | 2.3 (0.1-56.6) | 0.6 |
| AG | 33 | 31 | 2.45 (0.1-64.6) | 0.6 |
| GG | 1 | 1 | | 0.87 |
| MTRR 66 AA | 5 | 28 | 30.7 (3.9-239) | 0* |
| AG | 58 | 93 | 11.4 (1.9-66.5) | 0.01* |
| GG | 11 | 2 | | 0 |
| RFC GG | 25 | 16 | 0.15 (0.05-0.5) | 0* |
| GA | 27 | 57 | 0.83 (0.33-2.1) | 0.68 |
| AA | 22 | 50 | | 0 |
| SHMT 1420 CC | 45 | 51 | 2.2 (0.5-10.4) | 0.31 |
| CT | 18 | 69 | 15.1 (3.1-72.7) | 0* |
| TT | 11 | 3 | | 0 |
| TS 3R | 45 | 86 | 2.2 (0.7-7) | 0.17 |
| 3R2R | 22 | 31 | 1.6 (0.5-5.6) | 0.42 |
| 2R | 7 | 1 | | 0.31 |

* $p < 0.05$

## DISCUSSION

Leukaemia is one of the most prevalent childhood cancers worldwide and also in Malaysia (1). Onset at a young age and a short latency period between the appearance of mutations and identification of tumor cells are unique to childhood leukaemia patients (20).

Distribution of MTHFR polymorphisms differs between ethnic groups as we observed a high frequency of MTHFR 1298 variant AC (71%) in our population of

childhood leukaemia patients compared with the Filipino population (47.7%) (21). However, the frequency of MTHFR 677 T-allele (43.5%) is lower compared with Japanese population (66.7%) (12). Among the ethnic groups in Malaysia, we observed different distributions of MTHFR in the Indian population (9.2%), Chinese (7.8%) and Malay (83%).

From a previous study, the MTHFR 677 TT and MTHFR 1298 CC variants were reported to decrease the risk of colorectal cancer (22). Various studies reported both MTHFR variants to decrease the susceptibility to lymphoid leukaemia in children and adults (2, 6, 11, 12, 13). Using a recessive model, we observed that the T-allele of MTHFR 677 decreased the leukaemia risk (OR = 0.33; 95% CI, 0.19-0.55) in children. According to a meta-analysis done by Robien et al., in 2003, many studies have shown that MTHFR 677TT decreased leukaemia risk especially in childhood ALL (23). However, the relationship between MTHFR 1298 variant with childhood leukaemia is still controversial. In Korean populations, there is interaction between MTHFR 677 CC variant and MTHFR 1298 CC variant in chronic myeloid leukaemia (CML), acute myeloid leukaemia (AML), and multiple myeloma (MM) population ($p < 0.05$) (24) .

The polymorphisms of MTHFR 1298 AC and CC variants were higher in our patients compared to the control group indicating the role of this polymorphism in increasing leukaemia risk in childhood. This finding concurred with the study done in the Philippines (21). We also observed that the interaction between MTHFR 677 and MTHFR 1298 with other folate-related genes increased leukaemia risk especially in patients who presented with the combination alelle variant of MTHFR 677 CC and MTHFR 1298 CC (22).

Theoretically, lower activity of MTHFR will lead to pooling of methylene tetrahydrofolate (methylene-THF) that will enhance the fidelity of DNA synthesis. The increases in the methylene-THF pool leads to an increase in the availability of methyl groups for the conversion of uracil to thymidine, causing less mis-incorporation of uracil than otherwise that might have if there was inadequate thymidine for DNA synthesis (25, 26). Taking it all together, it is postulated that the relationship between MTHFR polymorphism and the risk of cancer is modifiable by other nutrients involved in the MTHFR metabolic pathway. It is also speculated that during times of high supply of dietary methyl, the 677TT polymorphism confers a protective effect by way of a higher intracellular methylene-THF pool. However, when dietary folate is low and presence of methyl-THF is depleted by high alcohol consumption, the protective effect of 677TT is abolished. The reduction of the methylene-THF pool will then lead to abnormal DNA synthesis. Methylation in this case would also be another mechanism by which carcinogenesis appears, since the methyl-THF pool would have been depleted by poor supply and the influence of the 677TT polymorphism (25). For that reason, it must be appreciated that the gene-nutrient interaction in the MTHFR polymorphism-folate scenario is not straightforward.

As for RFCs that play a role as folate transporter, distribution of RFC1 80 variant in a Japanese 281 childhood case-control group showed highest distribution among heterozygous mutants (43.8%) compared with other genotypes (27). De Marco in 2001, reported almost the same distribution of this heterozygous mutant genotype in patients with neural tube defects in Italy (28). In our study, RFC polymorphisms seemed to be related to an increased risk of childhood leukaemia. The RFC transport system plays pivotal roles as methotrexate (MTX) (29) and folate transporters hence the effectiveness of chemotherapy with these agents is closely linked to the levels and activity of RFC transport in both tumors and normal tissues (30, 31). The RFC1 80 AA polymorphism was observed to increased MTX transport in plasma indicating effective transportation of MTX for the variant (27). However, this study did not elucidate the relationship between the transport system and the development of leukaemia.

The TS gene regulation occurs not only at the transcriptional level but also at the translation level (18). The TS is an important target for a variety of chemotherapeutic agents including 5-fluorouracil (5-FU), 5-FU prodrugs (capecitabine), and novel folate-based TS inhibitors (raltitrexed and pemetrexed) which are used for colorectal and other solid tumors treatment (32). The TS alleles polymorphisms are represented by 28-based pair tandem double or triple repeats, creating genotypes defined as 2R/2R, 2R/3R and 3R/3R respectively. The TS genes with the 3R allele were found to have greater expression activities than the 2R sequence in a transient expression assay in cancer cells (18). Taken together, the polymorphisms might have altered TS gene expression (18) and translation efficiency (33).

In our study, 69.5% of the childhood leukaemia group carried the 3R variant compared to 57.3 % in the control group, and this difference is not significant ($p = 0.64$). Non-significant difference in distribution of TS polymorphisms was observed in a Japanese study (23). Skibola et al. (16) in 2002 and de Jonge et al in 2009 (5) reported in their studies the protective effect of TS 3R variant in childhood leukaemia patients. In our study we observed that the TS 3R2R (OR = 0.79; 95% CI, 0.22-2.92) and 2R2R variants (OR = 0.39; 95% CI, 0.09-1.58) to be associated with a decreased risk of childhood leukaemia in the Malaysian population. An interaction between TS 2R variant and SHMT 1420 CC was observed in increasing the risk of malignant lymphoma (17), while decreased risk of leukaemia in adults was observed in TS 3R variant and SHMT1 interaction (16). However, in our study, we could not confirm the protective effect of interaction between 3R variant and SHMT1 gene. Futher studies are needed to elucidate the effect of TS gene polymorphisms.

SHMT1 gene catalyzes the reversible conversion of serine and tetrahydofolate (THF) to glycine and methylene-THF providing the cells with most of the one-carbon units reqiured for the synthesis of thymidine, purines, cholines and methionine (32). In our study, the SHMT1 allele-T variant showed a higher distribution (56%) compared with SHMT allele-C variant. Both the Japanese (17) and British populations (16) had a higher proportion with the SHMT allele-C variant. We also observed an increased risk of leukaemia in for the SHMT1 CT variant (OR = 2.33; 95 % CI, 0.56-9.64) and SHMT1 TT (OR = 13.7; 95% CI, 3.24-58.05), an observation which is different from the British and Japanese studies which showed decreased risk in adult leukaemia for the same variant (16). This might be due to the different ethnic composition of the Malaysian population compared to Japanese and Caucasian populations.

MTHFD1 G1958A is also known as tri-functional enzyme that converts THF in three different ways (34). Even though the function and the relationship of MTHFD1 gene with the risk of childhood leukaemia are still unclear, we believe that MTHFD1, which plays a role in the formation of purines through formyl-THF (34) formation also play a role in leukaemogenesis as do other folate-related gene polymorphisms. The frequency of MTHFD1 1958 GG variant in childhood leukaemia in the Malaysian population is 51.9% in comparison to those with neural tube defects (17.6%) in the Italian population (15). In this study, the MTHFD1 1958 GA variant (OR = 0.42; 95% CI, 0.20-0.90; p = 0.03) showed decreased risk of childhood leukaemia. However, due to limited number of references to support our finding, the function of MTHFD1 remains questionable.

The MTR gene plays an essential role in determining the homosisteine level in methtionine metabolism. It plays a critical role in maintaining adequate intracellular S-adenosyl-methionine (SAM) levels for DNA methylation and it also has a tumour suppressor effect. The MTR polymorphism A2576C converts an aspartic acid to a glycine residue and has been predicted to alter enzyme activity that may affect DNA methylation process (35). MTR requires activation by MTRR (36). The elevated homocysteine level causes DNA methylation which is involved in carcinogenesis (37). In this study, the frequencies of MTR gene polymorphisms were not significantly different between childhood leukaemia patients and controls and this is similar to that reported by Gemmati et al in 2004 (2). In the same report, the MTRR 66 AG genotype was found to be most common in the Italian population which is similar to the observation in our study with a 75.8% distribution of the genotype. We also observed an interaction between MTR 2756 AA and MTRR 66 GG variants in decreasing leukaemia risk (OR = 0.04; 95% CI, 0.007-0.3).

Even though statistical analysis show that our hypothesis of the involvement of folate-related genes in the development of leukaemia in Malaysian childhood is valid, low frequency distribution in carrier alleles and mutant alleles might have cause some discrepancies. Results observed in our study may have been affected by the ethnic composition of our subjects. However, we were not able to study the relationship between ethnicity and folate-related gene polymorphisms as the distribution ratio of the 3 ethnic groups among our study subjects was not evenly distributed (Malay: Chinese : Indian = 11 : 1.2 : 1).

In conclusion, we observed different distribution of folate-related gene polymorphisms in the Malaysian population and compared this distribution to other populations in other regions. In this study, the relationship of these genes with the risk of childhood leukaemia in the Malaysian population was observed, increasing our understanding of the function and interaction of these genes in folate metabolism pathway.

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