The Association of Methylenetetrahydrofolate Reductase Genotypes with the Risk of Childhood Leukemia in Taiwan

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Abstract

Background

Acute lymphoblastic leukemia (ALL) is the most prevalent type of pediatric cancer, the causes of which are likely to involve an interaction between genetic and environmental factors. To evaluate the effects of the genotypic polymorphisms in methylenetetrahydrofolate reductase (MTHFR) on childhood ALL risk in Taiwan, two well-known polymorphic genotypes of MTHFR, C677T (rs1801133) and A1298C (rs1801131), were analyzed to examine the extent of their associations with childhood ALL susceptibility and to discuss the MTHFR genotypic contribution to childhood ALL risk among different populations.

Methodology/Principal Findings

In total, 266 patients with childhood ALL and an equal number of non-cancer controls recruited were genotyped utilizing PCR-RFLP methodology. The MTHFR C677T genotype, but not the A1298C, was differentially distributed between childhood ALL and control groups. The CT and TT of MTHFR C677T genotypes were significantly more frequently found in controls than in childhood ALL patients (odds ratios=0.60 and 0.48, 95% confidence intervals=0.42–0.87 and 0.24–0.97, respectively). As for gender, the boys carrying the MTHFR C677T CT or TT genotype conferred a lower odds ratio of 0.51 (95% confidence interval=0.32–0.81, P=0.0113) for childhood ALL. As for age, those equal to or greater than 3.5 years of age at onset of disease carrying the MTHFR C677T CT or TT genotype were of lower risk (odds ratio=0.43 and 95% confidence interval=0.26–0.71, P=0.0016).
Conclusions

Our results indicated that the MTHFR C677T T allele was a protective biomarker for childhood ALL in Taiwan, and the association was more significant in male patients and in patients 3.5 years of age or older at onset of disease.

Introduction

Acute lymphoblastic leukemia (ALL) is now the most common form of pediatric leukemia, accounting for 25–30% of all childhood malignancies [1]. The annual incidence rate worldwide of childhood ALL is approximately 10 cases per 100,000, with peak incidence occurring at approximately 2 to 5 years of age [2]. While the clinical, pathological and immunophenotypic features of the disease are well documented, the etiology of ALL has not been fully clarified [1]. In the literature, certain environmental factors (i.e., ionizing radiation, parental use of alcohol and tobacco, and virus exposure) have been identified as potential risk factors for the development of childhood ALL, but only ionizing radiation has been confirmed thus far [3]. However, several lines of evidence now suggest that genetic factors may play a significant role in the development of childhood ALL. For instance, inherited genetic disorders, such as Down syndrome and Fanconi anemia, have been associated with an enhanced ALL risk [4, 5]. Additionally, genetic mutations in several cancer-related genes, such as p53, N-ras, and PHF6, have frequently been identified in ALL patients [6]; and finally, only a small fraction of children who are exposed to environmental factors go on to develop ALL, indicating the potential for a genetic predisposition to develop childhood ALL.

To reveal the environmental and genomic factors together with the interactions among them is useful for evaluation and prevention of cancer risk. It is believed that one group of the candidate genes are those encoding enzymes related to the metabolism of identified carcinogens. Among these genes, methylenetetrahydrofolate reductase (MTHFR) is a folate-metabolism enzyme in charge of the conversion of 5, 10-methylene-tetrahydrofolate into 5-methyltetrahydrofolate, homocysteine remethylation, and biosynthesis of DNA and RNA [7]. The regulatory effects of MTHFR on DNA methylation, DNA replication, DNA repair and cell division make MTHFR a potential candidate for a cancer-predisposition gene. It is reasonable that rapidly proliferating malignancies have a higher requirement for DNA synthesis and could be more susceptible to folate deficiency and resultant DNA damage. Low dietary folate and MTHFR deficiency induced the formation of intestinal tumors in a BALB/c mice model [8].

Previous investigations of MTHFR variations focused on the catalytic domain and the two polymorphisms, C677T (rs1801133) and A1298C (rs1801131), which may determine its enzymatic activity [9, 10]. In the case of the C677T polymorphism, the cytosine base at position 677 changes to a thymidine base, which in turn affects the amino acid at position 222, as it is changed from alanine to valine. The MTHFR enzyme TT variants from the polymorphism become thermo labile, resulting in a loss of its activity with elevated temperature [11]. The modified protein loses its flavin adenine dinucleotide cofactor more quickly and has a lower stability. The mutation effect can be suppressed by the addition of folate, which causes a higher flavin adenine dinucleotide affinity and an increase in MTHFR stability [11]. The MTHFR A1298C polymorphism is localized in the coding regulatory domain [12]. In the literature, it was reported that heterozygotes and rare homozygotes of MTHFR C677T variant exert 60% and 30% of wild-type enzyme activity, respectively [10]. As for A1298C, the rare homozygous have 60% of wild-type activity [10]. In 2004, it was found that cancer cells which expressed
677T MTHFR were of lower activity than those express 677C MTHFR. Also the expression of mutant MTHFR 677T would increase the sensitivity of cancer cells to the cytotoxicity of 5FU. In null mice model, the expression of 677T MTHFR enhanced the growth rates of xenografts than those expressed wild-type 677C MTHFR. Consistent with the evidence observed in cell models, the 677T xenografts were more sensitive to 5FU treatment than those of 677C in mice model [13]. Many studies investigating the MTHFR variant have found positive associations with solid cancers, such as colorectal cancer [14], breast cancer [15], oral cancer [16, 17], and lung cancer [18].

Over the past decade, there has been a growing interest in the possible association between folate-related polymorphisms and the risk of developing lymphoid malignancies, including childhood ALL. First, Skibola [19] and Matsuo [20] in 1999 and 2001, respectively, reported that people with variant MTHFR genotypes had a significantly lower susceptibility to adult ALL and malignant lymphoma. In the same period, Franco [21] and Wiemels [22] provided similar pilot results for pediatric leukemia. The purpose of this study, therefore, was to analyze the genetic polymorphisms of both MTHFR C677T and A1298C in a representative pediatric population sample (control/case = 266/266), to investigate the correlation between MTHFR genotypes and childhood ALL in Taiwanese children, and to then summarize all of the relevant updated literature.

Materials and Methods

Study population and sample collection

Our study was approved by the Institutional Review Board of China Medical University Hospital, and written informed consent was obtained from one or both the parent of all participants. Two hundred and sixty-six patients diagnosed with childhood ALL (all patients under 18 years of age) were recruited between Apr 2005 to Jan 2010 from the general surgery outpatient clinics within the Pediatric Departments at China Medical University Hospital and National Taiwan University Hospital, Taiwan, Republic of China. All of the clinical characteristics of these ALL patients, including their histological details, were identified by expert surgeons. All subjects voluntarily participated, completed a questionnaire with the help of parents or guardians and provided peripheral blood samples. The questionnaires recorded their disease history, diet and sleep lifestyles and the disease history, diet and behavioral lifestyle, social-economic status of the parents. An equal number of age-matched non-cancer healthy volunteers were selected for use as a control group following initial random sampling from the Health Examination Cohort established from Apr 2005 to Jan 2010 as previously published [23]. The registered health practitioners in the hospital provide a multidisciplinary team approach of health assessment for the volunteers. Most of the volunteers underwent health examinations every 5 to 6 months. A total of 457 volunteers age under 18 years were recruited into this study and chosen were cancer free by the age at diagnosis of the case child with the International Classification of Disease, ninth revision (ICD-9) codes. Finally, 266 participants were included for analysis in the study since we have to match the population structure (number, age and gender) with our case population. The overall agreement rate in the study was above 85%.

Genotyping assays

Genomic DNA was prepared from peripheral blood leukocytes using a QIAamp Blood Mini Kit (Blossom, Taipei, Taiwan), long-term stored at -80°C, diluted and aliquotted for genotyping as working stock at -20°C [23–25]. Genotyping for MTHFR C677T and A1298C of all subjects was carried out by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) assays as previously published [17, 25–27]. The primers for MTHFR C677T were
forward 5'- TGA AGG AGA AGG TGT CTG CGG GA-3' and reverse 5'- AGG ACG GTG CGG TGA GAG TG-3'. The primers for \textit{MTHFR} A1298C were forward 5'- GGG AGG AGC TGA CCA GTG CAG-3' and reverse 5'- GGG GTG AGG CCA GGA GCA G-3'. The underlined C mismatched base in the forward primer were used to create a partial \textit{Fnu4H I} cutting site, which will be completed in the presence of the C allele at the A1298C polymorphic site. The following cycling conditions were performed: 5 min of initial denaturation at 95°C, 35 cycles of 30 sec of denaturation at 95°C, 30 sec of annealing at 54°C and 1 min of elongation at 72°C, and 7 min of final extension at 72°C. The 198-bp PCR product of \textit{MTHFR} C677T and 138-bp PCR product of \textit{MTHFR} A1298C were subject to enzyme digestion with \textit{Hinf I} and \textit{Fnu4H I} (New England, Biolabs, Beverly, MA USA), respectively for 4 h and then visualized by ethidium bromide-stained 3% agarose gel electrophoresis under UV light. On digestion with \textit{Hinf I}, the PCR product of \textit{MTHFR} C677T arising from the C allele was uncut (198 bp), whereas the T allele was cut into fragments of 175 bp and 23 bp. On digestion with \textit{Fnu4H I}, the PCR product of \textit{MTHFR} A1298C arising from the A allele was uncut (138 bp), whereas the C allele was cut into fragments of 119 bp and 19 bp [17, 25, 27]. The success rate of PCR-RFLP is 100%, and the genotypes of five percent of the participants in both the control and patient groups were analyzed by PCR direct sequencing (Genomics BioSci & Tech Co., Taipei). The consistency between direct sequencing and PCR-RFLP was 100%.

**Statistical analyses**

Only those participants having both genotypic and clinical data (control/case = 266/266) were selected for final analysis. The descriptive statistics of patients and controls were presented as the mean and standard deviations (SDs) or as percentages. The Pearson’s chi-square test or Fisher’s exact test (when any cell was less than five) was used to compare the distribution of the genotypes. Associations were expressed and evaluated as odds ratios (ORs) with 95% confidence intervals (95%CIs). Interactions between the genotypes and onset age or gender were examined by using the likelihood ratio test. Statistical tests were deemed significant when the P-value was less than 0.05. All statistical analysis were performed with SAS 9.2 and SPSS 17.

**Results**

The frequency distributions for the age and gender of 266 childhood ALL patients and 266 non-cancer controls are shown in Table 1. The characteristics of the patients and controls were well matched (P > 0.05) (Table 1).

The genotype frequencies for the \textit{MTHFR} C677T and A1298C in the controls and childhood ALL patients are shown in Table 2. The genotype frequencies of the two \textit{MTHFR} SNPs of the controls were in Hardy-Weinberg Equilibrium (p = 0.9007 and 0.8886, respectively). The genotypic frequency distributions for \textit{MTHFR} C677T were significantly different between childhood ALL and control groups (P = 0.0076), while those for the A1298C polymorphism were not significantly different (P > 0.05) (Table 2). Those who carried CT, TT, CT or TT genotypes had significantly reduced risk of ALL with ORs of 0.60, 0.48, and 0.58 respectively compared to those with the CC genotype (95% CI = 0.42–0.87, 0.24–0.97 and 0.41–0.82, respectively). The conclusion that can be deduced from Table 2 is that the \textit{MTHFR} C677T T allele seems to be a protective biomarker for childhood ALL in Taiwan.

Because age and gender are the predominant risk factors for developing childhood ALL, the interactions between the \textit{MTHFR} genotype and age and gender were further analyzed and presented in Table 3. The average age of onset for the 133rd and 134th subjects in the control and patient groups was 3.5 years; thus, we further stratified the groups into <3.5 and ≥3.5 year-old subgroups. Noticeably, in the elder (≥3.5 years) group, subjects with CT or TT genotypes for
MTHFR C677T had lower risks for developing childhood ALL than those with the homozygous CC genotype (P for trend = 0.0016, OR = 0.48 and 0.22, CI = 0.28–0.80 and 0.07–0.69 for CT and TT, respectively); however, this was not the case for the younger (<3.5 years) group (Table 3). As for gender, boys with CT or TT genotypes for MTHFR C677T were less likely to develop childhood ALL than those with the homozygous CC genotype (P for trend = 0.0113, OR = 0.54 and 0.32, CI = 0.33–0.89 and 0.11–0.94 for CT and TT, respectively), but this was not the case for the girls (Table 3). In summary, analyses revealed an interaction between the age of onset and gender among MTHFR C677T genotypes in the childhood ALL susceptibility (P values for interaction = 0.0378 and 0.2524 for ≥3.5 years versus < 3.5 years and girls versus boys, respectively).

Discussion

ALL is more common among children than adults. Among the patients, the precursor B subtype accounted for approximately 75% to 90% of the cases, while the remaining 10% to 25% were precursor T lymphoblastic leukemia [2]. Lymphocytic leukemia cells are rapidly dividing cells that require higher folate supplementation and are more vulnerable to folate deficiency. Previous research has demonstrated that maternal folate supplementation during pregnancy can be effective in reducing the risk of childhood ALL in babies [28]. The two most common genotypes associated with childhood ALL were C677T and A1298C.

Table 2. Distribution of the MTHFR genotypes among 266 childhood ALL patients and 266 controls.

| Genotype      | Controls | Patients | P-value* | OR (95% CI)b |
|---------------|----------|----------|----------|--------------|
| C677T rs1801133 |          |          |          |              |
| CC            | 134      | 169      | 0.0076*  | 1.00 (Reference) |
| CT            | 109      | 83       | 0.60 (0.42–0.87)* | 0.60 (0.42–0.87)* |
| TT            | 23       | 14       | 0.48 (0.24–0.97) * | 0.48 (0.24–0.97) * |
| CT+TT         | 132      | 97       | 0.58 (0.41–0.82) * | 0.58 (0.41–0.82) * |
| A1298C rs1801131 |        |          | 0.8984   |              |
| AA            | 171      | 168      | 1.00 (Reference) | 1.00 (Reference) |
| AC            | 85       | 86       | 1.03 (0.71–1.49) | 1.03 (0.71–1.49) |
| CC            | 10       | 12       | 1.22 (0.51–2.90) | 1.22 (0.51–2.90) |
| AC+CC         | 95       | 98       | 1.05 (0.74–1.50) | 1.05 (0.74–1.50) |

* Based on Pearson’s chi-square test
b OR: odds ratio; CI: confidence interval
* Statistically significant
Table 3. Distribution of the MTHFR C677T and A1298C genotypes stratified by age and gender

| Characteristics | MTHFR C677T | OR (95% CI) | P<sub>trend</sub><sup>a</sup> | MTHFR A1298C | OR (95% CI) | P<sub>trend</sub><sup>a</sup> |
|----------------|-------------|-------------|----------------|-------------|-------------|----------------|
| Onset age      |             |             |                 |             |             |                 |
| < 3.5 years    |             |             |                 |             |             |                 |
| CC             | 70 (52.63)  | 78 (58.65)  | 1.00 (Reference)| 83 (62.41)  | 86 (64.66)  | 1.00 (Reference) |
| CT             | 53 (39.85)  | 45 (33.83)  | 0.76 (0.46–1.27)| 44 (33.08)  | 40 (30.08)  | 0.88 (0.52–1.48) |
| TT             | 10 (7.52)   | 10 (7.52)   | 0.90 (0.35–2.28)| 6 (4.51)    | 7 (5.26)    | 1.13 (0.36–3.49) |
| CT+TT          | 63 (47.37)  | 55 (41.35)  | 0.76 (0.48–1.27)| 50 (37.59)  | 47 (35.34)  | 0.91 (0.55–1.50) |
| ≥ 3.5 years    |             |             |                 |             |             |                 |
| CC             | 64 (48.12)  | 91 (68.42)  | 1.00 (Reference)| 88 (66.16)  | 82 (61.65)  | 1.00 (Reference) |
| CT             | 56 (42.11)  | 38 (28.57)  | 0.48 (0.28–0.80)*| 41 (30.83)  | 46 (34.59)  | 1.20 (0.72–2.02) |
| TT             | 13 (9.77)   | 4 (3.01)    | 0.22 (0.07–0.69)*| 4 (3.01)    | 5 (3.76)    | 1.34 (0.35–5.17) |
| CT+TT          | 69 (51.88)  | 42 (31.58)  | 0.43 (0.26–0.71)*| 45 (33.84)  | 51 (38.35)  | 1.22 (0.74–2.01) |
| Gender         |             |             |                 |             |             |                 |
| boys           |             |             |                 |             |             |                 |
| CC             | 76 (51.35)  | 100 (67.57) | 1.00 (Reference)| 96 (64.86)  | 90 (60.81)  | 1.00 (Reference) |
| CT             | 60 (40.54)  | 43 (29.05)  | 0.54 (0.33–0.89)*| 47 (31.76)  | 51 (34.46)  | 1.16 (0.71–1.89) |
| TT             | 12 (8.11)   | 5 (3.38)    | 0.32 (0.11–0.94)*| 5 (3.38)    | 7 (4.73)    | 1.49 (0.46–4.88) |
| CT+TT          | 72 (48.65)  | 48 (32.43)  | 0.51 (0.32–0.81)*| 52 (35.14)  | 58 (39.19)  | 1.19 (0.74–1.91) |
| girls          |             |             |                 |             |             |                 |
| CC             | 58 (49.15)  | 69 (58.47)  | 1.00 (Reference)| 75 (63.56)  | 78 (66.10)  | 1.00 (Reference) |
| CT             | 49 (41.53)  | 40 (33.90)  | 0.69 (0.40–1.18) | 38 (32.20)  | 35 (29.66)  | 0.89 (0.51–1.55) |
| TT             | 11 (9.32)   | 9 (7.63)    | 0.69 (0.27–1.77) | 5 (4.24)    | 5 (4.24)    | 0.96 (0.27–3.46) |
| CT+TT          | 60 (50.85)  | 49 (41.53)  | 0.69 (0.41–1.15) | 43 (36.44)  | 40 (33.90)  | 0.89 (0.52–1.53) |

<sup>a</sup> P for trend based on chi-square test.
<sup>b</sup> P for interaction based on likelihood ratio test; NS, non-significant.
<sup>c</sup> OR, odds ratio; CI, confidence interval.
<sup>*</sup> Statistically significant.

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SNPs of the MTHFR gene may determine MTHFR enzyme activity and the availability of folate to the whole body and may ultimately be linked to childhood ALL risk. For this reason, we investigated their relationships with the susceptibility to developing childhood ALL in Taiwan. We found that the T variant genotypes of MTHFR C677T were significantly associated with a lower susceptibility to childhood ALL (Table 2). These findings are consistent with previous research that identified the T allele to be a protective factor [21, 22, 29–36], but not with those studies which identified the T allele as a risk factor [37–39]. Additionally in some studies the T allele has been shown to have no association with childhood ALL [40–54]. Table 4 contains a summary of the findings from all of the literature investigating the association between MTHFR genotypes and childhood leukemia risk with a representative sample size (control/case larger than 70/70) and a non-redundant population. Among the studies, allele T was associated with decreased risk in populations of Taiwan, Serbia, China, Netherlands, Greece, Canada, and UK [22, 30–32, 34, 36], while with increased risk in populations of India [38]. Others proposed that the T allele was not associated with childhood ALL [40–54]. However, even the people in the same country, for instance Brazil, there was a dramatically different susceptibility to cancer, showing both decreased [21, 35] and increased risk [37]. In India, the same inconsistency could be observed [38, 39, 42, 45]. This kind of inconsistency may be mainly caused by different genetic background and environmental exposure status in addition to sampling bias. Therefore, all the molecular epidemiologists are revealing the genomic and environmental factors contribute to childhood ALL. The limited sample sizes in childhood ALL, compared to studies using larger amounts of samples collected among patients of common cancers, may be one of the factors contributing to inconsistent conclusions. For the low incidence of childhood ALL, this is unavoidable. In a current meta-analysis investigating the contributions of MTHFR C677T and A1298C genotypes to childhood ALL, it was demonstrated that the T allele of MTHFR C677T was associated with a lower risk of childhood ALL in both Asians and Caucasians, while A1298C was not observed to have any effect [55]. The current study was the first to utilize a Taiwanese sample, and the relationship was consistently positive for MTHFR C677T and negative for A1298C (Table 2).

We have further analyzed the relationship between the C677T genotype and childhood ALL risk according to the subject age and gender. Interestingly, the interaction between MTHFR C677T and age is clear; specifically children ≥3.5 years old at age of onset of disease with a CT or TT genotype had a lower risk of childhood ALL than those with the CC genotype. This relationship was not found for the group of children <3.5 years old at age of onset of disease (Table 3). Additionally, no such age difference was observed in analyses of the A1298C genotype. In 2013, Jiang and his colleagues extended the Meta-analysis to 37 individual studies investigating adult ALL. They found that TT genotype was associated with a lower risk of ALL (OR = 0.776, 95% CI: 0.687–0.877, p < 0.001). After stratification by ethnicity, the significance only existed among Caucasians (OR = 0.715, 95% CI: 0.655–0.781, p < 0.001), at borderline among Asian (OR = 0.711, 95% CI: 0.591–1.005, p = 0.055), but not among others (OR = 0.913, 95% CI: 0.656–1.271, p = 0.590) [56]. The mechanisms of MTHFR involved in the etiology and progression of ALL may be different for adults and children. Although no statistically significance was found in the girls-only analysis in Table 3, there is a similar trend to that observed for the boys (Table 3). The enlarged sample size may provide more realistic answer. Thus, as for the different contribution of MTHFR genotypes to ALL susceptibility between boys and girls, further investigations are in urgent need. In the literature, studies have revealed significant differences in the serum or plasma folate levels among people with various C677T genotypes, but not among those with various A1298C genotypes [57, 58]. Further measurement and analysis of the folate levels among children of different ages, together with their diet intake of folic acid, may help us to better understand the etiology.
We also tested for a gender-dependent effect on determining childhood susceptibility to ALL. The protective impact of the T allele at MTHFR C677T with respect to cancer risk appeared to be stronger for boys than the girls (Table 3) but this difference was not statistically significant. The incidence of pediatric hematological malignancies worldwide has increased for boys but not for girls [59, 60]. While the complete underlying mechanism has not been discovered, sex hormones and steroids reportedly play a part in the control of the proliferation of leukemic cells. Supporting the idea of gender differences in susceptibility to childhood ALL, it was found that 17-β-estrogen had a stronger inhibitory effect than testosterone on human monoblastic U937 cells [61]. In addition, the TT genotype at MTHFR C677T conferred higher...
plasma homocysteine levels than the CC genotype selectively in folate-dependent boys, which provides additional evidence for gender differences [62, 63].

In conclusion, this study documented the evidence of a relationship between the genotypes of MTHFR and childhood ALL risk and investigated age- and gender-interactions with the genotype to determine childhood ALL susceptibility. The presence of the T allele of C677T was not only a detectable and predictive biomarker for childhood ALL but also a protective determinant for older patients and boys.

Author Contributions
Conceived and designed the experiments: DTB CMH. Performed the experiments: WSC HXJ CLH CEM. Analyzed the data: JSP CMH CWT. Contributed reagents/materials/analysis tools: JSP YNH CWT. Wrote the paper: JSP DTB.

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