Determining the Association between Methylenetetrahydrofolate Reductase (MTHFR) Gene Polymorphisms and Genomic DNA Methylation Level: A Meta-Analysis

Li Wang, Shaofang Shangguan, Shaoyan Chang, Xin Yu, Zhen Wang, Xiaolin Lu, Lihua Wu, and Ting Zhang*

Background: The methylenetetrahydrofolate reductase (MTHFR) polymorphism is a risk factor for neural tube defects. C677T and A1298C MTHFR polymorphisms produce an enzyme with reduced folate-related one carbon metabolism, and this has been associated with aberrant methylation modifications in DNA and protein. Methods: A meta-analysis was conducted to assess the association between MTHFR C677T/A1298C genotypes and global genomic methylation. Results: Eleven studies met the inclusion criteria. Of these, 10 were performed on C677T MTHFR genotypes and 6 were performed on A1298C MTHFR genotypes. Our results did not indicate any correlation between global methylation and MTHFR A1298C, C677T polymorphisms. Conclusion: The results of our study provide evidence to assess the global methylation modification alterations of MTHFR polymorphisms among individuals. However, our data did not found any conceivable proof supporting the hypothesis that common variant of MTHFR A1298C, C677T contributes to methylation modification.


Key words: methylenetetrahydrofolate reductase (MTHFR); MTHFR C677T; MTHFR A1298C; genomic DNA methylation; meta-analysis

Introduction
Folate deficiency has been linked to many different pathologies, including cancer and birth defects (Czeizel and Dudas, 1992; Bhargava and Tyagi, 2014). Whereas some underlying mechanisms have been proposed, there are many more to be determined. It appears that folic acid and other one-carbon intermediates may determine clinical programming effects by means of DNA and protein methylation modifications (Lamprecht and Lipkin, 2003; Stover, 2009; Bailey et al., 2010).

The enzyme methylenetetrahydrofolate reductase (MTHFR) is the rate-limiting enzyme in the folate metabolism cycle. It catalyzes the reduction of 5,10 methylene tetrahydrofolate (THF) to 5-methyl THF; the 5-methyl THF form of the vitamin converts the amino acid homocysteine to methionine. Methionine is further converted and provides a methyl group that is important in a variety of biological reactions, including the methylation of DNA.

Because of the important role of the MTHFR gene in one carbon metabolism, MTHFR gene polymorphisms were hypothesized to play an essential role in inherited DNA methylation profiles through folic acid metabolism (Christensen et al., 2010; Kim et al., 2010; Wallace et al., 2010). MTHFR single nucleotide polymorphisms (SNPs) can decrease the function of the enzyme and, therefore, the body’s ability to methylate. At least 23 polymorphisms in the MTHFR gene have been identified and are thought to be associated with an increased susceptibility to neural tube defects (NTDs), occlusive vascular disease, Alzheimer’s disease and other forms of dementia, colon cancer, and acute leukemia (Posey et al., 1996; Martinez de Villarreal et al., 2001; Cunha et al., 2002; Sailsree et al., 2010; Saberi et al., 2012; de Arruda et al., 2014).

Two of these polymorphisms (MTHFR C677T and A1298C) are well understood and have been tested in a variety of diseases. The common 677 C>T and 1298 A>C polymorphisms in the MTHFR gene are hypothesized to cause production of a thermolabile enzyme with reduced function, or to affect the conversion of MTHF to BH4, and eventually cause genomic DNA hypomethylation (Yamada et al., 2001). Some studies have explained the association between MTHFR polymorphisms and types of cancer by suggesting that hypomethylation is induced by the polymorphism and consequently activates proto-oncogenes (Izmirli, 2012). However, other studies have provided inconsistent results on the relationship between MTHFR polymorphisms and genomic DNA hypomethylation in
patients (Arakawa et al., 2012; Hanks et al., 2013; de Arruda et al., 2014). Until now, no specific meta-analyses have been carried out to explore the relationship between MTHFR genotype and global methylation levels. Given the important role of MTHFR in the folate metabolism cycle, understanding the magnitude of the impact that C677T and A1298C MTHFR polymorphisms may have on methylation modifications could be critical in assessing the population-level risk of tumors and birth defects. Therefore, the current meta-analysis was carried out to estimate the magnitude of association of the most common MTHFR polymorphisms (C677T and A1298C) with global DNA methylation.

**Materials and Methods**

**DNA METHYLATION AND MTHFR GENOTYPING ANALYSIS**

Experimental data in this study were obtained and extracted from our own lab, all samples came from Shanxi province, China. Global DNA methylation was detected with the Methylamp Global DNA Methylation Quantification Ultra Kit, and LINE-1 methylation analysis was performed using Sequenom MassARRAY platform, as previously described (Wang et al., 2010). MTHFR variant calling was performed as published before (Yu et al., 2014).

**LITERATURE SEARCH**

We performed a literature search in PubMed, Embase, and Medline using the following terms, "MTHFR," "global methylation/genomic methylation/global DNA methylation/genomic DNA methylation/5-methylcytosine," and "polymorphism/SNP/genotype/gene polymorphism/genetic risk," limiting our search to publications in the English language and on human studies up to December 31, 2015. A second search was then performed using the same terms and by assigning all terms within Title/Abstract. The results were compared with the first result to make sure no related literature was missed. For studies involving LINE-1 methylation levels as a marker of global methylation, a further search was carried out using the following terms: "MTHFR," AND "polymorphism/SNP/genotype/gene polymorphism/genetic risk," and "LINE-1 methylation/long interspersed nuclear element methylation." References of included literature were checked and related literatures were included. These steps were performed independently by two researchers (LW. and YX.) and were cross-verified. Any differences in opinion were resolved through team discussion.

**INCLUSION CRITERIA AND EXCLUSION CRITERIA**

As we did not expect many data from randomized controlled trials, we included both cohort and case–control studies. We included studies that compared global DNA methylation levels and LINE-1 methylation levels in human cases. Only novel data were included. Studies were excluded if there was no control group, for example, case series and case reports. Data from populations with extra folate intervention were excluded because folate could provide more methyl groups. Review papers were excluded. Animal and cell model studies were also excluded.

**DATA EXTRACTION**

For each study, information such as: authors, year of publication, country of origin, sample type, number of cases with gender, study design, genotyping, methylation level, and analysis method was extracted. In some cases, additional folate or folate analogues were used in the design, and these data were not included in the meta-analysis. Some studies that presented DNA methylation results without mean methylation or without standard deviation or variance were excluded in this analysis. Data with only 95% confidence interval were transferred to standard deviation in hands by calculator tools in Review Manager 5.1. All the findings were then grouped by a specific data extraction template.

**META-ANALYSIS**

Review Manager 5.1 software was used to conduct meta-analyses. Considering methylation levels were measured using different methods, a random effects model was used in all analyses of the mean methylation level; where

<table>
<thead>
<tr>
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<th>Global Methylation level%a (n²)</th>
<th>LINE-1 Methylation level%a (n²)</th>
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</thead>
<tbody>
<tr>
<td>A1298C aa</td>
<td>6.24 ± 2.46 (22)/7.48 ± 1.30 (5)</td>
<td>59.10 ± 4.98 (20)/54.58 ± 5.69 (6)</td>
</tr>
<tr>
<td>A1298C ac</td>
<td>6.02 ± 1.97 (5)/5.84 ± 4.03 (4)</td>
<td>57.36 ± 3.76 (4)/54.43 ± 5.41 (4)</td>
</tr>
<tr>
<td>A1298C cc</td>
<td>3.58 ± 1.95 (2)/5.63 ± 2.89 (4)</td>
<td>60.13 ± 2.47 (2)/58.92 ± 7.89 (3)</td>
</tr>
<tr>
<td>C677T cc</td>
<td>5.35 ± 1.97 (8)/6.76 (1)</td>
<td>59.80 ± 2.70 (7)/53.86 (1)</td>
</tr>
<tr>
<td>C677T ct</td>
<td>6.42 ± 2.73 (12)/6.17 ± 3.15 (8)</td>
<td>59.51 ± 4.07 (11)/54.91 ± 6.16 (8)</td>
</tr>
<tr>
<td>C677T tt</td>
<td>6.00 ± 2.51 (8)/6.80 ± 2.56 (4)</td>
<td>58.59 ± 6.71 (7)/55.54 ± 5.91 (4)</td>
</tr>
</tbody>
</table>

*aMethylation level shows as methylation level in controls/methylation level in NTDs.

*bNumber of samples shown in bracket.
feasible, a fixed effect model was performed when the study heterogeneity was less than 50%. Stratified analyses were performed by source of case samples (peripheral blood or not).

Results
GENOMIC DNA METHYLATION AND LINE-1 METHYLATION AMONG MTHFR GENE POLYMORPHISMS IN DISEASE TISSUES
MTHFR gene polymorphisms and methylation modifications are involved in the pathogenesis of NTDs. To assess if the MTHFR genotype affects global methylation, our previous data were extracted to compare methylation levels with C677T and A1298C MTHFR. Both genomic DNA methylation and LINE-1 methylation were compared in brain tissue of control and a NTD fetus. As shown in the results in Table 1, no significant difference in methylation levels was found among MTHFR polymorphisms.

CHARACTERISTICS OF THE STUDIES
The literature search identified a total of 253 potential relevant articles, with 83 articles remaining after the second search. Of these, 33 articles were excluded as duplicates and 8 articles were excluded after reading the title or abstract because of obvious irrelevance. In addition, 3 articles were excluded as nonhuman research; 3 articles were excluded because they had folate intervention; 25 articles were excluded because they lacked mean data; 1 article was excluded because it did not have any control data; 1 article was excluded because there were no heterozygous data; 2 articles were excluded because there were no separately heterozygous data; 4 articles were included after a reference review.

In total, 13 articles met all the primary inclusion criteria, and 12 studies for MTHFR C677T, 7 studies for MTHFR A1298C were included in the final meta-analysis. A flow chart describing the process of study inclusion/exclusion is displayed in Figure 1. Selected characteristics of these studies related to the association with methylation are summarized in Table 2. Tested sample types include peripheral blood cells and normal disease tissues. The mean age of the total population ranged from 0 to 88 years. Several methylation assay methods were used, including the Methyl Flash Methylated DNA Quantification Kit, Global DNA Methylation Quantification Ultra Kits, [3H]methyl-SAM assay, LC-MS/LC mCyt/mgDNA, in vitro methyl acceptance assay,[3H]-Dctp assay, high-performance capillary electrophoresis, Global DNA Methylation Quantification Ultra Kit, and Epityper MassArray (Sequenom) assay.

META-ANALYSIS RESULTS
For C677T MTHFR genotyping and methylation assays, the meta-analysis included pooled data from 1147 individuals. Overall, this meta-analysis showed that the C677T MTHFR
polymorphism was not associated with global methylation level (for TT vs. CC: $z = 0.25$; $p = 0.80$; for CT vs. CC: $z = 1.04$; $p = 0.30$). Heterogeneity between studies was observed in the overall comparisons as well as in subgroup analyses (Fig. 2). Publication bias was explored using funnel plots; results are shown in Supplementary Figure S1, which is available online. Similarly, no associations were found in subgroup analyses when sample type was limited to peripheral blood cells (Fig. 3).

Discussion

To our knowledge, this is the first meta-analysis investigating the association between MTHFR polymorphisms and global DNA methylation levels. No significant evidence was found to indicate that global DNA methylation level was regulated by MTHFR polymorphisms in individuals, even in samples originating from peripheral blood cells.

In recent years, population and experimental studies have provided evidence to support the role of folate...
FIGURE 2. Forest plot analyses for MTHFR A1298C and MTHFR C677T genotyping and global methylation. The random effects odds ratio with the corresponding 95% confidence interval (CI) is shown. Total indicates the total number of samples. Tau2, the estimated between-study heterogeneity variance of log odds ratios; Chi^2, chi-squared test, it assesses whether observed differences in results are compatible with chance alone; df, degrees of freedom, the percentage of the variability in effect estimates that is due to heterogeneity rather than sampling error (chance); I^2, heterogeneity index (0–100).
metabolism in healthy development, through regulation of methylation modifications of the genome (Crott et al., 2008; Wang et al., 2010; Crider et al., 2012; Serra-Juhe et al., 2015). MTHFR genotypes are believed to alter folate metabolism in cells (Kim et al., 2010; Tsang et al., 2015) and subsequently modify methylation. Although several hypotheses have been proposed, as yet there is no clear evidence for a link between MTHFR polymorphisms and DNA methylation status. Our data come from high risk NTD regions, and no correlation was observed between MTHFR genotype and methylation modification. For this reason, it is not possible to assess if MTHFR genotypes could genuinely affect methylation modification by folate metabolism and increase the risk of diseases.

MTHFR C677T is one of the most common polymorphisms connected with birth defects and cancer across ethnic groups and regions. The MTHFR 677TT polymorphism leads to the amino acid alanine being replaced by valine (p.Ala222-Val) and the production of a thermolabile variant of MTHFR with 30% less enzyme activity (Sharp and Little, 2004). However, no alteration in global DNA methylation with MTHFR C677T polymorphism was observed in this meta-analysis. Another common polymorphism is MTHFR A1298C. The C polymorphism leads to glutamic acid being replaced by alanine (p.Glu429-Ala) and affects the conversion of MTHF to BH4 (Yamada et al., 2001). Our results indicated no correlation was observed between global methylation and MTHFR A1298C polymorphism.

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>MTHFR 677TT</th>
<th>MTHFR 677CC</th>
<th>Std. Mean Difference</th>
<th>IV, Random, 95% CI</th>
</tr>
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<tbody>
<tr>
<td>Mean</td>
<td>SD</td>
<td>Total</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Simonetta Frios 2002</td>
<td>32.23</td>
<td>38.5</td>
<td>105</td>
<td>62.24</td>
</tr>
<tr>
<td>Alexandra S. Weiner 2014</td>
<td>4.323</td>
<td>1.525</td>
<td>80</td>
<td>5.103</td>
</tr>
<tr>
<td>Sabrina Narayan 2004</td>
<td>23.311</td>
<td>4.561</td>
<td>25</td>
<td>22.805</td>
</tr>
<tr>
<td>Francesco Graziano 2006</td>
<td>10.474</td>
<td>2.720</td>
<td>40</td>
<td>6.881</td>
</tr>
<tr>
<td>R Castro 2004</td>
<td>29.3</td>
<td>3.33</td>
<td>9</td>
<td>21.68</td>
</tr>
<tr>
<td>Lor Latho Stern 2004</td>
<td>12.615</td>
<td>1.636</td>
<td>10</td>
<td>7.843</td>
</tr>
<tr>
<td>Total (95% CI)</td>
<td>269</td>
<td>450</td>
<td>100.0%</td>
<td>0.34 [-0.26, 0.93]</td>
</tr>
<tr>
<td>Heterogeneity: Tau^2 = 0.46; Chi^2 = 54.47, df = 5 (P &lt; 0.00001); I^2 = 91%</td>
<td></td>
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<td></td>
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<tr>
<td>Test for overall effect: Z = 1.10 (P = 0.27)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>MTHFR 677CT</th>
<th>MTHFR 677CC</th>
<th>Std. Mean Difference</th>
<th>IV, Random, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>SD</td>
<td>Total</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>R Castro 2004</td>
<td>21.3</td>
<td>4.67</td>
<td>43</td>
<td>21.8</td>
</tr>
<tr>
<td>Sabrina Narayan 2004</td>
<td>22.955</td>
<td>4.244</td>
<td>84</td>
<td>22.359</td>
</tr>
<tr>
<td>Total (95% CI)</td>
<td>127</td>
<td>134</td>
<td>100.0%</td>
<td>-0.01 [-0.25, 0.24]</td>
</tr>
<tr>
<td>Heterogeneity: Tau^2 = 0.00; Chi^2 = 2.7, df = 1 (P = 0.60); I^2 = 0%</td>
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<tr>
<td>Test for overall effect: Z = 0.06 (P = 0.96)</td>
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</table>

FIGURE 3. Meta-analysis for MTHFR A1298C & C677T polymorphism and global methylation level in peripheral blood cells. The random effects odds ratio with the corresponding 95% confidence interval (CI) is shown. Total indicates the total number of samples. Tau2, the estimated between-study heterogeneity variance of log odds ratios; Chi^2, chi-squared test, it assesses whether observed differences in results are compatible with chance alone; df, degrees of freedom, the percentage of the variability in effect estimates that is due to heterogeneity rather than sampling error (chance); I^2, heterogeneity index (0-100).
Given that methods for methylation assays have greatly changed over the years with the development in technology, all data included in this study were obtained from different methods and the random effect was taken as priority method for analysis. However, for a continuous variable evaluation, incorporating random effects increased the likelihood of accounting for inter-study heterogeneity and addressed the potential correlation among results reported.

Methylation modification can be tissue specific, and so to remove biases induced by different tissue samples, studies in peripheral blood cells were analyzed for further comparison. However, there was still no alteration in global DNA methylation, even if the sample type was limited to the same origin. Our study is subject to some limitations. The results were based on data abstracted from previous publications; however, this does not necessarily eliminate the robust estimate of association, and instead it allowed us to estimate the genetic effect of MTHFR in a larger sample size and thereby increased its power. Besides, differences between assay methods occur because global methylation modifications are represented by different forms, such as mCyt, methyl, or LINE-1 methylation. It is difficult to avoid the limited literature used in this study.

In conclusion, the results of our study provide evidence to assess the global methylation modification alterations of MTHFR among individuals. Whereas it is possible that the common variant MTHFR A1298C, C677T did not contribute to methylation modification it might still predispose to disease. Further studies should be conducted to identify and understand methylation regulation as a result of MTHFR polymorphisms.

Acknowledgments
The work described has not been submitted elsewhere for publication, in whole or in part, and all the authors listed have approved the manuscript that is enclosed. The experimental study was reviewed and approved by the Ethics Board of Capital Institute of Pediatrics, and all participants gave informed consent.

References


