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Potential Role of Methylene tetrahydrofolate Reductase Mutations in Perinatal Stroke Outcomes.

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Potential Role of Methylenetetrahydrofolate Reductase Mutations in Perinatal Stroke Outcomes

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ISP Committee Members: MJ Harbert, MD and John Crawford, MD, Department of Neuroscience

Abstract

Perinatal stroke is a focal cerebrovascular event occurring during fetal or neonatal life before 28 days after birth that occurs in approximately 1 out of every 2,500 term births. Perinatal stroke is associated with lifelong morbidity, but because the developing brain is capable of tremendous plasticity, developmental outcomes can vary greatly among patients. While some pro-thrombotic conditions have been implicated as risk factors for early ischemic events, there is less data about the impact these genetic factors have on developmental outcomes. One particularly interesting pro-thrombotic trait is the methylenetetrahydrofolate reductase (MTHFR) gene with polymorphisms at nucleotides 677 and 1298, which lead to increased serum homocysteine levels and has been shown to potentially increase the risk of a number of cardiovascular diseases, including ischemic stroke. To explore the potential association between MTHFR mutations and outcomes of perinatal stroke, 13 children with perinatal stroke were categorized into three genotype groups based on the presence of C677T and A1298C mutations in MTHFR which were correlated with neurodevelopmental outcomes. There was no significant increased allele frequency or genotype frequency of MTHFR in perinatal stroke subjects compared to the general population. Given the small sample size, no significant difference in lesion size or language and cognitive outcomes among the genotype groups could be detected; however, there was a trend toward worse outcomes with increasing mutant MTHFR polymorphisms which may warrant further study.

Introduction

There are two periods in life during which the risk of stroke is the highest: in the elderly and in the fetus or newborn infant – stroke at these times is also associated with increased mortality, although the neonatal brain has a remarkable potential for recovery if therapeutic measures are promptly employed. Perinatal stroke is defined as a focal cerebrovascular event occurring during fetal or neonatal life before 28 days after birth, which shows pathological or radiological evidence of arterial or venous infarction or hemorrhage of the brain. The etiology of the ischemic injury can be used to classify the lesion as arterial ischemic stroke (AIS), cerebral sinovenous thrombosis (CSVT), or hemorrhagic stroke (HS). The incidence of AIS is 1 in every 2,500 term births while the prevalence of CSVT is approximately 1 per 100,000 children each year, often presenting as seizures or encephalopathy in the first weeks of life. The actual incidence of these injuries may be even higher as 50% of perinatal ischemic strokes are asymptomatic in the neonatal period, and typically present as motor asymmetry at 4-6 months. In addition, male children and African-American children appear to have a higher risk of perinatal stroke for reasons which are not well understood.

Children who have had perinatal stroke are free from comorbidities present for stroke in adults (e.g. atherosclerosis, diabetes, hypertension, and smoking), so understanding the risk factors may lead to important insights about the genetic basis of stroke. There are also other risk factors specifically associated with the fetal and neonatal period that do not apply to adult stroke; though a causal relationship is as yet undefined. Pregnant women are especially vulnerable to thrombotic and thromboembolic complications because normal pregnancy is a pro-coagulant and pro-inflammatory condition, perhaps an evolutionary adaptation to prevent potential hemorrhage during birth. In addition, the fetus has a high hematocrit, which increases blood viscosity, and an immature blood coagulation system that leads to developmental hemostasis, an innate hypercoagulability of blood. Pro-thrombotic disorders (or thrombophilias) are fairly common, and have been shown to confer higher risk for perinatal stroke when combined with these other environmental factors. Several pro-thrombotic factors that have been
implicated in the pathogenesis of perinatal stroke include anticardiolipin antibodies, antithrombin deficiency, protein C deficiency, factor V Leiden deficiency, increased lipoprotein A, and homozygous C677T polymorphism in the methylenetetrahydrofolate reductase (MTHFR) gene.\(^2\)\(^4\)

Methylenetetrahydrofolate reductase is an important enzyme that catalyzes the remethylation of homocysteine (Hcy) to methionine by reducing 5-methylenetetrahydrofolate to methyl donor methyldihydrofolate.\(^5\) Because there is close coupling of folate metabolism and methylation of Hcy to methionine, plasma Hcy concentration is proportional to levels of folate in serum and red blood cells.\(^5\)

There is a very common point mutation in the MTHFR gene at nucleotide 677 (C \(\rightarrow\) T substitution which converts an alanine to a valine residue in the protein), which converts MTHFR into a thermolabile variant with reduced activity.\(^6\) The distribution of MTHFR 677 polymorphisms in the general population has been extensively studied and varies with ethnicity.\(^7\),\(^15\) In the mutant homozygotes, the thermolabile MTHFR causes plasma Hcy concentration to be moderately elevated (about 20\%) and mean serum folate concentrations to be lower than normal CC homozygotes.\(^5\),\(^7\) Many studies have established that elevated Hcy plasma concentration is an independent risk factor for cardiovascular disease (ischemic heart disease, deep vein thrombosis, pulmonary embolism, and stroke) and increased mortality from coronary artery disease.\(^5\)

Homocysteinurias are rare autosomal recessive disorders that cause greatly elevated serum Hcy levels (>100 \(\mu\)mol/L compared to normal 0-15 \(\mu\)mol/L) which leads to cardiovascular disease in half of homozygotes by the age of 30.\(^7\) However, whether the moderately elevated serum Hcy seen in MTHFR mutations can be shown to cause cardiovascular disease or not is controversial.\(^8\) One meta-analysis of both genetic studies and prospective studies shows that lowering homocysteine concentrations by 3\(\mu\)mol/L could reduce the risk of ischemic heart disease by 16\%, deep vein thrombosis by 25\%, and stroke by 24\%.\(^7\) Other studies have shown that the homozygous mutant TT MTHFR genotype does not significantly increase the risk of premature death or venous thrombosis in women, and randomized trials of folic acid supplements appear to be inconclusive.\(^6\),\(^8\),\(^9\) Taking all of the cohort studies, genetic polymorphism studies, and randomized trials together, some researchers have concluded that causality has been established.\(^8\) An additional polymorphism in MTHFR has also been described at the 1298 nucleotide – the A1298C substitution which is also associated with decreased enzyme activity but is not as well studied, and the combined effect of these two mutations has not been well established.\(^16\) It is important to conclusively determine the role of MTHFR in ischemic stroke because the treatment is simple and benign: a 0.8 mg/day supplement of folic acid can lead to the 3 \(\mu\)mol/L decrease in serum Hcy that may effectively reduce risk.\(^5\) Pregnant women should also be scanned for MTHFR mutations because they may increase the risks of surgical delivery or neural tube defects, and may require higher than the normal recommended dose of folic acid.\(^5\)

While MTHFR mutations may be implicated in the causation of cerebrovascular disease, their impact on outcomes of cerebrovascular disease is not well known. For perinatal stroke patients, understanding what leads to varying outcomes is critical because the morbidity of childhood stroke persists for a lifetime.\(^3\) More than 95\% of perinatal stroke children survive into adulthood, but motor deficits, cognitive, and language impairments may be present.\(^5\) On the other hand, children with unilateral focal lesions often demonstrate the tremendous plasticity of the developing brain, with often no evidence of decline in cognitive function over time in children without ongoing seizures.\(^10\) Perhaps genetic factors including the number of MTHFR mutations can influence whether or not perinatal stroke children will have favorable outcomes. In San Diego County alone, the incidence of perinatal stroke is approximately 15-20 per year with a diverse ethnic background representative of California and the US as a whole;\(^23\) the presence of MTHFR mutations in these patients was determined by retrospective examination of medical records. The purpose of this study was to determine how lesion volume and developmental outcomes in this population were affected by MTHFR status.
Methods

Subjects
Participants were recruited as part of a larger longitudinal study of developmental outcome in children with perinatal stroke and controls. The perinatal stroke infants examined in this study were recruited through referrals from pediatric neurologists in southern California (primarily from San Diego County). Inclusion criteria for recruited perinatal stroke patients were: radiological evidence of focal acute infarction or remote presumed perinatal infarction (unilateral or bilateral), term gestation, and no other conditions that may have caused generalized or multifocal brain damage (including severe hypoxic-ischemic encephalopathy, bacterial meningitis, etc). Socioeconomic status (SES) was assessed using the Hollingshead Four Factor Index of Social Status to account for this potential confounding variable in developmental progress. Complete medical and family histories were obtained from the parents of the subjects. Medical records were reviewed for results of hypercoagulability genetic testing particularly for the MTHFR mutations (C677T and A1298C) and blood homocysteine levels. MTHFR mutation status was characterized as wild-type, single heterozygous (either a single C677T or A1298C mutation), or compound heterozygous (heterozygous for both the 677 and 1298 polymorphisms); there were no homozygous mutations among the subjects.

Procedure and Measures
All participating children were given a complete neurological exam to assess motor development. To assess language development, the Preschool Language Scale (PLS-3) was administered to all participants. PLS-3 is a language task that yields standardized language scores for both auditory comprehension and expressive communication that provide standardized percentile ranks and age equivalents for infants and children from birth through 6 years of age. Overall early cognitive development was measured using the Bayley Scales of Infant Development (BSID-II), which includes tasks of increasing difficulty with age (up to 42 months) and provides a standardized score for mental and psychomotor development. The size (by volume) and location of the focal lesions in the perinatal stroke patients were determined by a clinical neuroradiologist measuring primary lesion length, width, and height on Magnetic Resonance Imaging (MRI) scans.

Statistical Analyses
To analyze whether or not MTHFR 677 and 1298 genotype frequencies were higher in this sample of perinatal stroke subjects compared to published controls, a literature review was conducted on published manuscripts with the MTHFR genotype distribution clearly stated. A pooled analysis of control groups of 6 large population-based studies of the MTHFR allele frequencies of C677T and A1298C polymorphisms in several countries (including a global meta-analysis) was conducted. Allele frequencies were pooled into the three genotype groups used in this study: wild-type (CC/AA), single heterozygote (CT/AA or CC/AC), and subjects with two or more mutations (which includes compound heterozygotes and homozygous mutants). A Fisher’s exact test was used to compare the frequencies of these genotype groups between the study sample and the pooled control population allele distribution.

Lesion volume, three PLS-3 standardized scores for language development (auditory comprehension, expressive communication, and total language), and two BSID-II standardized scores (mental development index and psychomotor development index) were analyzed among the three MTHFR groups using SPSS (version PASW 18). Potential MTHFR genotype group demographic differences in age at testing, socioeconomic status, sex, lesion side, and presence of neonatal or late seizures were analyzed using independent t-tests. Given the small sample size (normal distribution not assumed), the Kruskal-Wallis H test was used to determine whether there were statistically significant differences among the three groups in all of the outcomes measures listed above. As a follow-up, Bonferroni (equal variances assumed) or Dunnett’s T3 (equal variances not assumed) was used to determine differences between two of the three subgroups (wild-type vs. single heterozygote, wild-type vs. compound heterozygote, and single vs. compound heterozygote) based on Levene’s test of variances.
Results
13 perinatal stroke subjects age 5 to 31 months with known MTHFR genotypes were included in this study: 3 with wild-type MTHFR alleles (23.1%), 7 with a single heterozygous polymorphism for MTHFR (53.8%), and 3 compound heterozygotes (23.1%). Of the several hundred perinatal stroke subjects in the overall longitudinal study, 36 were screened for hypercoagulability genetic testing and 19 had records of MTHFR status – the final 13 included in this study had complete clinical data and outcome measures. There were no significant differences among the MTHFR genotype groups in the demographic variables (age at testing, sex, or socioeconomic status) or potential confounding clinical features (side of lesion, neonatal or late seizures). Homocysteine levels were not reported for almost all of the subjects, so it was not included in subsequent analysis.

Comparison of MTHFR allele and genotype frequencies
6 published studies from around the world with genotype information for the MTHFR 677 and 1298 loci were included in the analysis of general population allele frequency. Control populations from each of these studies were pooled to calculate overall allele frequencies, and compared to the current study sample (Table 1). There was no significant difference between the allele frequencies observed in the current study and those in the control population obtained in the prior studies (Fisher’s exact $\chi^2 = 4.10$, df = 5, $p = 0.487$).

<table>
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</tr>
</thead>
<tbody>
<tr>
<td>Sazci et al. 2005</td>
<td>Turkey</td>
<td>1684</td>
<td>725 (42.9)</td>
<td>791 (47.4)</td>
<td>168 (9.6)</td>
<td>735 (43.7)</td>
<td>786 (46.3)</td>
<td>163 (10)</td>
</tr>
<tr>
<td>Weisberg et al. 1998</td>
<td>Canada</td>
<td>274</td>
<td>120 (43.8)</td>
<td>116 (42.3)</td>
<td>38 (13.9)</td>
<td>159 (58.0)</td>
<td>89 (32.5)</td>
<td>26 (9.5)</td>
</tr>
<tr>
<td>Hubacek et al. 2014</td>
<td>Czech</td>
<td>2486</td>
<td>1068 (43.0)</td>
<td>1116 (44.9)</td>
<td>302 (12.2)</td>
<td>1145 (46.1)</td>
<td>1066 (42.9)</td>
<td>275 (11.1)</td>
</tr>
<tr>
<td>Ogino et al. 2003</td>
<td>Global (US, Turkey, Australia, UK, Netherlands)</td>
<td>5389</td>
<td>2552 (47.4)</td>
<td>2221 (41.2)</td>
<td>616 (11.4)</td>
<td>2564 (47.6)</td>
<td>2328 (43.2)</td>
<td>497 (9.2)</td>
</tr>
<tr>
<td>Balcerzyk et al. 2014</td>
<td>Poland</td>
<td>106</td>
<td>47 (44.3)</td>
<td>48 (45.3)</td>
<td>11 (10.4)</td>
<td>51 (48.1)</td>
<td>46 (43.4)</td>
<td>9 (8.5)</td>
</tr>
<tr>
<td>Wu et al. 2014</td>
<td>Global (Czech, India, Australia, China)</td>
<td>5383/994*</td>
<td>2466 (45.8)</td>
<td>2296 (42.7)</td>
<td>621 (11.5)</td>
<td>521 (52.4)</td>
<td>380 (38.2)</td>
<td>93 (9.4)</td>
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<tr>
<td>Total</td>
<td></td>
<td>15322/10933</td>
<td>6978 (45.5)</td>
<td>6588 (43.0)</td>
<td>1756 (11.5)</td>
<td>5175 (47.3)</td>
<td>4695 (42.9)</td>
<td>1063 (9.7)</td>
</tr>
<tr>
<td>Current</td>
<td></td>
<td>13</td>
<td>5 (38.5)</td>
<td>8 (61.5)</td>
<td>0 (0)</td>
<td>8 (61.5)</td>
<td>5 (38.5)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

Table 1: Analysis of MTHFR allele frequencies at the 677 locus (CC, CT, TT corresponding to amino acid sequences AA, AV, VV) and the 1298 locus (AA, AC, CC corresponding to EE, EA, AA). Percentages are listed in parentheses. *Wu et al. performed a meta-analysis with some papers involving only the 677 locus, leading to different control sample sizes. There was no significant difference in allele frequency between the current study and the pooled general population in the 6 prior studies (Fisher’s exact $\chi^2 = 2.48$, df = 2, $p = 0.292$).

5 studies further characterized MTHFR genotypes into all 9 possible genotype combinations of both polymorphisms (C677C/A1298A, C677C/A1298C, C677C/C1298C, C677T/A1298A, C677T/A1298C, C677T/C1298C, T677T/A1298A, T677T/A1298C, T677T/C1298C). These genotype frequencies were categorized into the 3 groups used in this study: wild-type (CC/AA), single heterozygous (CT/AA or CC/AC), and two or more mutations (CT/AC, TT/--, --/CC). Table 2 shows the pooled genotype frequencies compared to the frequencies found in the current sample. The frequencies of these three MTHFR genotype groups was not significantly different between the current subjects and the general population (Fisher’s exact $\chi^2 = 2.48$, df = 2, $p = 0.292$).
Table 2: Analysis of MTHFR polymorphism genotype group frequencies in 5 published studies. Percentages are listed in parentheses. There was no significant difference in genotype group frequency between the current study and the pooled general population.

<table>
<thead>
<tr>
<th>Paper</th>
<th>n</th>
<th>Wild-type</th>
<th>Single heterozygote</th>
<th>Compound heterozygote/homozygote (2+ mutations)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sazci et al. 2005</td>
<td>1684</td>
<td>178 (10.6)</td>
<td>812 (48.2)</td>
<td>694 (41.2)</td>
</tr>
<tr>
<td>Weisberg et al. 1998</td>
<td>274</td>
<td>47 (17.2)</td>
<td>122 (44.5)</td>
<td>105 (38.3)</td>
</tr>
<tr>
<td>Hubacek et al. 2014</td>
<td>2486</td>
<td>278 (11.2)</td>
<td>1100 (44.2)</td>
<td>1108 (44.6)</td>
</tr>
<tr>
<td>Ogino et al. 2003</td>
<td>5389</td>
<td>838 (15.6)</td>
<td>2345 (43.5)</td>
<td>2206 (40.9)</td>
</tr>
<tr>
<td>Balcerzyk et al. 2014</td>
<td>106</td>
<td>11 (10.4)</td>
<td>58 (54.7)</td>
<td>37 (34.9)</td>
</tr>
<tr>
<td>Total</td>
<td>9939</td>
<td>1352 (13.6)</td>
<td>4437 (44.6)</td>
<td>4150 (41.8)</td>
</tr>
<tr>
<td>Current</td>
<td>13</td>
<td>3 (23.1)</td>
<td>7 (53.8)</td>
<td>3 (23.1)</td>
</tr>
</tbody>
</table>

MTHFR Genotype Group Association with Lesion Volume and Outcomes

Lesion volume tended to increase with the number of mutant MTHFR alleles among the 3 groups (note that a primary lesion volume was not calculated for one of the compound heterozygote subjects because there were multifocal and bilateral infarcts noted on her MRI). There was also a trend toward decreasing standardized scores in all domains of language, cognitive, and motor development associated with increasing copies of mutant MTHFR alleles (Figure 1). However, none of these differences between the 3 MTHFR groups reached statistical significance with either the Kruskal-Wallis H test or post-hoc analysis (Tables 3 and 4). There were two outliers in the single heterozygote group, but the results of analysis did not change when these were excluded.

Figure 1: Summary of language and cognitive outcomes by MTHFR genotype. PLS AC = auditory comprehension score, PLS EC = expressive communication, PLS TL = total language, Bayley MDI = mental development index, Bayley PDI = psychomotor development index

<table>
<thead>
<tr>
<th>MTHFR Genotype Group</th>
<th>Mean Standardized Score (± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PLS AC</td>
</tr>
<tr>
<td>WT (n = 3)</td>
<td>99.67±10.27</td>
</tr>
<tr>
<td>Single heterozygote (n = 7)</td>
<td>90.43±5.49</td>
</tr>
<tr>
<td>Compound heterozygote (n = 3)</td>
<td>73.67±8.97</td>
</tr>
</tbody>
</table>

Kruskal-Wallis (χ², p-value) 2.39, 0.303

Table 3: Summary of interaction between MTHFR genotype group and mean standardized scores on PLS-3 and BSID-II measures of language and cognitive development. There were no significant differences in these outcomes based on the Kruskal-Wallis H test (df = 2). PLS AC = auditory comprehension score, PLS EC = expressive communication, PLS TL = total language, Bayley MDI = mental development index, Bayley PDI = psychomotor development index
Comparisons Post-hoc independent t-tests (p-values)

<table>
<thead>
<tr>
<th></th>
<th>PLS AC</th>
<th>PLS EC</th>
<th>PLS TL</th>
<th>Bayley MDI</th>
<th>Bayley PDI</th>
<th>Lesion volume (cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single vs. WT</td>
<td>0.41</td>
<td>0.99</td>
<td>0.66</td>
<td>0.53</td>
<td>0.39</td>
<td>0.58</td>
</tr>
<tr>
<td>Compound vs. WT</td>
<td>0.13</td>
<td>0.52</td>
<td>0.24</td>
<td>0.26</td>
<td>0.45</td>
<td>0.09</td>
</tr>
<tr>
<td>Compound vs. Single</td>
<td>0.14</td>
<td>0.25</td>
<td>0.16</td>
<td>0.32</td>
<td>0.10</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Table 4: Bonferroni or Dunnett’s T3 tests for comparison between MTHFR genotype groups. There were no significant differences in the means of PLS-3 or BSID-II standardized scores for developmental outcomes. PLS AC = auditory comprehension score, PLS EC = expressive communication, PLS TL = total language, Bayley MDI = mental development index, Bayley PDI = psychomotor development index.

Discussion

The present study is the first to examine the effect of methylenetetrahydrofolate reductase (MTHFR) polymorphisms on lesion size and developmental outcomes in children who have had a perinatal stroke. The results indicate that there are no significant differences between wild-type, single MTHFR heterozygotes at either 677 or 1298, and subjects with two or more mutations in MTHFR (in our sample, all compound heterozygotes). There appeared to be a trend toward increasing lesion size and decreasing PLS-3 scores in all domains and Bayley MDI (particularly for compound heterozygotes), but these did not approach statistical significance due to the small sample sizes obtained in this study. The Bayley PDI score was the only measure that did not follow this trend, with a higher score for single heterozygotes than the wild-type subjects; however, this still did not approach significance on post-hoc analysis.

Several prior studies have sought to determine whether or not mutations in the MTHFR gene increase susceptibility to a variety of cardiovascular and thrombotic diseases. A recent study did not demonstrate any effect of the MTHFR A1298C polymorphism on the incidence of pediatric stroke in mother-child pairs in Poland, and found no synergistic effect of the C677T and A1298C mutations. There have been no large scale studies specifically examining the effect of MTHFR mutations on the incidence of perinatal stroke. Because these polymorphisms are relatively common worldwide and vary significantly with ethnicity, large sample sizes are needed to truly assess allele frequency in the perinatal stroke population. Nevertheless, our limited sample did not demonstrate a significantly increased mutant allele frequency compared to the pooled control population in several large-scale genetic studies worldwide.

The primary limitation of the current study is the small sample size. Not all perinatal stroke patients are tested for MTHFR mutations because its clinical significance remains unclear at this time, and no control subjects are genetically tested for hypercoagulability mutations so there were no matched samples for comparison. Another potential limitation is the lack of longitudinal follow-up for the subjects – although there were no significant difference in the age at testing between the groups, it is difficult to assess the changes in different domains of development over time especially given the plasticity demonstrated in other studies.

In conclusion, we did not find a significant effect of MTHFR genotype on language, cognitive, and psychomotor outcomes after a perinatal stroke. There was also no observed difference in the MTHFR allele frequency between perinatal stroke children and the general population. Despite the small number of subjects included in the analysis, there is an intriguing trend toward larger lesion volumes and worse outcomes in subjects with more MTHFR mutations that was particularly notable and approached significance in the compound heterozygote group. Prior studies only involving lesion volume and outcomes have failed to detect clear associations between lesion size and developmental trajectories, but perhaps this is due to interaction between lesion characteristics and genetic factors that influence recovery from strokes (including pro-thrombotic states like MTHFR mutations). Because MTHFR mutations may be clinically treatable with folate supplementation, the results of this study warrant further analysis with a larger sample size, which can be accomplished with a prospective study to obtain MTHFR genetic status more consistently on all infants with perinatal stroke. A longitudinal study trending the cognitive...
development of these subjects over time and noting any effect on neuroplasticity as observed in many prior studies\textsuperscript{10} may also yield more useful information.

Acknowledgements

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References


