Maternal 677CT/1298AC genotype of the MTHFR gene as a risk factor for cleft lip

V Shotelersuk, C Iittiwut, P Siriwan, A Angspatt

Subjects and Methods

The study sample consisted of 109 CL/P patients, 67 of their mothers, 45 of their fathers, and 202 controls. Of 109 patients, 14 were familial. All of them were studied under the auspices of the Thai Red Cross, a national charity organisation devoted to providing clinical care for the poor. Subjects were recruited between 2000 and 2002 from seven centres in Thailand (Nakornratchasem, Nan, Uthaithanee, Maehongsorn, Trang, Srakaew, and Bangkok). As preoperative evaluations, every patient was examined by a geneticist (VS) for any presence of associated anomalies suggestive of syndromic variants. In addition, a family history and epidemiological data were obtained and will be reported elsewhere. After receiving their informed consent, blood samples for DNA analysis were obtained at the time of blood typing and haematocrit determination. Only one patient in families with more than one affected subject was included in the case group. All syndromic cases were excluded. Only the cases of non-syndromic CL/P (normal growth, normal development, and no other major anomalies) were analysed in this report.

Statistical analysis

Standard chi-square and p values were calculated by a program available at http://quantmr2.psy.ohio-state.edu/kris/chisq/chisq.htm. Haplotype frequencies were estimated by the EH program downloaded from http://linkage.rockefeller.edu/ott/eh.htm. The transmission disequilibrium test (TDT) analysis was carried out on subjects with heterozygous informative CL/P patients and their parents. The control samples were blood donors with no oral clefts in Bangkok and Nakornratchasem collected in the same period. The study was approved by the institutional review board in Thailand. DNA was extracted by standard procedures and was amplified using the polymerase chain reaction (PCR). Genotyping for the MTHFR 677CT and 1298A>C polymorphisms was performed by restriction digestion of PCR products with HinfI and MboII, respectively.

Key points

- Previous data have shown an association between the 677C>T polymorphism in the MTHFR gene in either non-syndromic cleft lip with or without cleft palate (CL/P) patients or their mothers and an increased risk of the anomaly, but this finding remains controversial. No studies have investigated 1298A>C, the second most common polymorphism in MTHFR, in CL/P patients and their parents.
- We investigated 109 CL/P patients, 67 of their mothers, 45 of their fathers, and 202 controls for the 677C>T and 1298A>C polymorphisms. We found no association between any of the patients' genotypes and CL/P. Transmission disequilibrium test (TDT) analysis also showed no evidence for the association.
- However, a significantly higher frequency of the compound heterozygous 677CT and 1298AC genotype was detected in mothers of CL/P patients with an odds ratios of 4.43 (95% confidence interval 1.33 to 15.10).
- These results indicate an effect of the maternal genotype, rather than in the affected subjects.

The control samples were blood donors with no oral clefts in Bangkok and Nakornratchasem collected in the same period. The study was approved by the institutional review board in Thailand.
RESULTS

The observed frequencies of the 677C, 677T, 1298A, and 1298C genotypes in affected subjects, their mothers, their fathers, and controls are given in table 1. The observed distribution of genotypes among controls was compared with that expected according to Hardy-Weinberg equilibrium: no difference was found (for the 677 locus, according to Hardy-Weinberg equilibrium: no difference was found). The observed distribution of genotypes among controls was compared with that expected according to Hardy-Weinberg equilibrium (data not shown).

The haplotype frequencies of the patients, their parents, and controls also followed Hardy-Weinberg equilibrium (data not shown).

The haplotype frequencies of the patients, their parents, and controls calculated from the EH program are shown in table 2. All of the 677CT/1298AC genotypes were expected to be 677C-1298C and 677T-1298A haplotypes. No 677T-1298C haplotype was detected. This finding agrees with a previous observation that the polymorphism was found in every population tested and its frequency among Asians is quite similar to that of Europeans, but higher than in Africans.

We found no association between any of the patients' genotypes and CL/P. TDT analysis was carried out on subjects with heterozygous informative parents but also showed no evidence for the association. This finding is consistent with a previous report.

However, when we analysed maternal genotypes and CL/P in their offspring, we found the odds ratio calculated for mothers having the 677CT/1298AC genotype compared to the normal 677CC/1298AA genotype was 4.43 (95% CI 1.33 to 15.10). There were approximately 12% (eight of 67) of mothers with such a genotype; therefore, folate supplementation in a pregnant woman's diet may benefit these 12% of Thai children who are susceptible to CL/P owing to the 677CT/1298AC genotype in their mother. No such relationship could be found with any genotypes in fathers of affected offspring. This observation is in agreement with two recent independent association studies showing that there is an association between a maternal polymorphism, 677C>T, in the MTHFR gene in mothers of affected subjects and an increased risk of CL/P with risk ratios of 2.51 (1.00 to 6.14) and 4.09 (1.32 to 11.57).

A previous study showed that the activity of the MTHFR enzyme in subjects with 677CT/1298AC (47.7%) is higher than that of 677TT/1298AA (24.8%). However, we found that the detected risk of the 677TT/1298A mothers was no higher than that of the 677CT/1298AC mothers. Although the higher risk is associated with the 677TT/1298AC genotype, it is not statistically significant (95% CI 0.30 to 31.28). A possible interpretation is that the lower activity of the enzyme increases the susceptibility to fetal loss or to giving birth to children with multiple associated malformations, without

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Frequencies of MTHFR alleles in patients with CL/P, their parents, and controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTHFR allele</td>
<td>677C*</td>
</tr>
<tr>
<td>CL/P patients (n=109)</td>
<td>0.89 (193)</td>
</tr>
<tr>
<td>Mothers (n=67)</td>
<td>0.83 (111)</td>
</tr>
<tr>
<td>Fathers (n=45)</td>
<td>0.87 (78)</td>
</tr>
<tr>
<td>Controls (n=202)</td>
<td>0.88 (354)</td>
</tr>
</tbody>
</table>

*The number of subjects is indicated in parentheses. †Reference category.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Calculated frequencies of the MTHFR haplotypes in patients with CL/P, their parents, and controls from the EH program</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haplotype</td>
<td>677C-1298A</td>
</tr>
<tr>
<td>CL/P patients</td>
<td>0.61</td>
</tr>
<tr>
<td>Mothers</td>
<td>0.52</td>
</tr>
<tr>
<td>Fathers</td>
<td>0.66</td>
</tr>
<tr>
<td>Controls</td>
<td>0.61</td>
</tr>
</tbody>
</table>

*χ² = 2T; T = (Ln(L)case) + (Ln(L)control) - (Ln(L)case+control). †Reference category.

<table>
<thead>
<tr>
<th>Table 3</th>
<th>TDT analysis in CL/P patients</th>
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<tbody>
<tr>
<td>Haplotype</td>
<td>Transmitted</td>
</tr>
<tr>
<td>677C-1298A</td>
<td>17</td>
</tr>
<tr>
<td>677C-1298C</td>
<td>12</td>
</tr>
<tr>
<td>677T-1298A</td>
<td>6</td>
</tr>
<tr>
<td>677T-1298C</td>
<td>0</td>
</tr>
</tbody>
</table>

*χ² were analysed using a k-1/4 correction (where k is the number of alleles). Degree of freedom = (number of rows - 1) × (number of columns - 1) = 1 × 2 = 2.

DISCUSSION

We have established MTHFR genotypes in 109 CL/P patients, their parents (67 mothers and 45 fathers), and 202 controls. The frequency of the 677T allele in our controls was 12%, which is comparable to the frequencies of 14% in the control population reported in independent studies in Thailand. Our results agree with a previous observation that the polymorphism was found in every population tested and its frequency among Asians is quite similar to that of Europeans, but higher than in Africans. The frequency of the 1298C allele in our controls was 12%, which is similar to that of white populations (27-33%).

No 677T-1298C haplotype was detected. This finding agrees with an observation that a subject with the 677TT genotype always had a 1298AA genotype and a subject with the 1298CC genotype always had a 677CC genotype (table 4). In addition, it supports the recent finding that the 677T occurred on a founder haplotype of 1298A.

We found no association between any of the patients' genotypes and CL/P. TDT analysis was carried out on subjects with heterozygous informative parents but also showed no evidence for the association. This finding is consistent with a previous report. However, when we analysed maternal genotypes and CL/P in their offspring, we found the odds ratio calculated for mothers having the 677CT/1298AC genotype compared to the normal 677CC/1298AA genotype was 4.43 (95% CI 1.33 to 15.10). There were approximately 12% (eight of 67) of mothers with such a genotype; therefore, folate supplementation in a pregnant woman's diet may benefit these 12% of Thai children who are susceptible to CL/P owing to the 677CT/1298AC genotype in their mother. No such relationship could be found with any genotypes in fathers of affected offspring. This observation is in agreement with two recent independent association studies showing that there is an association between a maternal polymorphism, 677C>T, in the MTHFR gene in mothers of affected subjects and an increased risk of CL/P with risk ratios of 2.51 (1.00 to 6.14) and 4.09 (1.32 to 11.57).

A previous study showed that the activity of the MTHFR enzyme in subjects with 677CT/1298AC (47.7%) is higher than that of 677TT/1298AA (24.8%). However, we found that the detected risk of the 677TT/1298A mothers was no higher than that of the 677CT/1298AC mothers. Although the higher risk is associated with the 677TT/1298AC genotype, it is not statistically significant (95% CI 0.30 to 31.28). A possible interpretation is that the lower activity of the enzyme increases the susceptibility to fetal loss or to giving birth to children with multiple associated malformations, without
increasing the risk of CL/P alone. Another explanation would be a limited number of mothers with the 677TT/1298AA genotype in our study owing to its low frequency. Although not statistically significant, a higher risk is associated with the 677TT/1298AA controls (odds ratio = 4.65), which is also greater than that observed for the 677TT/1298AA mothers. Further studies with more numbers of fathers are needed to determine the association.

Three previous studies, however, showed no association between the maternal 677TT genotype and CL/P.17–21 Our results do not contradict these reports because the three studies did not determine the genotype at the 1298 position. Moreover, the inconsistent result could be caused by the difference in populations studied with diverse genetic backgrounds and environmental factors. In addition, the timing of sample collections, whether it was before or after folic acid fortification of foods, started in the USA in 1998, could affect the frequencies of the polymorphisms in subjects with some anomalies.22

These findings indicate a possible involvement of the folate pathway in the causation of CL/P and support an influence of the maternal genotype, rather than an effect of the embryo’s genotype. The 677C>T polymorphism converts an alanine to a valine residue at position 222 making the MTHFR thermolabile with reduced activity to 65% in heterozygotes and 30% in homozygous nor the heterozygous state for the 1298A>C is associated with raised plasma homocysteine or decreased homozygous nor the heterozygous state for the 1298A>C is associated with raised plasma homocysteine or decreased plasma folate levels, the combined heterozygosity for 677C>T and 1298A>C is associated with higher homocysteine and lower plasma folate concentrations.23 Nonetheless, the real detailed biological mechanism remains to be elucidated.

So far, this is the first report on the investigations of both 677C>T and 1298A>C polymorphisms in the MTHFR gene of patients with CL/P and their parents. Our results indicate an effect of the maternal genotype, rather than that of the affected subjects.

ACKNOWLEDGEMENTS
We wish to thank the patients and their families for their participation in the study; and the medical staff of the Thai Red Cross, the Craniofacial Centre of King Chulalongkorn Memorial Hospital, and the Provincial Hospitals of Nakornpathom, Chai Nat, and Sakaew for their excellent care of their patients. This work was supported by grants from the Thailand Research Fund, the National Center for Genetic Engineering and Biotechnology, and the Molecular Biology Projects, Faculty of Medicine, Chulalongkorn University.

Table 4 Prevalence and calculated odds ratios with 95% CI of the MTHFR polymorphisms in patients with CL/P, their parents, and controls

<table>
<thead>
<tr>
<th>MTHFR 677/1298 genotype</th>
<th>Observed frequency†</th>
<th>Odds ratio (95% CI)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CL/P patients (n=109)</td>
<td>Mothers (n=67)</td>
</tr>
<tr>
<td>CC/AA</td>
<td>0.30 (33)</td>
<td>0.25 (17)</td>
</tr>
<tr>
<td>CC/AC</td>
<td>0.41 (45)</td>
<td>0.37 (25)</td>
</tr>
<tr>
<td>CC/CC</td>
<td>0.06 (6)</td>
<td>0.05 (4)</td>
</tr>
<tr>
<td>CT/AA</td>
<td>0.20 (22)</td>
<td>0.16 (11)</td>
</tr>
<tr>
<td>CT/AC</td>
<td>0.03 (3)</td>
<td>0.12 (8)</td>
</tr>
<tr>
<td>TT/AA</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>TT/AC</td>
<td>0.03 (2)</td>
<td>0.04 (2)</td>
</tr>
<tr>
<td>TT/CC</td>
<td>NO</td>
<td>NO</td>
</tr>
</tbody>
</table>

*The number of subjects is indicated in parentheses. †No = not observed. ‡Odds ratio not determined. §The 95% CI is not determined owing to the invalidity of the Cornfield 95% confidence limits for odds ratio.

REFERENCES
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