Association of PEMT rs4244593 Polymorphism with Non-syndromic Cleft Lip and Palate in the Indian Population

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ABSTRACT

Introduction: Non-syndromic cleft lip with or without cleft palate (NSCLP) is a multifactorial threshold trait (MFT) involving both genetic and environmental factors. Choline, methionine and folate metabolism are interrelated in converting the homocysteine to methionine. Phosphatidylethanolamine N-methyltransferase (PEMT) is involved in biosynthesis of choline. Methods: We studied the PEMT rs4244593 SNP to assess its effect on NSCLP risk in the South Indian population. Blood samples of 142 cases with NSCLP and 141 controls were collected and genotyped using PCR-RFLP. Statistical analysis of the results was performed by calculating OR, and 95% CI via χ² test. Results: Proportions of genotypes were 16.9 % AA, 64.8 % AC, 16.9 % CC in cases and 35.5 % AA, 47.5 % AC, 17.0 % CC in controls. The C allele frequency was 50.7% for cases and 40.8% for controls. An increased risk was found for co-dominant (AC vs. AA: OR =2.86, 95% CI =1.60 to 5.11, p<0.001; CC vs. AA: OR =2.26, 95% CI =1.08 to 4.72, p=0.029), dominant (AC+CC vs. AA: OR =2.70, 95% CI =1.55 to 4.72, p<0.001) and allelic models (C vs. A: OR =1.49, 95% CI =1.07 to 2.08, p=0.018). Conclusions: Although our results indicate that the PEMT rs4244593 polymorphism is one of the important genetic determinants of NSCLP risk in South Indian subjects, in the absence of mechanistic studies, this polymorphism cannot be considered as a determinant of NSCLP risk. Additional studies with fully validated functional SNPs and larger sample sizes are needed to confirm our findings.

Key words: Choline orofacial cleft, PEMT protein, SNP

INTRODUCTION

Nonsyndromic cleft lip with or without cleft palate (NSCLP) is a multifactorial threshold trait (MFT) involving both genetic and environmental factors (Murthy & Bhaskar, 2009). Higher methionine trans-sulfuration and trans-methylation found respectively in the first and third trimesters during pregnancy suggest a higher requirement for methionine and folate during these periods (Dasarathy et al., 2010). Furthermore, pregnant women in the absence of folate supplementation showed increased levels of homocysteine during the second and third trimesters pregnancies, probably due to increased need for folate, and declining circulating folate levels (McNulty et al., 2013). Choline, a water soluble essential nutrient, plays an important role in the production of the

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phospholipid of cell membranes. Choline is the major source of labile methyl-
groups; one of its metabolites betaine mediates homocysteine methylation to
form methionine (Zeisel, 2006). Folate metabolism, methionine and choline are
interrelated at the methionine formation step (Finkelstein, 2000). According to
the Institute of Medicine (IOM), dietary reference intake (DRI) for choline is 550
mg/day and 425 mg/day for men and women respectively (Otten, Hellwig &
Meyers, 2006).

The availability of choline is crucial throughout the fetal development and has
a vital role in foetal brain development (Niculescu, Craciunescu & Zeisel 2005;
Cheng et al., 2008). Higher demand for choline by a developing foetus during
pregnancy could put the mother and, subsequently, the embryo and its
extraembryonic membranes at risk for choline deficiency (Zeisel et al., 1995).
Presence of some inhibitors of choline uptake and metabolism, produced
neural tube defects in neurulating mouse embryos grown in vitro (Fisher et al.,
2002). This indicates that the maternal genotype determines the phenotype of the
offspring. Maternal diets low in choline influence neural tube development (Fisher et al.,
2002) and increase the risk of having a new born with birth defects (Fisher et al.,
2002; Shaw et al., 2006). Although choline and choline esters can be obtained through
food (Otten et al., 2006), the human body is capable of synthesising choline through
de novo phosphatidylcholine biosynthesis catalysed by phosphatidylethanolamine
N-methyltransferase (PEMT, EC 2.1.1.17) (Hirata et al., 1978; Schneider & Vance 1979).
The gene encoding phosphatidylethanolamine N-methyltransferase is located on
chromosome 17p11.2. As polymorphisms in the human PEMT gene alter biosynthesis
of choline, some studies have been conducted to investigate the association
of diseases related to choline deficiency and PEMT gene polymorphisms (Zhang et
al., 2006; Bi et al., 2012; Li et al., 2009, Romeo, Cohen & Hobbs, 2006). In this study, we
examined the association between cleft lip and palate and PEMT rs4244593 (intron 3)
genotypes in a South Indian population.

METHODS

Subjects
We evaluated 142 unrelated patients with NSCLP [123 cleft lip and palate (CLP) and 19
cleft palate only (CPO)] and 141 individuals as control from Cleft and Craniofacial
Centre, Sri Ramachandra University, Chennai, India. All cases were enrolled in
the study from May 2012 to April 2014 and were evaluated by two different surgeons
for their individual phenotypic features; this was cross-verified through their
medical records. Unrelated individuals without clefts or family history of clefting
for three generations were considered as controls. The subjects who presented
with congenital malformations or major developmental disorders were excluded
from the study. This study was approved by the Institutional Ethics Committee of
Sri Ramachandra University, Chennai, India, and written informed consent was
obtained from all study subjects. As many of the children were minors, consent
was obtained from their parents or legal guardians.

Genotyping
Blood samples (3 ml) were collected into an EDTA vacutainer (Becton Dickinson,
Franklin Lakes, NJ) for DNA extraction. Genomic DNA was isolated from leucocytes
using phenol-chloroform extraction and ethanol precipitation (Sambrook & Russell,
2001). PEMT rs4244593 SNP genotyping was performed following polymerase
chain reaction-restriction fragment length polymorphism method (Mostowska et al.,
2010b). Briefly, 637 basepair (bp) fragment
of PEMT rs4244593 region was amplified with the primers of 5'-CTG CCT CCT CAC GAC CTG TA-3' and 3'-GCG TGG TTC ACT CIT TC-3'. The PCR products were digested with restriction enzyme TaqI (Fermentas Life Sciences, Germany) at 65°C for 4 h for PEMT rs4244593 genotyping and detected by electrophoresis on 2% agarose gel. Upon digestion, 637-bp PCR product remained uncleaved for the C allele and but was cleaved into smaller fragments of 401-bp and 236-bp in the case of the A allele. The digested products were stained with ethidium bromide and visualised under UV light.

**Statistical analysis**

Allele frequencies were calculated based on the observed number of the two different alleles, A and C. Hardy-Weinberg equilibrium (HWE) analysis was performed by comparison of observed and expected genotype frequencies using chi-squared goodness-of-fit test. Association between PEMT rs4244593 polymorphism and different cleft phenotypes (NSCLP, CLP and CPO) was analysed by χ²-test.

To compare all three cases, we considered the same control group (n=141). The risk estimates were examined in four hypothetical risk models, co-dominant, dominant (AC+CC vs. AA), recessive (AC+AA vs. CC) and allelic (C vs. A) models. Statistical analysis was performed using SPSS statistical software version 16.0 (SPSS Inc, Chicago, Illinois) for Windows. A two-sided p-value <0.05 was considered to be statistically significant. From the HapMap 20kb up and downstream SNPs around rs4244593 were extracted and LD maps were constructed using Haploview (Barrett et al., 2005).

**RESULTS**

Genotype and allelic distribution for the PEMT rs4244593 polymorphism are presented in Table 1. Proportions of genotypes were 16.9 % AA, 64.8 % AC, 16.9 % CC in cases and 35.5 % AA, 47.5 % AC, 17.0 % CC in controls. The C allele frequency was 50.7% for cases and 40.8% for controls. The Hardy-Weinberg equilibrium test showed that the genotypic distribution was in equilibrium in the controls.

### Table 1. Genotype distribution and allele frequencies of the PEMT rs4244593 in cleft lip and palate

<table>
<thead>
<tr>
<th>Genotype distribution</th>
<th>Control (%)</th>
<th>Overall clefts (%)</th>
<th>CLP (%)</th>
<th>CPO (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>50 (35.5)</td>
<td>24 (16.9)</td>
<td>19 (15.5)</td>
<td>5 (26.3)</td>
</tr>
<tr>
<td>AC</td>
<td>67 (47.5)</td>
<td>92 (64.8)</td>
<td>81 (65.9)</td>
<td>11 (57.9)</td>
</tr>
<tr>
<td>CC</td>
<td>24 (17.0)</td>
<td>26 (18.3)</td>
<td>23 (18.7)</td>
<td>3 (15.8)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Allele frequency</th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>A allele</td>
<td>167 (59.2)</td>
<td>140 (49.3)</td>
<td>119 (48.4)</td>
<td>21 (55.3)</td>
</tr>
<tr>
<td>C allele</td>
<td>115 (40.8)</td>
<td>144 (50.7)</td>
<td>127 (51.6)</td>
<td>17 (44.7)</td>
</tr>
</tbody>
</table>

Test for HWE

| Chi-square | 0.037 | 12.44 | 12.48 | 0.555 |
| P-value    | 0.847 | <0.001| <0.001| 0.456 |
Table 2. Results of association tests with PEMT rs4244593 SNP in cleft lip and palate

<table>
<thead>
<tr>
<th>PEMT rs4244593 A/C</th>
<th>OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall clefts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>AC</td>
<td>2.86 (1.60-5.11)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CC</td>
<td>2.26 (1.08-4.72)</td>
<td>0.029</td>
</tr>
<tr>
<td>AC+CC vs. AA</td>
<td>2.70 (1.55-4.72)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AC+AA vs. CC</td>
<td>0.91 (0.50-1.69)</td>
<td>0.776</td>
</tr>
<tr>
<td>A allele</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>C allele</td>
<td>1.49 (1.07-2.08)</td>
<td>0.018</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CLP</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>AC</td>
<td>3.18 (1.71-5.91)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CC</td>
<td>2.52 (1.16-5.49)</td>
<td>0.018</td>
</tr>
<tr>
<td>AC+CC vs. AA</td>
<td>3.01 (1.65-5.47)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AC+AA vs. CC</td>
<td>0.89 (0.47-1.68)</td>
<td>0.722</td>
</tr>
<tr>
<td>A allele</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>C allele</td>
<td>1.55 (1.10-2.19)</td>
<td>0.013</td>
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<table>
<thead>
<tr>
<th>CPO</th>
<th></th>
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<tbody>
<tr>
<td>AA</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>AC</td>
<td>1.64 (0.54-5.03)</td>
<td>0.381</td>
</tr>
<tr>
<td>CC</td>
<td>1.25 (0.28-5.67)</td>
<td>0.772</td>
</tr>
<tr>
<td>AC+CC vs. AA</td>
<td>1.54 (0.52-4.52)</td>
<td>0.431</td>
</tr>
<tr>
<td>AC+AA vs. CC</td>
<td>1.09 (0.29-4.05)</td>
<td>0.893</td>
</tr>
<tr>
<td>A allele</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>C allele</td>
<td>1.18 (0.59-2.33)</td>
<td>0.642</td>
</tr>
</tbody>
</table>

(P=0.847). The frequency of the C allele of the PEMT rs4244593 polymorphism was 40.8% among control individuals. The genotype and allele frequencies were significantly different between cases and controls (Table 2). Table 2 represents the OR and 95% CI that were calculated to assess the effect of the PEMT rs4244593 variant on NSCLP risk. Both heterozygous (OR=2.86; 95% CI: 1.60-5.11; p<0.001) and homozygous (OR=2.26; 95% CI: 1.08-4.72; p=0.029) genotypes significantly increased NSCLP risk. In the dominant model, the risk of NSCLP remained the same as for the co-dominant model but the level of significance increased (OR=2.70; 95% CI: 1.55-4.72; p<0.001). Whereas in the recessive model (AC+AA vs. CC), no significant risk for this variant (OR=0.91; 95% CI: 0.50-1.69; P=0.776) was observed. The allelic model also showed a significant positive association between C allele and susceptibility to oral clefts, but this effect was modest (OR=1.49; 95% CI: 1.07-2.08; p<0.018). In subgroup analysis, the rs4244593 variant showed a similar trend of association for CLP group (Table 2). The PEMT rs4244593 polymorphism did not show a significant association with CPO risk in three different models (Table 2). The PEMT rs4244593 variant allele frequencies collected from various populations are presented in Figure 1. Data revealed that the frequency of the PEMT rs4244593
Figure 1. The frequency of PEMT rs4244593 SNP in the current study compared to the world populations.

Note: Forest plot represents minor allele frequency (MAF) with 95% confidence interval (CI). Data from previous studies is denoted by the citations. N: sample size; CC, AG and AA are genotypes; HWEp: Hardy-Weinberg equilibrium p value.

Minor allele in Indian populations are showing slight proximity with the frequencies of East Asian (p > 0.05) and European populations (p > 0.05), but it is fairly dissimilar from African populations (p < 0.001). Calculation of LD in the 20 kb up and downstream regions from PEMT rs4244593 loci in HapMap populations revealed that the East Asian populations showed larger LD blocks followed by the European populations. These blocks are very small in African populations (Figure 2).

DISCUSSION

The PEMTTrs4244593 minor allele frequency (C allele) in our controls was 40.8%, which is slightly less than that (47%) found in the GIH population of HapMap (http://hapmap.ncbi.nlm.nih.gov). This discrepancy may be due to the variations in source of samples included in the HapMap. The highest minor allele frequency of C allele was observed in African populations followed by European and Asian populations of HapMap. The PEMT rs4244593 polymorphism is significantly associated with cleft lip and palate in co-dominant, dominant and allelic models.

Some of the enzymes like cystathionine beta-synthase (CBS), methionine synthase reductase (MTR) and 5,10-methylene tetrahydrofolate reductase (MTHFR) are directly involved in folate-Hcy metabolism, while PEMT is indirectly coupled to folate-Hcy metabolism, bymodulating its metabolite profile through the choline pathway. Previous studies showed higher dietary intakes of choline decreased risk of CLP (Shaw et al., 2006). In contrast to this higher plasma choline increased CLP risk (Shaw et al., 2009) and neural tube defects (Shaw et al., 2004). PEMT knockout mice showed substantially diminished concentrations of docosahexaenoic acid (DHA) in plasma...

*PEMT* gene is highly polymorphic; direct sequencing of *PEMT* gene in 48 Japanese individuals revealed 98 SNPs (Saito et al., 2001). The rs12325817 (−744 G/C) SNP is located in the promoter region of *PEMT* gene and is associated with increased risk to choline deficiency syndrome in women (da Costa et al., 2006). The exact function of this SNP is not known because it does not fall within a mapped transcription factor binding site nor is it assessed in the HapMap study (Figure 2) (Frazer et al., 2007). A functionally significant SNP (rs7946: Val175Met) in *PEMT* exon 6 region results in a 30% loss of function and is associated with increased risk of non-alcoholic fatty liver disease (Song et al., 2005; Dong et al., 2007). Polymorphisms of *PEMT* were not independently correlated with NSCLP risk in the Polish population. However, considerable epistatic interaction between the polymorphisms of MTHFR (Ala222Val), MTR (A2756C) and *PEMT* (rs1646406), indicate interaction between choline and folate metabolisms in the pathogenesis of NSCLP (Mostowska et al., 2010b). Similar epistatic interaction between rs1801133

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**Figure 2.** Linkage disequilibrium profiles in different world populations studied in International HapMap Project.

*Note:* Colour coding represents the D'/LD values and the values in cells are r² multiplied by 100.
of MTHFR and rs4244593 of PEMT in endometriosis-associated infertility has been reported (Szczepanska et al., 2011). Another Polish population study, the gene–gene interaction analysis revealed a significant epistatic interaction of BHMT2 (rs673752), PEMT (rs12325817), and PCYT1A (rs712012) with maternal NSCLP susceptibility (Mostowska et al., 2010a). In contrast to the present study, the above two studies (Mostowska et al., 2010b, Szczepanska et al., 2011) considered “A” allele as risk allele in their analysis because it is a minor allele in the Polish population. Further, the PEMT rs4244593 is an A to C transversion, and C allele is the minor allele and therefore the risk allele in this study.

In conclusion, our analyses suggest that PEMT rs4244593 may contribute to the risk of NSCLP in the Indian population. In the absence of mechanistic studies, additional studies with fully validated functional SNPs and larger sample sizes are needed to confirm our findings.

Conflict of interest
There is no conflict of interest.

ACKNOWLEDGEMENTS
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REFERENCES


