Maternal Polymorphism MTHFR A1298C not C677T and MSX1 as the Risk Factors of Non-syndrome Cleft Lips / Palate in Sasak Tribe Indonesia

Yayun Siti Rochmah¹*, Lusi Suwarsi², Stefani Harumsari³, Agung Sosiawan⁴, Siti Fatimah-Muis⁵, Sultana MH Faradz²

1. Department of Oral and Maxillofacial Surgery, Faculty of Dentistry Sultan Agung Islamic University Semarang, Indonesia.
2. Center for Biomedical Research (CEBIOR), Faculty of Medicine Diponegoro University, Semarang Indonesia.
3. Department of Medical Biology, Faculty of Medicine Sultan Agung Islamic University, Semarang Indonesia.
4. Department of Dental Public Health, Faculty of Dental Medicine Airlangga University, Surabaya Indonesia.
5. Department of Nutrition, Faculty of Medicine Diponegoro University, Semarang Indonesia.

Abstract
The etiology of orofacial cleft as Non-syndromic cleft lips with or without palate (NSCL/P) are complex which including genetic and environmental factors. To investigate maternal polymorphism of MTHFR C677T, A1298C and MSX1 as the risk factor of NSCL/P in Sasak Tribe, Lombok Indonesia.

The study was a case control study involving 148 subjects from Sasak Tribe, consisting of 35 children with NSCL/P-mother pairs and 39 healthy children-mother pairs as controls. EDTA blood was drawn from all subjects. Molecular analyses of MTHFR C677T, A1298C and MSX1 polymorphisms were done using PCR-RFLP. The risk factors were analyzed statistically using OR and Chi square test.

Children with at least one copy of the MTHFR 1298C allele had a higher risk of NSCL/P (p=0.036, OR 2.7, 95% CI (1.1-7.0)). Maternal polymorphisms MTHFR C677T and MSX1 were not found to be risk factors of NSCL/P (p>0.05). New sequence variation of c.469 + 12G>A was found near the splice site region of exon 1 MSX1 in an affected child.

MTHFR A1298C polymorphism increases the risk of NSCL/P in Sasak Tribe, Lombok, Indonesia. A novel sequence of MSX1 c.469 + 12G>A was found. Further study with higher sample size to fulfill minimum number of subjects for genetic study may find more novel polymorphisms.

Keywords: MTHFR C677T, MTHFR A1298, MSX1, Orofacial cleft.

Received date: 31 October 2017

Introduction
Non syndromic cleft lips with or without cleft palate are the common orofacial cleft among newborn worldwide. Cleft and palate reconstructive surgery in Sasak children have been carried out a special emphasize in maxillofacial surgeon community service activity. In the last six years a total of 313 Sasak children with clefts of the lips and/or palate (CL/P) recieverd maxillofacial surgery done by Dentistry charity foundation. Familial and twin study provided a fascinating signal for genetic factor yield in NSCL/P. The dominant factors affecting in NSCL/P varies and correlates with geographic, racial and ethnic background. Review by Lesmana (2016) for contributing genes in NSCL/P were TGFβ2, TGFβ3, IRF6, AXIN2, PVRL1, CRISPLD2, FOX1, SHH, MTHFR, MMPs, DHFR, FGF, GREM1, SATB2, MID1, WNT9B, and PAX9. However some studies in Indonesian population were not always confirmed.

Folate supplementation or dietary during pregnancy has been reported in several studies that may prevent or reduce the risk of NSCL/P. Methylene tetrahydrofolatereductase (MTHFR) is a vital enzyme in folate metabolism and DNA synthesis. MTHFR C677T and A1298C are the most common single nucleotide polymorphisms (SNPs) which decrease of enzyme activity. Those SNPs have been considered as suspected genetic factor for NSCL/P risk. Numerous studies...
have shown inconsistent results for genotype-phenotype interaction between MTHFR polymorphism and NSCL/P.\textsuperscript{10,11}

Loss of function in human Homologous Homeobox 1 (MSX1) gene yields as cleft lip or palate. MSX1 produces a protein which plays a function in protein signaling during embryogenesis particularly in the craniofacial development and odontogenesis.\textsuperscript{3,12} The risk for NSCL/P increases in the existence of environmental interaction between maternal exposure to smoking and alcohol and MSX1 polymorphism.\textsuperscript{13} However, not all research proved the contribution of MSX1 to the development of NSCL/P. It was thought that ethnicity plays important role in the development of NSCL/P.\textsuperscript{14,15} The reported study was the first study to explore genetic factors as risk for orofacial cleft in Sasak Tribe. The aim of this study was to investigate maternal polymorphism of MTHFR C677T, A1298C and MSX1 as the risk factors of NSCL/P in Sasak Tribe.

Materials and methods

The study was designed as a case control study involving 148 pairs of mother-child subjects from Sasak Tribe, consisted of 35 pairs with NSCL/P and 39 pairs of healthy controls. Subjects were recruited from Dr Soejono Hospital East Lombok. The diagnosis and type of clefts were evaluated by detailed physical examination LAHSAL (Labial-Alveolar-Hard palate-Soft Palate-Labial) and categorized as Cleft lips (CL), Cleft lips and palate (CLP) and Cleft palate (CP). DNA was obtained using salting out method from EDTA blood for all subjects. MTHFR C677T, A1298C and MSX1 polymorphisms were analyzed using PCR-RFLP.\textsuperscript{16} The product was digested using restriction enzyme (Table 1). Sanger sequencing was carried out for confirmation of sequence changes.

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Primer</th>
<th>Enzyme</th>
<th>Product size</th>
<th>RFLP Fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTHFR C677T</td>
<td>MTHFR C677T</td>
<td>HinfI</td>
<td>188 bp</td>
<td>CC (188 bp)</td>
</tr>
<tr>
<td>MTHFR A1298C</td>
<td>MTHFR A1298C</td>
<td>BsmI</td>
<td>188 bp</td>
<td>AA (188 bp)</td>
</tr>
<tr>
<td>MSX1 C348T</td>
<td>MSX1 C348T</td>
<td>TaqI</td>
<td>188 bp</td>
<td>CC (188 bp)</td>
</tr>
</tbody>
</table>

Table 1. Primers and PCR profile for MTHFR and MSX1 polymorphisms analysis.

Results

Figure 1. PCR-RFLP pattern of MTHFR C677T, MTHFR A1298C, MSX1 and its sequencing.

A. PCR-RFLP of MTHFR C677T and its sequencing using reverse sequence (complimentary nucleotide) demonstrated a variation of MTHFR C677T : (A1) Genotype TT (homozygous) in subject M01, (A2) Genotype CT (heterozygous) in subject C01 and (A3) Genotype CC (WT) in subject M02.
B. PCR-RFLP of MTHFR A1298C and its sequencing using reverse sequence (complimentary nucleotide) demonstrated a variation of MTHFR A1298C : (B1) Genotype CC (homozygous) in subject C13, (B2) Genotype AC (heterozygous) in subject M13, and (B3) Genotype AA (WT) in subject 21.
C. PCR-RFLP of MSX1 and its sequencing using forward sequence (complimentary nucleotide) demonstrated a variation of MSX1 C348T : (C1) p.Gly116= (rs34165410), (C2) C458A; p.Pro153Gln (rs.104893854), (C3) C469 + 12G>A (New finding).

Abbreviation: Bl = blank; C=children and M=mother.
Maternal Polymorphism MTHFR A1298C was detected in MSX1. Sanger sequencing was done and discovered an affected child with new MSX1 missense mutation in intron region (Figure 1). Genotype of MTHFR 677-CC (71.4%), 1298-AA (45.7%) and MSX1 330-TT (82.9%) were more frequently observed in mother of cases (Table 2). The genotype of MTHFR C677T, MTHFR A1298 and MSX1 were not shown statistically different in NSCL/P cases and controls (Table 2). 

**Discussion**

Maternal MTHFR A1298C was statistically proven as a risk factor of NSCL/P in Sasak (Table 2). Children who have inherited mutant allele “C” from their parents were in higher risk for NSCL/P in Lombok. This study found that there was an association between mutant allele status with the risk of NSCL/P in Sasak children cases and controls (Table 2). In contrast to A1298C, result from present study suggest no association between MTHFR C677T and NSCL/P. The higher frequencies of A1298C than C677T mutant allele in NSCL/P children was proven in this study (62.9% vs 34.3%, respectively). Children who had homozygous mutant allele MTHFR C677T were observed with more severe phenotype than heterozygous (Figure 2).

Table 2. Polymorphisms of MTHFR and MSX1 in presence of mutant allele and NSCL/P.

<table>
<thead>
<tr>
<th>Suspected gene</th>
<th>Samples</th>
<th>NSCLP</th>
<th>Control</th>
<th>P</th>
<th>OR (CI 95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTHFR C677T</td>
<td>Mother</td>
<td>n=35</td>
<td>n=39</td>
<td>0.81</td>
<td>0.9 (0.5-2.5)</td>
</tr>
<tr>
<td>- MT</td>
<td>10 (28.6)</td>
<td>10 (25.6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- WT</td>
<td>25 (71.4)</td>
<td>29 (74.4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Children</td>
<td>n=35</td>
<td>n=39</td>
<td></td>
<td>0.57</td>
<td>1.3 (0.5-3.6)</td>
</tr>
<tr>
<td>- MT</td>
<td>11 (31.4)</td>
<td>10 (25.6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- WT</td>
<td>24 (68.6)</td>
<td>29 (74.4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTHFRA1298C</td>
<td>Mother</td>
<td>n=35</td>
<td>n=39</td>
<td>0.04*</td>
<td>2.7 (1.1-7.0)</td>
</tr>
<tr>
<td>- MT</td>
<td>19 (54.3)</td>
<td>16 (41.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- WT</td>
<td>16 (45.7)</td>
<td>23 (58.9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Children</td>
<td>n=35</td>
<td>n=39</td>
<td></td>
<td>0.03*</td>
<td>2.7 (1.1-7.0)</td>
</tr>
<tr>
<td>- MT</td>
<td>22 (62.9)</td>
<td>12 (31.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- WT</td>
<td>13 (37.1)</td>
<td>27 (68.9)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Polymorphisms of MTHFR and MSX1 in presence of mutant allele and NSCL/P.

| Abbreviation: MT = mutant-type; WT = wild-type. |

Maternal MSX1 C330T polymorphism was not a risk factor for NSCL/P in Sasak Tribe (Table 2). This finding is supported the study by Saskia et al (2011) that no association observed between MSX1 C330T and NSCL/P in Deuteromelayu, Indonesia. This is evidence that MSX1 polymorphism is heterogeneous depending on the ethnic origin and not highly related to NSCL/P. High consanguinity marriage may influence to heterogeneity of polymorphism among ethnic group. The present study found a new sequence variation in MSX1 c.469 +12G>A. This intron changes is predicted as polymorphism by MutationTaster2 because of the location near the splice site region of exon 1 MSX1. Although this study was directed to genes role as contributing factors to NSCL/P, the age of the subjects when the operation was done influence a great deal on the result of operation. The already operated subject will be followed up by foundation for second operation in the future to have optimal result. It is well accepted by expertise in cleft lip/palate that operations alone will not give optimal result if not supported by comprehensive esthetic treatment.

**Conclusions**

Maternal MTHFR A1298C polymorphism increases the risk of NSCL/P in Sasak Tribe, Lombok, Indonesia. A novel sequence of MSX1 c.469 +12G>A was found, which is different with other previous study. Further study with higher sample size to meet the number of proper...
genetic study may found more novel polymorphisms and its consequences to the disease.

Acknowledgements

We would like to thank Dr. H. Karsito, SpPD (Dr R. Soejono Hospital, Lombok), Drg Ahmad Hariadi SpBM (Departement of Oral and Maxillofacial Surgery Airlangga University), Dr Yantoko SpBP-RE (YSS Foundation Jakarta), Staff of Center for Biomedical Research Diponegoro University, Prof.Dr.Ign.Riawanto SpBD for support and review. This research was funded by Sultan Agung Islamic University.

Declaration of Interest

The authors declared that there are no conflict of interest whatsoever.

References

3. Hozyasz KK. The search for risk factors that contribute to the etiology of non-syndromic cleft lip with or without cleft palate (CL/P) in the Polish population. Pediatria Polska. 2010;85:609-23.