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

Yayun Siti Rochmah[1], Lusi Suwarsi[2], Stefani Harumsari[3], Agung Sosiawan[4], Siti Fatimah-Muis[5], Sultana MH Faradz[2]*

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 MSX1as 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 sequenceof MSX1 c.469 + 12G>A was found. Further study with higher sample size to fullfill minimum number of subjects for genetic study may found more new novel polymorphisms.

Clinical article (J Int Dent Med Res 2018; 11(1): pp. 120-123)

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

Received date: 31 October 2017 **Accept date:** 04 December 2017

Introduction

Non syndromic cleft lips with or without cleft palate are the common orofacial cleft among newborn worldwide.[1]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) recieved maxillofacial surgery done by Dentistry charity foundation.[2] Familial and twin study provided a fascinating signal for genetic factor yield in NSCL/P.[3] The dominant factors affecting in NSCL/P varies and correlates with geographic, racial and ethnic background.[4,5] 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.[6] 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.[7,8] *Methylenetetrahydrofolatereductase (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.[9] Those SNPs have been considered as suspected genetic factor for NSCL/P risk. Numerous studies

Corresponding author:
Sultana MH Faradz,
Center for Biomedical Research (CEBIOR),
Faculty of Medicine, Diponegoro University,
Semarang Indonesia
E-mail: sultanafaradz@gmail.com

have shown inconsistent results for genotype-phenotype interaction between *MTHFR* polymorphism and NSCL/P.[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.[3,12] The risk for NSCL/P increases in the existence of environmental interaction between maternal exposure to smoking and alcohol and *MSX1* polymorphism.[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.[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 detail 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 methode from EDTA blood for all subjects. *MTHFR* C677T, A1298C and *MSX1* polymorphisms were analyzed using PCR- RFLP.[16] The product was digested using restriction enzyme (Table 1). Sanger sequencing was carried out for confirmation of sequence changes.

Polimorphism	Primer	Enzyme	Product size	RFLP Fragment
MTHFR C677T	5'-TGA AGG AGA AGG TGT CTG CGG GA-3' 5'-AGG ACG GTG CGG TGA GAG TG-3' - Denaturation : 1m, 94° - Annealing : 45s , 55°C - Extension : 30s, 72 °C	*Hinf1*	198 bp	CC (198 bp) CT (198 bp and 175 bp) TT (175 bp and 23 bp)
MTHFR A1298C	5'-CAA GGA GGA GCG GCT CTG GAA GA-3' 5'-CCA CTC CAG CAT CAC TCA CT-3' - Denaturation : 1m, 94° - Annealing : 45s , 55°C - Extension : 30s, 72 °C	*MbOII*	128 bp	AA (72 bp and 28 bp) AC (100 bp,72 bp, and 28 bp) CC (100 bp and 28 bp)
MSX1	5'-CGG CTG CTG ACA TGA CTT C- 3' 5'-GCC TGG GTT CTG GCT ACT AC - 3' - Denaturation : 30s, 95° - Annealing : 30s , 60°C - Extension : 30s, 72 °C	*MbOII*	483 bp	TT (387 bp), TC (96 bp and 387) bp CC(96 bp)

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

Ethics: This study was approved by Ethic Committee of Health Research from Faculty of Medicine, Diponegoro University – Dr. Kariadi Hospital, Semarang. Informed Consents were obtained from all of mother subjects.

Statistics: Samples were classified into mutant-type (MT) and wild-type (WT) in individuals inherit of mutant and common allele, respectively. Chi Square test was used to determine of polymorphism of having NSCL/P between affecteds and controls. The risk factors were analyzed statistically using Odd Ratio, 95% Confidence Interval.

Results

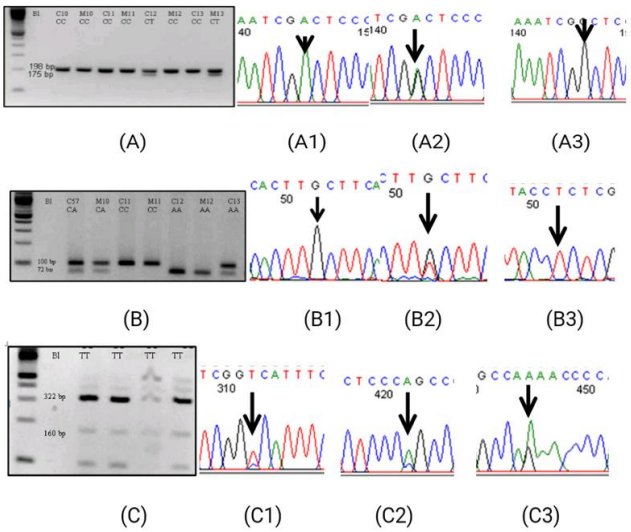

Figure 1. PCR-RFLP pattern of MTHFR C677T, MTHFR A1298C, MSX1 and its sequencing.
A. PCR-RFLP of MTHFR C677T and its sequencing using reverse sequence (complimentary nucleotide) demonstrated a variation of MTHFR C677T : (A1). Genotype TT (homozygous) in subject M01, (A2) Genotype CT (heterozygous) in subject C01 and (A3) Genotype CC (WT) in subject M02.
B. PCR-RFLP of MTHFR A1298C and its sequencing using reverse sequence (complementary 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 foward sequence (complementary nucleotide) demonstrated a variation of MSX1 C348T : (C1) p.Gly116= (rs34165410), (C2)C458CA; p.Pro153Gln (rs.104893854), (C3) c.469 + 12G>A (New finding).
Abbreviation: Bl = blank; C=children and M=mother

The genotyping of MTHFR C677T, A1298C are presented in Figure 1: A and B. Sanger sequencing verified the existence of C>T changes in MTHFR C677T and A>T changes in MTHFR A1298C (Figure 1). PCR-RFLP MSX1 found T330C (T>C) polymorphism in the 4 affected subjects. However, nonspecific band

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).

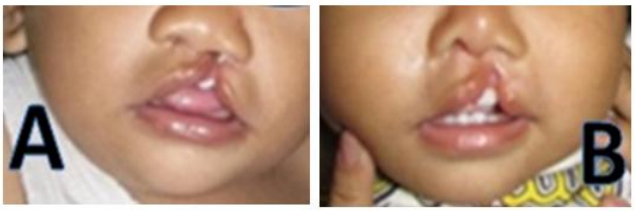

Figure 2. Showed that difference phenotype from polymorphism MTHFR C677T between A (child genotype CT heterozygote mutant and mother genotype TT homozygote mutant) and B (child genotype CT heterozygote mutant and mother genotype CC wildtype).

This difference in phenotype could be responsible that the mothers who had genotype polymorphism homozygote would inherit children with more severe phenotype than heterozygote. The explanation may also come from maternal dietary intake of folate during pregnancy that could influence the embryogenesis and the phenotype modulation of the fetus thorough epigenetic mechanism.

Suspected gene	Sampels		*p*	OR (CI 95%)
	NSCLP N(%)	Control N(%)		
MTHFR C677T				
Mother	n=35	n=39		
- MT	10 (28.6)	10 (25,6)	0.81	0.9 (0.5-2.5)
- WT	25 (71.4)	29 (74.4)		
Children	n=35	n=39		
- MT	11 (31.4)	10 (25,6)	0.57	1.3 (0.5-3.6)
- WT	24 (68.6)	29 (74.4)		
MTHFRA1298C				
Mother	n=35	n=39		
- MT	19 (54.3%)	16 (41%)		
- WT	16 (45.7%)	23 (59%)	0.04*	2.7 (1.0-7.0)
Children	n=35	n=39		
- MT	22 (62,9%)	12 (31%)	0.03*	2.7 (1.1-7.0)
- WT	13 (37.1%)	27 (69%)		
MSX1				
Mother	n=35	n=39		
- MT	6 (17.1%)	1 (2.6%)	0,617	0.4(0.04-3.6)
- WT	29 (82.9%)	38(97.4%)		
Children	n=35	n=39		
- MT	4 (11.4%)	4 (7.7%)	0,101	2.5(0.6-10.8)
- WT	31 (88.6%)	35(92.3%)		

Table 2. Polymorphisms of MTHFR and MSX1 in presence of mutant allele and NSCL/P.
* Statistically significant
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.[17] 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 heterogenocity of polymorphism among ethnic group.[18] 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.[19] The already operated subject will be followed up by foundation for second operation in the future to have optimal result.[20] It is well accepted by expertise in celft lip/palate that operations allone will not give optimal result if not supported by comprehensive esthetic treatment.[21]

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 new 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.Riwanto 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 whatsover.

References

1. The Rotary Club of Mataram. Rotary and European Surgeons Transform Hundreds of Life August 27th [cited; Available from: http://www.thelombokguide.com/issue_109_full.html#rotary__european_surgeons.
2. Dixon MJ, ML Marazita, TH Beaty,JC Murray. Cleft lip and palate: synthesizing genetic and environmental influences. Nat Rev Genet. 2011;12:167–78.
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.
4. Elavenil P, C Murugavel, K Kannadasan, VBK Raja, A Gnanam, G Kanimozhi,D Davis. Folic acid in cleft lip, alveolus and palate prevention: Awareness among dental professionals. Indian J Dent Res. 2010;21:360-3.
5. Goetzinger KR, AL Shanks, AO Odibo, GA Macones, AG Cahill, Advanced Maternal Age and the Risk of Major Congenital Anomalies, Am J Perinatol. 2017;34(3):217-22
6. Lesmana S, Auerkari Elza I., Genes Contributing in Cleft Lip and Cleft Palate: A Literature Review, Journal of International Dental and Medical Research. 2016; 9 , (Special Issue U.I. 1st International Workshop on Dental Research) : 441-8
7. Li SS, J Li, Z Xiao, AG Ren,L Jin. Prospective study of MTHFR genetic polymorphisms as a possible etiology of male infertility. Genet Mol Res. 2014;13:6367–74
8. Luo YL, YL Cheng, P Ye, W Wang, XH Gao,Q Chen. Association between MTHFR polymorphisms and orofacial clefts risk: a meta-analysis. Birth Defects Res A Clin Mol Teratol. 2012;94
9. Pan Y, W Zhang, J Ma, Y Du, D Li, Q Cai et al. Infants' MTHFR polymorphisms and nonsyndromic orofacial clefts susceptibility: a meta-analysis based on 17 case-control studies. Am J Med Genet A. 2012;158(1):2162-9.
10. Aisaiti A, D Aji, Z Jie,G Awuti. PAX9 and MSX1 gene mutations are responsible for non-syndromic oligodontia in Uyghur population. Int J Clin Exp Med. 2016;9:5525-31.
11. Veros C,Z Iakovidou-Kritsi. The genetic basis of cleft lip and cleft palate. Aristotle University Medical Journal. 2016;43:25-36.
12. Ulucan K, A Akcay, B Aksoy, M Boyraz, D Kirac, D Ergec et al. Coding Regions of MSX1 do not Contribute to Non-Syndromic Cleft Lip With/Without Palate in Turkish Patients. IJCP. 2014;3:12-15.
13. Deepak C, A Venkastesan,A Ramanathan. In search of mutations of MSX1 gene in Indian nonsyndromic cleft palate patients. J Ind Orthod Soc. 2012;46:43-7
14. Brandalize APC, E Bandinelli, JB Borba, TM Félix, I Roisenberg, L Schüler-Faccini. Polymorphisms in genes MTHFR, MTR and MTRR are not risk factors for cleft lip/palate in South Brazil. Braz J Med Biol Res. 2007;40:787-91
15. Fall CHD, HS Sachdev, C Osmond, MC Restrepo-Mendez, C Victora, R Martorell et al. Association between maternal age at childbirth and child and adult outcomes in the offspring: a prospective study in five low-income and middle-income countries (COHORTS collaboration. Lancet Glob Health. 2015;3:366-77
16. Yi P, P Igor. Pogribny, S. Jill James. Multiplex PCR for simultaneous detection of 677 C→T and 1298 A→C polymorphisms in methylenetetrahydrofolate reductase gene for population studies of cancer risk, Cancer letter, 2002;181(2):209–13.
17. N Saskia, Melani A, Yazid H, Variasi Genetik Regio Ekson 1 C330T Gen MSX1 Pada Kejadian Celah Bibir dan Langit-langit Non Sindromik (CB/L NS) Subras Deutromelayu. Indonesian Journal of Oral and Maxillofacial Surgeons. 2011;1:34-44.
18. Hussein MA and A. Abdughani,Genetic of Non-syndromic Cleft Lip and Palate, Open access Scientific Report. 2012;1(1).
19. Yastani D, Purbiati M, Astuti IA, Krisnawati, Dentocraniofacial Morphology in Unilateral and Bilateral Cleft Lip and Palate Following Labioplasty and Palatoplasty; Analysis at First and Second Cervical Vertebral Maturation Stage, Journal of International Dental and Medical Research. 2016; 9, (Special Issue U.I. 1st International Workshop on Dental Research): 282-6.
20. Ariawan D, DA Maharani, Rahardjo A, Tofani I, A Review of Speech Function and Maxillary Growth in Cleft Palate Patients, Journal of International Dental and Medical Research. 2016; 9 (Special Issue, U.I. 1st International Workshop on Dental Research):420-6.
21. Amila Z, Nakas E, Prohic S, Interdisciplinary approach in a treatment of the patient with cleft lip and palate – Case Report, Journal of International Dental and Medical Research. 2010; 3(1); 29-33.