



Sequence Variants in Exon 1 of *MSX1* Gene Associated With Nonsyndromic Cleft Lip/Palate (NS CL/P) Among Indonesian Patients

Ani Melani Maskoen^{1*}, Saskia Lenggogeni Nasroen², Harmaz Yazid³,
Prima Nanda Fauziah⁴, Eky Setiawan Soeria Soemantri⁵

¹Oral Biology Department, Faculty of Dentistry, Universitas Padjadjaran, Bandung, Indonesia

²Dentistry Study Program Department, Faculty of Medicine, Jenderal Achmad Yani University Cimahi, Indonesia

³Oral and Maxillofacial Surgery Department, Faculty of Dentistry, Universitas Padjadjaran, Bandung, Indonesia

⁴Department of Medical Laboratory Technology, School of Health Sciences Jendral Achmad Yani Cimahi, Indonesia

⁵Orthodontic Department, Faculty of Dentistry, Universitas Padjadjaran, Bandung, Indonesia

Abstract : *Background*: Orofacial cleft, especially nonsyndromic cleft lip/palate (NS CL/P), is a common congenital abnormalities that affect between 1 in 700 - 1000 live births worldwide. The etiology of NS CL/P is multifactorial that involves the interaction of both genetic and environmental factors. Drosophila muscle segment homeobox homolog-1 (*MSX1*) gene has been proposed as one of strong candidate genes associated with NS CL/P. The patterns of *MSX1* gene mutation may vary according to race and geographical region. The purpose of this study was to detect and analyze the mutation in exon 1 of *MSX1* gene in the form of sequence variants as the risk factor that being an etiological role in the development of NS CL/P in Indonesian patients.

Methods: This study was case control design using samples from 48 NS CL/P subjects and 43 control subjects. PCR and Sanger Sequencing Technique were used to resolved the aim of the study. Statistical analysis which was used to determine significantly of differences from sequence variants frequency among NS CL/P subject and control subject was χ^2 . The odds ratio was used to determine a risk factor of NS CL/P.

Results: The study results showed that 2 sequence variants in exon 1 of *MSX1* gene were identified as C101G/rs36059701 and C330T/rs34165410. In C101G, the frequency of G mutant allele was 50% in both NS CL/P subject and control subject. This difference was not significant ($\chi^2=0,019$; $p > 0,05$). In C330T, the frequency of T mutant allele frequency was 48.1% in NS CL/P subject and 51.9% in control subject and this difference was not significant ($\chi^2=0,326$; $p > 0,05$). The odds ratio from all mutant alleles did not show significant result statistically means that the risk factor can not be determined.

Results: The study results showed that 2 sequence variants in exon 1 of *MSX1* gene were identified as C101G/rs36059701 and C330T/rs34165410. In C101G, the frequency of G mutant allele was 50% in both NS CL/P subject and control subject. This difference was not significant ($\chi^2=0,019$; $p > 0,05$). In C330T, the frequency of T mutant allele frequency was 48.1% in NS CL/P subject and 51.9% in control subject and this difference was not significant ($\chi^2=0,326$; $p > 0,05$). The odds ratio from all mutant alleles did not show significant result statistically means that the risk factor can not be determined.

Keywords : nonsyndromic cleft lip/palate, exon 1 of *MSX1* gene, Indonesian.

Introduction

Cleft lip with or without cleft palate (CL/P) is the most common congenital malformation in craniofacial region (Koillinen, 2003). The worldwide overall incidence of clefts is estimated to be 1 in 700 - 1000 with wide variability among races and regions so its prevalence varies depending on racial and ethnic backgrounds, geographic origin, and socioeconomic status (Lee et al, 2008). Low incidences are seen among black people while the highest incidences are seen among American Indians, Japanese and Chinese. Birth prevalence of CL/P is generally higher in Asian populations compared to European populations [1]. The exact prevalence of CL/P in Indonesia is still unknown.

Most CL/P are of the nonsyndromic type that does not accompany syndromes, and this nonsyndromic type is known to affect as much as 70% of the whole CL/P phenotypes [2]. Approximately 30% of CL/P cases are syndromic and have additional characteristic features that can be subdivided into categories of chromosomal abnormalities, > 350 recognizable Mendelian single gene disorders, teratogenic effects and various unclassified syndromes [3]. Nonsyndromic oral clefts can be defined as complex traits, since they do not exhibit classic Mendelian recessive or dominant inheritance attributable to any single locus, but show strong familial aggregation and have a substantial genetic component [2]. Nonsyndromic (NS) CL/P might be due to mutations in a number of different genes. Therefore, this study into CL/P focuses on understanding the etiology of the nonsyndromic form. The genetic characteristics of NS CL/P are complicated by an uncertain mode of inheritance, incomplete penetrance, and heterogeneity both within and among populations [4].

NS CL/P is a common congenital anomaly with significant medical, psychological, social, and economic ramifications [5]. Gene-gene and gene-environmental interactions have been believed to cause CL/P [2] and genetic may play an important role as the etiology of CL/P. Palatogenesis is a complex process that involves many genes [1]. Several loci and genes have been suggested as candidates. The current list of candidate genes for NS CL/P includes transforming growth factor beta 3 gene (*TGFβ3*), orofacial cleft 1 gene (*OFC1*), transforming growth factor alpha gene (*TGFα*), *Drosophila* muscle segment homeobox 1 gene (*MSX1*), retinoic acid receptor alpha gene (*RARα*), orofacial cleft 2 gene (*OFC2*), orofacial cleft gene 3 (*OFC3*) and methylenetetrahydrofolate reductase (*MTHFR*). In recent years, *MSX1* has been emerging as an especially strong candidate for NS CL/P based on the complete secondary cleft palate, complete failure of incisor development, and the foreshortened maxilla phenotype shown in the mouse knockout model [2]. Numerous reports indicate that mutations or polymorphisms of *MSX1* might also constitute risk factors for isolated forms of orofacial clefts, supporting its critical role in craniofacial development [6].

Although the exact action of *MSX1* remains uncertain, it appears to promote growth and inhibit differentiation. Some previous studies showed a significant association between C 101G (nucleotide "C" of the 101 basepairs (bp) region was substituted for "G") and C330T (nucleotide "C" of the 330 basepairs (bp) region was substituted for "T") in exon 1 *MSX1* gene and NS CL/P especially in Asian populations [1].

Sequence variants related to NS CL/P in *MSX1* gene may vary according to race and geographical region, so it is possible for NS CL/P among Deuteromalayan subrace in Indonesia to have distinct characteristics of the *MSX1* gene through sequence variants in the region of exon 1 of *MSX1* gene as Indonesia has so many different ethnic but most of them is Deuteromalayan subrace which include the ethnic of Javanese, Sundanese, Malayan, Bugis, Minang and so on, except the ethnic of Batak, Toraja, Papua and Gayo.

Experimental

Materials

The samples were collected from 48 NS CL/P patients from Deuteromalayan subrace which include all those patients who come Bandung, Cilegon, Malang and Makassar and 43 normal subjects from Deuteromalayan race without family history of craniofacial clefts.

This study based on molecular epidemiology with case control study which is done in Molecular Biology Laboratory, Health Research Unit Faculty of Medicine/ Hasan Sadikin Hospital in Bandung and Geneka Laboratory in Molecular Biology Institute of Eijkman, Jakarta.

Methods

DNA isolation. DNA was isolated from venous blood of each subjects using DNA isolation kit from Phamacia, then 200 ng of DNA template was using for Polymerase Chain Reaction (PCR) step. PCR was performed by using the primers of *F*5'-GGCTGCTGACATGACTTCTT-3' and *R*:5'-AGGTCTGGAACCTTCTTCCTG-3' (Lace B, 2006) which cover the exon 1 segment. The PCR product of exon 1 *MSX1* gene described as a segment was obtained for 91 subjects (48 NS CL/P subjects and 43 normal subjects). The size of this PCR product was 646 bp (Figure 1).

DNA Sequencing. DNA sequencing was performed by using dideoxy Sanger method in Geneka Laboratory in Molecular Biology Institute of Eijkman, Jakarta. The Sequencing result aligning with reference sequence from gene bank used Bioedit software.

Statistical method. Statistical analysis which was used to determine significantly of differences from sequence variants frequency among NS CL/P subject and control subject was χ^2 . The odds ratio (OR) was used to determine a risk factor of NS CL/P. A *p* value of less than 0.05 was considered to be statistically significant. All of the analyses were performed using SPSS programme, version 11.

Results and Discussion

The ethnic characteristic from all total samples of 91 were: 54,9% of Sundanese, 27,5% of Javanese, 4,4 % of Bugis, 7% of Minang, and 5,5% of Malayan.

1 2 3

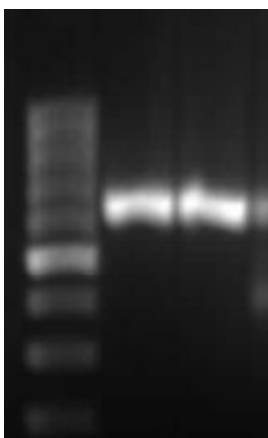


Figure 1. Initial PCR product of exon 1 *MSX1* gene: (1) 100 bp ladder, (2) & (3) PCR product (646 bp)

After obtaining the initial PCR products of exon 1 *MSX1* gene (646 bp), samples were then analyzed by dideoxy Sanger method of sequencing. The sequencing result from all subjects shows that there were sequence variants found in the form of substituting C nucleotide into G nucleotide (C101G) and substituting C nucleotide into T nucleotide (C330T). According to the data from the gene bank in ensembl genome browser, sequence variant numbering with rs36059701 for C101G and rs34165410 for C330T.

Based on sequencing result, in C101G it was found that there were the feature of normal CC genotype, heterozygous mutant CG genotype but no homozygous mutant of GG genotype found (Figure 2) and in C330T it was found that there were the feature of normal CC genotype, heterozygous mutant CT genotype and homozygous mutant of TT genotype (Figure 3).

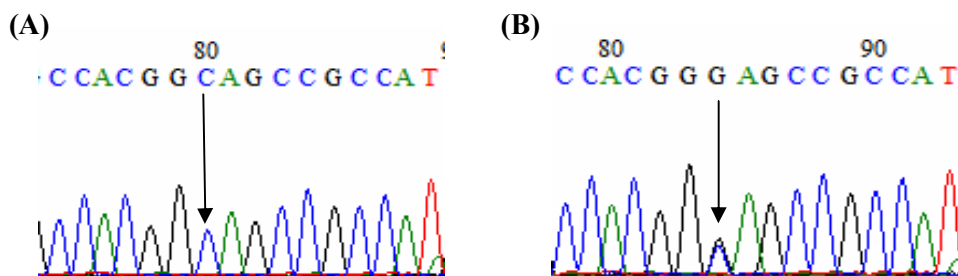


Figure 2. The feature of C101G exon 1 of *MSX1* gene : (A) normal CC genotype (arrow), (B) heterozygous mutant CG

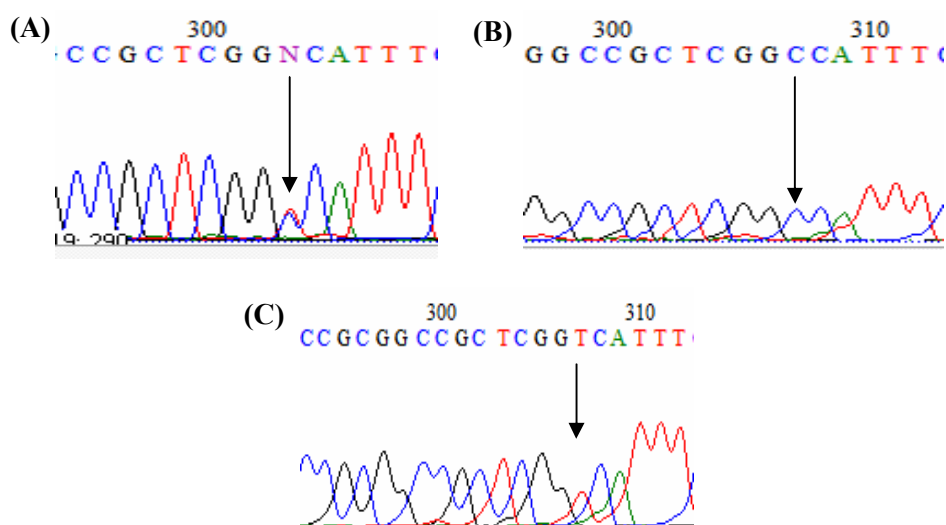


Figure 3. The feature of C330T exon 1 of *MSX1* gene: (A) normal CC genotype (arrow), (B) heterozygous mutant CT genotype at the same position with A, (C) homozygous mutant TT genotype at the same position with A and B.

Statistical analysis which was used to determine significantly of differences from sequence variants frequency among NS CL/P subject and control subject, was χ^2 . The odds ratio (OR) was used to determine a risk factor of NS CL/P. A P value of less than 0.05 was considered to be statistically significant. All of the analyses were performed using SPSS programme, version 11. χ^2 analysis through all the subjects was done to compare allelic frequency of G mutant allele and C normal allele and also to compare genotype frequency of homozygous normal CC genotype and heterozygous mutant CG genotype in C101G; and also to compare allelic frequency of T mutant allele and C normal allele and also genotype frequency of homozygous normal CC genotype, heterozygous mutant CT genotype and homozygous mutant TT genotype in C330T, and all comparison is done in between NS CL/P subjects and normal subjects. All of statistical analysis of allelic frequency from both of C101G and C330T from 48 NS. CL/P subjects and 43 normal subjects shown in Table 1 and 2.

Table 1. Allelic frequency of nucleotide C and G from C101G/ rs36059701 in NS CL/P subjects compared with normal subjects

Allele	Subject		χ^2	p	Odds Ratio	CI 95%
	CB/L NS (%)	Normal (%)				
C	52,7	47,3	Undetected			
G	50	50	0,019	0,889	0,889	0,170 - 4,656

C : cytosine (normal allele); G : guanine (mutant allele)

Table 2. Allelic frequency of nucleotide C and T from C330T/rs34165410 in NS CL/P subjects compared with normal subjects

Allele	Subject		χ^2	p	Odds Ratio	CI 95%
	CB/L NS (%)	Normal (%)				
C	52,9	47,1	0,013	0,910	1,122	0,151 - 8,329
T	48,1	51,9	0,326	0,568	0,769	0,312 - 1,895

C : cytosine (normal allele); T; thymine (mutant allele)

Table 3. Genotype frequency of nucleotide C and G from C101G/ rs36059701 in NS CL/P subjects compared with normal subjects

Genotype	Subject		χ^2	p	Odds Ratio	CI 95%
	CB/L NS (%)	Normal (%)				
CC	52,9	47,1	0,019	0,889	1,125	0,215 - 5,893
CG	50	50	0,019	0,889	0,889	0,170 - 4,656
GG	0	0	undetected			

CC: homozygous normal genotype; CG: heterozygous mutant genotype; GG: homozygous mutant genotype

Table 4. Genotype frequency of nucleotide C and T from C330T/rs34165410 in NS CL/P subjects compared with normal subjects

Genotype	Subject		χ^2	p	Odds Ratio	CI 95%
	CB/L NS (%)	Normal (%)				
CC	54,7	45,3	0,326	0,568	1,300	0,528 - 3,210
CT	47,8	52,2	0,299	0,584	0,768	0,298 - 1,980
TT	50	50	0,013	0,910	0,891	0,120 - 6,617

CC: homozygous normal genotype; CT: heterozygous mutant genotype; TT: homozygous mutant genotype

All of statistical analysis of genotype frequency from both of C101G and C330T from 48 NS CL/P subjects and 43 normal subjects shown in Table 3 and 4.

Discussion

Identification of the genes involved in the development of the human craniofacial region can serve as a first step towards developing a better understanding of diagnosis, treatment and preventions of developmental anomalies of this region [7].

The etiology and pathogenesis of cleft formation have been extensively studied but it is still poorly understood. On the basis of mouse studies, cleft palate seems to be either a growth or a fusion failure of the secondary palate. In humans, some families with NSCL/P show an autosomal dominant model of inheritance but, in most cases, the model of inheritance is not clearly mendelian. Newborns are at greatest risk when both

parents are affected. Risk for a newborn developing cleft palate when one parent has cleft versus neither parent with cleft varies depending on the number of normal siblings. When one parent and one child are affected, the chance of a second child having a cleft palate is 13%. When both parents are normal (without cleft), but two of the children have clefts, the chance of a third child being affected is 19%. When one parent has a cleft palate and two offspring are normal, the chance of the third child being born with a cleft palate is the lowest (3.5%) [8]. These facts clearly show that CL/P has a strong genetic component. Numerous previous studies have suggested that many extrinsic factors might influence cleft formation. Thus, NS CL/P is considered to be genetically complex, multifactorial diseases [9]. In this present study, we collected all samples without linked it into environmental factor, so it was purely only genetics point of view.

The *MSX1* gene maps to chromosome 4p16, consist of two exon and one intron, has recently been proposed as another strong candidate gene with allelic variants potentially contributing to nonsyndromic forms of CL/P [3]. Animal models indicate that this gene plays a role in craniofacial anomalies, leading to a clefting phenotype. Van den Boogaard et al. identified a stop codon in the *MSX1* gene in a three-generation Dutch family with tooth agenesis and combinations of NS CL/P and NS CLP, providing further evidence for the involvement of this gene in orofacial clefting [10]. Mice lacking functional *MSX1* gene develop a cleft of the secondary palate and tooth agenesis [11]. The *MSX1* gene is expressed at diverse sites of epithelial-mesenchymal interaction during vertebrate embryogenesis, and has been implicated in signalling processes between tissue layers. *MSX1* gene play an important role in inductive epithelial-mesenchymal interactions leading to vertebrate organogenesis and especially palatal fusion and it has been proposed that the development of CL/P is because of insufficient palatal mesenchyme [12]. Although the exact action of *MSX1* remains uncertain, it appears to promote growth and inhibit differentiation [1].

The majority of such mutations have been found in the exon 1 of *MSX1* gene, suggesting the presence of hidden regulatory elements in this exon thus causing conservation of the sequence. Several sequence variants frequencies in exon 1 of *MSX1* gene showed differences between populations [13]. Therefore, exon 1 (together with neighbouring noncoding sequences) was initially scanned for mutations in previous study through NS CL/P among Lithuanian and some previous study in Caucasian also revealed that all eight different sequence variants identified in the coding and noncoding parts of the *MSX1* gene have already been described as functionally neutral polymorphisms, including one mutation expected to result in amino acids conversion, i. e. C101G substituting alanine for glycine (p.A34G) [3].

Based on previous study done by Morkuniene et al (2006), there were sequence variant identified in exon 1 of *MSX1* gene in NS CL/P patients in the form substituting nucleotide "C" into "G" of the 101 base pair (bp) region (C101G) resulted in substituting amino acid of alanine for glycine [3]. There was a specific mutation identified in exon 1 of *MSX1* gene in NS CL/P in the form substituting nucleotide "A" into "G" of the 253 base pair (bp) region (A253G) and insertion of nucleotide "G" of the 255 bp, this substitution affected protein sequences and made one missense mutation (Thr85Ala) [2]. This sequence variant was identified in 27 of the total 36 subjects who had NS CL/P in Korean. This finding is contrary to our study as there was no same sequence variant found among Indonesian with NS CL/P.

Study from Park et al through Korean population revealed that a total of 14 sequence variants were identified near or in *MSX1* related to NS CL/P, 6 of which were sufficiently polymorphic to be analyzed and sequencing concentrated on regions of *MSX1* that are thought to be functionally important. Most of these sequence variants found in exon 1 and in exon 2 only one sequence variant identified mainly 1170G/A (3'UTR) [1]. Some previous studies showed a significant association between C330T and CL/P especially in Asian populations (Park et al, 2006). Based on the previous study, sequence variants frequency of C330T was 4.9%, versus previous publication for Asians had 19% and Caucasian 16% [13]. C330T is located in coding region (exon 1), without amino acid substitution (gly19gly) so when there is sequence variant the code will substitute from GGC into GGT [14]. Even though there is no change in amino acid sequence through sequence variant of C330T in this gene, but this sequence variant suppose to have a role to change transcription efficiency leads to some changes in quality, structure or protein function of this gene.

In this present study we focusing to find sequence variants in exon 1 of *MSX1* gene associated with NS CL/P among Deuteromalayan subrace in Indonesia. The study results showed that 2 sequence variants in exon 1 of *MSX1* gene were identified as C101G/rs36059701 and C330T/rs34165410. In C101G, the frequency of G mutant allele was 50% in both NS CL/P subject and control subject. This difference was not significant

($\chi^2=0,019$; $p > 0,05$). In C330T, the frequency of T mutant allele frequency was 48.1% in NS CL/P subject and 51.9% in control subject and this difference was not significant ($\chi^2=0,326$; $p > 0,05$). The odds ratio from all mutant alleles did not show significant result statistically means that the risk factor can not be determined. This findings suggest that these sequence variants do not have a role in the etiology of NS CL/P among Deuteromalayan subrace in Indonesia and could not be considered to be a risk factor that being an etiological role in CL/P development in Indonesian patients with NS CL/P.

This is contrary to the previous studies done in Korean population and Asian in general and these differences could be attributed to genetic differences in population and this may suggest the possibility of involvement of other segment from this gene or other genes as NC CL/P is polygenetic nature like *TGF β 3*, *RAR α* , *BCLX3*, *TGF α* , *MTHFR*, *PVLRI* are involved in the etiology of NS CL/P.

Acknowledgement

This research was funded by the Indonesian Ministry of National Education, through the program of *Hibah Penelitian Strategi Nasional (Stranas)*

References

1. Park J, Park BY, Kim HS, Lee JE, Suh I, Nam CM et al., *MSX1* polymorphism associated with risk of oral cleft in Korea: evidence from case-parent trio and case control studies, *Korean J Med*, 2006, 38(2), 120-125.
2. Lee HK, Kim SS, Son WS, Characteristics of *MSX1* gene in Korean nonsyndromic cleft lip and palate individuals, *Korean J Orthod*, 2008, 38(2), 133-43
3. Morkuniene A, Steponaviciute D, Kasnauskiene J, Kucinkas V., Nucleotide sequence changes in the *MSX1* and *IRF6* genes in Lithuanian patient with nonsyndromic orofacial clefting. *ACTA MEDICA LITUANICA*, 2006, 13(4), 219-225
4. Jagomagi T, Nikopensius T, Krjutskov K, Tammekivi V, Viltrop T, Saag M et al., *MTHFR* and *MSX1* contribute to the risk of nonsyndromic cleft lip/palate. *Eur J oral Sci*, 2010, 118, 213-220
5. Lidral CA, Romitti PA, Basart AM, Doetschman T, Leysens NJ, Daark-Hirsch S, et al, Association of *MSX1* and *TGF β 3* with nonsyndromic clefting in humans. *Am J Hum Genet*, 1998, 63(2), 557-568
6. Mostowska A, Biedziak B, Trzeciak WH, A novel C.581C>T transition localized in a highly conserved homeobox sequence of *MSX1*: it is responsible for oligodontia?. *J Appl Genet* 47(2), 2006, 159-164
7. Singh VP and Ramu D, Association of *MSX1* 799 G>T variant with nonsyndromic cleft lip/palate in South Indian adolescent patients. *Int J Paediatric Dentistry*, 2012, 22, 228-231
8. Abubaker AO, Benson KJ, Oral and maxillofacial surgery secrets. Philadelphia : Hanley & Belfus, Inc, 2001, 252
9. Koillinen H., Molecular genetics of non-syndromic cleft palate and van der woude syndrome, Helsinki University Biomedical Dissertation, 2003, No. 41.
10. da Silva AL, Ribeiro LA, Cooper ME, Marazita ML, Morretti-Ferreira D, Transmission analysis of candidate genes for nonsyndromic oral clefts in Brazilian parent-child triad with recurrence. In : *Genetics and Molecular Biology*, 2006, 29, 3, 439-442.
11. Wong FK, Hagg U., An update on the aetiology of orofacial clefts, *Hong Kong Med J*, 2005, 10(5)
12. Vodolatskiĭ, M.P., Evsev'eva, M.E. and Rekvava, Z.A., Phenotypical analysis of children with congenital cleft of upper lip and palate, *Stomatologija*, 2007, 87(1), 74-76.
13. Lace B, Vasiljeva I, Dundure I, Barkane B, Akota I, Krumina A. Mutation analysis of the *MSX1* gene exon and intron in patients with nonsyndromic cleft lip and palate. *Stomatologija, Baltic Dental and Maxillofacial Journal*, 2006, 8, 21-4.
14. Jezewski PA, Vieira AR, Nishimura C, Ludwig B, Johnson M, O'Brien SE et al. Complete sequencing shows a role for *MSX1* in non-syndromic cleft lip and palate. *J Med Genet*, 2003, 40, 399-407
