치의학박사 학위논문

Association between *MSX1* SNPs and nonsyndromic cleft lip with or without cleft palate in the Korean population

한국인에서 *MSX1* 유전자의 단일염기다형성과 구순구개열의 연관성

2013 년 8 월

서울대학교 대학원
치의과학과 치과교정학 전공

김 나 영

Association between *MSX1* SNPs and nonsyndromic cleft lip with or without cleft palate in the Korean population

지도교수 백 승 학

이 논문을 치의학박사 학위논문으로 제출함

2013 년 5 월

서울대학교 대학원

치의과학과 치과교정학 전공

김 나 영

김나영의 치의학박사 학위논문을 인준함

2013 년 6 월

위 원 장 _____ (인)

부 위 원 장 _____ (인)

위 원 _____ (인)

위 원 _____ (인)

위 원 _____ (인)

ABSTRACT

Association between *MSX1* SNPs and nonsyndromic cleft lip with or without cleft palate in the Korean population

Na Young Kim, DDS, MSD

Department of Orthodontics, Graduate School,
Seoul National University
(Directed by Professor Seung-Hak Baek, DDS, MSD, PhD)

The purpose of this study was to investigate the contribution of *MSX1* gene to the risk of nonsyndromic cleft lip with or without cleft palate (NS-CL/P) in the Korean population. The samples consisted of 142 NS-CL/P families (9 with cleft lip, 26 with cleft lip and alveolus, and 107 with cleft lip and palate; 76 trios and 66 dyads). Three single nucleotide polymorphisms (SNPs: rs3821949 (G>A), rs12532 (A>G), and rs4464513 (T>G)) were tested for association with NS-CL/P case-parent trios using transmission disequilibrium test (TDT) and conditional logistic regression models (CLRMs). Minor allele frequency, heterozygosity, χ^2 test for Hardy-Weinberg equilibrium, and pairwise linkage disequilibrium (LD) at each SNP were computed. The family- and haplotype-based association test programs were used to perform allelic and genotypic TDTs for individual

SNPs and to fabricate sliding windows of haplotypes. Genotypic odds ratios (GORs) were obtained from CLRMs using R software. Although the family-based TDT indicated a meaningful association for rs3821949 (G>A) (*P*=0.028), the haplotype analysis did not reveal any significant association with rs3821949 (G>A), rs12532 (A>G), or rs4464513 (T>G). The A allele at rs3821949 (G>A) had a significant increased risk of NS-CL/P (GOR, 1.64; 95% confidence interval, 1.03-2.63; *P*=0.0384, additive model). A positive association is suggested between *MSX1* rs3821949 (G>A) and NS-CL/P in the Korean population. The results of this study may increase the genetic understanding of the etiological role of the *MSX1* gene in Korean NS-CL/P.

Key Words: *MSX1* SNP; Nonsyndromic Cleft Lip with or without Palate; Korean; Association Analysis

Association between *MSX1* SNPs and nonsyndromic cleft lip with or without cleft palate in the Korean population

Na Young Kim, DDS, MSD

Department of Orthodontics, Graduate School, Seoul National University
(Directed by Professor Seung-Hak Baek, DDS, MSD, PhD)

한국인에서 *MSX1* 유전자의 단일염기다형성과 구순구개열의 연관성

서울대학교 대학원 치의과학과 치과교정학 전공
(지도교수: 백 승 학)

김 나 영

-CONTENTS-

I. INTRODUCTION

Nonsyndromic cleft lip and/or palate (NS-CL/P) is a common congenital craniofacial deformity in humans and is known to be caused by a combination of genes and environmental interactions. The frequency of NS-CL/P is higher in Asian populations (1/500 or higher) than in Caucasian (1/1,000) or African populations (1/2,500).[1-4]

The muscle segment homeobox1 (*MSX1*) gene at 4p16.1 encodes a DNA-binding sequence and is expressed in spatially-restricted regions of the head during early development. Mutations in this gene have been known to be associated with NS-CL/P, Witkop syndrome, Wolf-Hirschhorn syndrome, and autosomal dominant hypodontia.[5-7] In animals, homozygous *Msx1*-deficient transgenic mice exhibit cleft palate, deficiency of the alveolar bone in the mandible and maxilla, incisor development failure, and arrest of molar development.[8,9] Gong[10] showed that there was misregulation in the expression of the *Msx1* gene in embryos of A/WySn mice with cleft palate.

Complete sequencing of *MSX1* in humans has revealed several novel mutations, and it is estimated that approximately 2% of NS-CL/P patients carry mutations in this gene.[6,7,11] There have been several association and linkage studies between *MSX1* gene variants and NS-CL/P in humans.[12-18] However, in terms of the Korean population, only one article has been published for association and linkage study between *MSX1* single nucleotide polymorphisms (SNP) and NS-CL/P.[19]

Park et al.[19] reported significant evidence of linkage in the presence of disequilibrium for 1170G/A of exon 2 and the disease risk decreased with the presence of the A allele (AA genotype: odds ratio, 0.26; 95% confidence interval [CI], 0.10-0.99). However, their study has several limitations as follows: They analyzed only novel SNPs with low occurrence frequency. The samples included cleft lip (CL), cleft lip and palate (CLP), and cleft palate only (CPO). However, CPO has been regarded as a separated identity etiologically and embryologically from clefts involving the lip with or without the palate.[20] In addition, when trio-case with CPO (n=8) was excluded, the numbers of trio-case with CL/P was relatively small (n=44; 14 with CL, and 30 with CLP).

Therefore, it is needed to determine the association and linkage relationship about tag SNPs in the Korean population and to confine the samples with NS-CL/P in order to get results for epidemiologic studies of cleft patients. The purpose of this study was to investigate the contribution of the *MSX1* gene to the risk of NS-CL/P in the Korean population, whose samples are independent form those of Park et al.[19] We tested three tag single nucleotide polymorphism (SNP) markers in and around the *MSX1* gene in Korean NS-CL/P case-parent families using the transmission disequilibrium test (TDT) and conditional logistic regression models (CLRM).

II. REVIEW OF LITERATURE

FUNCTION OF THE *MSX1* GENE AND RELATED DISEASES

The *MSX1* gene, a member of homeobox genes, is critical of the limb-pattern formation, craniofacial development, and odontogenesis during early development stage. [21-23]

Nonsense mutation (Ser104stop) in exon 1 of *MSX1* was appeared in a Dutch family with hypodontia and oral cleft patients.[5] Ser202stop in exon 2 of *MSX1* were related with Witkop syndrome, also known as "tooth and nail syndrome".[24]

In addition, deletion of *MSX1* were reported in some patients with Wolf-Hirschhorn syndrome, a multi-organ syndrome caused by deletions of the short arm of chromosome 4 (4p).[25]

ANIMAL STUDY OF THE *Msx1* GENE ABOUT OROFACIAL CLEFT

Since *Msx1* has a critical role in mediating epithelial-mesenchymal interactions during craniofacial bone and tooth development,[8] *Msx1 (-/-)* mice exhibited a secondary cleft palate, a deficiency of alveolar mandible and maxilla, a failure of tooth development during transition from bud to cap stage, and abnormalities of the nasal, frontal, and parietal bones, and of the malleus in the middle ear.[8]

In dHAND-null mice embryos, Thomas et al.[9] showed that expression of *Msx1* was not detectable in the mesenchyme of dHAND (basic helix-loop-helix transcription factor)-

null branchial arches but unaffected in the limb bud.

Gong et al.[10] showed that phenotypic and molecular changes in A/WySn mice produced craniofacial defects. They also exhibited that A/WySn mice embryos with cleft had misregulation of the expression of the *Msx1* gene and a persistence of expression in the distal growing tips of the midfacial processes.[10]

HUMAN STUDY OF THE *MSX1* GENE ABOUT OROFACIAL CLEFT

Direct sequencing

Jezewski et al.[6] performed a sequencing of the *MSX1* gene in 917 cases including European, Asian, and native South American ancestry. They found missense mutations in conserved amino acids and point mutations in conserved regions.[6]

Suzuki et al.[7] identified transmission distortion for alleles of the *MSX1* gene in the Vietnamese population. In their study, two missense mutations, including one (P147Q), were found in approximately 2% of the population.[7]

Tongkobpetch et al.[11] performed mutation analysis for the coding regions of the *MSX1* gene in 100 nonsyndromic CL/P cases of the Thai population. The 799G>T (G267C) and 832C>T (P278S) variants were predicted to be 'probably damaging'by PolyPhen (http://www.bork.embl-heidelberg.de/PolyPhen/), changed (either created or eliminated) themselves as potential ESEs [ESEfinder software (http://www.rulai.cshl.edu/tools/ESE/)] for serine-arginine proteins. However, these variants were not present in 162 control

individuals of the Thai ethnic background.[11] In contrast to previous reports of P147Q variant in the Vietnamese[7] and in the Filipino populations,[26] association between the P147Q variant and CL/P in Thai population was not detected.[11] Therefore, they concluded that it was not pathogenic.[11]

Association and linkage studies between *MSX1* gene variants and NS-CL/Pin humans

Lidral et al.[12] performed case-control analysis and nuclear-family based analysis to find candidate genes for oral cleft (*TGFA, BCL3, DLX2, MSX1,* and *TGFB3)* in a predominantly Caucasian population. Since significant LD was found between CL/P and both *MSX1* and *TGFB3* and between CPO and *MSX1,* these genes were suggested to be involved in the pathogenesis of oral cleft.[12]

In a study to identify both genetic and environmental risk factors in NS-CL, CLP, and CPO cases in Maryland (n=171) and unaffected controls (n=182), Beaty et al.[13] reported that *MSX1* showed significant differences in allele frequencies between CPO cases and controls and significant evidence of linkage disequilibrium with a susceptibility gene controlling risk for CPO.[13]

Fallin et al.[14] reported that the fourth allele of the intronic CA repeat yields the strongest magnitude of association with oral clefts in single-marker and haplotype-based transmission disequilibrium tests (TDTs) of 206 complete NS-CL, CLP, and CPO trios in Maryland and Washington, DC. According to their study, association between the *MSX1*

gene and oral cleft risk occurs most strongly among CPO families.[14]

In a population-based study of orofacial clefts in Norway with 262 case-parent trios, Jugessur et al.[15] showed a weak association of the *MSX1*-CA variant with CPO. The risk was increased as much as 9.7-fold among children homozygous for both the *MSX1*-CA A4 allele and the *TGFA* A2 allele [95% confidence interval (CI)=2.9-32].[15] The effect of *TGFA* genotype was even stronger among children homozygous for the *MSX1*-CA A4 allele, increasing the possibility of interaction between *TGFA* and *MSX1* genes.[15]

To detect transmission distortion of *MSX1* and *TGFB3* in South American children from their respective mothers, Vieira et al.[16] performed a transmission disequilibrium test (TDT) analysis. They showed significant evidence of association with *MSX1* (CL/P, *P* =0.004; CLP + CPO, *P*=0.037; and all data sets combined, *P*=0.001).[16] In likelihood ratio test analysis, they also revealed that "cleft lip only" showed meaningful associations with *MSX1* (*P*=0.04) and "cleft palate only" with *TGFB3* (*P*=0.02).[16]

Moreno et al.[17] tried to replicate previously suggestive linkage to 10 loci and evaluated additional candidate genes in 49 Colombian and 13 Ohio families. In their study, significant association results were obtained in the regions 1p36 (*P*=0.046), 6p23-25 (*P*=0.020), and 12q13 (*P*=0.046) of the Colombian families. [17] In addition, several families yielded LOD scores ranging from 1.09 to 1.73, for loci at 4p16, 6p23-25, 16q22-24, and 17q13.[17] These differences between the two populations suggest that there is specific locus heterogeneity in different populations.[17]

Schultz et al.[18] performed parametric linkage analysis (LOD score), nonparametric linkage analysis (SIMIBD), and transmission disequilibrium test (TDT) analysis in the 36 Filipino families (1,066 total individuals). As a result, five markers showed possible results from the 36 families.[18] The parametric LOD scores for the *MSX1*-CA and *D4S1629* were more than 1.0, the SIMIBD *P* values for *D6S1029* and *RFC1 were* marginal ($P<0.06$), while the SIMIBD *P* value for *TGFA* was significant ($P=0.01$).[18]

In linkage disequilibrium analysis of the Korean populations with 52 CL/P and CPO cases and 96 controls, Park et al.[19] reported significant evidence of linkage disequilibrium for 1170 G/A of exon 2. When the GG genotype was set as a reference group at 1170G/A, the risk of disease decreased with the presence of the A allele (AA genotype: OR=0.26, 95% CI=0.10-0.99).[19]

III. MATERIALS AND METHODS

Subject

The sample population consisted of 142 Korean NS-CL/P families (90 males and 52 females; 9 with cleft lip, 26 with cleft lip and alveolus, and 107 with cleft lip and palate; 76 trios and 66 dyads, Table 1). Five orthodontists performed clinical investigation to diagnose NS-CL/P; the individuals with a cleft have no other physical and/or developmental anomalies. For mutation analysis and case-control studies, peripheral venous blood samples of patients and their parents were collected at either Seoul National University Dental Hospital (SNUDH) or Samsung Medical Center (SMC).

Extraction of genomic DNA and genotyping

Genomic DNA samples were extracted from peripheral venous blood lymphocytes using a commercial DNA extraction kit (Quiagen Inc., Valencia, CA, USA) and were genotyped using VeraCode Technology® (Illumina Inc., San Diego, CA, USA) at SNP Genetics Inc. (Seoul, Korea).

Selection of SNPs

SNP markers located from 2kb ~ 5′ to 2kb ~ 3′ of the *MSX1* gene were obtained from literature review and the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/). Among tag-SNPs covering all SNPs in the *MSX1* gene, three SNP markers with minor allele frequency (MAF) greater than 1% in the Japanese population (rs3821949 (G>A),

rs12532 (A>G), and rs4464513 (T>G)) were selected using the web-based program TAG SNP selection (TagSNP; http://snpinfo.niehs.nih.gov/guide.htm#snptag) .[27]

These SNPs achieved high "design scores" (a predictor of usable genotypes provided by Illumina Inc., San Diego, CA, USA). Their heterozygosity was greater than 0.1 in the Japanese population (www.hapmap.org/index.html.en). The genotype call rate and sample call rate were considered acceptable at ≥95%. Primers for each SNP were synthesized using Oligator technology (Illumina Inc.).

Statistical analysis

The MAF, heterozygosity, and χ^2 test for the Hardy-Weinberg equilibrium (HWE) at each SNP were computed using the genotypes of parents. Pairwise linkage disequilibrium (LD) was computed as both D' and r^2 for all SNPs using the Haploview program (http://www.broad.mit.edu/mpg/haploview/index.php/).[28-30]

The family-based association test program was used to perform allelic and genotypic transmission disequilibrium tests (TDTs) for individual SNPs and the haplotype-based association test program was used to fabricate sliding windows of haplotypes consisting of two and three SNPs (http://www.biostat.harvard.edu/fbat/default.html).[30,31] The permutation option[32] was used to obtain empirical *P* values for observed versus expected transmission and to compute the $-\log_{10}$ *P* value for each SNP/haplotype within the *MSX1* gene.

Genotypic odds ratios (GORs) for heterozygotes and homozygotes under additive, dominant, and recessive models were calculated separately for individual SNPs. A matched case-control dataset was generated with each NS-CL/P case matched to three possible pseudo-control subjects created from the non-transmitted parental allele.[30] The GORs were obtained from conditional logistic regression models for matched sets using publicly available subroutines in the R software (www.r-project.org).

Ethics statement

The study protocol was reviewed and approved by the institutional review board at each institution (SNUDH IRB CRI-G07002 and SMC IRB #2007-08-086, respectively). Informed consent was received from each subject before the sampling.

IV. RESULTS

Demographic information for proband gender and cleft type

One hundred forty-two Korean NS-CL/P families had 76 trios and 66 dyads. The cases included 90 males and 52 females; 9 with cleft lip (CL), 26 with cleft lip and alveolus (CLA), and 107 with cleft lip and palate (CLP) patients (Table 1).

MAF, heterozygosity, HWE, and LD analyses

Minor allele frequency (MAF) means the frequency at which the rare allele occurs in a given population. The MAF for rs4464513 (T>G) was lowest (0.295), while the MAFs for rs12532 (A>G) and rs3821949 (G>A) showed values of 0.359 and 0.484, respectively. H (heterozygosity) for rs3821949 (G>A), rs12532 (A>G) and rs4464513 (T>G) took the value of 0.499, 0.460 and 0.416. All of three SNPs had the high level of a genetic informative power. None of the three SNPs exhibited any evidence of deviation from HWE (Table 2). Among three SNPs, the values of Pairwise LD (D'/r^2) for pairs of rs3821949 (G>A)-rs12532 (A>G), rs12532 (A>G)-rs4464513 (T>G), and rs3821949 (G>A)-rs4464513 (T>G) were 0.56/0.16, 0.74/0.21, and 0.97/0.71, respectively. DNA recombination has occurred in all three SNPs and rs3821949 (G>A)-rs4464513 (T>G)showed relatively higher LD value than the other two pairs.

TDT analyses for individual markers and haplotypes

Although the family-based TDT using individual SNPs indicated a significant

association for rs3821949 (G>A) (P=0.028, Table 2), the haplotype analysis did not reveal any significant association with rs3821949 (G>A), rs12532 (A>G), or rs4464513 (T>G) (Table 2). Because three SNPs used in this study are tag SNPs which are independent of each other, there was no synergic interaction between these SNPs and significance in haplotype analysis could not be exhibited.

Genotypic odds ratios (GOR)

The A allele at rs3821949 (G>A) had a significant increased risk of NS-CL/P in an additive model (GOR, 1.64; 95% CI, 1.03-2.63; P=0.0384, Table 3).

V. DISCUSSION

Single marker analysis and genotypic odds ratio analysis in the present study exhibited that rs3821949 (G>A) has a meaningful *P* value (*P*=0.028, Table 2) and an increased risk of NS-CL/P under an additive model (GOR, 1.64; 95% CI, 1.03-2.63; *P*=0.0384, Table 3). Huang et al.[33] also reported similar results for rs3821949 (G>A) in the Han Chinese population living in Western China. However, if the number of samples were to be increased, there may be a more significant association in rs3821949 (G>A) when considering the relatively low frequency (2%) of *MSX1* mutations in NS-CL/P patients.[6,7] There is no reported result about association between *MSX1* rs3821949 (G>A) and NS-CL/P in non-Asian population until now. Therefore, it is needed to confirm the ethnic differences in other populations.

In this study, the A allele at rs3821949 (G>A) appears to increase the risk of NS-CL/P, while the G allele is under-represented (Tables 2 and 3). Huang et al.[33] suggested that the cleft lip and palate (CLP) patients showed a significant difference in allele frequency of rs3821949 (G>A) (GG vs. GA/AA) compared to cleft lip (CL) and cleft palate only (CPO) patients. Therefore, further study is needed to investigate whether the association of SNP in rs3821949 (G>A) is different according to cleft type with a large number of cases.

Numerous previous studies have investigated the role of the *MSX1* gene in the etiology of NS-CL/P in different human populations.[12,33-36] Among the proposed pathogenic mutations, a rare SNP, P147Q, has been a mutation of interest. It has been found in

approximately 2% of Vietnamese[7] and 1.2% of Han Chinese[33]. However, Tongkobpetch et al.[11] reported that the P147Q mutation could not be pathogenic because there was no association between the P147Q variant and NS-CL/P in the Thai population. Since this study tested only three SNPs whose MAF was greater than 1.0% in the Japanese population (rs3821949 (G>A), rs12532 (A>G), and rs4464513 (T>G)),[27] the P147Q variant was not included as a SNP in the *MSX1* gene in this study. Therefore, further study of the association of the P147Q variant with NS-CL/P in the Korean population might be needed.

The cause and time of formation of clefts vary; cleft lip and alveolus (CLA) results from fusion failure between the medial nasal process and maxillary process in the primary palate (lip and premaxilla), which takes place during the fourth to the seventh week of gestation; cleft palate (CP) results from fusion failure between the palatal processes in the secondary palate, which develops during the seventh to the twelfth week.[20,30] Therefore, the developmental classification between CLA and CLP is needed for epidemiologic studies of cleft patients.[30] However, considering the number of samples in the present study (cleft in the primary palate [n=35; 9 with cleft lip and 26 with cleft lip and alveolus] and cleft in the primary and secondary palate [n=107 with cleft lip and palate], Table 1), it is needed to increase the number of the cleft patients with lip and/or alveolus (CL and CLA) for investigating the possibility of difference in association and linkage between *MSX1* SNPs and cleft type.[30]

VI. CONCLUSIONS

In conclusion, the results from this study suggest a positive association between *MSX1* rs3821949 (G>A) and NS-CL/P in the Korean population. It may provide a better understanding of the etiological role of the *MSX1* gene in NS-CL/P and potential options for genetic counseling.

ACKNOWLEDGMENTS

I thank all participants who donated samples and acknowledge Yu-Jin Seo (Seoul National University School of Dentistry), Ah-Young Lee (Samsung Medical Center, The Institute of Oral Health Science), Jung Sun Cho (Hallym University College of Medicine) for assisting during this work; and Duk Hwan Kim, Yong Ick Ji, Eunhyun Jung, and Se Young Cho for their contributions concerning DNA preparation for genotyping (Center for Genome Research, Samsung Biomedical Research Institute, Seoul, Korea).

I declare no conflict of interest.

REFERENCES

1. Vanderas AP. Incidence of cleft lip, cleft palate, and cleft lip and palate among races: a review. Cleft Palate J 1987;24:216-25.
2. Wyszynski DF, Beaty TH, Maestri NE. Genetics of nonsyndromic oral clefts revisited. Cleft Palate Craniofac J 1996;33:406-17.
3. Mossey PA, Little J. Epidemiology of oral clefts: an international perspective. In: Cleft Lip & Palate, From Origin to Treatment. Wysznski DF, editor. Oxford, Oxford University Press, 2002:127-58.
4. Kim S, Kim WJ, Oh C, Kim JC. Cleft lip and palate incidence among the live births in the Republic of Korea. J Korean Med Sci 2002;17:49-52.
5. van den Boogaard MJ, Dorland M, Beemer FA, van Amstel HK. *MSX1* mutation is associated with orofacial clefting and tooth agenesis in humans. Nat Genet 2000;24:342-3.
6. Jezewski PA, Vieira AR, Nishimura C, Ludwig B, Johnson M, O'Brien SE, Daack-Hirsch S, Schultz RE, Weber A, Nepomucena B, Romitti PA, Christensen K, Orioli IM, Castilla EE, Machida J, Natsume N, Murray JC. Complete sequencing shows a role for *MSX1* in non-syndromic cleft lip and palate. J Med Genet 2003;40:399-407.
7. Suzuki Y, Jezewski PA, Machida J, Watanabe Y, Shi M, Cooper ME, Viet le T, Nguyen TD, Hai H, Natsume N, Shimozato K, Marazita ML, Murray JC. In a Vietnamese population, *MSX1* variants contribute to cleft lip and palate. Genet Med 2004;6:117-25.

8. Satokata I, Maas R. *Msx1* deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development. Nat Genet 1994;6:348-56.

9. Thomas T, Kurihara H, Yamagishi H, Kurihara Y, Yazaki Y, Olson EN, Srivastava D. A signaling cascade involving endothelin-1, dHAND and *msx1* regulates development of neural-crest-derived branchial arch mesenchyme. Development 1998;125:3005-14.

10. Gong SG. Phenotypic and molecular analyses of A/WySn mice. Cleft Palate Craniofac J 2001;38:486-91.

11. Tongkobpetch S, Siriwan P, Shotelersuk V. *MSX1* mutations contribute to nonsyndromic cleft lip in a Thai population. J Hum Genet 2006;51:671-6.

12. Lidral AC, Romitti PA, Basart AM, Doetschman T, Leysens NJ, Daack-Hirsch S, Semina EV, Johnson LR, Machida J, Burds A, Parnell TJ, Rubenstein JL, Murray JC. Association of *MSX1* and TGFB3 with nonsyndromic clefting in humans. Am J Hum Genet 1998;63:557-68.

13. Beaty TH, Wang H, Hetmanski JB, Fan YT, Zeiger JS, Liang KY, Chiu YF, Vanderkolk CA, Seifert KC, Wulfsberg EA, Raymond G, Panny SR, McIntosh I. A case-control study of nonsyndromic oral clefts in Maryland. Ann Epidemiol 2001;11:434-42.

14. Fallin MD, Hetmanski JB, Park J, Scott AF, Ingersoll R, Fuernkranz HA, McIntosh I, Beaty TH. Family-based analysis of *MSX1* haplotypes for association with oral clefts. Genet Epidemiol 2003;25:168-75.

15. Jugessur A, Lie RT, Wilcox AJ, Murray JC, Taylor JA, Saugstad OD, Vindenes HA, Abyholm F. Variants of developmental genes (*TGFA, TGFB3*, and *MSX1*) and their

associations with orofacial clefts: a case-parent triad analysis. Genet Epidemiol 2003;24:230-9.

16. Vieira AR, Orioli IM, Castilla EE, Cooper ME, Marazita ML, Murray JC. *MSX1* and *TGFB3* contribute to clefting in South America. J Dent Res 2003;82:289-92.
17. Moreno LM, Arcos-Burgos M, Marazita ML, Krahn K, Maher BS, Cooper ME, Valencia-Ramirez CR, Lidral AC. Genetic analysis of candidate loci in non-syndromic cleft lip families from Antioquia-Colombia and Ohio. Am J Med Genet A 2004;125A:135-44.
18. Schultz RE, Cooper ME, Daack-Hirsch S, Shi M, Nepomucena B, Graf KA, O'Brien EK, O'Brien SE, Marazita ML, Murray JC. Targeted scan of fifteen regions for nonsyndromic cleft lip and palate in Filipino families. Am J Med Genet A 2004;125A:17-22.
19. Park JY, Park BY, Kim HS, Lee JE, Suh I, Nam CM, Kang DR, Kim S, Yun JE, Go EN, Jee SH, Beaty TH. *MSX1* polymorphism associated with risk of oral cleft in Korea: Evidence from case-parent trio and case-control studies. Yonsei Med J 2007;48:101-8.
20. Conway H, Wagner KJ. Incidence of clefts in New York City. Cleft Palate J 1966;3:284-90.
21. Bensoussan-Trigano V, Lallemand Y, Saint Cloment C, Robert B. *Msx1* and *Msx2* in limb mesenchyme modulate digit number and identity. Dev Dyn. 2011;240:1190-202.
22. Roybal PG, Wu NL, Sun J, Ting MC, Schafer CA, Maxson RE. Inactivation of *Msx1* and *Msx2* in neural crest reveals an unexpected role in suppressing heterotopic bone formation in the head. Dev Biol. 2010;343:28-39.

23. Thesleff I, Nieminen P. Tooth morphogenesis and cell differentiation. Curr Opin Cell Biol. 1996;8:844-50.

24. Jumlongras D, Bei M, Stimson JM, Wang WF, DePalma SR, Seidman CE, Felbor U, Maas R, Seidman JG, Olsen BR. A nonsense mutation in *MSX1* causes Witkop syndrome. Am J Hum Genet. 2001;69:67-74.

25. Nieminen P, Kotilainen J, Aalto Y, Knuutila S, Pirinen S, Thesleff I. J Dent Res. *MSX1* gene is deleted in Wolf-Hirschhorn syndrome patients with oligodontia. 2003;82:1013-7.

26. Vieira AR, Avila JR, Daack-Hirsch S, Dragan E, Félix TM, Rahimov F, Harrington J, Schultz RR, Watanabe Y, Johnson M, Fang J, O'Brien SE, Orioli IM, Castilla EE, Fitzpatrick DR, Jiang R, Marazita ML, Murray JC. Medical sequencing of candidate genes for nonsyndromic cleft lip and palate. PLoS Genet. 2005;1:e64.

27. Xu Z, Taylor JA. SNPinfo: integrating GWAS and candidate gene information into functional SNP selection for genetic association studies. Nucleic Acids Res 2009;37:W600-5.

28. Ardlie KG, Kruglyak L, Seielstad M. Patterns of linkage disequilibrium in the human genome. Nat Rev Genet 2002;3:299-309.

29. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 2005;21:263-5.

30. Lee JK, Park JW, Kim YH, Baek SH. Association between *PAX9* SNPs and nonsyndromic cleft lip with or without cleft palate in the Korean population. J Craniofac Surg 2012;23:1262-6.

31. Rabinowitz D, Laird NM. A unified approach to adjusting association tests for

population admixture with arbitrary pedigree structure and arbitrary missing marker information. Hum Hered 2000;50:211-23.

32. Horvath S, Xu X, Laird NM. The family based association test method: strategies for studying general genotype-phenotype associations. Eur J Hum Genet 2001;9:301-6.
33. Huang YQ, Ma J, Ma M, Deng Y, Li YD, Ren HW, Zhao GZ, Guo SS, Wang YY, Zhang GX, Shi B. Association between *MSX1* variants and oral clefts in Han Chinese in western China. DNA Cell Biol 2011;30:1057-61.
34. Jugessur A, Murray JC. Orofacial clefting: recent insights into a complex trait. Curr Opin Genet Dev 2005;15:270-8.
35. Blanco R, Chakraborty R, Barton SA, Carreño H, Paredes M, Jara L, Palomino H, Schull WJ. Evidence of a sex-dependent association between the *MSX1* locus and nonsyndromic cleft lip with or without cleft palate in the Chilean population. Hum Biol 2001;73:81-9.
36. Singh VP, Ramu D. Association of *MSX1* 799>T variant with nonsyndromic cleft lip/palate in South Indian adolescent patients. Int J Paediatr Dent 2012;22:228-31.

Table 1. Demographic information of gender and cleft type

Type of malformation		Trios		Dyad	
		Male	Female	Male	Female
CL	unilateral	2	1	2	3
	bilateral	1	0	0	0
CLA	unilateral	9	2	7	3
	bilateral	2	1	1	1
CLP	unilateral	17	16	19	12
	bilateral	17	8	13	15
Sum		48	28	42	34
		76		66	

CL, cleft lip only; CLA, cleft lip and alveolus; CLP, cleft lip and palate.

Each numerical number in Table 1 is the number of actual patient with CL, CLA, and CLP.

This table was reorganized from the Data of previous study which was performed by Lee et al. (2009)[30]

Table 2. Marker information and transmission disequilibrium test (TDT) results for three single nucleotide polymorphisms (SNPs) in the *MSX1* gene in cleft lip with or without cleft palate (CL/P) in 142 CL/P families

SNP	*M/m**	*MAF*	*HWP (P)*	*T/NT*†	*Allele (P)*‡	*Haplotype (P)*‡	
						2	3
rs3821949 (G>A)	G/**A**	0.484	0.961	46:28	0.028**§**	0.0867	0.1947
rs12532 (A>G)	**G**/A	0.359	1.000	37:33	0.553	0.2236	
rs4464513 (T>G)	**G**/T	0.295	0.568	34:24	0.152		

* Over-transmitted alleles are in bold type.

†Transmission/non-transmission counts from heterozygous parents.

‡Significant *P* values for individual SNP and global *P* values for sliding windows of haplotypes of two and three SNPs from TDT analyses.

MAF, minor allele frequency; HWE, Hardy-Weinberg equilibrium; TDT, transmission disequilibrium test; SNP, single nucleotide polymorphism; §, $P<0.05$.

Table 3. Genotypic odds ratios (GORs) for heterozygotes and homozygotes for individual SNPs in 76 NS-CL/P trios

SNP	Genotype	N*	Additive model		Dominant model		Recessive model	
			GOR (95% CI)	*P* value†	GOR (95% CI)	*P* value†	GOR (95% CI)	*P* value†
rs3821949 (G>A)		n*=76	1.64 (1.03-2.63)	0.0384‡	2.03 (0.94-4.38)	0.0715	1.56 (0.80-3.04)	0.1929
	G/G	94						
	G/A	184						
	A/A	82						

LD represents linkage disequilibrium; CI, confidence interval.

*'N' and 'n' refer to the number of subjects carrying the genotype and the number of case/pseudo-control sets generated, respectively.

†, *P* values of χ^2 tests for the conditional logistic regression model for each SNP

‡, $P<0.05$

국문 초록

한국인에서 *MSX1* 유전자의 단일염기다형성과 구순구개열의 연관성

김 나 영

서울대학교 대학원 치의과학과 치과교정학 전공

(지도교수: 백 승 학)

본 연구의 목적은 한국인 비증후군성 구순구개열 (non-syndromic cleft lip with or without palate; NS-CL/P) 환자의 발생 위험에 대한 muscle segment homeobox1 (*MSX1*) 유전자의 기여도를 연구하기 위함이다.

환자군은 142 명의 한국인 비증후군성 구순구개열 환자와 그 부모 (구순열 9 명, 구순치조열 26 명, 구순구개열 107 명; 76 trios, 66 dyads)로 구성되었다. 세 가지 단일염기 다형성 (SNPs: rs3821949 (G>A), rs12532 (A>G), and rs4464513 (T>G)) 에 대해 transmission disequilibrium test (TDT) 와 conditional logistic regression model (CLRM) 을 이용하여 NS-CL/P 환자와 부모 trio 와의 연관성을 분석하였다. 각 SNP 에 대해 Minor allele frequency, heterozygosity, χ^2 test for Hardy-Weinberg equilibrium 과 pairwise linkage disequilibrium (LD)을 계산하였다. Family-based association test 와 haplotype-based association test 프로그램으로 각 SNP 에 대한 allelic TDT 와 genotypic TDT 를 계산하였고, haplotype 의 sliding windows 를

구성하였다. Genotypic odd ratios (GORs)는 R software 를 이용하여 additive, dominant, recessive 세 가지 유전 모델의 가정 하에 CLRM 을 이용하여 계산하였다.

이로부터 다음과 같은 결과를 얻었다.

1. Family-based TDT 에서는 rs3821949 (G>A)가 의미 있는 상관관계를 보였으나 (P=0.028), haplotype-based TDT 에서는 rs3821949 (G>A), rs12532 (A>G), rs4464513 (T>G) 모두에서 유의성 있는 결과가 나타나지 않았다.
2. GOR 분석에서는 additive model 하에서 rs3821949 (G>A)의 A allele 은 NS-CL/P 환자의 발생 위험을 유의성 있게 증가시켰다 (GOR, 1.64; 95% confidence interval, 1.03-2.63; P=0.0384).

본 연구의 결과 *MSX1* 유전자의 SNP (rs3821949 (G>A)) 은 한국인에서 NS-CL/P 의 발생 위험을 증가시킬 수 있다는 유전적 이해에 도움을 줄 수 있다고 생각한다.

주요어: *MSX1* 단일염기다형성; 비증후군성 구순구개열; 한국인; 연관성 분석

학 번: 2007-30587