

Interaction Effect of RsaI and BamHI Polymorphisms of TGF α , BMP2 and BMP4 on the Occurrence of Non-Syndromic Cleft Lip and Palate in Iranian Patients

Saba Samadi¹, Asghar Ebadifar^{2*}, Hamid Reza Khorram Khorshid³, Koorosh Kamali⁴,
and Mohammadreza Badiie⁵

1. General Dentist, Tehran, Iran

2. Dentofacial Deformities Research Center, Research Institute of Dental Sciences, Department of Orthodontic, Faculty of Dentistry, Shahid Beheshti University of Medical Sciences, Tehran, Iran

3. Genetic Research Centre, University of Social Welfare and Rehabilitation Sciences, Tehran, Iran

4 Department of Public Health, Faculty of Public Health, Zanjan University of Medical Sciences, Zanjan, Iran

5. Dentofacial Deformities Research Center, Research Institute of Dental Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Abstract

Background: Orofacial cleft is the most common congenital defect of the maxillofacial region. Its non-syndromic type is multi-factorial, and several genes are involved in its occurrence. This study aimed to assess the interaction effect of RsaI and BamHI polymorphisms of *Transforming Growth Factor-alpha (TGF α)* gene and Bone Morphogenetic Protein-2 (BMP2) and BMP4 variants on the occurrence of Non-Syndromic Cleft Lip and Palate (NSCLP) in the Iranian population.

Methods: This case-control study was conducted on 120 children with NSCLP and 215 healthy children. Genotyping of the TGFA/BamHI (rs11466297), TGFA/RsaI (rs37322-48), BMP4 (rs17563) and BMP2 (rs235768) was performed by Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) methods. Logistic regression was applied to determine the effective factors and the interaction effect of different variants on the occurrence of NSCLP.

Results: Gender of patients had no significant association with the occurrence of NSCLP ($p=0.335$). Multiple logistic regression showed that the interaction effect of the aforementioned polymorphisms on the occurrence of NSCLP was not statistically significant ($p=1.000$).

Conclusion: Although the individual effect of each of the BMP4, BMP2, RsaI and BamHI variants on the occurrence of NSCLP in the Iranian population has been previously confirmed, their interaction does not play a role in this respect.

Avicenna J Med Biotech 2018; 10(4): 248-252

Keywords: Bone morphogenetic proteins, Cleft Lip and palate, Polymorphism

Introduction

Orofacial cleft is the most common congenital defect of the maxillofacial region. It has the highest prevalence in the East Asia such as China and Japan with 1/500 live births and the least prevalence among the African-Americans with 0.21 to 0.41/1000 live births^{1,2}. The prevalence of orofacial cleft in Iran is 1.05 to 1.9/1000 live births³⁻⁵. Orofacial cleft decreases the quality of life of patients and their family and negatively affects the speech, oral health and mental health of patients⁶. The non-syndromic form is multifactorial, and environmental factors such as cigarette smoking, alcohol consumption, folate consumption, infections and viruses as well as genetics may be involved in its

occurrence^{1,7}. Nonetheless, the molecular mechanism of action of these factors has not been well recognized. Several genes may be involved in the occurrence of Cleft Lip and Palate (CLP). TGF α is a member of the epidermal growth factor superfamily, which codes a protein with the same name. During craniofacial development, TGF α is expressed in the internal border of the epithelium of fusing palatal shelves and stimulates the synthesis of extracellular matrix and migration of mesenchymal cells, which further strengthens the palatal tissue. RsaI (rs3732288) and BamHI (rs11466297) are the two common variants of this gene^{8,9}. Ardingier *et al* evaluated the role of *Transforming Growth Factor-*

* Corresponding author:
Asghar Ebadifar, Ph.D.,
Dentofacial Deformities Research
Center, Research Institute of
Dental Sciences, Shahid Beheshti
University of Medical Sciences,
Tehran, Iran
Tel: +98 9122173808
E-mail:
a.ebadifar@sbmu.ac.ir
Received: 17 May 2017
Accepted: 19 Jul 2017

alpha (TGFA) gene variants in the occurrence of CLP¹⁰. Ebadifar *et al* showed that polymorphism of TGFA variants plays a role in the occurrence of CLP¹¹.

Evidence shows that Bone Morphogenetic Proteins (BMPs) and their antagonist, Noggin, play a role in fusion of the upper lip and the primary palate¹². The two subgroups of BMP2 and BMP4 play a fundamental role in craniofacial development and are specifically expressed in epithelial and mesenchymal cells of the palatal shelves. The significance of BMP2 (rs235768) and BMP4 (rs17563) variants in the occurrence of CLP has been emphasized in previous studies^{13,14}. Saket *et al* indicated that polymorphism of BMP2 (rs235768) and BMP4 (rs17563) variants plays an important role in the occurrence of CLP in the Iranian population¹⁵. Blanco *et al* demonstrated that although the interaction between BMP4 and IRF6 is mild, presence of specific haplotypes indicates higher risk of occurrence of CLP¹⁶.

Molecular studies have shown that interaction of gene-gene such as Sonic Hedgehog (SHH), bone morphogenetic proteins, the homeobox containing genes *Barx1* and *Msx1* can control the initiation, outgrowth and specification of the facial processes¹⁷⁻¹⁹. Evidence suggests genetic predisposition of NSCLP, caused by the interactions of multiple interacting genes²⁰. Song *et al* showed that the interaction of PAX9 and IRF6 plays a potentially important role in the occurrence of CLP²¹. Lidral *et al* found that the 4-2-2-2 haplotype for MSX1 CA-X1.1-X1.3-X2.1-X2.4 was most frequently transmitted among both CLP and CP cases²². Jugessur *et al* raised the possibility of interaction between TGFA, TGFB3, and MSX1. The effect of this TGFA TaqI genotype was stronger among children homozygous for the MSX1-CA A4 allele, raising the possibility of interaction between these two genes²³. Since the individual role of polymorphism of the aforementioned variants has been previously confirmed in the occurrence of CLP in the Iranian population^{11,15}, this study aimed to assess the interaction effect of RsaI and BamHI polymorphisms of TGFA and BMP2 and BMP4 on the occurrence of Non-Syndromic Cleft Lip and Palate (NSCLP) in the Iranian population.

Materials and Methods

This case-control study was approved in the ethics committee of Shahid Beheshti University of Medical

Sciences (IR.SBMU.RIDS.REC.1395.195). The study group included blood samples of 120 children with NSCLP born at Mofid Hospital from 2013 to 2016. Patients with other skeletal and facial malformations such as congenital lip pits, cardiac diseases or neurologic anomalies were excluded. The control group included 215 healthy children born in Tehran city between 2013 to 2016. Written informed consent was obtained from the parents. The mothers of children were requested to sign the consent form and fill out a questionnaire regarding the presence of congenital anomalies, medication intake, cigarette smoking, tobacco use and intake of folate during the three months before and one month after conception and pregnancy.

In brief, 3 ml of peripheral blood was collected in tubes containing 200 µl of 0.5 M EDTA, and genomic DNA was extracted from the peripheral blood using the salting out method²⁴. Genotyping of the TGFA/BamHI (rs11466297), TGFA/RsaI (rs3732248), BMP4 (rs17563) and BMP2 (rs235768) was performed by Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) methods, according to a previous study²⁴. A total volume of 25 µl containing 30 ng of genomic DNA, 10 pmol of each primer, 1 µl dNTPs mix (Fermentas, Life Science), 2.5 µl 10×buffer and 0.5 U of Taq DNA polymerase (Fermentas Life Science, Lithuania) with 1.5 mM MgCl₂ was prepared in 0.5 ml Eppendorf microtube for amplification of the target sequences. Amplification conditions started with an initial denaturation step of 4 min at 95 °C, followed by 33 cycles of 45 s of denaturation (94 °C), 30 s of annealing (60 °C) and 40 s of extension (72 °C), ended by a final extension for 5 min (72 °C) and finally cooling to 4°C. All PCR products were subjected to 8% polyacrylamide gel electrophoresis and stained with silver nitrate. Table 1 shows the primer sequences and the pattern of restriction fragments.

Statistical analysis

Data were analyzed using SPSS 18 (SPSS Inc., Chicago, USA). Chi square and Fisher's exact test with Open Epi Version 2.2 (free statistical software) were performed to compare genotype and allele frequency among the study groups. Logistic regression was used to determine the effect of influential factors and the interaction effect of different variants on the occurrence of CLP. $p < 0.05$ was considered statistically significant.

Table 1. Primer sequences, product size and RE fragments for the BamHI, RsaI, BMP4 and BMP2

SNPs	Global MAF	Primer(5'→3)	Product Size (bp)	RE fragments (bp)
TGFA/ BamHI (rs11466297 A/C)	C=0.0238	F: GCCTGGCTTATTTGGGGATT R: AAGGGCAAGGAAACACAGG	174	A allele=120+54 C allele=174
TGFA/RsaI (rs3732248 C/T)	A=0.2075	F: TGCCTTCCTTCTGCTATCACT R: CAGAGCCAATGTCACCAAGT	153	C allele=91+75 T allele=166
BMP4(rs17563)	C = 0.3257	F: CACCATTTCATTGCCCAAC R:AGTTTGGCTGCTTCTCCC	179	AA: 165 TT: 86 + 79 AT: 165 + 86 + 79
BMP2(rs235768)	T = 0.2332	F: GAAACGAGTGGGAAAAACAACC R: GAGACACCTGTTTCTCTCCA	165	TT: 118 + 61 CC: 179 TC: 179 + 118 + 61

Interaction Effect of RsaI and BamHI Polymorphisms on the Occurrence of Non-Syndromic Cleft Lip and Palate

Table 2. Genotype and allele frequency of the TGF α BamHI and RsaI polymorphisms in the case and control groups

Genotype/allele	Case group	Control group	p-value	OR (95% CI)
BamHI (rs11466297)				
GG	97 (93.3%)	185 (92%)	Reference group	
GC	5 (4.8%)	16 (8%)	0.322	0.6 (0.2-1.7)
CC	2 (1.9%)	0(0%)	0.241	Undefined
G	199 (95.7%)	386 (96%)	Reference allele	
C	9 (4.3%)	16 (4%)	0.838	1.1 (0.47-2.5)
RsaI (rs3732288)				
CC	70 (66%)	112 (58.3%)	Reference group	
CT	30 (28.3%)	60 (31.3%)	0.409	0.8 (0.5-1.4)
TT	6 (5.7%)	20 (10.4%)	0.127	0.5 (0.2-1.3)
C	170 (80.2%)	284 (74%)	Reference allele	
T	42 (19.8%)	100 (26%)	0.087	0.7 (0.5-1.05)

Table 3. The genotype and allele frequency of the BMP2 and BMP4 polymorphisms in the case and control groups

Genotype/allele	Case group	Control group	p-value	OR (95% CI)
BMP2 (rs235768)				
TT	35 (32.1%)	124 (59.3%)	Reference group	
TA	65 (59.6%)	74 (39.4%)	0.561	0.2 (0.13-3)
AA	9 (8.3%)	11(5.3%)	0.002	2.7 (1.4-5.1)
T	135 (61.9%)	322 (77%)	Reference allele	
A	83 (38.1%)	96 (23%)	0.001	2 (1.4-2.9)
BMP4 (rs17563)				
TT	22 (20.2%)	74 (35.4%)	Reference group	
TC	70 (64.2%)	96 (45.9%)	0.585	1.3 (0.5-3].4)
CC	17 (15.6%)	39 (18.7%)	0.056	2.1 (0.98-4.5)
T	114 (52.3%)	244 (58.4%)	Reference allele	
C	104 (47.7%)	174 (41.6%)	0.142	1.3 (0.9-1.8)

Results

There were 59.3% males and 40.7% females in the case and 43.6% males and 56.4% females in the control group. The results showed that gender had no significant association with the occurrence of CLP ($p=0.335$).

Table 2 shows the genotype and allele frequency of TGF α variants namely RsaI and BamHI in the case and control groups. Table 3 shows the genotype and allele frequency of BMP2 and BMP4 in the two groups. Table 4 demonstrates the interaction effect of RsaI, BamHI, BMP2 and BMP4 polymorphisms on the occurrence of NSCLP. Multiple logistic regression showed that the interaction effect of polymorphisms of the BMP family, TGF α family and combination of all four

on the occurrence of NSCLP was not statistically significant ($p=1.000$).

Discussion

NSCLP is a common genetic defect that occurs due to the interaction of different genes and environmental factors²⁵. Several genes have been implicated in the occurrence of this defect among which *FGF8*, *FGFR1*, *MSX1*, *BMP* and *TGF α* can be named^{26,27}.

BMP4 and BMP2 are the main two subgroups of BMP superfamily, which play an important role in regulation of osteogenesis and chondrogenesis. They are expressed in epithelial and mesenchymal cells in the palatal shelves during palatogenesis¹². RsaI and BamHI are the two common variants of TGF α that induce

Table 4. Interaction effect of BamHI, RsaI, BMP4 and BMP2 polymorphisms on the occurrence of NSCLP

Interaction terms	p-value
BMP2 c.893 T>A(rs235768) & BMP4 c.538 C>T (rs17563)	0.997
BMP4 c.538 C>T (rs17563) & TGF α (BamHI)	1.000
BMP4 c.538 C>T (rs17563) & TGF α (RsaI) rs3732248	1.000
BMP2 c.893 T>A(rs235768) & TGF α (BamHI)	1.000
BMP2 c.893 T>A(rs235768) & TGF α (RsaI) rs3732248	1.000
TGF α (BamHI) & TGF α (RsaI) rs3732248	1.000
BMP2 c.893 T>A (rs235768) & BMP4 c.538 C>T (rs17563) & TGF α (RsaI) rs3732248	1.000
BMP2 c.893 T>A(rs235768) & TGF α (BamHI) & TGF α (RsaI) rs3732248	1.000
BMP2 c.893 T>A(rs235768) & BMP4 c.538 C>T (rs17563) & TGF α (BamHI) & TGF α (RsaI) rs3732248	1.000

extracellular matrix synthesis and migration of mesenchymal cells. They also play a pivotal role in strengthening of the palatal tissue^{8,9}. Several studies have evaluated the relationship of the aforementioned genes and occurrence of CLP. The results of previous studies are controversial, which may be attributed to the difference in sample size, genetic background and environmental factors. Ebadifar *et al* showed that a correlation exists between polymorphism of BamHI variant and occurrence of CLP in the Iranian population such that the frequency of AC genotype and C allele was significantly higher in the patient group (12.4 and 8%, respectively)¹¹. Ardinger *et al* found a significant correlation between the polymorphism of BamHI and Tag 1 variants and the occurrence of CLP in the American population⁸. On the other hand, Lidral *et al* found no significant correlation between *TGF α* gene and the occurrence of CLP in a non-Caucasian population²⁸.

Regarding *BMP* gene, Saket *et al* showed that the frequency of BMP4 and BMP2 genotypes was 70 and 59.8% higher in CLP patients compared to the values in the control group, respectively; this indicates a positive correlation between variants of this gene and occurrence of CLP in the Iranian population¹⁵. Jianyan *et al* indicated that the frequency of BMP4 polymorphism was twice the value in the control group in patients with CLP in a Chinese population²⁹. On the other hand, Wang *et al* did not find a significant association between this polymorphism and occurrence of CLP in a Chinese population³⁰. Hu *et al*, in their meta-analysis stated that BMP4 increases the risk of occurrence of CLP in the Chinese population while it has a positive inhibitory effect on the occurrence of CLP in the Brazilian population³¹.

The role of BMP4, BMP2, RsaI and BamHI polymorphisms in the occurrence of CLP in the Iranian population has been previously confirmed^{11,15}. Thus, this study assessed the interaction effect of these polymorphisms on the occurrence of CLP. The results showed that the interaction effect of two polymorphisms of *BMP* gene and *TGF α* gene had no significant effect on the occurrence of NSCLP. On the other hand, the overall and pairwise interaction effects of the four variants on the occurrence of CLP were not significant either.

Some previous studies have shown the interaction effect of different variants on the occurrence of NSCLP. Song *et al* showed that combinations of rs2073485 (GG) and rs17176643 (aC+CC), rs2235371 (CC) and rs17176643 (aC+CC) and also rs2236909 (aG+aa) and rs17176643 (aC+CC) were significantly correlated with the occurrence of NSCLP¹⁷. Sull *et al* demonstrated that the interaction of *TGF α* and *IRF6* may play a role in the occurrence of NSCLP³². Difference between the results of aforementioned studies and ours may be attributed to different study populations and the tested variants.

Conclusion

Although the individual role of BMP4, BMP2, RsaI and BamHI variants has been shown in the occurrence of NSCLP separately in various studies in Iranian population^{11,15}, this study showed that their interaction effect on the occurrence of NSCLP was not significant.

Acknowledgement

The authors would like to thank Dr. Rouzrokh, the administrator of Mofid Hospital and the personnel of this hospital for their sincere cooperation in the conduction of this study. This study was part of a thesis for doctorate degree in dental surgery by Saba Samadi and was financially supported by a grant from Dentofacial Deformities Research Center, Research Institute of Dental Sciences, Shahid Beheshti University of Medical Sciences.

References

- Dixon MJ, Marazita ML, Beaty TH, Murray JC. Cleft lip and palate: understanding genetic and environmental influences. *Nat Rev Genet* 2011;12(3):167-178.
- Conway JC, Taub PJ, Kling R, Oberoi K, Doucette J, Jabs EW. Ten-year experience of more than 35,000 orofacial clefts in Africa. *BMC Pediatr* 2015;15:8.
- Mirfazeli A, Kaviany N, Hosseinpour KR, Golalipour MJ. Incidence of cleft lip and palate in gorgan - northern iran: an epidemiological study. *Oman Med J* 2012;27(6):461-464.
- Kianifar H, Hasanzadeh N, Jahanbin A, Ezzati A, Kianifar H. Cleft lip and palate: A 30-year epidemiologic study in north-east of Iran. *Iran J Otorhinolaryngol* 2015; 27(78):35-41.
- Cooper ME, Ratay JS, Marazita ML. Asian oral-facial cleft birth prevalence. *Cleft Palate Craniofac J* 2006;43 (5):580-589.
- Mossey PA, Little J, Munger RG, Dixon MJ, Shaw WC. Cleft lip and palate. *Lancet* 2009;374(9703):1773-1785.
- Setó-Salvia N, Stanier P. Genetics of cleft lip and/or cleft palate: association with other common anomalies. *Eur J Med Genet* 2014;57(8):381-393.
- Vieira AR. Association between the transforming growth factor alpha gene and nonsyndromic oral clefts: a HuGE review. *Am J Epidemiol* 2006;163(9):790-810.
- Lu XC, Yu W, Tao Y, Zhao PL, Li K, Tang LJ, et al. Contribution of transforming growth factor α polymorphisms to nonsyndromic orofacial clefts: a HuGE review and meta-analysis. *Am J Epidemiol* 2014;179(3):267-281.
- Ardinger HH, Buetow KH, Bell GI, Bardach J, VanDemark DR, Murray JC. Association of genetic variation of the transforming growth factor-alpha gene with cleft lip and palate. *Am J Hum Genet* 1989;45(3):348-353.
- Ebadifar A, Hamed R, Khorram Khorshid HR, Salimnejad, Kamali K, Aghakhani Moghadam F, et al. Association of transforming growth factor alpha polymorphisms with nonsyndromic cleft lip and palate in Iranian population. *Avicenna J Med Biotechnol* 2015;7(4):168-172.

12. Nie X, Luukko K, Kettunen P. BMP signalling in craniofacial development. *Int J Dev Biol* 2006;50(6):511-521.
13. Kempa I, Ambrozaitytė L, Stavusis J, Akota I, Barkane B, Krumina A, et al. Association of BMP4 polymorphisms with non-syndromic cleft lip with or without cleft palate and isolated cleft palate in Latvian and Lithuanian populations. *Stomatologija* 2014;16(3):94-101.
14. Araújo TK, Simioni M, Félix TM, de Souza LT, Fontes MÍ, Monlleó IL, et al. Preliminary analysis of the non-synonymous polymorphism rs17563 in BMP4 gene in Brazilian population suggests protection for nonsyndromic cleft lip and palate. *Plast Surg Int* 2012;2012:247104.
15. Saket M, Saliminejad K, Kamali K, Moghadam FA, Anvar NE, Khorram Khorshid HR. BMP2 and BMP4 variations and risk of non-syndromic cleft lip and palate. *Arch Oral Biol* 2016;72:134-137.
16. Blanco R, Colombo A, Pardo R, Suazo J. Haplotype-based gene-gene interaction of bone morphogenetic protein 4 and interferon regulatory factor 6 in the etiology of non-syndromic cleft lip with or without cleft palate in a Chilean population. *Eur J Oral Sci* 2017;125(2):102-109.
17. Lee SH, Fu KK, Hui JN, Richman JM. Noggin and retinoic acid transform the identity of avian facial prominences. *Nature* 2001;414(6866):909-912.
18. Ashique AM, Fu K, Richman JM. Endogenous bone morphogenetic proteins regulate outgrowth and epithelial survival during avian lip fusion. *Development* 2002;129(19):4647-4660.
19. Hu D, Marcucio RS, Helms JA. A zone of frontonasal ectoderm regulates patterning and growth in the face. *Development* 2003;130(9):1749-1758.
20. Mehrotra D. Genomic expression in non syndromic cleft lip and palate patients: a review. *J Oral Biol Craniofac Res* 2015;5(2):86-91.
21. Song T, Wu D, Wang Y, Li H, Yin N, Zhao Z. SNPs and interaction analyses of IRF6, MSX1 and PAX9 genes in patients with non-syndromic cleft lip with or without palate. *Mol Med Rep* 2013;8(4):1228-1234.
22. Lidral AC, Romitti PA, Basart AM, Doetschman T, Leysens NJ, Daack-Hirsch S, et al. Association of MSX1 and TGFB3 with nonsyndromic clefting in humans. *Am J Hum Genet* 1998;63(2):557-568.
23. Jugessur A, Lie RT, Wilcox AJ, Murray JC, Taylor JA, Saugstad OD, et al. Variants of developmental genes (TGFA, TGFB3, and MSX1) and their associations with orofacial clefts: a case-parent triad analysis. *Genet Epidemiol* 2003;24(3):230-239.
24. Koshy L, Anju AL, Harikrishnan S, Kutty VR, Jissa VT, Kurikesu I, et al. Evaluating genomic DNA extraction methods from human whole blood using endpoint and real-time PCR assays. *Mol Biol Rep* 2017;44(1):97-108.
25. Sivertsen A, Wilcox AJ, Skjaerven R, Vindenes HA, Abyholm F, Harville E, et al. Familial risk of oral clefts by morphological type and severity: population based cohort study of first degree relatives. *BMJ* 2008;336(7641):432-434.
26. Leslie EJ, Marazita ML. Genetics of cleft lip and cleft palate. *Am J Med Genet C Semin Med Genet* 2013;163C(4):246-258.
27. Leslie EJ, Murray JC. Evaluating rare coding variants as contributing causes to non-syndromic cleft lip and palate. *Clin Genet* 2013;84(5):496-500.
28. Lidral AC, Murray JC, Buetow KH, Basart AM, ScheererH, Shiang R, et al. Studies of the candidate genes TGFB2, MSX1, TGFA, and TGFB3 in the etiology of cleft lip and palate in the Philippines. *Cleft Palate Craniofac J* 1997;34(1):1-6.
29. Jianyan L, Zeqiang G, Yongjuan C, Kaihong D, Bing D, Rongsheng L. Analysis of interactions between genetic variants of BMP4 and environmental factors with non-syndromic cleft lip with or without cleft palate susceptibility. *Int J Oral Maxillofac Surg* 2010;39(1):50-56.
30. Wang H, Zhou X, Cui Y, Liu J, Wang W. Relationship between nonsyndromic cleft lip with or without cleft palate (NSCL/P) and genetic polymorphisms of BMP4. *Jiangsu Med J* 2012;38(8):897-900.
31. Hu YY, Qin CQ, Deng MH, Niu YM, Long X. Association between BMP4 rs17563 polymorphism and NSCL/P risk: a meta-analysis. *Dis Markers* 2015;2015:763090.
32. Sull JW, Liang KY, Hetmanski JB, Wu T, Fallin MD, Ingersoll RG, et al. Evidence that TGFA influences risk to cleft lip with/without cleft palate through unconventional genetic mechanisms. *Hum Genet* 2009;126(3):385-394.