

Association of the *WNT3* Variations and the Risk of Non-Syndromic Cleft Lip and Palate in a Population of Iranian Infants

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Abstract

Background: Nonsyndromic cleft lip and/or palate (NSCL/P) is the most common orofacial birth defect, often attributed to ethnic and environmental differences. Up to now, linkage analyses and genome-wide association studies have identified several genomic susceptibility regions for NSCL/P. The *WNT* genes including *WNT3* are strong candidates for NSCL/P, since they are involved in regulating mid-face development and upper lip fusion. This study tested association of the *WNT3* polymorphisms, rs-3809857 G/T and rs9890413 G/A, with the risk of NSCL/P in a population of Iranian infants.

Methods: The allelic and genotypic frequencies for each participant were determined in 113 unrelated Iranian subjects with NSCL/P and 220 control subjects using PCR and restriction fragment length polymorphism (RFLP) methods. A p-value of ≤ 0.05 was considered statistically significant.

Results: The *WNT3* rs3809857 GT genotype was significantly lower ($p=0.039$, OR=0.55, 95% CI=0.30–0.97) in the NSCL/P (21.2%) than the control group (30.42%). For the *WNT3* rs9890413 G/A polymorphism, neither genotype nor allele frequencies were significantly different between the case and control groups.

Conclusion: Our results indicated that the *WNT3* rs3809857 GT genotype may have a protective effect against NSCL/P in Iranian population.

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Keywords: Genome wide association study, Cleft lip/Palate, Polymorphism, *WNT3*

Introduction

Clefts of the lip and/or palate (CL/P) are one of the most common birth defects which are typically classified in syndromic and more common non-syndromic (NSCL/P) forms¹. NSCL/P is a complex malformation caused by the interaction of multiple genes and environmental factors²; however, its etiology still remains poorly characterized¹. Genetic factors are thought to contribute to the development of this disorder, because the risk of recurrence of CL/P within a family is higher than for the general population^{3,4}.

Up to now, several distinct genetic and environmental risk factors have been identified and confirmed for the NSCL/P^{1,5,6}. Many candidate genes and loci have also been associated with facial clefts, through various genetic approaches. Until now, over 40 genes, including interferon regulatory 6 (*IRF6*), Msh homeobox ho-

molog 1 (*MSX1*), transforming growth factor alpha (*TGFα*), and domain containing 2 (*CRISPLD2*) as well as the 8q24 and 17q22 loci have been suggested to be associated with the etiology of NSCL/P¹.

The *WNT* gene family plays an important role in murine craniofacial embryogenesis⁷. Canonical WNT signaling is activated during midfacial morphogenesis in mice⁸. Although not implicated by Genome-Wide Association (GWA) studies, Single Nucleotide Polymorphism (SNP) within the *WNT* genes has been reported to be associated with NSCL/P, and the haplotype of the *WNT* genes may explain the etiology of NSCL/P⁹. SNPs in *WNT3A*, *WNT5A*, and *WNT11* are significantly associated with NSCL/P¹⁰. Previous studies have shown that two SNPs in the *WNT3A* gene, rs752107 and rs3121310, were significantly associated

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with NSCL/P in a Chinese population¹¹. In another study in a northeast Chinese population C392T SNP in the *WNT10A* gene was associated with NSCL/P¹².

According to our knowledge, a few studies have evaluated the association between the *WNT3* gene and NSCL/P in Caucasian populations. The *WNT3* rs3809857 was highly associated with NSCL/P in a Polish population⁹. The *WNT3* rs142167 and rs9890413 were shown to be associated with NSCL/P in a population of Caucasian ancestry¹³.

The aim of this study was to evaluate the association between the two SNPs of *WNT3*, rs3809857 and rs9890413, with the risk of NSCL/P using PCR and Restriction Fragment Length Polymorphism (RFLP) in an Iranian population.

Materials and Methods

Subjects

This case-control study tested the association of the *WNT3* SNPs with the risk of NSCL/P in a population of Iranian infants. The allelic and genotypic frequencies were determined in 113 unrelated newborns with NSCL/P and 220 newborns as control subjects without clefting.

A clinical examination was performed to look for dysmorphic features such as lip pits. Cases were excluded from the study if there was evidence of other facial or skeletal malformations (such as lip pits, congenital heart lesion, etc), metabolic or neurologic disorders or anomalies of other organ systems. Samples were recruited from Mofid Hospital, a referral pediatric center in Tehran, Iran during 2013-2015. Ethical approval for the study was obtained from the Ethics Committee of the School of Dentistry, Shahid Beheshti University of Medical Sciences. Informed consent was obtained from all parents.

DNA extraction and genotyping

Four to ten ml of peripheral blood samples were collected in tubes containing 200 μ l of 0.5 M EDTA and genomic DNA was extracted from peripheral blood using the salting out method as described previously¹⁴. DNA concentration was determined by measuring the absorbance at 260 nm. DNA purity was measured by calculating the ratio of absorbance at 260/280 nm.

Genotyping of the *WNT3* rs3809857 G/T and rs9890413 G/A SNPs in the *WNT3* was performed by PCR-RFLP. PCR-RFLP allows rapid detection of point mutations based on unique patterns of restriction enzyme cutting in specific regions of DNA. After the

amplification of genomic sequences by PCR, the mutation was discriminated by digestion with specific restriction endonucleases and was identified by gel electrophoresis. This method is especially useful in studies of complex genetic diseases¹⁵.

Briefly, a total volume of 20 μ l containing 30 ng of genomic DNA, 10 pmol of each primer, 0.5 μ l dNTPs mix, 2 μ l of 10 $\times$ buffer and 1 U of Taq DNA polymerase with 1.5 mM MgCl₂ and sterile distilled water up to 20 μ l were mixed for amplification of the target sequences (All reagents were from Bioneer, South Korea). Amplification conditions started with an initial denaturation step of 4 min at 94°C, followed by 30 cycles of denaturation at 94°C (45 s), annealing of 61°C (40 s) and 72°C extension (30 s), ended by a final 72°C extension for 5 min. The PCR products of the rs3809857 G/T and rs9890413 G/A SNPs were digested with the BamHI and MspI restriction enzymes at 37°C overnight (New England BioLabs, Beverly, MA, USA). The PCR products were subjected to 8% polyacrylamide gel electrophoresis and stained with silver nitrate. The primer sequences and the pattern of restriction fragments are shown in table 1.

Statistical Analysis

A p-value of ≤ 0.05 was considered statistically significant. The Chi square (χ^2) and Fisher's exact tests using the SPSS statistical software (Version 11.0, Chicago, IL, USA) were performed to compare genotype and allele frequencies in the study groups. The sample was stratified by gender, and genotype and allele frequencies were compared between the study groups.

Results

Descriptive analysis showed that among all 113 NSCL/P infants, 54 (47.8%) and 53 (46.9%) were boys and girls, respectively, while among them six had unknown gender (5.3%). Of all 220 healthy controls, 217 had known gender: 111 males (50.5%) and 106 females (48.2%), while three (1.3%) had unknown gender.

The distributions of genotype using chi-square showed that in the control group for the *WNT3* rs9890413 G/A was in Hardy-Weinberg equilibrium (p-value=0.115).

The allelic and genotypic frequencies of the *WNT3* SNPs are shown in table 2. The *WNT3* rs3809857 GT genotype was significantly lower (P=0.039, OR=0.55, 95% CI=0.30-0.97) in the NSCL/P (21.2%) than the control group (30.42%). For the *WNT3* rs9890413 G/A

Table 1. Primer sequences and their related PCR product sizes for the *WNT3* rs3809857 and rs9890413 polymorphisms

Variants	Primer sequences (5'→3')	Size (bp)	Restriction enzymes	RFLP fragments (bp)
rs3809857	GGTCATCGTCTCTGCATGTG	285	BamHI	GG:285
	CTTCCTTCTTGCAGCACTCG3			GA: 150+135+285 AA: 150+135
rs9890413	CTCTCTTCCTGCCCCAGTC	177	MspI	GG:78+99
	AGGACAGGGCTAGGGAGTG			GA: 177+78+99 AA: 177

Table 2. Genotype distribution and allele frequency of the *WNT3* rs3809857 polymorphism in the case and control groups

Genotype/ allele groups	Cases (n=113)	Controls (n=217)	p-value	OR (95% CI)
GG	60 (53.1%)	91 (41.9%)	Reference genotype *	
GT	24 (21.2%)	66 (30.4%)	0.039	0.55 (0.30-0.97)
TT	29 (25.7%)	60 (27.7%)	0.269	0.73 (0.42-1.30)
G	144 (63.7%)	248 (57.1%)	Reference allele **	
T	82 (36.3%)	186 (42.9%)	0.103	0.76 (0.55- 1.06)

* The reference genotype has the highest frequency in the genotypes group. ** The reference allele has the higher frequency between the two alleles.

Table 3. Genotype distribution and allele frequency of the rs9890413 polymorphism in the case and control groups

Genotype/ allele groups	Cases (n=113)	Controls (n=220)	p-value	OR (95% CI)
AA	71 (62.8%)	138 (62.7%)	Reference genotype *	
AG	34 (30.1%)	71 (32.3%)	0.778	0.9 (0.56-1.53)
GG	8 (7.1%)	11 (5%)	0.476	1.4 (0.54- 3.60)
A	178 (78.8%)	347 (78.9%)	Reference allele **	
G	48 (21.2%)	93 (21.1%)	0.976	1.01 (0.70-1.50)

* The reference genotype has the highest frequency in the genotypes group. ** The reference allele has the higher frequency between the two alleles.

SNP, neither genotype nor allele frequencies were significantly different between the case and control groups (Table 3).

When the samples were stratified by gender, the *WNT3* rs3809857 GT genotype in the male sub-group was significantly lower than the one in the case and the control group (p-value=0.037, OR=0.42, 95% CI=0.20-0.96). When stratified by gender, the *WNT3* rs9890413 did not differ significantly between cases and controls in the male and female sub-groups.

Discussion

NSCL/P is a genetically complex disorder caused by the interaction of multiple genetic and environmental factors¹⁶. Among non-syndromic clefts, cleft lip/palate is twice more frequent in males than in females, while the cleft lip is twice as frequent in females¹⁷. The genes contributing to the etiology of NSCL/P have been investigated by linkage analysis, genomic rearrangements, candidate genes analysis and genome-wide association studies¹⁸. So far, several candidate genes including *MSX1*, *FGFR1*, *FGF8*, *BMP4* as well as *WNT* gene family have successfully been associated with the CL/P^{10,19}.

In the present study, the two *WNT3* gene polymorphisms (rs3809857 and rs9890413) were investigated to determine if any of them are implicated in the etiology of NSCL/P. Our results showed that the *WNT3* rs3809857 was associated with NSCL/P. The *WNT3* rs3809857 GT genotype was significantly lower (p=0.039, OR=0.55, 95% CI=0.30-0.97) in the NSCL/P than the control group. This result means that the individuals with the GT genotype showed approximately a

two-fold decreased risk of NSCL/P. However, no association between the *WNT3* rs9890413 G/A polymorphism and NSCL/P was found. When stratified by gender, the *WNT3* rs9890413 did not differ significantly between cases and controls in the male and female sub-groups. When stratified by gender, the *WNT3* rs3809857 GT genotype in the male sub-group was significantly lower in the case than the control group (p-value=0.037, OR=0.42, 95% CI=0.20-0.96).

There are a few studies which evaluated the association of *WNT* genes with the risk of NSCL/P. Our results are consistent with the results of the studies conducted by Mostowska *et al*⁹ and Lu *et al*²⁰. This indicated that the common G allele may be a risk factor, and the minor T allele may be a protective factor for NSCL/P²⁰. The minor allele frequency (MAF) of *WNT3* rs9890413 in *WNT3* northeast China (MAF=0.03) is lower than the one in the European American (MAF=0.363), and Caucasian populations (MAF=0.32). This distinction may be attributed to the population heterogeneity between the northeast China and the western countries²⁰.

The *WNT* gene family plays a critical role in the development of the lip and ectoderm. The *WNT* gene family consists of structurally related conserved genes which encode secreted signaling glycoproteins proteins that play a fundamental role in developmental and cell-biological processes^{21,22}. The binding of Wnt ligands to Frizzled receptors, a family of G protein-coupled receptor, activates several distinct intracellular signaling pathways, such as the best understood canonical Wnt/b-catenin pathway²³. Liu and Millar²⁴ reported that the dynamic activation of the WNT/beta-catenin signaling

pathway was correlated with the occurrence of a cleft lip and cleft palate. Abnormal Wnt signaling has been associated with many human diseases, such as cancer, degenerative diseases, or osteoporosis^{22,25,26}. Wnt signaling also plays a crucial role in various aspects of craniofacial development, which was suggested by studies of phenotypes observed in knockout mouse embryos^{23,26}. Mutations and markers in genes encoding Wnt pathway²⁷ components might be correlated with oral congenital anomalies, including cleft lip and palate²⁴. Cleft lip with cleft palate may be induced by genetic inactivation of Lrp6, a co-receptor of the *WNT*/beta-catenin signaling pathway²⁸.

NSCL/P risk factors differ among populations which confirm the importance of testing putative susceptibility variants in different genetic backgrounds²⁹. Epidemiological data support a role for the environmental risk factors in the development of orofacial clefts. Maternal smoking has been consistently associated with an increased risk of clefting, with a population-attributable risk estimated as high as 20% and an odd ratio of 1.3 for the CL/P³⁰. Nutrition during pregnancy has been suggested as another contributing factor based on observational and interventional studies using folate supplements as a preventive measure³¹. The beneficial effect of folate use, however, remains controversial and has not been consistently replicated¹⁸.

Conclusion

Mutations and polymorphisms in components of Wnt signaling in NCL/P are not strong. Genome-wide association studies for CL/P did not confirm any role of the *WNT* signaling genes in the etiology of NSCL/P. Accordingly, further studies are required to explore the role of *WNT* genes during human craniofacial development and to identify possible functional variants and/or haplotypes in these genes that may influence the risk of NSCL/P in different populations⁹.

In conclusion, the *WNT3* rs3809857 SNP was associated with the non-syndromic CL/P and the *WNT3* GT genotype may have a protective effect against NSCL/P in Iranian population.

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Conflict of Interest

The authors declare no conflicts of interest.

References

1. 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.
2. Gorlin RJ, Cohen MM, Hennekam RCM. Syndromes of the head and neck. 4th ed. New York: Oxford University Press; 2001. 1215 p.
3. Rajabian MH, Sherkat M. An epidemiologic study of oral clefts in Iran: analysis of 1669 cases. *Cleft Palate Craniofac J* 2000;37(2):191-196.
4. Aldhore KA, Böhmer AC, Ludwig KU, Esmail AH, Al-Hebshi NN, Lippke B, et al. Nonsyndromic cleft lip with or without cleft palate in arab populations: genetic analysis of 15 risk loci in a novel case-control sample recruited in Yemen. *Birth Defects Res A Clin Mol Teratol* 2014;100(4):307-313.
5. Grosen D, Chevrier C, Skytthe A, Bille C, Mølsted K, Sivertsen A, et al. A cohort study of recurrence patterns among more than 54,000 relatives of oral cleft cases in Denmark: support for the multifactorial threshold model of inheritance. *J Med Genet* 2010;47(3):162-168.
6. Beaty TH, Taub MA, Scott AF, Murray JC, Marazita ML, Schwender H, et al. Confirming genes influencing risk to cleft lip with/without cleft palate in a case-parent trio study. *Hum Genet* 2013;132(7):771-781.
7. Brugmann SA, Goodnough LH, Gregorieff A, Leucht P, ten Berge D, Fuerer C, et al. Wnt signaling mediates regional specification in the vertebrate face. *Development* 2007;134(18):3283-3295.
8. Lan Y, Ryan RC, Zhang Z, Bullard SA, Bush JO, Maltby KM, et al. Expression of Wnt9b and activation of canonical Wnt signaling during midfacial morphogenesis in mice. *Dev Dyn* 2006;235(5):1448-1454.
9. Mostowska A, Hozyasz KK, Biedziak B, Wojcicki P, Lianeri M, Jagodzinski PP. Genotype and haplotype analysis of *WNT* genes in non-syndromic cleft lip with or without cleft palate. *Eur J Oral Sci* 2012;120(1):1-8.
10. Chiquet BT, Blanton SH, Burt A, Ma D, Stal S, Mulliken JB, et al. Variation in *WNT* genes is associated with non-syndromic cleft lip with or without cleft palate. *Hum Mol Genet* 2008;17(1):2212-2218.
11. Yao T, Yang L, Li PQ, Wu H, Xie HB, Shen X, et al. Association of Wnt3A gene variants with non-syndromic cleft lip with or without cleft palate in Chinese population. *Arch Oral Biol* 2011;56(1):73-78.
12. Feng C, Duan W, Zhang D, Zhang E, Xu Z, Lu L. A C392T polymorphism of the Wnt10a gene in nonsyndromic oral cleft in a northeastern Chinese population. *Br J Oral Maxillofac Surg* 2014;52(8):751-755.
13. Menezes R, Letra A, Kim AH, Küchler EC, Day A, Tanure PN, et al. Studies with Wnt genes and nonsyndromic cleft lip and palate. *Birth Defects Res A Clin Mol Teratol* 2010;88(11):995-1000.
14. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988;16(3):1215.
15. Ota M, Fukushima H, Kulski JK, Inoko H. Single nucleotide polymorphism detection by polymerase chain

- reaction-restriction fragment length polymorphism. *Nat Protoc* 2007;2(11):2857-2864.
16. 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.
 17. Mossey PA, Little J, Munger RG, Dixon MJ, Shaw WC. Cleft lip and palate. *Lancet* 2009;374(9703):1773-1785.
 18. Leslie EJ, Marazita ML. Genetics of cleft lip and cleft palate. *Am J Med Genet C Semin Med Genet* 2013;163 C(4):246-258.
 19. 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.
 20. Lu YP, Han WT, Liu Q, Li JX, Li ZJ, Jiang M, et al. Variations in WNT3 gene are associated with incidence of non-syndromic cleft lip with or without cleft palate in a northeast Chinese population. *Genet Mol Res* 2015;14 (4):12646-12653.
 21. Wodarz A, Nusse R. Mechanisms of Wnt signaling in development. *Annu Rev Cell Dev Biol* 1998;14:59-88.
 22. Macdonald BT, Tamai K, He X. Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell* 2009;17(1):9-26.
 23. Gordon MD, Nusse R. Wnt signaling: multiple pathways, multiple receptors, and multiple transcription factors. *J Biol Chem* 2006;281(32):22429-22433.
 24. Liu F, Millar SE. Wnt/beta-catenin signaling in oral tissue development and disease. *J Dent Res* 2010;89(4): 318-330.
 25. Nusse R. Wnt signaling in disease and in development. *Cell Res* 2005;15(1):28-32.
 26. Luo J, Chen J, Deng ZL, Luo X, Song WX, Sharff KA, et al. Wnt signaling and human diseases: what are the therapeutic implications? *Lab Invest* 2007;87(2):97-103.
 27. Mani P, Jarrell A, Myers J, Atit R. Visualizing canonical Wnt signaling during mouse craniofacial development. *Dev Dyn* 2010;239(1):354-363.
 28. Song L, Li Y, Wang K, Wang YZ, Molotkov A, Gao L, et al. Lrp6-mediated canonical Wnt signaling is required for lip formation and fusion. *Development* 2009;136(18): 3161-3171.
 29. Martinelli M, Girardi A, Cura F, Nouri N, Pinto V, Carinci F, et al. Non-syndromic cleft lip with or without cleft palate in Asian populations: Association analysis on three gene polymorphisms of the folate pathway. *Arch Oral Biol* 2016;61:79-82.
 30. Shi M, Wehby GL, Murray JC. Review on genetic variants and maternal smoking in the etiology of oral clefts and other birth defects. *Birth Defects Res C Embryo Today* 2008;84(1):16-29.
 31. Wehby GL, Murray JC. Folic acid and orofacial clefts: a review of the evidence. *Oral Dis* 2010;16(1):11-19.