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.

Keywords: Genome wide association study, Cleft lip/Palate, Polymorphism, WNT3
with NSCL/P in a Chinese population. In another study in a northeast Chinese population C392T SNP in the WNT3 gene and NSCL/P in Caucasian populations. The WNT3 rs9890413 was highly associated with NSCL/P in a Polish population. The WNT3 rs142167 and rs9890413 were shown to be associated with NSCL/P in a population of Caucasian ancestry.

The aim of this study was to evaluate the association between the two SNPs of WNT3, rs9890413 and rs-9890413, 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 µl of 0.5 M EDTA and genomic DNA was extracted from peripheral blood using the salting out method as described previously. 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 rs9890413 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.

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

<table>
<thead>
<tr>
<th>Variants</th>
<th>Primer sequences (5′→3′)</th>
<th>Size (bp)</th>
<th>Restriction enzymes</th>
<th>RFLP fragments (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs9890413</td>
<td>GGTCATCGTCCTGTCAATGTG</td>
<td>285</td>
<td>BamHI</td>
<td>GG:285</td>
</tr>
<tr>
<td></td>
<td>CTTCCCTCTTGACGACACTCG3</td>
<td></td>
<td></td>
<td>GA:150+135+285</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AA:150+135</td>
</tr>
<tr>
<td></td>
<td>CTCTCTCTCCGGCCCAGTC</td>
<td>177</td>
<td>MspI</td>
<td>GG:78+99</td>
</tr>
<tr>
<td></td>
<td>AGGGCAAGGGCCTAGGGAGTG</td>
<td></td>
<td></td>
<td>GA:177+78+99</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AA:177</td>
</tr>
</tbody>
</table>

Statistical Analysis

A p-value of ≤0.05 was considered statistically significant. The Chi square (χ²) 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 rs9890413 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
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 factors. 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 females. The genes contributing to the etiology of NSCL/P have been investigated by linkage analysis, genomic rearrangements, candidate genes analysis and genome-wide association studies. So far, several candidate genes including MSX1, FGFR1, FGFR8, BMP4 as well as WNT gene family have successfully been associated with the CL/P.

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 than the control group (p-value=0.039, OR=0.55, 95% CI=0.30-0.97).

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. and Lu et al. This indicated that the common G allele may be a risk factor, and the minor T allele may be a protective factor for NSCL/P. 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 countries.

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. 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 pathway. Liu and Millar reported that the dynamic activation of the WNT/beta-catenin signaling pathways...
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. 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. Mutations and markers in genes encoding Wnt pathway components might be correlated with oral congenital anomalies, including cleft lip and palate susceptibility variants in different genetic backgrounds, which confirm the importance of testing putative suspects using folate supplements as a preventive measure.

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-based odds ratio of 1.3 for the CL/P. Nutrition during pregnancy has been suggested as another contributing factor based on observational and interventional studies of phenotypes observed in knockout mouse embryos. Mutations and markers in genes encoding Wnt pathway might be correlated with the occurrence of a cleft lip and palate. Abnormal Wnt signaling has been associated with many human diseases, such as cancer, degenerative diseases, or osteoporosis. 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. Mutations and markers in genes encoding Wnt pathway components might be correlated with oral congenital anomalies, including cleft lip and palate susceptibility variants in different genetic backgrounds, which confirm the importance of testing putative suspects using folate supplements as a preventive measure.

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.

Acknowledgement

We would like to thank Dr. Roozrokh, Dean of Mofid Hospital, and Mofid Hospital staff for their kind helps in recruiting study subjects.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of Interest

The authors declare no conflicts of interest.

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