Association of Transforming Growth Factor Alpha Polymorphisms with Nonsyndromic Cleft Lip and Palate in Iranian Population

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Abstract

Background: Cleft lip with or without cleft palate (CL/P) is one of the most common congenital anomalies and the etiology of orofacial clefts is multifactorial. Transforming growth factor alpha (TGFA) is expressed at the medial edge epithelium of fusing palatal shelves during craniofacial development. In this study, the association of two important TGFA gene polymorphisms, BamHI (rs11466297) and RsaI (rs3732248), with CL/P was evaluated in an Iranian population.

Methods: The frequencies of BamHI and RsaI variations were determined in 105 unrelated Iranian subjects with nonsyndromic CL/P and 218 control subjects using PCR and RFLP methods, and the results were compared with healthy controls. A p-value of <0.05 was considered statistically significant.

Results: The BamHI AC genotype was significantly higher (p=0.016) in the patients (12.4%) than the control group (5.0%). The BamHI C allele was significantly higher (p=0.001; OR=3.4, 95% CI: 1.6-7.4) in the cases (8.0%) compared with the control group (2.5%).

Conclusion: Our study showed that there was an association between the TGFA BamHI variation and nonsyndromic CL/P in Iranian population.

Keywords: Association Study, Cleft lip/palate, Polymorphism, Transforming Growth Factor Alpha

Introduction

Cleft lip with or without cleft palate (CL/P) is one of the most common birth defects 1. The worldwide prevalence of CL and CL/P is 3.28 and 6.64 per 10,000 cases, respectively 2,4. Genetic factors are thought to contribute to the development of this disorder, because the risk of recurrence of CL/P within a family is approximately 28-40-fold greater for the general population 5,6. Nonsyndromic cleft in humans is most likely due to combination of genetic and environmental factors 7-9. Population based candidate gene studies as well as linkage disequilibrium has been used to identify the etiology of CL/P so as to predict its occurrence and to prevent it from occurring in the future. 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 the diagnosis, prevention and treatment of developmental anomalies of this region 9,10.

The association between CL/P and specific alleles in the transforming growth factor alpha (TGFA) gene suggests that TGFA could be a candidate gene for CL/P 11-15.

In 1989, Ardinger et al published the first association study of CL/P with five candidate genes which were involved in palate formation. Analysis of 80 unrelated patients from Iowa showed that there were significant associations of CL/P with TaqI and BamHI RFLPs at the TGFA locus 34. Holder et al in a British
population \(^24\), Tanabe \(et\ al\) in a Japanese population \(^{30}\) and Stoll \(et\ al\) in the French population \(^{25}\) indicated that the \(TGFA\) gene variant contributes to the occurrence of nonsyndromic CL/P. However, this is contrary to a study done by Lidor et al. in the Philippines \(^{36}\), which may be due to genetic differences in different populations.

\(TGFA\) is, both structurally and functionally, similar to Epidermal Growth Factor (EGF), and induces a mitogenic response by binding to and stimulating the tyrosine kinase activity of EGF receptor \(^{16,17}\). During mitogenic response by binding to and stimulating the Epidermal Growth Factor (EGF), and induces a mitogenic response.

Furthermore, in palatal cultures, \(TGFA\) promotes synthesis of extracellular matrix and migration of mesenchymal cells to ensure the strength of the fused palate during seam disruption \(^{20,24}\).

The \(TGFA\) gene is located on chromosome 2p13 \(^{11}\), contains six exons and spans 80 \(kb\) of genomic DNA. Three common polymorphisms of the \(TNFA\) gene (RsaI, and TaqI in intron 5 and BamHI in exon 6) have been investigated with susceptibility to the CL/P \(^{25-27}\). During craniofacial development, \(TGFA\) is expressed at the medial edge epithelium of fusing palatal shelves \(^{18,19}\). In palatal cultures, \(TGFA\) promotes synthesis of extracellular matrix and migration of mesenchymal cells to ensure the strength of the fused palate during seam disruption \(^{20,24}\). The aim of the present study was to investigate the association of the two common polymorphisms of the \(TGFA\) gene, BamHI and RsaI, in the development of nonsyndromic CL/P in an Iranian population for the first time.

**Materials and Methods**

**Subjects**

To determine the possible role of BamHI and RsaI polymorphisms in the \(TGFA\) gene in developing oral clefts in an Iranian population, a case-control study was performed. A sample of 105 newborns with nonsyndromic CL/P and 218 control subjects were included. A clinical examination to look for dysmorphic features (such as lip pits) was undertaken. The exclusion criteria of this study were 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 pediatrics center in Tehran, Iran in 2013-15. A control group consisted of 218 Iranian newborns, without cleft, who were born in or around Tehran between the years 2013 and 2015 were selected and their blood samples were stored. Ethical approval for the study was obtained from the Ethics Committee of the Dental Research Center of the University of Shahid Beheshti. Informed consent was obtained from all parents.

**DNA extraction and genotyping**

Five ml of peripheral blood samples were collected in tubes containing 200 \(\mu\)l of 0.5 M EDTA and genomic DNA was extracted from peripheral blood using the salting out method \(^{32}\). Genotyping of the BamHI (rs11466297) and RsaI (rs3732248) polymorphisms in the \(TGFA\) gene was performed by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) methods, according to the previous study. The primer sequences are shown in table 1. Briefly, a total volume of 25 \(\mu\)l containing 30 ng of genomic DNA, 10 \(pmol\) of primer, 1 \(\mu\)l dNTPs mix (Fermentas, Life Science), 2.5 \(\mu\)l 10×buffer and 0.5 \(U\) of Taq DNA polymerase (Fermentas Life Science, Lithuania) with 1.5 \(mM\) MgCl2 was prepared in the 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 denaturation (94°C), 30 s annealing (60°C) and 40 s extension (72°C), ended by a final extension for 5 min (72°C) and finally cooling to 4°C. The PCR products of the rs11466297 and rs3732248 polymorphisms were digested with the IU BamHI and Rsal restriction enzymes at 37°C overnight, respectively (New England BioLabs, Beverly, MA, USA). All PCR products were subjected to 8% polyacrylamide gel electrophoresis and stained with silver nitrate. The pattern of restriction fragments for both BamHI and RsaI are shown in table 1.

**Statistical analysis**

Chi square (\(\chi^2\)) and Fisher’s exact test with Open Epi Version 2.2 (free statistical software) were performed to compare genotype and allele frequencies in the study groups. The \(p<0.05\) were considered statistically significant. Statistical significance was corrected for multiple testing comparisons.

**Results**

The samples consisted of 105 patients with cleft lip with or without cleft palate and 218 healthy controls. The CL/P samples consisted of 65 males (62.0%) and 40 females (38.0%). A positive family history of cleft was observed in 38 CL/P cases (36.19%). There were 34 (32.3%) patients with unilateral CL/P, 27 (25.7%) with bilateral CL/P, 15 (14.2%) cleft lip only and 29 (27.6%) with cleft palate only. The distributions of genotypes using chi-square showed that in both case

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Global MAF</th>
<th>Primer Sequence (5→3)</th>
<th>Product Size (bp)</th>
<th>RFLP Fragments (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BamHI (rs11466297 A/C)</td>
<td>C=0.0238</td>
<td>F: GCCGTGCTTTATTTTGCGGATT R: AAGGGCCACCGGAAACACACGCG</td>
<td>174</td>
<td>A allele=120+54</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C allele=174</td>
</tr>
<tr>
<td>RsaI (rs3732248 C/T)</td>
<td>A=0.2075</td>
<td>F: TGGCTTCCGCTTTATTTTGCGGATT R: CAGGACGCGGCACGCGAAGT</td>
<td>166</td>
<td>C allele=91+75</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>T allele=166</td>
</tr>
</tbody>
</table>

* Global Minor Allele Frequency
TGFA Polymorphisms and Nonsyndromic CL/P

Table 2. The genotype and allele frequencies of the TGFA BamHI and RsaI polymorphisms in nonsyndromic CL/P patients and controls

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Genotype/Allele</th>
<th>Cases (n=105)</th>
<th>Controls (n=218)</th>
<th>p-value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BamHI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(rs11466297)</td>
<td>AA</td>
<td>90 (85.7%)</td>
<td>207 (95.0%)</td>
<td>Reference Genotype</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AC</td>
<td>13 (12.4%)</td>
<td>11 (5.0%)</td>
<td>0.016</td>
<td>2.1 (1.2-6.3)</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>2 (1.9%)</td>
<td>0 (0.0%)</td>
<td>0.187*</td>
<td>undefined'</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>193 (92.0%)</td>
<td>425 (97.5%)</td>
<td>Reference Allele</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>17 (8.0%)</td>
<td>11 (2.5%)</td>
<td>0.001</td>
<td>3.4 (1.6-7.4)</td>
</tr>
<tr>
<td>RsaI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(rs3732248)</td>
<td>CC</td>
<td>68 (64.8%)</td>
<td>127 (58.3%)</td>
<td>Reference Genotype</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>32 (30.5%)</td>
<td>69 (31.6%)</td>
<td>0.582</td>
<td>0.87 (0.7-2.1)</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>5 (4.7%)</td>
<td>22 (10.1%)</td>
<td>0.090</td>
<td>0.42 (0.6-3.3)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>168 (80.0%)</td>
<td>323 (74.0%)</td>
<td>Reference Allele</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>42 (20.0%)</td>
<td>113 (26.0%)</td>
<td>0.099</td>
<td>0.71 (0.8-6.1)</td>
</tr>
</tbody>
</table>

* Fisher’s exact test p-value

Discussion

TGFA was chosen as a candidate gene in the preliminary association studies of CL/P, because it is expressed in palatal tissue in culture 16,30. It subsequently revealed that TGFA was present at high levels in epithelial tissue of the medial edge of the palatal shelves at the time of shelf fusion 17. The role of TGFA in lip and palate development was then evaluated in different populations.

This study was performed to examine whether the TGFA BamHI and RsaI polymorphisms were associated with the increased risk of CL/P in an Iranian population including 105 CL/P patients and 218 controls. Our results showed that there was a significant difference in the genotype distribution and allele frequency of the BamHI polymorphism between the case and control groups. The BamHI AC genotype was significantly higher (p=0.016; OR=2.1, 95% CI:1.2-6.3) in the patients (12.4%) than the control group (5.0%). The BamHI C allele was significantly higher (p=0.001; OR=3.4, 95% CI:1.6-7.4) in the cases (8.0%) compared with the control group (2.5%). In contrast, no significant difference in the genotype and allele frequencies of the RsaI polymorphism was found between the case and control groups.

and control groups, for the TGFA BamHI polymorphism, they were in Hardy-Weinberg equilibrium (p>0.05). For the TGFA RsaI polymorphism, the distributions of genotypes in the case group were in Hardy-Weinberg equilibrium (p=0.625). The genotype distributions and allele frequencies of the TGFA BamHI and RsaI polymorphisms are shown in table 2. The results of the genotyping for the BamHI and RsaI RFLP are shown in figures 1 and 2. Our results showed that there was a significant difference in the genotype distribution and allele frequency of the BamHI polymorphism between the case and control groups. The BamHI AC genotype was significantly higher (p=0.016; OR=2.1, 95% CI:1.2-6.3) in the patients (12.4%) than the control group (5.0%). The BamHI C allele was significantly higher (p=0.001; OR=3.4, 95% CI:1.6-7.4) in the cases (8.0%) compared with the control group (2.5%). In contrast, no significant difference in the genotype and allele frequencies of the RsaI polymorphism was found between the case and control groups.

Figure 1. TGFA BamHI RFLP. Three genotypes from CL/P cases demonstrating the wild type (W), Heterovariant (H) and Homovariant (V). After digestion with the restriction enzyme BamHI, the amplified product was completely digested with one restriction site and two specific bands of 120 bp and 54 bp were indicated in wild type genotype.

Figure 2. TGFA RsaI RFLP. Three genotypes from CL/P cases demonstrating the wild type (W), Heterovariant (H) and Homovariant (V). After digestion with the restriction enzyme RsaI, the amplified product was completely digested with one restriction site and two specific bands of 91 bp and 75 bp were indicated in wild type genotype.
spective, which are very close to the global minor allele frequencies (0.024 and 0.208, respectively).

Ardinger et al (1989) investigated the possible association of five candidate genes including TGFA, Nuclear Receptor subfamily 3 group C member 1 (NR3C1), Epidermal Growth Factor (EGF), Epidermal Growth Factor Receptor (EGFR) and estrogen receptor (ESR) in an American population with nonsyndromic CL/P. They found a significant association between the TGFA BamHI and TaqI polymorphisms and the occurrence of cleft. Their results suggest that TGFA gene or adjacent DNA sequences may contribute to the development of a portion of cases with CL/P 31. Holder et al (1992) studied the three variations of TGFA (BamHI, TaqI and RsaI) in a British population with CL/P, and they found a significant association between the TaqI polymorphism and occurrence of cleft 24. Stoll et al (1997) evaluated the two polymorphisms of TGFA in unrelated Australians with CL/P and a significant association between the TGFA TaqI and BamHI polymorphism and CL/P was confirmed 30. Lidral et al (1997) evaluated the association of four candidate genes TGFA, TGFB2, TGFB3, homeobox 7 (MSX1) variations in a population from Philippines; however, no evidence for association of TGFA with nonsyndromic CL/P was found in non-Caucasian population 35. Tanabe et al (2000) assessed the association of polymorphisms of candidate genes TGFA, TGFB and gamma-aminobutyric acid type A receptor beta3 (GABRB3) with nonsyndromic CL/P in Japanese patients, and they found that the TGFA and TGFB2 polymorphisms were associated with CL/P 30.

Conclusion

In conclusion, our study showed that there was an association between the TGFA BamHI variation and nonsyndromic CL/P in Iranian population. Since common environmental exposures especially maternal smoking could play a role in the CL/P etiology, it is suggested that further works be done to explore the role of possible gene-environment interaction in the etiology of CL/P.

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Conflict of Interest

The authors report no conflicts of interest.

References


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