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 7-8. 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 Lidral 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 to Epidermal Growth Factor (EGF), and induces a


tions.

may be due to genetic differences in different popula-

polymorphisms and the risk of nonsyndromic CL/P

of the present study was to investigate the association

TGFA in the development of nonsyndromic

of the two common polymorphisms of the

was performed. A sample of 105 newborns with

clefts in an Iranian population, a case-control study

was formed to compare genotype and allele frequencies in

Epi Version 2.2 (free statistical software) were per-

for multiple testing comparisons.

Statistical analysis

Chi square (χ²) and Fisher’s exact test with Open

Epi Version 2.2 (free statistical software) were per-

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

materials and Methods

Subjects

To determine the possible role of BamHI and Rsal
polymorphisms in the TGFA gene in developing oral

lefts 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 dysmor-

phic 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, con-

genital heart lesion, etc), metabolic or neurologic dis-

orders or anomalies of other organ systems. Samples

were recruited from Mofid Hospital, a referral pediat-

rics 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 µl of 0.5 M EDTA and ge-
mic DNA was extracted from peripheral blood using

the salting out method 32. Genotyping of the BamHI

(rs11466297) and Rsal (rs3732248) polymorphisms in

the TGFA gene was performed by polymerase chain

reaction (PCR) and restriction fragment length poly-

morphism (RFLP) methods, according to the previous

study. The primer sequences are shown in table 1.

Briefly, 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 Sci-

ence, 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, fol-

led 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 over-

night, respectively (New England BioLabs, Beverly,

MA, USA). All PCR products were subjected to 8% poly-

acrylamide gel electrophoresis and stained with

silver nitrate. The pattern of restriction fragments for

the two common polymorphisms of the TGFA gene,

BamHI and Rsal, in the development of nonsyndromic

CL/P in an Iranian population for the first time.

Materials and Methods

Table 1. Primer sequences and their PCR product sizes, restriction enzymes, and RFLP fragments for the TGFA BamHI and Rsal polymorphisms

<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: GCCTGCGTTATTTGGGGATT R: AAGGGCAAGGAAACACAGG</td>
<td>174</td>
<td>A allele=120+54 C allele=174</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>33</td>
</tr>
<tr>
<td>Rsal (rs3732248 C/T)</td>
<td>A=0.2075</td>
<td>F: TGGCTTCTTCTGTATATC   R: CAGGCCAATGTCACCAAGT</td>
<td>166</td>
<td>C allele=91+75 T allele=166</td>
</tr>
</tbody>
</table>

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

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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 CL/P patients (8.0%) compared with the control group (2.5%). This result suggests that the C allele may be a risk factor for CL/P in Iranian population. In contrast, no significant difference in the genotype and allele frequencies of the RsaI polymorphism was found between the case and control groups.

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 (rs11466297 A/C) and RsaI (rs3732248 C/T) variations are associated with the increased risk of CL/P in an Iranian population including 105 CL/P patients and 218 controls. Our results showed that TGFA BamHI polymorphism was associated with the CL/P in Iranian population. The frequency of the BamHI AC genotype in the patients (12.4%) was approximately twice more than that of 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.

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

* Fisher’s exact test p-value

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.
respectively, 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 35. 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. Chenevix-Trench et al (1992) studied the two polymorphisms of TGFA, TGFB2 polymorphisms were associated with CL/P 30. Japanese patients, and they found that the T

receptor beta3

mucosal CL/P. They found a significant association between the TaqI polymorphism and occurrence of cleft 24. Stoll et al (1989) investigated the possible association of five candidate genes including CL/P was confirmed 34. 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.

Acknowledgement

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Conflict of Interest

The authors report no conflicts of interest.

References


