Original Article

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.

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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 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 *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]. The results of the association studies of *TGFA* gene polymorphisms and the risk of nonsyndromic CL/P have been contradictory and conflicting [28-31]. 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 *µl* 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 *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* $MgCl_2$ 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 RsaI 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 (χ^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 1. Primer sequences and their PCR product sizes, restriction enzymes, and RFLP fragments for the *TGFA* BamHI and RsaI polymorphisms

SNPs	Global MAF*	Primer Sequence (5′→ 3)	Product Size (*bp*)	RFLP Fragments (*bp*)	
BamHI (rs11466297 A/C)	C=0.0238	F: GCCTGGCTTATTTGGGGATT R: AAGGGCAAGGAAACACAGG	174	A allele=120+54 C allele=174	33
RsaI (rs3732248 C/T)	A=0.2075	F: TGCCTTCCTTCTGCTATCACT R: CAGAGCCAATGTCACCAAGT	166	C allele=91+75 T allele=166	33

* Global Minor Allele Frequency

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

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

* Fisher's exact test p-value

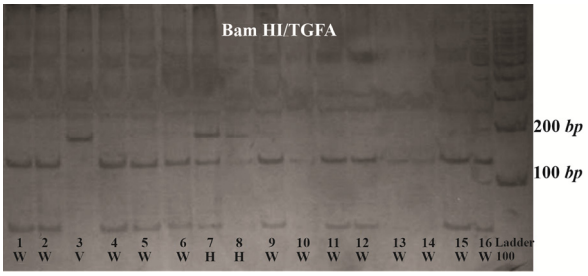

Figure 1. *TGFA* BamHI RFLP. Three genotypes from CL/P cases demonstrating the wild type (W), Heterovariant (H) and HomoVariant (V). After digestion with the restriction enzyme BamHI, the amplified product was completely digested with one restriction site and two specific bands of 120 *bp* and 54 *bp*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

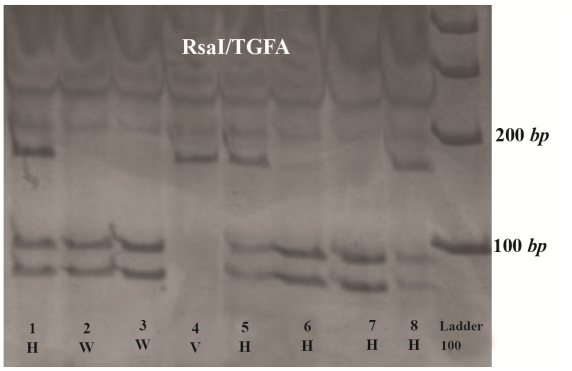

Figure 2. *TGFA* RsaI RFLP. Three genotypes from CL/P cases demonstrating the wild type (W), Heterovariant (H) and Homovariant (V). After digestion with the restriction enzyme RsaI, the amplified product was completely digested with one restriction site and two specific bands of 91 *bp* and 75 *bp* were indicated in wild type genotype.

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 Rsa1 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.

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 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. The minor allele frequencies in the control groups, for the BamHI and RsaI polymorphisms were C=0.025 and A=0.260, re-

spectively, 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 [33]. 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* (1992) detected a significant association with BamHI and not with TaqI in a French population of Alsatian ancestry with CL/P. They concluded that *TGFA* may be a modifier gene, not a major gene that may play a role in the development of bilateral cleft in some individuals [25]. Chenevix-Trench *et al* (1992) studied 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 [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 T*GFA* 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.

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