

Parental cigarette smoking, transforming growth factor-alpha gene variant and the risk of orofacial cleft in Iranian infants

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ABSTRACT

Objective(s): We investigated the influence of genetic variation of the transforming growth-factor alpha (*TGFA*) locus on the relationship between smoking and oral clefts.

Materials and Methods: In this study 105 Iranian infants with non-syndromic cleft lip/palate and 218 controls with non-cleft birth defects were examined to test for associations among maternal exposures, genetic markers, and oral clefts. Maternal and parental smoking histories during pregnancy were obtained through questionnaire. DNA was extracted from newborn screening blood samples, and genotyping of the BamHI polymorphism in the *TGFA* gene was performed by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) methods.

A number of factors including gender of the newborns, type of oral cleft, consanguinity of the parents, as well as the mother's age and education were evaluated as potential confounders and effect modifiers.

Results: Maternal smoking, in the absence of paternal smoking, was associated with an increased risk for CL/P (OR = 19.2, 95% CI = [(6.2-59.5)]) and cleft palate only (OR = 48.7, 95% CI = [(8-29.3)]). If both parents smoked, risks were generally greater (OR = 55.6, 95% CI = [12-20.25]). Analyses for the risk of clefting from maternal smoking, stratified by the presence or absence of the *TGFA*/BamHI variant, revealed that the risk of clefting among the infants with the *TGFA*/BamHI variant when their mothers smoked cigarettes was much greater than the infants who had non-smoker mothers ($P=0.001$, OR=10.4, 95% CI=[3.2,33.6]).

Conclusion: The results of this study indicate that first-trimester maternal smoking and infant *TGFA* locus mutations are both associated with nonsyndromic cleft lip and/or palate (CL/P).

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Introduction

Cleft lip and palate (CL/P) is a common birth defect which its risk factors include genes, environment, and their interaction (1, 2), gender (3), geographic location (4), nationality (4), and nutritional (5) and periconceptional consumption of folic acid (6-10). It has also been reported that tobacco use (11), antiepileptic drugs, and possibly alcohol consumption (12, 13), as well as birth weight, increase the incidence rate of oral clefts in newborns. Several studies have also reported that racial/ethnic factors (14, 15) and consanguinity (16-19) have an effect on the incidence rate of oral clefts, but findings have been inconclusive. Smoking has attracted special attention because of its common exposure (20), and common environmental exposures could

play a role in the CL/P etiology if they accompany with other etiologic factors (21-23).

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 (4, 24). Identification of the genes involved in the development of the human craniofacial region can be considered as a first step towards developing a better understanding of the diagnosis, prevention and treatment of developmental anomalies of this region (25).

Studies of segregation analysis have been extended by using candidate genes and linkage disequilibrium (24, 25). The association detected between CL/P and specific alleles in the transforming growth-factor

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alpha (*TGFA*) gene strongly supports *TGFA* as a major gene component in CL/P (26,27). Subsequent work demonstrated that during craniofacial development *TGFA* was presented at high levels in epithelial tissue of the medial edge of the palatal shelves at the time of shelf fusion (28). 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 (26-27).

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.

Studies of gene-environment interaction effects have become increasingly important for complex traits such as clefting, whose etiology probably involves both genes and environmental factors (29, 30). The first reports about the interaction between maternal smoking and *TGFA* genotype in CLP etiology have been published within the last few years. A statistically significant interaction between maternal smoking and infant *TGFA* genotype for isolated cleft palate (CP) was found by Hwang *et al* (31). A meta-analysis of 24 case-control and cohort studies of the association between maternal smoking during pregnancy and offspring oral clefts identified statistically significant associations between maternal smoking and CL/P and between maternal smoking and cleft palate only (CPO) (32). Thus, we conducted a case series study of Iranian infants born with an orofacial cleft, to investigate whether mothers who smoked cigarettes or fathers who smoked had an increased risk for having offsprings with orofacial clefts. We also investigated the influence of genetic variation of the infant's *TGFA* locus on the relation between maternal smoke exposures and clefting, to evaluate a possible gene-environment interaction among Iranian infants.

Materials and Methods

Samples were recruited from Mofid Hospital in Tehran, Iran, in 2013–2015. The control group consisted of DNA samples from 218 Iranians, without clefting (102 males and 116 females) whose blood samples were available. The case group consisted of 105 infants (65 males and 40 females) with nonsyndromic CL/P (76 cleft lip and palate and 29 cleft lip only). A clinical examination to look for dysmorphic features (such as lip pits) was undertaken. Cases with evidence of other facial or skeletal malformations were excluded from the study. Ethical approval for the study was obtained from the Ethics Committee of the Dental Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran. Informed consent was

obtained from all parents. Maternal smoking information in/at first trimester was obtained. The questionnaire addressing the relevant clinical and demographic factors for each case and control subject was completed by both the pediatrician and a nurse during an interview with the parents. The questionnaire data included infant's birth date and gender, type of oral cleft, consanguinity of the parents, paternal smoking, as well as the mother's age at pregnancy, education, and smoking status.

Cigarette smoke exposure

In the questionnaire, to assess maternal smoking, women were asked for the number of cigarettes they smoked daily for the first-trimester maternal smoking. To assess paternal smoking, a woman was asked how many cigarettes per day her infant's biological father had smoked from 3 months before through 3 months after the gestation.

TGFA genotyping

Genomic DNA was extracted from peripheral blood lymphocytes according to the method described by Hwang (33).

SNP genotyping was performed using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. The Polymerase Chain Reaction (PCR) primer was designed based on the SNP flanking sequence described in the Ensembl genome browser.

The *BamHI* polymorphic site is located within exon VI, thus, for screening of the *BamHI* variant in the *TGFA* gene, exon VI of the gene was amplified by PCR using standard conditions along with modified primers (Forward Primer: gcttgcttatttggggatt 3' and Reverse Primer: 5'aaagggaaggaaacacagg'3). DNA fragments were separated and visualized by electrophoresis using 8% polyacrylamide gels. 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.

Statistical analyses

Data were analyzed by SPSS 11.5 (SPSS Inc. Chicago, USA). Descriptive results are shown as number and frequency. The odds ratio along with its 95% confidence interval was used to estimate the risk using logistic regression analysis. For each phenotypic case group, analyses were performed for maternal cigarette smoking, paternal smoking, and both parents' smoking. Analyses were also performed for maternal cigarette smoking for each phenotypic group with or without the uncommon (A2) *TGFA* allele. Maternal age and education were considered as potential covariates. Analyses were performed controlling for the influence of maternal education (less than a high school graduate, high

Table 1. Multivariate analysis to assess the effect of study variables on congenital cleft lip and/or palate malformation in the case group (n= 105) and control group (n= 218) of a case-control study

Study variables	Case group (n=105) (%)	Control group (n=218)(%)	OR (CI 95%)	P-value
Age at pregnancy (years):				
>25	45.7	40.8	1	ref*
25-35	51.4	49.5	3.3(0.3-2.9)	0.006
<35	2.9	9.6	17.5(1.04-7)	0.001
Maternal education:				
Not a high school graduate	58.1	33.9	1	Ref*
High school graduate	34.3	41.3	3.3(1.4-7.85)	0.006
College graduate	7.6	24.8	17.5(3.4-90.4)	0.001
Male child	61.9	46.8	0.95(0.43- 2.1)	0.892
Familial marriage	43.8	6	18.3(2.4-89.4)	0.001
Maternal smoking	41	5	14.7(5.4-75.4)	0.001
Paternal smoking	23.8	31.2	NA	0.254

*Reference group

school graduate and college graduate), age (<25, 25–35, >35 years), and infant's sex. *P*-values less than 0.05 were considered statistically significant.

Results

The 105 cases consisted of 76 with CL/P and 29 with isolated CP. The association of the evaluated risk factors with the occurrence of the cleft lip and/or palate CL/P is depicted in Table 1. The mean maternal age at pregnancy in the case and control groups were 25.9 and 25.1 years, respectively. There was a significant association between maternal age and increased risk for oral clefts (OR=17.5, CI 95% [1.04-7], *P*=0.001). CL/P case mothers were more likely to have had male infants as compared with control mothers, but there was no significant association between infant gender and oral clefts (*P*=0.95). Consanguineous marriages in parents of infants with a CL/P were 43.8%, whereas this rate was 6% in the control group. There was a significant association between consanguineous marriages and an increased risk of oral clefts (OR = 18.3, CI 95% [2.4- 89.4], *P*= 0.001). CL/P case mothers were less likely either to have attended a college or graduated from a college (*P*=0.001). A total of 5% of mothers in

the control group and 41% in the case group reported smoking before conceiving or during pregnancy. Case mothers reported smoking during the month before conception more frequently than did control mothers (41% vs. 5%). There was significant association between maternal smoking and increased risk for oral clefts (OR = 14.7, 95% CI = [5.4-75.4], *P*= 0.001), but there was no significant association between paternal smoking and oral clefts (*P*= 0.254).

Table 2 shows risk estimates associated with maternal smoking in the absence or presence of paternal smoking and vice versa. Among nonsmoking women, we found no evidence of an increased risk for clefting from paternal smoking. In the case of paternal smoking, in the absence of maternal smoking, the odds ratios were 0.45 and 11.2 with 95% CIs [0.2, 1.05] and [3.1, 40.3] for CL/P and isolated CP, respectively.

In contrast, maternal smoking, in the absence of paternal smoking, was associated with an increased risk for CL/P and CPO (OR = 19.2, 95% CI= [6.2, 59.5]; OR = 48.7, 95% CI = [8-293]), respectively. If both parents smoked, however, risks were generally greater than if only the mother smoked (OR = 55.6, 95% CI= [12-256]).

Table 2. Risk estimates for maternal and paternal cigarette smoking by case groupings

Paternal no. of cigarettes smoked/ day	*N	Maternal no. of cigarettes smoked/day		*N	OR &95% CI	P-value
		0	1-19			
Isolated CL±P (n= 76)	38	ref**		20	19.2 (6.2-59.5)	0.021
Isolated CP (n= 29)	7	0.45 (0.2-1.05)	0.254	11	6 (2.2-16.6)	0.005
	3	ref		4	48.7 (8- 293)	0.041
	14	11.2 (3.1-40.3)	0.031	8	55.6 (12-256)	0.023

*Number of case mothers/fathers in a given category. Number in parentheses is the number of control mothers/fathers.**Reference group

Table 3. Risk estimates for maternal cigarette smoking from 1 month before through 3 months after the conception, by case groupings and *TGFA* genotypes

	Maternal no. of cigarettes smoked/day	TGF-alpha(Bam H1)			
		AA		AC+CC	
		N*	OR & 95% CI	N*	OR & 95% CI
Isolated CL±P (n = 76)	0	42	ref**	3	2.6 (0.6-12.1)
	1-19	28	1.5 (0.9-2.8)	3	1.6 (0.4-6)
Isolated CP (n = 29)	0	15	ref	2	4.8 (0.8-28.8)
	1-19	5	0.8 (0.28-2.3)	7	10.4 (3.2-33.6)***

*Number of case mothers/fathers in a given category. Number in parentheses, number of control mothers/fathers. **Reference group
*** $P=0.001$

The BamH1 *TGFA* polymorphism was genotyped for 76 CL/P cases, 29 isolated CP cases, and 218 controls. Compared with controls, genotype logistic regression analysis showed BamH1 genotype was associated with increased CL/P susceptibility ($P=0.001$, OR = 3.4, 95% CI =1.6, 7.4).

Our previous study showed that 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%) [34].

Analyses for the risk of clefting from maternal smoking, stratified by the presence or absence of the BamH1 *TGFA* polymorphism, revealed that the risk of clefting among infants with the BamH1 *TGFA* polymorphism was much greater than for infants with the common allele when their mothers smoked cigarettes (Table 3). Thus, maternal first-trimester smoking and infant *TGFA* locus mutations are associated with isolated CP and that a synergistic effect of these two risk factors occurs ($P=0.001$, OR = 10.4, 95% CI =3.2, 33.6]).

Discussion

The results of this study suggest that maternal, and not paternal, smoking during early pregnancy is associated with a 14.6-fold increased risk for orofacial cleft defect in infants.

Several studies have examined the effect of maternal smoking on the risk for oral clefts. Using a Swedish registry, Kallen (34) performed a large case-control analysis with a total of 1,834 oral cleft cases and found a statistically significant association between maternal smoking and both CL/P (OR = 1.64, 95% CI = [1.33 to 2.02]) and CP (OR = 1.42, 95% CI = [1.06 to 1.90]). Wyszynski *et al* (32) performed a meta-analysis utilizing the results from 11 published studies and found an overall OR of 1.29 (95% CI = [1.18, 1.42]) for any oral cleft in children of women who smoked. Their attributable risk of 11% suggests that smoking is a general risk factor for all oral clefts, either with isolated CL/P or with CP alone. The increased risk observed for maternal smoking is consistent with Kallen *et al* (34) and Wyszynski *et al* (32) and contrasts Saxen (35), Evans *et al* (36), Hemminki *et al* (37), and Shiono *et al* (38),

which may be due to genetic differences in that population.

A study in 2015 (39) evaluated the relationship between maternal passive smoking and nonsyndromic orofacial clefts, and compared the associations between passive and active smoking. Results of this systematic review showed that maternal passive smoking exposure results in a 1.5 fold increase in the risk of orofacial clefts, similar to the magnitude of risk reported for active smoking. But the results of our study suggest that maternal, and not paternal, smoking during early pregnancy is associated with a 14.6-fold increased risk for orofacial cleft, which may be due to differences in the distribution of cleft types, adjustment for covariates, broad geographic region, or study bias/quality.

No previous study has examined the clefting risk to offspring from both parents' smoking in the Iranian population.

Our study showed that the risk of orofacial clefting is influenced by the interaction between genotype (*TGFA*) and maternal smoking. Also, there was a strong association between maternal smoking and a genetic variant of *TGFA* in the risk for clefting. Similar to our results, Hwang *et al* (31) showed that there was an increase in the number of infants with CP who carried the *TGFATaq1* variant compared with controls with an isolated, non-cleft birth defect, and this difference was greatly increased among infants of mothers who smoked. The biological mechanisms of how maternal smoking and *TGFA* genotype interact in the etiology of oral clefts remain unknown. It is very likely that several genes are involved in the etiology of nonsyndromic oral clefts and that these may interact with one another or with environmental risk factors, such as maternal smoking.

The teratogenic effects of smoking have been well documented. Cigarettes contain N-nitroso compounds and polycyclic aromatic hydrocarbons such as benzo (a) pyrene, some of which are known or suspected teratogens in laboratory animals (11, 40, 41).

The first reports about the interaction between maternal smoking and *TGFA* genotype in CL/P etiology have been published by Hwang *et al* (31); the study describes the association between CP, CL/P, and *TGFA* and shows there was an increase in the number of infants with CP who carried the

TGFATaq1 variant compared with controls with an isolated, noncleft birth defect, and this difference was greatly increased among infants of mothers who smoked.

Epidemiologic evidence pointing to maternal smoking during pregnancy are strongly suggested to be involved in the pathogenesis of nonsyndromic orofacial clefts (22, 42, 43-63). Several mechanisms for the detrimental actions of tobacco use have been proposed. It has been postulated (20) that women who smoked during pregnancy had compromised uteroplacental blood flow that could result in poor fetal development. Carbon monoxide affects oxygen transfer to the placenta, and nicotine constricts the uterine wall resulting in hypoxia. Hypoxia has been shown experimentally to induce orofacial cleft defects (44, 45) as well as other malformations.

Furthermore, cigarette smoking may decrease a pregnant woman's serum folate and is probably associated with an increased risk for clefting (46, 47).

Ebadifar *et al* in 2015 (48) evaluated the association of *MTHFR* gene single nucleotide polymorphisms (C677T and A1298C) and maternal supplementary folate intake with orofacial clefts in the Iranian population. They found that children carrying the 677TT variant of the *MTHFR* gene may have an increased risk of CL/P and the risk associated with this allele was obviously higher when the mothers did not use folic acid, which supports the fact that folic acid may play a role in the etiology of CL/P(47).

As mentioned earlier, *TGFA* is the most widely studied candidate gene associated with the risk for oral clefts since it was present at high levels in epithelial tissue of the medial edge of the palatal shelves at the time of shelf fusion (26). Thus, smoking may affect the expression of *TGFA* involved in palatogenesis (30-32). Hence, allelic variants in BamH1/*TGFA* demonstrated the influence of smoking on CL/P risk. Moreover, how the evidence relates to the observations made with the gene variant of *TGFA* is unknown but may prove informative to those investigating mechanisms underlying the relation between *TGFA* and lip and palate formation/closure.

Based on this study, oral clefts are more common in males, and this result was not statistically significant indicating there is no association between gender and oral clefts. This result is similar to other studies in Pakistan (49), Scotland (50), and Ireland (51).

According to our results, there was a significant association between maternal age and oral clefts, which was in accordance with studies done by Herkrath and coworkers (52) and is in contrast to a study by Jagomagi and colleagues (53), Abramowicz (54), and Fathololumi and colleagues (55).

Based on our findings, consanguinity was significantly associated with an increase in the rate of oral clefts. This result is similar to other reports (53-55), but several studies in; Iran (48, 56), Pakistan (49), and South India (57) have reported a non-significant association between familial matrimony and orofacial clefts.

We observed that there is a significant association between maternal education and the risk of oral clefts, and that CL/P case mothers had lower educational levels. Our finding is similar to a study in India by Reddy and colleagues (57) and is in contrast to a study by Lebby and colleagues (58). This difference in results could be related to differences in environmental exposure, racial and geographic differences (53-58).

Conclusion

To conclude, the demonstration of an interaction between maternal smoking and BamH1 variant may make it possible for risk counselors to identify couples for whom behavior modification may help substantially reduce oral cleft risk. As the genetic constitution is not modifiable, these findings emphasize the importance of preconception counseling of mothers-to-be on amendable lifestyle factors, such as smoking and dietary habits, in order to reduce the prevalence of CL/P in future generations. Thus, our data support the advice of cessation of smoking during pregnancy. The limitation of this study was the sample size. These data should be further investigated in larger studies and in other populations as well. It seems likely that future progress in the understanding of the etiologic basis for CL/P will come from such studies of gene-environment interaction.

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