

TGFA/Taq I polymorphism and environmental factors in non-syndromic oral clefts in Southern Brazil

Liliane Todeschini de Souza^(a)
 Thayne Woycinck Kowalski^(a)
 Ana Paula Vanz^(b)
 Roberto Giugliani^(b)
 Têmis Maria Félix^(a)

^(a)Laboratório de Medicina Genômica, Centro de Pesquisa Experimental, Hospital de Clínicas de Porto Alegre - HCPA, Porto Alegre, RS, Brazil.

^(b)Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre - HCPA, Porto Alegre, RS, Brazil.

Abstract: We report a study of *TGFA/Taq I* polymorphisms and environmental factors in non-syndromic oral cleft in Southern Brazil. Non-syndromic cleft case-parent triads were recruited to participate. Clinical data was collected with an emphasis on tobacco and alcohol use during pregnancy. DNA was extracted from peripheral blood and *TGFA/Taq I* polymorphisms were analyzed by PCR/RFLP with *Taq I* restriction enzyme. Association of clefts and *TGFA/Taq I* polymorphisms was determined using a transmission disequilibrium test (TDT). Association of environmental factors, clefts, and genotypes was evaluated with Fisher's exact test. The minor allele frequency was 0.064. We found no evidence of association between *TGFA/Taq I* polymorphisms and clefting (TDT $p = 0.335$). We also found no association between *TGFA/Taq I* polymorphisms and environmental factors (alcohol and/or tobacco). Therefore, no evidence was found that *TGFA/Taq I* polymorphisms play a role in clefting in this population. No evidence was found that tobacco or alcohol exposure during pregnancy was related to clefting, however a larger sample size is needed to confirm these results.

Descriptors: Cleft Lip; Cleft Palate; Polymorphism, Genetic; Environmental Exposure.

Introduction

Orofacial clefts are the most common craniofacial birth defects in humans, with an average worldwide prevalence of 1 in 700 live births. Orofacial clefts represent a significant public health problem that is an immediate and long-term medical and economic burden as well as a social impact on patients and their families. Affected children need multidisciplinary care from birth until adulthood and have a higher morbidity and mortality throughout life.¹

Non-syndromic orofacial clefts, which include cleft lip with or without cleft palate (CL ± P), and cleft palate only (CP) affect speech, hearing, appearance, and cognition.¹ Cleft lip is most frequent in males, and cleft palate in females.¹

Non-syndromic oral clefts have a complex etiology. They are caused by a multifactorial inheritance including both genetic and environmental factors.² Epidemiological and experimental data suggest that environmental risk factors including poor nutrition, exposure to medicinal drugs such as phenytoin, and maternal tobacco smoking and alcohol consump-

Declaration of Interests: The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

Corresponding Author:
 Têmis Maria Félix
 E-mail: tfelix@hcpa.ufrgs.br

Submitted: Mar 12, 2012
 Accepted for publication: Jun 22, 2012
 Last revision: Jul 06, 2012

tion during pregnancy might be important in clefting.^{1,3} Despite some disparities, studies report moderate but statistically significant association between clefting and maternal use of tobacco and alcohol during pregnancy, especially for CL \pm P.^{4,5}

The transforming growth factor alpha (*TGFA*) gene is a well-studied candidate gene for oral clefting. *TGFA* is expressed during craniofacial development in the medial edge epithelium of the palatal shelves.⁶ The *TGFA* protein binds to epidermal growth factor receptor (EGFR) leading to a potent epithelial mitogen response. The *TGFA* gene acts synergistically with the *TGFB* protein to promote *in vitro* cell proliferation.² *TGFA* has been mapped to chromosome 2p13, comprises 80 kb, and consists of six exons coding for a polypeptide of 50 amino acids.^{7,8} The *TGFA* gene has a restriction fragment length polymorphism when treated with *Taq I* restriction enzyme located in intron 5 that is 1,602 bp in the 5' direction of the exon 6 acceptor site. A mutant allele shows a four-base (TAAT) deletion changing the 178 bp C1 allele to the 174 bp C2 allele.⁹

The first evidence for an association between specific *TGFA* alleles and non-syndromic CL \pm P came from a Caucasian population in the state of Iowa.¹⁰ The association has been confirmed in several populations from different regions of the world.⁷ A meta-analysis of studies published before 1997 showed a significant association between a *TGFA/Taq I* polymorphism and CL \pm P in a population of European descent.¹¹ These findings have been confirmed in a meta-analysis including other ethnic populations.⁷ Another study reported a three-fold risk for cleft palate for a child with two copies of the *TGFA/Taq I* C2 allele.¹²

The *TGFA* gene seems to have a small but important role in clefting, especially when associated with environmental factors.^{7,13} The aim of this study was to evaluate the association of the *TGFA/Taq I* polymorphism in non-syndromic oral cleft. We also analyzed the interaction of this polymorphism with the environmental factors of alcohol and tobacco use during pregnancy.

Methodology

This research was approved by the Research Ethics Committee of Hospital de Clínicas de Porto Alegre (04-307).

Case-parent triads were recruited at the Craniofacial and Genetics clinics at the Hospital de Clínicas de Porto Alegre (HCPA). Cases were included in this study if they presented with non-syndromic cleft lip, with or without cleft palate, or cleft palate only.

Data were collected from 175 case-parent triads (96 complete case-triads: proband, father and mother; and 79 incomplete case-parents: proband, father or mother). Informed consent was obtained from each subject. A questionnaire was used to gather data on environmental factors (first-trimester maternal use of alcohol and tobacco) as well as consanguinity, family history of malformation, and mother's medical history.

Blood samples for DNA were collected in EDTA tubes. DNA was extracted using an extraction kit according to manufacturer's instructions (Gentra Puregene, Quiagen Inc., Valencia, USA).

TGFA/Taq I polymorphisms were determined by polymerase chain reaction (PCR) followed by restriction-enzyme digestion. Primers were 5'-TCACTTCCCCTTTTCATCTG-3' (forward primer) and 5'-CGAGGAGGCTCCTGAGGTG-3' (reverse primer). PCR was in 25 μ L containing 10 μ M each primer, 10 μ M deoxynucleotide triphosphate, 50 μ M MgCl₂, 1.5 units *Taq* polymerase, and 20 ng/ μ L genomic DNA. PCR conditions were 94°C for 5 minutes, followed by 36 cycles of 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 10 seconds, with a final extension at 72°C for 5 minutes. Amplified DNA fragments were digested with 10 units of *Taq I* restriction enzyme and buffer (Life Technologies, Grand Island, USA), at 65°C for 3 hours. Fragments were visualized by 2% agarose-gel electrophoresis. The *TGFA/Taq I* polymorphism has a restriction site because of a TAAT deletion. The C1 allele has one fragment of 178 bp and the C2 allele has two fragments of 122 and 52 bp.

Statistical analysis was performed with a transmission disequilibrium test (TDT) using FBAT software (Family Based Association Test)¹⁴ to evaluate

the association of oral cleft with *TGFA/Taq I* polymorphisms. Fisher's exact test was used to evaluate all sets of comparisons. *P* values lower than 0.05 were considered significant.

Results

Of 175 probands, 91 (52%) were males and 84 (48%) were females. CL ± P was more frequent in males (56.5%), however CP was more frequent in females (71.4%). The highest proportion of cases were CL ± P (147 cases) followed by CP (28 cases). Table 1 shows the distribution of the genotype frequency of *TGFA/Taq I* polymorphisms in the probands, mothers, and fathers. The allele frequencies of the C1 and C2 alleles were 0.935 and 0.064, respectively. The TDT for the *TGFA/Taq I* polymorphisms was not significant for oral clefting ($p = 0.335$).

We observed tobacco smoking during pregnancy in 17 cases and alcohol consumption during pregnancy in 8 cases (Table 2). Comparing environmental factors with proband genotypes did not show significant differences in exposed and nonexposed children for either alcohol ($p = 0.588$) or tobacco ($p = 0.606$) (Table 2).

Table 1 - Genotype frequency of proband, father and mother.

Genotype	Proband n (%)	Father n (%)	Mother n (%)
C1C1	157 (89.6)	81 (82.6)	153 (88.5)
C1C2	16 (9.2)	15 (15.3)	18 (10.4)
C2C2	2 (1.2)	2 (2.01)	2 (1.1)
Total	175 (100)	98 (100)	173 (100)

Table 2 - Distribution of proband genotypes and phenotypes and correlation to maternal alcohol and tobacco use during pregnancy.

		Alcohol			p	Tobacco			p
		No	Yes	total		No	Yes	total	
Genotype	C1C1	150 (95.5%)	7 (4.5%)	157	0.588	141 (89.8%)	16 (4.5%)	157	0.606
	C1C2	15 (93.8%)	1 (6.3%)	16		15 (93.8%)	1 (6.3%)	16	
	C2C2	2 (100%)	zero	2		2 (100%)	zero	2	
	Total	167	8	175		158	17	175	
Phenotype	CL ± P	140 (95.2%)	7 (4.8%)	147	0.625	132 (89.8%)	15 (10.2%)	147	0.466
	CP	27 (96.4%)	1 (3.6%)	28		26 (92.9%)	2 (7.1%)	28	
	Total	167	8	175		158	17	175	

A comparison of proband phenotypes and environmental factors showed no significant difference between the CL ± P and CP groups by alcohol ($p = 0.625$) or tobacco use during pregnancy ($p = 0.466$) (Table 2).

Discussion

This study evaluated the association between *TGFA/Taq I* polymorphisms and two common environmental exposures (maternal cigarette smoking and alcohol consumption during pregnancy) and CL ± P and CP in Southern Brazil.

Our study data showed that CL was more frequent in males and CP was more frequent in females. We also observed a higher prevalence of cases of CL ± P than CP. These data were in accordance with the previous literature.¹

In this study, the C2 allele frequency was 0.06, similar to that reported in previous case-control studies performed in Brazil.^{15,16} Several studies (case-control and case-parent triads) found an association between a *TGFA/Taq I* polymorphism and clefting;^{10,12,17,18} however, other studies did not find an association.¹⁹⁻²² We did not find any evidence of association between *TGFA/Taq I* polymorphisms and oral cleft in the population studied. A previous case-control study performed in the Southern Brazilian population also found no association between the rare *TGFA C2* allele and clefting.¹⁵

The *TGFA/Taq I* polymorphism is predominantly associated in European populations. The Brazilian population represents an ethnic admixture of three different populations: Europeans, Africans, and Amerindians, making it difficult to determine

the ethnicity of cases and controls in our population.^{23,24} A negative association between *TGFA* and clefting in previous Brazilian studies could be due to selection of controls. The TDT approach used in our study tends to avoid population stratification, confirming that *TGFA* was not associated with non-syndromic oral cleft in the Southern Brazilian population; however the low C2 allele frequency and low number of heterozygotes could be responsible for this result. Other variants in the *TGFA* gene are also identified as contributing to clefting. Recently, two single nucleotide polymorphisms, rs382161 and rs3771475, showed significant excess maternal transmission, suggesting a parent-of-origin effect.¹³ In addition, the *TGFA Taq I* marker is located in an intron. This suggests that the marker is in linkage disequilibrium with an as yet unidentified causally relevant allele.²⁵

In this study, we found no significant association between exposure to alcohol and/or tobacco environmental factors during pregnancy and oral cleft in the population studied. These results could be due to a small sample size, especially the low number of children exposed to environmental factors in our population.

When we compared environmental factors with proband genotypes, we found no significant association. We also had no cases of C2C2 genotype in children exposed to alcohol or tobacco during pregnancy. Several other studies found no association between the C2 allele and maternal tobacco use in CL ± P or CP.^{26,27} A study on the effect of maternal tobacco use and association with allele C2 of the *TGFA* gene observed significant odds ratios for CP for low users of tobacco (less than 10 ciga-

rettes *per day*) (OR: 6.16; 95% CI 1.09–34.7) and for moderate-to-heavy users of tobacco (OR: 8.69; 95% CI 1.57–47.8).¹⁷ These data were subsequently confirmed for CL ± P (OR: 6.5; 95% CI: 1.3–35.2) and CP (OR: 9.2; 95% CI: 1.6–59.1) for mothers who used more than 18 cigarettes a day.²⁸

Maternal smoking is an established risk factor for oral cleft. A meta-analysis of 24 studies estimated that mothers who smoked during pregnancy had a 1.3-fold increased risk of having a baby with cleft lip, with or without cleft palate, and a 1.2-fold risk of cleft palate alone.⁴ High levels of alcohol consumption during pregnancy can affect fetal development. One study reported an increased risk of cleft lip with or without cleft palate associated with smoking and an increased risk of cleft palate associated with alcohol consumption.²⁹

Conclusions

We found no evidence that a *TGFA/Taq I* polymorphism played a role in clefting in a Southern Brazilian population. Identification of other genes and factors involved in the development of the human craniofacial region will help to better understand the genetic factors involved in oral cleft. We also found no evidence of an influence of tobacco and alcohol exposure in clefting, however the low prevalence of those environmental factors in our population could have contributed to these findings.

Acknowledgments

This study was sponsored by Fundo de Incentivo a Pesquisa e Eventos do Hospital de Clínicas de Porto Alegre (FIPE/HCPA) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

References

1. Mossey PA, Little J, Munger RG, Dixon MJ, Shaw WC. Cleft lip and palate. *Lancet*. 2009 Sep;374(9703):1773-85.
2. Jugessur A, Murray JC. Oral clefting: recent insights into a complex trait. *Curr Opin Genet Dev*. 2005 Jun;15(3):270-8.
3. Shi M, George LW, Murray JC. Review on genetic variants and maternal smoking in the etiology of oral clefts and other birth defects. *Birth Defects Res C Embryo Today*. 2008 Mar;84(1):16-29.
4. Little J, Cardy A, Munger R. Tobacco smoking and oral cleft: a meta-analysis. *Bull World Health Organ*. 2004 Mar;82(3):213-8.
5. Wyszynski DF, Duffy DL, Beaty TH. Maternal cigarette smoking and oral clefts: a meta-analysis. *Cleft Palate Craniofac J*. 1997 May;34(2):206-10.
6. Chevrier C, Bahuau M, Perret C, Iovannisci DM, Nelva A, Herman C, et al. Genetic susceptibilities in the association between maternal exposure to tobacco smoke and the

- risk of nonsyndromic oral cleft. *Am J Med Genet A*. 2008 Sep;146(18):2396-406.
7. Vieira AR. Association between the transforming growth factor alpha gene and nonsyndromic oral clefts: a HuGE review. *Am J Epidemiol*. 2006 May;163(9):790-810.
 8. Vieira AR, Orioli IM. Candidate genes for nonsyndromic cleft lip and palate. *ASDC J Dent Child*. 2001 Jul-Aug;68(4):272-9.
 9. Tanabe A, Taketani S, Endo-Ichikawa Y, Tokunaga R, Ogawa Y, Hiramotos M. Analysis of the candidate genes responsible for non-syndromic cleft lip and palate in Japanese people. *Clin Sci (Lond)*. 2000 Aug;99(2):105-11.
 10. Ardinger HH, Buetow AH, Bell GI, Bardach J, VanDemark DR, Murray JC. Association of genetic variation of the transforming growth factor alpha gene with cleft lip and palate. *Am J Hum Genet*. 1989 Sep;45(3):348-53.
 11. Mitchell LE. Transforming growth factor alpha locus and nonsyndromic cleft lip with or without cleft palate: a reappraisal. *Genet Epidemiol*. 1997;14(3):231-40.
 12. Jugessur A, Lie RT, Wilcox AJ, Murray JC, Taylor JA, Saugstad OD, et al. Variants of developmental genes (TGFA, TGFB3 and MSX1) and their associations with orofacial clefts: a case-parent triad analysis. *Genet Epidemiol*. 2003 Apr;24(3):230-9.
 13. Sull JW, Liang KY, Hetmanski JB, Wu T, Fallin MD, Ingersoll RG, et al. Evidence that TGFA influence risk to cleft lip with/ without cleft palate through unconventional genetics mechanisms. *Hum Genet*. 2009 Sep;126(3):385-94.
 14. Rabinowitz D, Laird N. A unified approach to adjusting association tests for population admixture with arbitrary pedigree structure and arbitrary missing marker information. *Hum Hered*. 2000 Jul-Aug;50(4):211-23.
 15. Bertoja AE, Alho CS, França E, Menegoto B, Robinson WM. TGFA/TaqI polymorphism in nonsyndromic cleft lip and palate patients from Rio Grande do Sul, Brazil. *Cleft Palate Craniofac J*. 2008 Sep; 45(5):539-44.
 16. Passos-Bueno MR, Gaspar DA, Kamiya T, Tescarollo G, Rabanéa D, Richieri-Costa A, et al. Transforming growth factor- α and nonsyndromic cleft lip with or without palate in Brazilian patients: results of a large case-control study. *Cleft Palate Craniofac J*. 2004 Jul;41(4):387-91.
 17. Hwang SJ, Beaty TH, Panny SR, Street NA, Joseph JM, Gordon S, et al. Association study of transforming growth factor alpha (TGFA) TaqI polymorphism and oral clefts: indication of gene-environment interaction in a population-based sample of infants with birth defects. *Am J Epidemiol*. 1995 Apr;141(7):629-36.
 18. Shaw GM, Wasserman CR, Murray JC, Lammer EJ. Infant TGF-alpha genotype, orofacial clefts, and maternal periconceptional multivitamin use. *Cleft Palate Craniofac J*. 1998 Jul;35(4):366-70.
 19. Beaty TH, Wang H, Hetmanski JB, Fan YT, Zeiger JS, Liang KY, et al. A case-control study of nonsyndromic oral clefts in Maryland. *Ann Epidemiol*. 2001 Aug;11(6):434-42.
 20. Christensen K, Olsen J, Norgaard-Pedersen B, Basso O, Stovring H, Milhollin-Johnson L, et al. Oral clefts, transforming growth factor alpha gene variants, and maternal smoking: a population-based case-control study in Denmark, 1991-1994. *Am J Epidemiol*. 1999 Feb;149(3):248-55.
 21. Lidral AC, Murray JC, Buetow KH, Basart AM, Schearer H, Shiang R, et al. Studies of the candidate genes TGFB2, MSX1, TGFA, and TGFB3 in the etiology of cleft lip and palate in the Philippines. *Cleft Palate Craniofac J*. 1997 Jan;34(1):1-6.
 22. Zhu J, Hao L, Li S, Bailey LB, Tian Y, Li Z. MTHFR, TGFB3 and TGFA polymorphisms and their association with the risk of non-syndromic cleft lip and cleft palate in China. *Am J Hum Genet A*. 2010 Feb;152(2):291-8.
 23. Alves-Silva J, Silva Santos M, Guimaraes PE, Ferreira AC, Bandelt HJ, Pena SD, et al. The ancestry of Brazilian mtDNA lineages. *Am J Hum Genet*. 2000 Aug;67(2):444-61.
 24. Carvalho-Silva DR, Santos FR, Rocha J, Pena SD. The phylogeography of Brazilian Y-chromosome lineages. *Am J Hum Genet*. 2001 Jan;68(1):281-6.
 25. Jugessur A, Farlie PG, Kilpatrick N. The genetics of isolated orofacial clefts: from genotypes to subphenotypes. *Oral Dis*. 2009 Oct;15(7):437-53.
 26. Romitti PA, Lidral AC, Munger RG, Daack-Hirsch S, Burns TL, Murray JC. Candidate genes for nonsyndromic cleft lip and palate and maternal cigarette smoking and alcohol consumption: evaluation of genotype-environment interactions from a population-based case-control study of oral clefts. *Teratology*. 1999 Jan;59(1):39-50.
 27. Zeiger JS, Beaty TH, Liang KY. Oral clefts, maternal smoking and TGFA: a meta-analysis of gene-environment interaction. *Cleft Palate Craniofac J*. 2005 Jan;42(1):58-63.
 28. Shaw GM, Wasserman CR, Lammer EJ, O'Malley CD, Murray JC, Basart AM, et al. Orofacial clefts, parental cigarette smoking, and transforming growth factor alpha gene variants. *Am J Hum Genet*. 1996 Mar;58(3):551-61.
 29. Lorente C, Cordier S, Goujard J, Aymé S, Bianchi F, Calzolari E, et al. Tobacco and alcohol use during pregnancy and risk of oral cleft. *Am J Public Health*. 2000 Mar;90(3):415-9.