TGFA/Taq I Polymorphism in Nonsyndromic Cleft Lip and Palate Patients From Rio Grande do Sul, Brazil

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Objective: **To test the TGFA/Taq I polymorphism in the development of nonsyndromic cleft lip and palate.**

Design and Setting: **The research was based on a case-control study, including nonsyndromic cleft lip and palate patients (140 individuals) and a control sample of unaffected individuals (142) to ascertain the absence or presence of genic mutation at the TGFA locus.**

Interventions: **The DNA of carriers of nonsyndromic cleft lip with or without cleft palate was obtained by buccal swab, and the DNA of the control group was extracted from peripheral blood leucocytes. TGFA/Taq I polymorphism was determined genetically by polymerase chain reaction using specific primers and fragment digestion with Taq I restriction enzyme.**

Results: **No significant association was detected when patients and controls were compared with the genotype for TGFA/Taq I polymorphism.**

Conclusion: **Mutations in *TGFA* gene have no association with nonsyndromic cleft lip and palate in the sample from Rio Grande do Sul. Therefore, based on this study, it is not possible to determine the role played by TGFA in the expression of cleft lip and palate.**

KEY WORDS: *cleft lip and palate, genetics, TGFA/Taq I polymorphism*

Cleft lip and palate (CLP) is the most common craniofacial malformation in humans, and its prevalence varies according to ethnic factors, geographic origin, and socioeconomic level (Slayton et al., 2003). North American natives display one of the highest prevalences for this malformation (3.6/1000), followed by Asians (2.11/1000—Japanese; 1.7/1000—Chinese), whites (1/1000), and African Americans (0.3/1000) (Wyszynski et al., 1996).

The Bari Indians, who live in the plains of the tropical forest in western Venezuela and northwestern Colombia, show the highest ever reported prevalence of cleft lip with or without cleft palate (CL±P). Twelve affected individuals were identified in a population of nearly 1200 inhabitants. However, this population constitutes a racial isolate group, which explains this high prevalence (Ballew et al., 1993).

The etiology of cleft lip and palate is complex and involves both genetic and environmental factors (Slayton et al., 2003). Clinically, these clefts cause feeding difficulties, occlusal disharmony, and maxillary hypoplasia during growth. Effective treatment consists of reconstructive and corrective surgeries, as well as associated orthodontic and phonoaudiologic treatments.

The CL±P development mechanism shows that the isolated cleft lip occurs similarly to cleft lip and palate, and both clefts differ from the formation mechanism of cleft palate only. In general, cleft lip and cleft lip and palate are analyzed together and the cleft palate separately (Vieira, 2006).

Nonsyndromic cleft lip and palate (CLP) seems to present a more complex etiology than that of CLP associated with a given syndrome. It is not a Mendellian simple inheritance, displaying a very familial aggregation, which reveals the presence of an important genetic component (Wyszynski et al., 1996). Many authors suggest a genetic and environmental etiology consistent with the threshold-effect multifactorial inheritance model. As occurs in a large number of congenital malformations, in this model a threshold divides a sample into two groups: normal individuals and affected individuals. The affected group ranges from moderate to severe. Such a variation can be explained by a genotypic threshold, that is, a minimum

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amount of genes necessary to give rise to malformations in a certain environment (Vieira and Orioli, 2001). Some studies support an oligogenic model in which 2 through 20 genes can be active (Moreno et al., 2004).

Since the late 1980s, studies have been carried out to test genes that could be involved in the etiology of CL±P. These genes include (with different degrees of success) TGFA (transforming growth factor alpha), TGFB 2 and 3 (transforming growth factor beta 2 and 3), MSXI (homeobox protein of group 7), BCL3 (lymphoma B-cell type), RARA (retinoic acid receptor, alpha), MTHFR (5,10-methhylenetetrahydrofolic reductase), and IFR6 (interferon regulator factor 6).

TGFA (codified by gene TGFA) is a secretion protein that binds to the epidermal growth factor receptor and is situated at the palate epithelium during palate closing (Jugessur et al., 2003; Passos-Bueno et al., 2004). This protein can act as a normal embryonary version of the EGF-related growth factor and is considered to be a powerful epithelial mitogen. The TGFA gene acts synergistically with TGFB protein promoting *in vitro* cell proliferation (Vieira and Orioli, 2001).

TFGA gene has 70 to 100 kilobases (kb), located at the short arm of chromosome 2 (2p13), coding for a polypeptide formed by 50 amino acids (Vieira and Orioli, 2001). The TFGA gene shows a restriction fragment length polymorphism when treated with Taq I restriction enzyme. The mutant allele shows a four-base (TAAT) deletion. In this case, it shows a 178-base pair (bp) C1 allele and a 174-bp C2 allele (Tanabe et al., 2000). TGFA/Taq I polymorphism is located at intron 5 and has 602 bp in the 5′ direction of the acceptor site of exon 6 (Vieira, 2006).

Several studies show a positive association between both alleles. However, the biological mechanism for explaining this association is still unclear, because the Taq I marker forms a polymorphic allele situated at an inexpressive region of the gene, the intron (Beaty et al., 1997).

Objectives

The objectives of this study were twofold: (1) to determine the frequency of C1 and C2 alleles of the TGFA/Taq I polymorphism in a sample of CLP patients from Rio Grande do Sul, Brazil; (2) to detect the presence of the TGFA/Taq I polymorphism in those patients and in normal or healthy individuals (control group) in order to verify the occurrence of an association between genetic mutations at the TGFA locus (C2 allele) and the malformation.

Materials and Methods

A sample of 140 CLP patients of both sexes, ages 7 months to 50 years, was studied. Informed consent was obtained from each subject and the research was approved by the institutional ethics council and SISNEP (Brazilian Ethics in Research System). All patients were examined clinically in detail to discard any type of associated malformation. A sample of buccal mucous cells was collected from each patient by swab scraping, followed by DNA extraction. Marrero et al. (2005) showed that the population of Rio Grande do Sul is heterogeneous. Although some individuals have the majority of their genome of European origin with correspondence between phenotype and ancestry, others reflect the history of extensive Native American and African admixture with dissociation between physical appearance and ancestry. This is because this state is known to have a large number of individuals classified as white as compared with other Brazilian states. Porto Alegre is the capital of Brazil's southernmost state. This city was founded in 1752 by 60 white couples from the Azores Islands. Currently, the population is still mainly of Portuguese descent, but Italians, Spaniards, and Germans also have contributed to its gene pool.

The control group was composed of 142 healthy individuals whose DNA was extracted for paternity tests at the Serviço de Genética Médica (Medical Genetics Service) of the Hospital de Clínicas de Porto Alegre, Brazil. The patients of the control group belong to a demographic admixture similar to that of CLP patients. They are white individuals of both sexes coming from the State of Rio Grande do Sul.

TGFA/Taq I polymorphism was determined genetically by polymerase chain reaction (PCR) in a thermal cycler using forward and reverse primers. For a reaction of 25 μL of final volume, we used 10 μM of each primer, 10 μM of desoxinucleotide triphosphate, 50 μM of MgCl2, 1.5 units of Taq polymerase, and 0.1 to 1 ng of DNA. The PCR conditions consisted of an initial denaturation at 94°C for 5 minutes, followed by 36 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds and extension at 72°C for 10 seconds, and a final extension cycle at 72°C for 5 minutes. For individuals' genotyping, 2 μL of the PCR product was digested with 10 units of Taq I restriction enzyme and buffer, both provided by the manufacturer, at 65°C for 3 hours, and the product of this digestion was submitted to 2% agarose-gel electrophoresis. The TGFA/Taq I genotype of each individual was then determined by fragment size in agarose gel.

The region of the TGFA gene digested by Taq I restriction enzyme exhibits two types of restriction fragments length polymorphism: one shows a Taq I restriction site due to the absence of TAAT bases (C2 allele), and another shows the presence of this sequence (C1 allele) (Tanabe et al., 2000; Table 1).

The genotype was visualized by agarose gel electrophoresis (2%)/ethidium bromide/Tris-Borate-EDTA Buffer (TBE) on an ultraviolet transilluminator, and the cleavage pattern can be visualized both in Figure 1B and in the schematic representation of Figure 1A.

TABLE 1 Representation of DNA Sequence of TGFA/Taq I Polymorphism in Both Alleles*

Sequence	*PCR*	*Digestion*
C1 Allele		
416 **TCACT TCCCCTTTTT CATCTG**TAAA AGGAGGAATT TGGCCTATGA	178 bp	178 bp
461 AGG*TCTCTA ATGACC*TTAA AACCCTTAGA TCCTATGATC TTCATTTTAAGT		no digestion
511 TTACCTTGTT TCCTGGATAT TTTCGCCAAC ATCCATGAAG ACATCAGGAT		
561 GTGGGGCCCA GCTTG**CGAGG AGGCTCTGAG GTG**		
C2 Allele		
416 **TCACT TCCCCTTTTT CATCTG**TAAA AGGAGGAATT TGGCCTATGA	174 bp	122 bp
461 AAGG*TCTC - - - -GACC*TTAA AACCCTTAGA TCCTATGATC TTCATTTTAAGT		52 bp
511 TTACCTTGTT TCCTGGATAT TTTCGCCAAC ATCCATGAAG ACATCAGGAT		
561 GTGGGGCCCA GCTTG**CGAGG AGGCTCTGAG GTG**		

* The primer region is in bold. The polymorphic region (italic and underline) occurs only in the C1 allele, because the C2 allele shows deletion of these four bases (TAAT), allowing Taq I enzyme to cleave the region, because this enzyme recognizes the TCGA sequence (in gray) where the cut occurs. In polymerase chain reaction (PCR) reaction, the amplified product has 178 base pairs (bp) at the C1 allele and 174 bp at the C2 allele. After digestion, the C1 allele remains the same size, and the C2 allele shows two fragments: one of 122 bp and another of 52 bp (Tanabe et al., 2000).

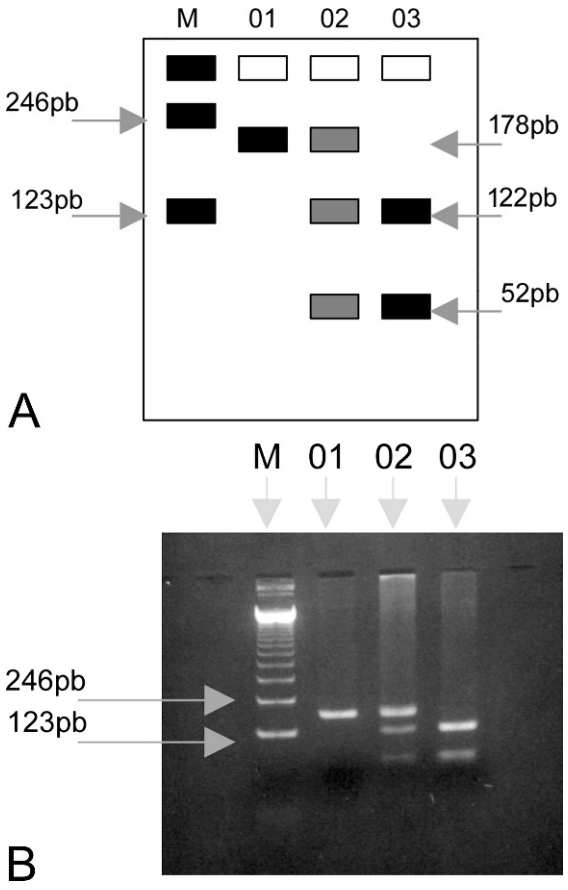

FIGURE 1 A: Schematic representation and B: photography of the ethidium-bromide 2% agarose gel electrophoresis analysis of the TGFA gene digestion. The product amplified by polymerase chain reaction (178 base pair [bp] and 174 bp) was digested with Taq I restriction enzyme. M = banding pattern of the 123-bp marker; 01 = banding pattern expected for the C1C1 homozygous individual in which Taq I enzyme does not recognize the cleavage site in both chromosomes; 02 = banding pattern expected for C1C2 heterozygous individuals in which Taq I enzyme recognizes the cleavage site in only one of the chromosomes; 03 = banding pattern expected for C2C2 homozygous individuals in which Taq I enzyme recognizes the cleavage site on both chromosomes.

Statistics

A chi-square test was used for the statistical analysis. The C2C2 individual was an isolated case and was excluded from the analysis to avoid imprecise results by any statistical test.

Results

With respect to TGFA/Taq I polymorphism (Table 2), most of the patients (114 or 81.4%) exhibited C1C1 genotype (i.e., two wild-type alleles). Twenty-five (17.9%) were genotyped as C1C2, one mutant allele and one wild-type allele. The presence of two mutant C2C2 alleles was detected only in one patient (0.7%).

Table 3 shows a comparison among the studies carried out by several authors with TGFA/Taq I polymorphic marker in nonsyndromic CL±P patients. As can be observed in the present study, C1 and C2 alleles showed frequencies of 0.90 and 0.10, respectively. The frequencies ranged from 0.85 to 0.95 for the wild-type allele (C1) and from 0.05 to 0.15 for the mutant allele.

In the present study, 5.3% of the individuals with C1C1 phenotype have CP and 94.7% have CL±P. The C1C2 genotype appears only in CL±P individuals. Nevertheless, no statistically significant association between the genotype and the cleft type was found (Table 4).

When the patients were compared with the controls (Table 5), we observed a lower prevalence of individuals with C1C1genotype (114; 81.4%) in the patients group than in the control group (121; 85.2%). The C1C2 genotype was

TABLE 2 Genotypic Distribution of Nonsyndromic Cleft Lip and Palate Patients for TGFA Polymorphism in the Sample From Porto Alegre Analyzed in 2005

Genotype	*No. of Patients*	*%*
C1C1	114	81.4
C1C2	25	17.9
C2C2	1	0.7
Total	140	100.0

TABLE 3 Frequency Distribution of C1 and C2 Alleles of TGFA/Taq I Polymorphism in Different Studies

Author (y)	*Sample*	*Number of Patients*	*Number of Controls*	*Type of Study*	*Genic Frequency*	
					C1	*C2*
Ardinger et al. (1989)	Iowa	78	98	Case-control	0.87	0.13
Hwang et al. (1995)	Maryland	183	284	Case-control	0.88	0.12
Lidral et al. (1997)	Iowa	1518	1552	Case-control	0.92	0.08
Lidral et al. (1998)	Iowa	502	428	Case-control	0.89	0.11
Christensen et al. (1999)	Denmark	302	567	Case-control	0.87	0.13
Tanabe et al. (2000)	Japan	43	73	Case-control	0.88	0.12
Jugessur et al. (2003)	Norway	262	524	Familial	0.85	0.15
Slayton et al. (2003)	Iowa	120	502	Case-control	0.90	0.10
Passos-Bueno et al. (2004)	São Paulo and Ceará (Brazil)	536	412	Case-control	0.95	0.05
Present study	Rio Grande do Sul (Brazil)	140	142	Case-control	0.90	0.10

the second most frequent: 25 (17.9%) in the case group and 21 (14.8%) in the control group. Furthermore, only one individual in the case group (0.4%) carried the C1C2 genotype, which was not found in the control group. No significant association was found when samples of patients and controls were compared with the genotype for the TGFA/Taq I polymorphism.

Table 6 summarizes studies on the gene TFGA in different populations using different techniques. In relation to the sample size evaluated in the present study, a sample of 142 individuals is consistent with other similar studies because the number of probands has ranged from 43 to 1518 in case-control studies. The absence of statistical association was prevalent both in the present investigation and in the studies analyzed.

Discussion

No association was detected among mutations (C2 allele) in the TGFA/Taq I polymorphism of CL±P patients in the studied population or in the studies by Moreno et al. (2004) except for subjects from Ohio.

The frequency of C1 (0.90) and C2 (0.10) alleles for the TGFA/Taq I locus is within the parameters expected in the Hardy-Weinberg equilibrium, which denotes the correct sample selection. As shown in Table 3, the results of the present investigation regarding the genic frequency (allele C2 = 0.10) are within the range of 0.5 to 0.15 observed in past studies (Jugessur et al., 2003; Passos-Bueno et al., 2004). Our study found the same data observed by Slayton et al. (2003), who reported a frequency of 0.90 for C1 allele in a population from Iowa using a sample of patients with similar size.

As can be seen in Table 6, several studies in white populations have shown an association between the TGFA/Taq I polymorphic marker and CLP. However, other studies, as well as the present investigation, did not show this association, as was shown in the study conducted by Passos-Bueno et al. (2004). According to these authors, TGFA is not a relevant modifier locus for the incidence of CL/P in the southeastern and northeastern regions of Brazil. The Brazilian population represents a trihybrid ethnic admixture of Euro-Brazilians, Afro-Brazilians, and Amerindians, making it very difficult to relate perfectly the ethnicity of cases and controls. Due to differences between colonization of the South (preferentially European) and Northeast (mainly African) regions and to the intensive migration from the Northeast to the Southeast, a difference of genotypic distribution should have occurred in Rio Grande do Sul, because mutations at TGFA gene are predominantly found in white populations. Therefore, we can suppose that some effects of the population stratification could lead to the absence of statistical significance of the case-control study in relation to C2/TGFA allele. This study, carried out at Pontifícia Universidade Católica do Rio Grande do Sul, confirms that the slight difference in the frequency of C2 allele between patients and controls is insufficient to consider that TGFA polymorphisms is responsible for CL±P in nonsyndromic subjects (Passos-Bueno et al., 2004).

Shiang et al. (1993) and Morküniené et al. (2007) found an association only in cases of isolated cleft palate. According to Jugessur et al. (2003), when an individual is homozygous for the C2 allele, the risk of having cleft palate is three times higher. In this study, the only individual with

TABLE 4 Distribution of Genotypes for TGFA Polymorphism Regarding Cleft Type of the Affected Individual in Terms of CL±P and CP in the Sample From Porto Alegre Analyzed in 2005*

Cleft	*Genotype*			*Total*
	C1C1	*C1C2*	*C2C2*	
CP	6 (5.3%)	—	1 (100.0%)	7 (5.0%)
CL±P	108 (94.7%)	25 (100.0%)	—	133 (95.0%)
Total	114 (100.0%)	25 (100.0%)	1 (100.0%)	139 (100.0%)

* CP = cleft palate; CL±P = cleft lip with or without cleft palate.

TABLE 5 Comparison of the Genotypes for TGFA Polymorphism Between Patients and Controls in the Sample From Porto Alegre Analyzed in 2005

Genotype	*Group*		*Total*
	Case	*Control*	
C1C1	114 (81.4%)	121 (85.2%)	235 (83.3%)
C1C2	25 (17.9%)	21 (14.8%)	46 (16.3%)
C2C2	1 (0.7%)	—	1 (0.4%)
Total	140 (100.0%)	142 (100.0%)	282 (100.0%)

TABLE 6 Comparison Between the Present Data and Previously Published Data in Relation to TGFA Gene, Either by Sequencing or RLFP

Author (y)	*Sample*	*Number of Patients*	*Number of Controls*	*Type of Study*	*Type of Analysis*	*Statistics*
Ardinger et al. (1989)	Iowa	78	98	Case-control	RFLP	S*
Hwang et al. (1995)	Maryland	183	284	Case-control	RFLP	S
Beaty et al. (1997)	Maryland	149	138	Case-control	RFLP	NS
Lidral et al. (1997)	Iowa	1518	1552	Case-control	RFLP	NS
Lidral et al. (1998)	Iowa	502	428	Case-control	RFLP	NS
Shaw et al. (1998)	California	571	640	Case-control	RFLP	S
Christensen et al. (1999)	Denmark	302	567	Case-control	RFLP	NS
Prescott et al. (2000)	United Kingdom	92	271	Familial	Sequencing	S
Tanabe et al. (2000)	Japan	43	73	Case-control	RFLP	S
Mitchell et al. (2001)	Denmark	266	473	Case-control	RFLP	NS
Beaty et al. (2002)	Maryland	269	538	Case-control	Sequencing	NS
Marazita et al. (2002)	China	145	526	Familial	Sequencing	NS
Jugessur et al. (2003)	Norway	262	524	Familial	RFLP	S
Slayton et al. (2003)	Iowa	120	502	Case-control	RFLP	NS
Field et al. (2004)	India	82	190	Familial	Sequencing	NS
Marazita et al. (2004)	Turkey	18		Familial	Sequencing	S
Passos-Bueno et al. (2004)	São Paulo and Ceará (Brazil)	536	412	Case-control	RFLP	NS
Schultz et al. (2004)	Philippines	126	218	Familial	Sequencing	NS
Suzuki et al. (2004)	Vietnam	175	350	Familial	Sequencing	NS
Moreno et al. (2004)	Columbia, Ohio	26	39	Familial	Sequencing	S
		123	198			NS
Morkūnienė et al. (2007)	Lithuania	112	224	Familial	Sequencing	S
Present study	Rio Grande do Sul (Brazil)	140	142	Case-control	RFLP	NS

* S = significant; NS = nonsignificant; RFLP = restriction fragment length polymorphism.

C1C2 genotype was a CP carrier. However, no one can state that this genotype causes the cleft because it is an isolated case. A larger sample of individuals with CP is required to prove this.

Clefts result from a lack of binding between the facial prominences and maxillae, due to the absence of mesenchymal mass fusion and the nonproliferation of the mesenchyme under the overlying epithelium. Therefore, the reason for finding no association between the TGFA gene and CL±P can be explained by the fact that this gene is related to epithelial yielding, and epithelial breaking would be only a consequence of the mesenchymal malformation. TGFB3 would be a better candidate gene for CLP because it acts in palate morphogenesis in interactions between epithelium and mesenchyme. TGFA can be a supporter or a modifier, as reported by Prescott et al. (2000), in CLP formation; in these cases it could act synergistically with other genes, such as TGFB3, and therefore to be one of the responsible genes for the malformation. Further studies are required to answer this question.

Studies carried out to detect the occurrence of association between CLP and genetic markers can contribute to our knowledge of the evolution of the pathology, as well as help to identify inherited characteristics that account for increasing an individual's susceptibility to multifactorial disorders like CLP. Studies on mutations in other genes (e.g., MSX1, IRF6, and TGFB3) can provide arguments to help surgeons and dentists in their clinical practice in buccofacial pathology control and prevention programs. Currently, the genes cannot be changed, but once they have been identified, it will be easier to control the modifications to avoid pathological situations. Therefore, the knowledge of genetic polymorphisms can be applied to obtain genetic indicators that will be useful to carriers of the studied malformation, as well as to their relatives, with respect to diagnosis, prevention, treatment, and genetic counseling.

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