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ORIGINAL ARTICLE

Lack of association between *IRF6* polymorphisms (rs2235371 and rs642961) and non-syndromic cleft lip and/or palate in a Brazilian population

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BACKGROUND: Interferon regulatory factor 6 (*IRF6*) gene has emerged as a potential susceptibility gene for non-syndromic cleft lip and/or palate (NSCL/P) in different populations. The aim of this study was to determine the association of *IRF6* rs2235371 and rs642961 polymorphisms with NSCL/P in a Brazilian population.

METHODS: Two hundred and twenty-eight patients affected by NSCL/P and 126 healthy individuals were genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay.

RESULTS: Overall genotype distributions of rs2235371 and rs642961 polymorphisms were as expected by Hardy-Weinberg equilibrium test. The rs2235371 polymorphic genotype GA was identified in 10.1% of the patients with NSCL/P and in 10.3% of the control group, revealing no statistical difference. Similarly, the frequency of rs642961 minor genotypes (GA and AA) was quite similar between control group (28.6%) and NSCL/P group (25.4%), without significant difference.

CONCLUSION: Our findings are consistent with a lack of involvement of *IRF6* rs2235371 and rs642961 polymorphisms in the NSCL/P pathogenesis in the Brazilian population.

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Keywords: IRF6 gene; polymorphism; non-syndromic cleft lip and/or palate

Introduction

Non-syndromic cleft lip and/or palate (NSCL/P) (OMIM 119530) is the most common craniofacial anomaly, and its prevalence may vary according to ethnic factors, geographic origin, and socioeconomic level (Mitchell *et al*, 2002; Slayton *et al*, 2003). Populations of Asian (2.11 per 1000) and native American (3.6 per 1000) ancestries show the highest birth prevalence of NSCL/P, and populations from Africa (0.3 per 1000) show the lowest (Wyszynski *et al*, 1996). In Brazil, NSCL/P prevalence is of 1.46 per 1000 live births (Martelli-Junior *et al*, 2006). NSCL/P etiology is complex with both environmental and genetic factors playing important roles. To date, only a few genes were clearly associated with NSCL/P, such as *PVRL1* (Sozen *et al*, 2001), *TGF-β3* (Vieira *et al*, 2003), *MSX1* (Van den Boogaard *et al*, 2000), *TBX22* (Braybrook *et al*, 2001), *FGFs* (Riley *et al*, 2007), *PTCH* (Mansilla *et al*, 2006), and interferon regulatory factor 6 (*IRF6*) (Zucchero *et al*, 2004).

IRF6 gene was initially targeted for investigation after mutations were detected in patients with van der Woude syndrome (OMIM 119300) (Kondo *et al*, 2002), an autosomal dominant disorder characterized by CL/P and pits in the lower lip (Paranaiba *et al*, 2008). Several studies have demonstrated that NSCL/P may be associated with variations on *IRF6* gene in multiple populations (Blanton *et al*, 2005; Ghassibe *et al*, 2005; Scapoli *et al*, 2005; Du *et al*, 2006; Vieira *et al*, 2007; Pegelow *et al*, 2008). In particular, the polymorphism rs2235371 (820G > A) replaces a valine by an isoleucine at amino acid position 274 (V274I) of the SMIR-binding domain of *IRF6* and is significantly associated with NSCL/P (Srichomthong *et al*, 2005; Jugessur *et al*, 2008; Tang *et al*, 2009). Rahimov *et al* (2008) demonstrated a strong association between cleft lip and *IRF6* rs642961 polymorphism (G > A, that disrupts the binding site of the transcription factor AP-2α in the *IRF6* promoter) and found that *IRF6* rs2235371 polymorphism is not associated with oral clefts independent of the rs642961 polymorphism. The aim of this study was to verify if *IRF6* rs2235371 and rs642961 polymorphisms are associated with NSCL/P in Brazilian patients.

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Materials and methods

Population

This study included 228 unrelated patients with NSCL/P. As control, 126 healthy individuals were recruited, which were matched to NSCL/P group according to gender and skin color. As the Brazilian population is formed by an intense admixture of Europeans, Africans, and native Indians, there is no clear differentiation among ethnic groups (Vieira *et al*, 2002). The samples were derived from individuals living in the state of Minas Gerais, which is mostly formed by an admixed population of Africans and Europeans (most from Portugal, Spain, and Italy), with very small percentage of native Brazilians.

The clefts were classified with the incisive foramen as reference (Spina, 1973), and divided in two groups: cleft lip with or without cleft palate (CL/P) (n = 177), combination of isolated cleft lip (CL) and cleft lip and palate (CLP), and isolated cleft palate (CP) (n = 51). All patients were carefully examined and screened for the presence of associated anomalies or syndromes by the team of the Center for Rehabilitation of Craniofacial Anomalies, University of Alfenas, Brazil, and only those identified with NSCL/P were included in this study. Written informed consents were obtained from all participants or their guardians, and the study was carried out with approval of the Human Research Ethics Committee of the University.

DNA analysis

Genomic DNA was extracted from oral mucosa cells as previously described (Aidar and Line, 2007). PCR reactions were performed with the following *IRF6* primers: forward 5′AGT GGC CTT CCT GAA TGC TG3′ and reverse 5′CTT GAC CTC CTC CAG ACT A3′ for rs2235371, and forward 5′TGC CAG CTA CTC AGC TTG GTT CAT3′ and reverse 5′ATA GAG CAT GCT GCC TTC TTC CCA3′ for rs642961. The *IRF6* rs2235371 and rs642961 polymorphisms were genotyped by restriction digestion of each PCR product with MboI and BstNI (New England Biolabs Inc., Ipswich, MA, USA), respectively. After incubation with restriction enzymes, the products were electrophoresed on 8% non-denaturing polyacrylamide gels containing 0.5 μg/ml of ethidium bromide. For the rs2235371 polymorphism, MboI endonuclease digests the G allele in five fragments (322, 177, 80, 35, and 33 bp), whereas the A allele adds another restriction site, allowing the 322 bp fragment to be digested into two smaller pieces of 235 and 87 bp (Figure 1). Incubation of the rs642961 PCR products with BstNI results in three fragments (213, 33, and 30 bp) for the G allele and two fragments (246 and 30 bp) for the A allele (Figure 2).

Statistical analysis

The distribution of the genotypes was evaluated by Hardy-Weinberg equilibrium test (Rodriguez *et al*, 2009). Chi-square test and 95% confidence interval (95% CI) for the odds ratios were calculated with the control group as reference.

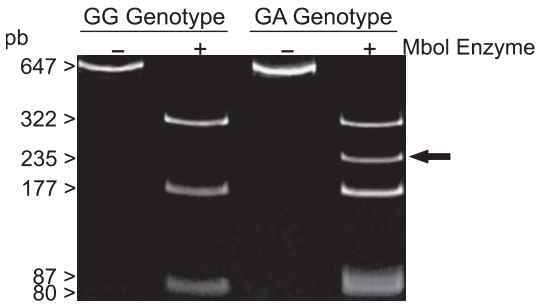

Figure 1 Restriction enzyme analysis of the *IRF6* rs2235371 polymorphism. Figure depicts representative samples from one individual with GG genotype and one with GA genotype of this study. Symbols on top (+/−) represent the incubation or not with MboI enzyme. Differentiation between the genotypes is possible by the identification of the 235 bp fragment (arrow) in the GA genotype. Small fragments (35 and 33 bp) are not seen in this image

Results

The genotypic frequencies for rs2235371 and rs642961 were in agreement with Hardy-Weinberg equilibrium. CL and CLP are typically grouped together in CL/P due to the similarities in both epidemiologic characteristics and embryologic timing. Thus, the frequencies of the polymorphic alleles of CL/P and isolated CP were compared with the control group.

For the rs2235371, the GG genotype was identified in 318 individuals (113 of the control group, 159 of the CL/P group, and 46 of the CP group), whereas the GA genotype was found in 36 individuals (13 of the control group, 18 of the CL/P group, and five of the CP group) (Table 1). The AA genotype was not identified in this particular Brazilian population. The rs642961 GG genotype was identified in 260 subjects (90 of the control group, 128 of the CL/P group, and 42 of the CP group), and the rs642961 GA genotype was observed in 90 (36 of the control group, 45 of the CL/P group, and nine of the CP group). The rs642961 AA genotype was detected in four patients affected by CLP (Table 2). We have not observed an association between rs2235371 polymorphic genotype and presence of cleft (Table 1), and there were

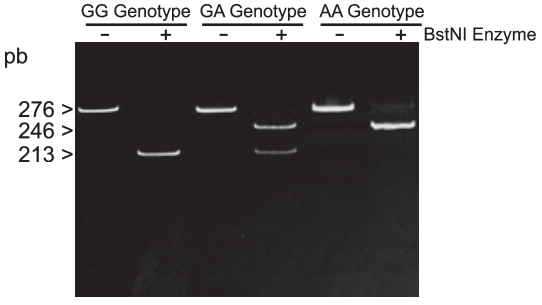

Figure 2 Restriction enzyme analysis of the *IRF6* rs642961 polymorphism. Representative samples from one individual with GG genotype, one with GA genotype and one with AA genotype is shown. Symbols on top (+/−) represent the incubation or not with BstNI enzyme. BstNI digests the A variant into two fragments (246 and 30 bp), while the G variant adds another restriction site, allowing the 246 bp fragment to be digested into two smaller fragments of 213 and 33 bp. Small fragments (33 and 30 bp) are not seen in this image

Table 1 Allelic distribution and frequency of rs2235371 polymorphism in the control, CL/P, and isolated CL groups

	Control group n *(%)*	*CL/P group* n *(%)*	*CP group* n *(%)*
Allele			
Allele G	239 (94.8)	336 (94.9)	97 (95.1)
Allele A	13 (5.2)	18 (5.1)	5 (4.9)
		$\chi^2 = 0.01$	$\chi^2 = 0.90$,
		$P = 0.96$	$P = 0.92$
Genotype			
GG	113 (89.7)	159 (89.8)	46 (90.2)
GA	13 (10.3)	18 (10.2)	5 (9.8)
AA	0	0	0
		$\chi^2 = 0.02$	$\chi^2 = 0.15$
		$P = 0.96$	$P = 0.70$
Comparison (GG *vs* GA)			
Odds ratio		0.98	0.94
95% CI		0.46 to 2.08	0.32 to 2.80

Control group was used as reference in all comparisons. CL/P, cleft lip with/without cleft palate; CP, isolated cleft palate.

Table 2 Allelic distribution and frequency of rs642961 polymorphism in the control and NSCL/P group

	Control group n *(%)*	*CL/P* n *(%)*	*CP* n *(%)*
Allele			
Allele G	216 (85.7)	305 (86.2)	93 (91.2)
Allele A	36 (14.3)	49 (13.8)	9 (8.8)
		$\chi^2 = 0.02$,	$\chi^2 = 1.95$,
		$P = 0.87$	$P = 0.16$
Genotype			
GG	90 (71.4)	128 (72.3)	42 (82.4)
GA	36 (28.6)	45 (25.4)	9 (17.6)
AA	0	4 (2.3)	0
		$\chi^2 = 3.12$,	$\chi^2 = 2.28$,
		$P = 0.21$	$P = 0.13$
Comparison (GG *vs* GA/AA)			
Odds ratio		0.87	0.53
95% CI		0.52 to 1.47	0.23 to 1.21

Control group was used as reference in all comparisons. CL/P, cleft lip with/without cleft palate; CP, isolated cleft palate.

no significant differences in rs642961 frequencies of genotypes between patients with or without clefts (Table 2). Furthermore, no clinical differences in the type of cleft or in its extension were observed between patients carrying or not the polymorphic alleles (Tables 1 and 2). The haplotype G-G was the most prevalent in the present Brazilian population, but no effects were observed with any of the tested haplotypes (Table 3).

Discussion

NSCL/P is a complex polygenic disease, and several modifying genes have been associated with its etiology (Vieira, 2008). Identification of new risk factors for NSCL/P development other than known environment factors would improve recognition of mothers at risk and could be relevant for genetic counseling. Mutations in *IRF6* cause a common form of syndromic cleft known as van der Woude syndrome (Kondo *et al*, 2002; Paranaiba *et al*, 2008), and mice deficient in *IRF6* develop cleft of the secondary palate (Ingraham *et al*, 2006). Furthermore, several studies demonstrated that *IRF6* DNA sequence variants are associated with NSCL/P in distinct populations (Blanton *et al*, 2005; Ghassibe *et al*, 2005; Scapoli *et al*, 2005; Vieira *et al*, 2007). Here, we demonstrated that the carrier rate of the polymorphic allele A of the *IRF6* rs2235371 was markedly low, and a similar distribution of alleles and genotypes was observed between control and NSCL/P groups. The frequency of the rs2235371 G allele in our population was 94.9% (94.8% for the control group, 94.9% for the CL/P group, and 95.1% for the CL group), which is comparable to the frequencies of 100% in Africans (Zucchero *et al*, 2004) and 90–100% in Europeans (Kondo *et al*, 2002; Zucchero *et al*, 2004). It is worth noting that Zucchero's study also included Brazilian individuals and found a prevalence of the G allele of 44.8% and a strong over-transmission of the A allele in patients with NSCL/P. These differences can be explained, at least in part, by the fact that the Brazilian population studied by Zucchero *et al* (2004) was

Table 3 Haplotype association between rs2235371 and rs642961 polymorphisms in the control and NSCL/P group

	Control group (n)	*Odds ratio 95% CI*	*CL/P* (n)	*Odds ratio 95% CI*	*CP* (n)	*Odds ratio 95% CI*
Haplotype						
G-G *vs* G-A	113/36	0.83	156/45	0.90	46/9	0.63
		0.52 to 1.36		0.54 to 1.49		0.30 to 1.34
A-G *vs* G-G	13/90	1.11	17/128	1.08	5/42	1.20
		0.54 to 2.34		0.50 to 2.35		0.42 to 3.47
A-G *vs* G-A	13/36	0.88	17/45	0.95	5/9	0.64
		0.40 to 1.97		0.41 to 2.21		0.17 to 2.36
G-G *vs* A-A	113/0	4.77	156/4	5.61	46/0	N/A
		0.61 to 37.41		0.76 to 41.51		
A-G *vs* A-A	13/0	5.09	17/4	5.93	5/0	N/A
		0.58 to 44.46		0.71 to 49.20		

Order of haplotypes: rs2235371 and rs642961. Control group was used as reference. CL/P, cleft lip with/without cleft palate; CP, isolated cleft palate. N/A, not applicable.

composed of native Indians, whereas our population was formed basically by an admixed of Caucasian European (particularly from Portugal, Spain, and Italy) and African ancestries. Interestingly, the G allele frequency in Italians is 100% (Zucchero *et al*, 2004). Furthermore, the G and A alleles are transmitted in an expected equilibrium in the Chilean population, which shows a gradient of ethnicity similar to ours (Suazo *et al*, 2008). Taken that the associated G allele is evolutionarily conserved and its frequency is very high in European, African, and Pakistani populations, it is unlikely that this polymorphism, by itself, is related with NSCL/P pathogenesis.

There are few studies describing the association of *IRF6* rs2235371 polymorphism and NSCL/P. Jugessur *et al* (2008) demonstrated in the Norwegian population a fetal relative risk of NSCL/P associated with a single dose of the A allele. Indeed, the authors demonstrated a significant reduction in the risk of NSCL/P development in children carrying the A allele (Jugessur *et al*, 2008). Similarly, the rs2235371 GG genotype was found at a significant low frequency in Han-Chinese patients with isolated CL, suggesting an increased risk for this type of cleft in rs2235371 polymorphic patients (Tang *et al*, 2009). In contrast, Srichomthong *et al* (2005) found a significantly higher frequency of the rs2235371 GG genotype in patients with NSCL/P compared with the controls. As in this study, a lack of association between rs2235371 polymorphism and NSCL/P was reported in populations from Germany and Chile (Hering and Grundmann, 2005; Suazo *et al*, 2008).

Recently, strong association between NSCL/P, in particular CL, and *IRF6* rs642961 polymorphism, and haplotype dependency of the rs2235371 G allele in relation to the polymorphic GA or AA genotypes of rs642961 were described (Rahimov *et al*, 2008). Thus, the presence of the A allele at *IRF6* rs642961 site, which alters the binding site of AP-2α transcription factor, is the true polymorphic allele associated with NSCL/P. We found a high prevalence of the polymorphic genotypes in the Brazilian population, but in disagreement with Rahimov's results, the genotype frequencies between patients and control subjects were quite similar. In addition, haplotype analysis showed no significant associations. Although the number of isolated CL is low in our sample, statistical analysis did not confirm an association of this type of cleft and *IRF6* rs642961 polymorphism (data not shown). Besides our small sample size, other possible causes of bias in our findings include assumption of Hardy-Weinberg equilibrium, unsegregated population with high ethnic admixture, and genotyping errors. Nevertheless all data were examined, at least, for two authors, and unclear results were double checked by repeating the PCR-RFLP analysis on new DNA samples. In Rahimov's study, the frequency of the minor allele of rs642961 was $\sim$25% in the European population and even higher (32%), compared to ours ($\sim$13%), in the Philippine population. It is well accepted that European populations such as from Norway, Denmark, and The Netherlands, which were included in Rahimov's study, are genetically homogenous in comparison with the Brazilian population, which may have contributed to our results. Interestingly, African populations demonstrated a very low frequency of the rs642961 minor allele (11%) (Rahimov *et al*, 2008).

In conclusion, our findings are consistent with a lack of involvement between NSCL/P and *IRF6* rs2235371 and rs642961 polymorphisms in the examined Brazilian population, demonstrating that their presences may not play an important role in the etiology of the NSCL/P isolately. The complex ethnic admixture of the Brazilian population may have contributed to such result.

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Conflict of interest

There is no conflict of interest.

Author contributions

Dr Paranaiba and Dr Bufalino were responsible for experiments, interpretation of the data, and manuscript preparation, Dr Barros was responsible for the sample collection, and Dr Martelli-Junior, Dr Graner, and Dr Coletta were responsible for the research design and manuscript revisions.

References

Aidar M, Line SR (2007). A simple and cost-effective protocol for DNA isolation from buccal epithelial cells. *Braz Dent J* **18:** 148–152.

Blanton SH, Cortez A, Stal S *et al* (2005). Variation in IRF6 contributes to nonsyndromic cleft lip and palate. *Am J Med Genet A* **137A:** 259–262.

Braybrook C, Doudney K, Marcano AC *et al* (2001). The T-box transcription factor gene TBX22 is mutated in X-linked cleft palate and ankyloglossia. *Nat Genet* **29:** 179–183.

Du X, Tang W, Tian W *et al* (2006). Novel IRF6 mutations in Chinese patients with Van der Woude syndrome. *J Dent Res* **85:** 937–940.

Ghassibe M, Bayet B, Revencu N *et al* (2005). Interferon regulatory factor-6: a gene predisposing to isolated cleft lip with or without cleft palate in the Belgian population. *Eur J Hum Genet* **13:** 1239–1242.

Hering R, Grundmann K (2005). The IRF6 p.274V polymorphism is not a risk factor for isolated cleft lip. *Genet Med* **7:** 209; (author reply 209–210).

Ingraham CR, Kinoshita A, Kondo S *et al* (2006). Abnormal skin, limb and craniofacial morphogenesis in mice deficient for interferon regulatory factor 6 (Irf6). *Nat Genet* **38:** 1335–1340.

Jugessur A, Rahimov F, Lie RT *et al* (2008). Genetic variants in IRF6 and the risk of facial clefts: single-marker and haplotype-based analyses in a population-based case-control study of facial clefts in Norway. *Genet Epidemiol* **32:** 413–424.

Kondo S, Schutte BC, Richardson RJ *et al* (2002). Mutations in IRF6 cause Van der Woude and popliteal pterygium syndromes. *Nat Genet* **32:** 285–289.

Mansilla MA, Cooper ME, Goldstein T *et al* (2006). Contributions of PTCH gene variants to isolated cleft lip and palate. *Cleft Palate Craniofac J* **43:** 21–29.

Martelli-Júnior H, Orsi-Júnior J, Chaves MR, Barros LM, Bonan PRF, Freitas JAS (2006). Estudo epidemiológico das fissuras labiais e palatais em Alfenas. *Minas Gerais - de 1986 a 1998* RPG. Revista de pós-graduação **13:** 31–35.

Mitchell LE, Beaty TH, Lidral AC *et al* (2002). Guidelines for the design and analysis of studies on nonsyndromic cleft lip and cleft palate in humans: summary report from a Workshop of the International Consortium for Oral Clefts Genetics. *Cleft Palate Craniofac J* **39:** 93–100.

Paranaiba LM, Martelli-Junior H, Oliveira Swerts MS *et al* (2008). Novel mutations in the IRF6 gene in Brazilian families with Van der Woude syndrome. *Int J Mol Med* **22:** 507–511.

Pegelow M, Peyrard-Janvid M, Zucchelli M *et al* (2008). Familial non-syndromic cleft lip and palate – analysis of the IRF6 gene and clinical phenotypes. *Eur J Orthod* **30:** 169–175.

Rahimov F, Marazita ML, Visel A *et al* (2008). Disruption of an AP-2alpha binding site in an IRF6 enhancer is associated with cleft lip. *Nat Genet* **40:** 1341–1347.

Riley BM, Mansilla MA, Ma J *et al* (2007). Impaired FGF signaling contributes to cleft lip and palate. *Proc Natl Acad Sci USA* **104:** 4512–4517.

Rodriguez S, Gaunt TR, Day IN (2009). Hardy-Weinberg equilibrium testing of biological ascertainment for Mendelian randomization studies. *Am J Epidemiol* **169:** 505–514.

Scapoli L, Palmieri A, Martinelli M *et al* (2005). Strong evidence of linkage disequilibrium between polymorphisms at the IRF6 locus and nonsyndromic cleft lip with or without cleft palate, in an Italian population. *Am J Hum Genet* **76:** 180–183.

Slayton RL, Williams L, Murray JC *et al* (2003). Genetic association studies of cleft lip and/or palate with hypodontia outside the cleft region. *Cleft Palate Craniofac J* **40:** 274–279.

Sozen MA, Suzuki K, Tolarova MM *et al* (2001). Mutation of PVRL1 is associated with sporadic, non-syndromic cleft lip/palate in northern Venezuela. *Nat Genet* **29:** 141–142.

Spina V (1973). A proposed modification for the classification of cleft lip and cleft palate. *Cleft Palate J* **10:** 251–252.

Srichomthong C, Siriwan P, Shotelersuk V (2005). Significant association between IRF6 820G- > A and non-syndromic cleft lip with or without cleft palate in the Thai population. *J Med Genet* **42:** e46.

Suazo J, Santos JL, Jara L *et al* (2008). Linkage disequilibrium between IRF6 variants and nonsyndromic cleft lip/palate in the Chilean population. *Am J Med Genet A* **146A:** 2706–2708.

Tang W, Du X, Feng F *et al* (2009). Association analysis between the IRF6 G820A polymorphism and nonsyndromic cleft lip and/or cleft palate in a Chinese population. *Cleft Palate Craniofac J* **46:** 89–92.

Van den Boogaard MJ, Dorland M, Beemer FA *et al* (2000). MSX1 mutation is associated with orofacial clefting and tooth agenesis in humans. *Nat Genet* **24:** 342–343.

Vieira AR (2008). Unraveling human cleft lip and palate research. *J Dent Res* **87:** 119–125.

Vieira AR, Karras JC, Orioli IM *et al* (2002). Genetic origins in a South American clefting population. *Clin Genet* **62:** 458–463.

Vieira AR, Orioli IM, Castilla EE *et al* (2003). MSX1 and TGFB3 contribute to clefting in South America. *J Dent Res* **82:** 289–292.

Vieira AR, Cooper ME, Marazita ML *et al* (2007). Interferon regulatory factor 6 (IRF6) is associated with oral-facial cleft in individuals that originate in South America. *Am J Med Genet A* **143A:** 2075–2078.

Wyszynski DF, Beaty TH, Maestri NE (1996). Genetics of nonsyndromic oral clefts revisited. *Cleft Palate Craniofac J* **33:** 406–417.

Zucchero TM, Cooper ME, Maher BS *et al* (2004). Interferon regulatory factor 6 (IRF6) gene variants and the risk of isolated cleft lip or palate. *N Engl J Med* **351:** 769–780.