RESEARCH ARTICLE

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IRF6 is a Risk Factor for Nonsyndromic Cleft Lip in the Brazilian Population

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Nonsyndromic cleft lip with or without cleft palate (NSCL/P) is a complex disorder with a worldwide incidence estimated at 1:700. Among the putative susceptibility loci, the *IRF6* gene and a region at 8q24.21 have been corroborated in different populations. To test the role of *IRF6* in NSCL/P predisposition in the Brazilian population, we conducted a structured association study with the SNPs rs642961 and rs590223, respectively, located at 5′ and 3′ of the *IRF6* gene and not in strong linkage disequilibrium (LD), in patients from five different Brazilian locations. We also evaluated the effect of these SNPs in *IRF6* expression in mesenchymal stem cells (MSC). We observed association between rs642961 and cleft lip only (CLO) ($P = 0.009$; odds ratio (OR) for AA genotype = 1.83 [95% Confidence interval (CI), 0.64–5.31]; OR for AG genotype = 1.72 [95% CI, 1.03–2.84]). This association seems to be driven by the affected patients from Barbalha, a location which presents the highest heritability estimate ($H^2 = 0.85$), and the A allele at rs642961 is acting through a dominant model. No association was detected for the SNP rs590223. We did not find any correlation between expression levels and genotypes of the two loci, and it is possible that these SNPs have a functional role in some specific period of embryogenesis.

Key words: cleft lip/palate; IRF6 transcription levels; rs642961; rs590223; mesenchymal stem cell; structured association; common disease-common variant; admixture population; heritability

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INTRODUCTION

Nonsyndromic cleft lip with or without cleft palate (NSCL/P) are among the most common congenital malformations [Gorlin et al., 2001], with an estimated worldwide prevalence of 1:700, varying across ethnicities [Vanderas, 1987; Loffredo et al., 2001; Mossey et al., 2009]. NSCL/P represents a significant burden for family and society, as affected individuals require multidisciplinary treatment until adult life. High heritability estimates and a 20–40 times increased recurrence risk in families with one affected patient suggest an important genetic component in NSCL/P etiology [Menegotto and Salzano, 1991; Christensen and Fogh-Andersen, 1993, Brito et al., 2011]. Worldwide studies have been conducted in order to dissect the genetics of NSCL/P, assuming a multifactorial model of inheritance with a common origin for the risk alleles (reviewed in Dixon et al., 2011). The two loci more consistently associated are the *IRF6* gene at 1q32.2 and a locus mapped to a gene desert at 8q24.21. The SNP rs2235371 (V274I) was the first marker in *IRF6* shown to be associated with NSCL/P, notably in Asian and South American populations [Zucchero et al., 2004]. This association was further reproduced in other populations, including those of European descent, and with other *IRF6* SNPs in linkage disequilibrium (LD) with V274I [Park et al., 2007; Jugessur et al., 2008; Huang et al., 2009; Blanton et al., 2010]. A second SNP, rs642961 (G>A), located in an *IRF6* enhancer, was reported as a likely cause

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of the association between the previously tested *IRF6* SNPs and NSCL/P and a more pronounced effect was observed in cleft lip only (CPO) patients than in cleft lip with cleft palate (CLP) patients. [Rahimov et al., 2008]. Only one in vivo study, conducted in a small sample, suggested that rs642961 genotypes modulate *IRF6* expression in an allele-dosage manner in skin lip from affected patients [Pan et al., 2010]. The SNP rs590223 (A>G) also is predicted to modulate in vivo the transcriptional levels of *IRF6* in liver cells [Schadt et al., 2008], and its association with NSCL/P is controversial [Jagomägi et al., 2010; Mostowska et al., 2010].

The extent of the contribution of *IRF6* SNPs for NSCL/P susceptibility in Brazilian patients is still unknown. A single previous study failed to find association of rs642961 in patients from the Southeastern region of Brazil [Paranaiba et al., 2010], while the SNP rs590223 has never been tested for association in Brazilian patients. The Brazilian population has a tri-hybrid origin, composed primarily by West Africans (brought as slaves between the 16th and 19th centuries), Europeans (settlers and immigrants), and Amerindians (native inhabitants; [Centro de Documentação e Disseminação de Informações (Brazil), 2007]), a factor that may introduce bias if not taken into account. Heritability of NSCL/P can be another confounding factor in our population, as it varies from 45 to 85% depending on the patient's geographical origin [Brito et al., 2011].

In the present study, our goal is to verify whether *IRF6* is associated with NSCL/P in Brazilian patients through a structured analysis of the SNPs rs642961 and rs590223 to control population admixture. We also discuss whether heritability of NSCL/P and clinical variability interfere in the detection of the association. Finally, we evaluate whether the genotype of the two SNPs were correlated with *IRF6* transcriptional expression levels in mesenchymal stem cells (MSC) derived from *orbicularis oris* muscle, as their transcriptome seems to reflect, at least in part, the dysregulated pathways of NSCL/P patients [Bueno et al., 2009].

MATERIALS AND METHODS

Patients and Controls

Patients were ascertained from medical programs of a non-governmental organization—Operation Smile (http://www.operationsmile.org/)—in five different Brazilian cities: Santarém-PA (n = 88), Barbalha-CE (n = 57), Fortaleza-CE (n = 225), Maceió-AL (n = 77), and Rio de Janeiro-RJ (n = 116), totaling 563 patients. Patients were classified as CPO if the cleft involved only the lip (unilateral or bilateral) with no involvement of the palate, but including those with alveolar defect, while those classified as CLP included unilateral and bilateral cleft lip associated with cleft palate. Detailed patients' ascertainment method and inclusion criteria were previously reported elsewhere [Brito et al., 2011]. We clinically excluded syndromic and cleft palate only patients. Only unrelated individuals were included in our sample.

The control group was provided by the Biobank of our institute, and consisted of 401 individuals (336 individuals ascertained in São Paulo and 65 from Fortaleza-CE, as detailed in Errera et al. [2006] and Orabona et al. [2009], respectively). Ethical issues prevented the collection of control samples in other locations. This study was approved by the Research Ethics Committee of the Institute of Biosciences, University of São Paulo, Brazil, and informed consent was obtained from the individuals or their legal tutors.

Association Analysis

To correct for the confounding effect of population structure, we used a panel of 40 ancestry-informative markers (AIMs) to characterize regional and individual contributions of African, European, and Amerindian ancestries. The AIMs consist of indel (insertion-deletion) polymorphisms spread across the autosomal chromosomes, and were accessed from Marshfield database (http://research.marshfieldclinic.org/), which holds a set of indel markers described by Weber et al. [2002]. Marker selection criteria were described in Bastos-Rodrigues et al. [2006], and also adopted in Santos et al. [2010] and Brito et al. [2011]. Our ancestry panel were previously validated by the verification that they allow the ancestry discrimination of the three parental populations (data available under request). An M13 tail labeled with FAM fluorochrome was added to every 5′ end of forward primers, so that each PCR product incorporated one tail, allowing multiplex PCR with 4 to 7 markers per reaction (primer sequences available upon request). Fragment analysis was performed with Gene Mapper software (Applied Biosystems, Foster City, CA), after capillary electrophoresis targeting the PCR products for the FAM-labeled M13 tail.

We next ran Structure 2.3.3 to estimate ancestry components of each sample, and thus infer possible roles of population stratification [Falush et al., 2007]. We assumed K = 3 (where K is the number of ancestry components that Structure estimates) based on the well-known tri-hybrid origin of Brazilians, and incorporated "learning samples" in the run, with pre-specified population of origin (European, African, and Amerindian) according to the Mammalian Genotyping Service database (Marshfield Clinic, Marshfield-WI), in order to assist the software to estimate ancestry of the admixed individuals.

The SNPs rs642961 and rs590223, located 42,563 bp apart (NCBI build 18) and in weak LD ($r^2 = 0.30$ in patients and 0.36 in controls) were genotyped with TaqMan® method (Applied Biosystems). Following the ancestry contribution estimations, we used STRAT to test each SNP for association, conditioning on the individual ancestries [Pritchard et al., 2000]. Considering the multiple testing, we accepted 0.0083 as threshold for significance in the analysis stratified by clinical variability, after Bonferroni correction. Odds ratios (OR) with 95% confidence inverval (CI) were estimated under additive model for the genotypes with one or two copies of the risk allele. Haplotype inferences were performed with Arlequin 3.5 software [Excoffier and Lischer, 2010].

Expression Analysis

The effect of both SNPs in *IRF6* expression levels was tested in MSC cultures established from *orbicularis oris* muscle fragments from an independent sample of 46 individuals (42 NSCL/P patients and 4 controls; median and average = 4 and 6.9 years old, respectively). Tissue samples were obtained from three different centers (Division of Plastic Surgery of University of São Paulo, SOBRAPAR

and Operation Smile). Informed consent was obtained from the patient's legal tutors.

Orbicularis oris muscle MSC cultures were established according to previously published protocol [Bueno et al., 2009], and expanded to 80% confluence. RNA and DNA extraction followed manufacturer's protocols (Macherey-Nagel, Germany). We confirmed the homogeneous mesenchymal immunophenotype of 10 cell cultures through flow cytometer analysis (EasyCyte Flow cytometer, Guava Technologies, Hayward, CA).

We used the Human Gene 1.0 ST microarray chips (Affymetrix, Santa Clara, CA) to measure whole gene expression of the 46 individuals, according to the manufacturer's protocol. Gene expression values were obtained with the three-step Robust Multi-array Average pre-processing method implemented in the software Expression Console (Affymetrix). As a validation, we also measured the relative expression of *IRF6* in 22/46 individuals through real-time PCR, and we observed a strong correlation between the two methods ($P = 0.001$, $r = 0.67$). These individuals were also genotyped for the two SNPs with TaqMan® method, and presented 100% of genotype concordance with those obtained through microarray.

RESULTS

Association Analysis

After excluding individuals with low AIM genotype assignment (<80%), we obtained a final sample of 471 patients (Santarém, n = 82; Barbalha, n = 52; Fortaleza, n = 163; Maceió, n = 62, and Rio de Janeiro, n = 112) and 391 controls. The two SNPs (Table I) and the indel markers (data not shown) were in Hardy–Weinberg equilibrium in controls.

The population structure analysis of our sample revealed a high contribution of European ancestry both in patients (61.2%) and in controls (71.1%; Table I). We also observed some variation in the ancestry contribution among the patient's groups, e.g., the highest Amerindian ancestry in Santarém (31.6%) and the highest African contribution in Rio de Janeiro (30.8%; Supplementary eTable I—See Supporting Information online). Individual ancestry contributions inferred by Structure are plotted in Figure 1.

Minor allele (A) frequency of rs642861 was higher in patients (0.19) than in controls (0.15) but no significant allelic association was observed in the total sample of patients ($P = 0.48$). We next stratified the analysis by "subphenotype" (CLO and CLP), and we observed a marginal association between rs642961 and CLO (90 patients; $P = 0.009$; $OR_{AA/GG} = 1.83$ [95% CI 0.64–5.31], $OR_{AG/GG} = 1.72$ [95% CI 1.03–2.84]), but no evidence of association with CLP (381 patients; $P = 0.62$, $OR_{AA/GG} = 1.62$ [95% CI 0.81–3.25], $OR_{AG/GG} = 1.12$ [95% CI 0.80–1.56]) The rs590223 SNP was not associated (NSCL/P, $P = 0.99$; CLO, $P = 0.85$; CLP, $P = 0.99$; Table I). The haplotype based analysis for rs642961–rs590223 did not present significant deviation from random distribution among cases and controls ($P = 0.95$).

Expression Analysis

We did not observe any significant effect neither for rs642961 (Spearman correlation test, $P = 0.15$, $r = 0.21$) nor for rs590223 ($P = 0.07$, $r = 0.27$) in the *IRF6* expression levels in *orbicularis oris* muscle MSC (Supplemntary eFigure 1; genotyping information in Supplementary eTable II – See Supporting Information online). Inter-individual variation of *IRF6* expression levels was not correlated with age in our sample (Spearman correlation, two-tailed $P = 0.74$, $r = 0.05$).

DISCUSSION

Recent studies have reported association of NSCL/P with many loci throughout the genome, but the majority of the studies conducted so far has analyzed non-Latin populations. The replication of those results in other populations and the investigation of the mechanisms by which the arising variants interfere in clefting susceptibility are crucial for a better understanding of the genetic architecture of NSCL/P.

In the present study, we did not detect association between rs642961 and NSCL/P, however, stratifying the analysis by clinical variability, we observed association in the CLO group ($P = 0.009$), thus confirming the previous observation of Rahimov et al. [2008] that the effect of this allele is more pronounced among CLO than in CLP patients. The A allele seems to be acting in a dominant model of

TABLE I. Estimated Ancestry Proportions of European, African, and Amerindian Parental Populations

			rs642961				rs590223			
	N	Ancestry contributions Eur/Afr/Am (%)	Observed genotype frequencies (%) AA/AG/GG	f(A) (%)	H–W *P*-value	Association test *P*-value	Observed genotype frequencies (%) GG/GA/AA	f(G) (%)	H–W *P*-value	Association test *P*-value
Controls	391	71/18/11	4/23/73	15	0.05	—	11/46/43	34	0.65	—
NSCL/P patients										
Total	471	61/22/17	6/26/68	19	0.004	0.48	11/47/42	34	0.43	0.99
CLO	90	61/22/17	6/33/61	22	0.74	0.009	10/53/37	37	0.16	0.85
CLP	381	61/22/17	5/25/70	18	0.001	0.68	44/45/11	34	0.82	0.99

Genotypic and allelic frequencies of rs642961 and rs590223, and *P*-values of Hardy–Weinberg test, and structured association test (at 95% confidence).
H–W, Hardy–Weinberg; NSCL/P, nonsyndromic cleft lip with or without cleft palate; CLO, cleft lip only; CLP, cleft lip and palate.

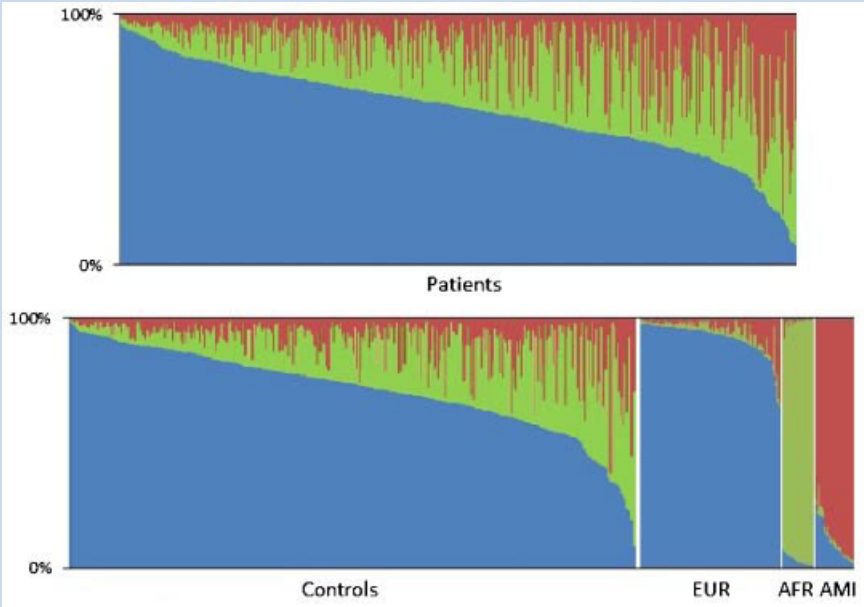

FIG. 1. Bar plot of contribution of European (blue), African (green), and Amerindian (red) ancestries to our sample, in which single columns represent an individual. NSCL/P patients in the upper panel; in the lower panel, controls, and parental populations (EUR, Europeans, AFR, Africans, AMI, Amerindians—used as "learning samples" to assist the ancestry estimations of admixed individuals in Structure, run assuming K = 3 subpopulations).

susceptibility, given the similar OR observed for homozygous (1.83 [95% CI 0.64–5.31]) and heterozygous (1.72 [95% CI 1.03–2.84]) genotypes, in accordance to previous data obtained among European ancestry NS CL/P patients [Rahimov et al., 2008; Birnbaum et al., 2009]. Although these findings might suggest different etiologies for CLO and CLP, their co-ocurrence within families is not uncommon, which in turn favors a common etiology for them. It is thus possible that CLO etiology might be genetically more homogenous than CLP, that is, only one or a few molecular pathways are deregulated among CLO patients.

We previously reported on a positive association of rs987525 SNP (8q24.21) in Brazil, driven by a subpopulation of patients with the highest heritability estimates (from Barbalha; $H^2 = 85\%$) as compared to other four regions of Brazil (Santarém, Fortaleza, Maceió, and Rio de Janeiro presented $H^2 = 45–71\%$; [Brito et al., 2012]). A stratified analysis by region of origin in the present study did not reveal a strong association with any group (Supplementary eTable I—See Supporting Information online), however, we observed a trend of positive association only in the Barbalha subset (Barbalha, $P = 0.02$; Santarém, $P = 0.59$; Fortaleza, $P = 0.66$; Maceió, $P = 0.44$; Rio de Janeiro, $P = 0.69$). In Barbalha, the frequency of the A allele/rs642961 among CLO patients is 0.5, which is much higher than among CLO patients from the other regions (Santarém = 0.21, Fortaleza = 0.20, Maceió = 0.22, Rio = 0.15), and it might be the contributing factor to the trend of association observed only in Barbalha. In support to this finding, we did not observe such substantial deviation of the rs642961 A allele frequency comparing CLP patients (Barbalha: 0.23, Santarém:0.22, Fortaleza: 0.18, Maceió: 0.14, Rio: 0.10) and CLO patients. These results further support the hypothesis that high-heritability populations are more suitable to find moderate-to-low-effect alleles in association mapping approaches.

The marginal association observed in the small CLO sample is in accordance with a common *IRF6* risk allele in populations across the world. Although the rs590223 SNP failed to reach positive association, it is still possible that other susceptibility variants at *IRF6* locus are also involved with NSCL/P susceptibility, independently of rs642961. For example, SNP rs2235371 but not rs642961, has been associated with NSCL/P in some population groups [Blanton et al., 2010]. Giving the low frequency of rs2235371 in our population (previously estimated at 0.05 in a set of 108 NS CL/P patients, unpublished data), we were not able to investigate its role in the orofacial clefting predisposition in our sample.

We verified a lack of correlation between *IRF6* expression levels and the SNPs rs642961 and rs590223 in the MSCs here tested, which in turn might be the consequence of the small sample size or the studied cell type might not be the best one to address this issue. It is possible that these alleles interfere in the proper expression of *IRF6* in critical moments of embryonic craniofacial development and different functional analysis will be necessary to conduct, in order to understand how they contribute to the occurrence of CLO or CLP.

In conclusion, despite the limitations of the small sample size of the CLO group, we found a weak association between the CLO group and rs642961, which risk allele seems to act in a dominant model. The CLO patients also explain the trend of association observed in Barbalha, the population subset with the highest heritability of NSCL/P. Our findings, together with the literature, support the model of a common risk allele in CLO in several

populational groups. Although the structured association approach minimizes the effects of population structure, we can never discard the possibility of false positive results in such heterogeneous population. We expect that the elucidation of the mechanisms through which rs642961 leads to NSCL/P predisposition will impact the translation of the association studies results into the clinic.

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