Linkage Disequilibrium Between *IRF6* Variants and Nonsyndromic Cleft Lip/Palate in the Chilean Population

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To the Editor:

The genetics of nonsyndromic cleft lip and palate (NSCLP) is complex. Several candidate genes have been associated with this phenotype, but replication studies showed conflicting results [Carinci et al., 2000; Murray, 2002]. Candidate gene analyses indicate that variants in a handful of genes taken together may account for approximately 15–20% of the genetic contribution to NSCLP [Zucchero et al., 2004; Vieira et al., 2005; Riley et al., 2007].

Association of NSCLP and *IRF6* SNPs has been reported [Zucchero et al., 2004]. Strong evidence of over transmission of the ancestral allele of the p.V274I polymorphism to patients with NSCLP was observed in Asians but not in groups of European descent. However, analysis of additional SNPs showed that linkage disequilibrium with NSCLP was also detectable in Iowa and Danish populations. Subsequently, several recent studies have reported genetic variants associated with NSCLP in numerous groups [Blanton et al., 2005; Scapoli et al., 2005; Srichomthong et al., 2005; Morkünienė et al., 2006; Park et al., 2007; Vieira et al., 2007].

The aim of this study was to evaluate the role of *IRF6* in NSCLP in the Chilean population, which represents an admixture of Amerindians and Spaniards [Rothhammer et al., 1968]. To accomplish this we performed an association study of *IRF6* SNPs and NSCLP using a case-parents trio design.

The study group comprised 150 unrelated case-parent trios ascertained through unrelated probands affected with NSCLP. Among the cases, 94 were sporadic (17 CL and 77 CL/P) and 56 had a positive family history (9 CL and 47 CL/P). No isolated cleft palate cases were included. Families were recruited from the School of Dentistry, University of Chile, the Dr. Alfredo Gantz Foundation, the Cleft Lip/Palate Center, Luis Calvo Mackenna Hospital and the Cleft Lip/Palate Center, Exequiel Gonzalez Cortes Hospital, located in the Santiago, Chile. Interviews of at least three family members were conducted to provide detailed information for pedigree construction. Each proband and their relatives were evaluated to exclude syndromic CLP. All the families enrolled were of admixed Chilean ancestry and included subjects presenting NSCLP as the unique familial disease. To exclude potential teratogenic influences, a history was taken to evaluate the presence of any neurological disorder in the family or the use of teratogens, such as phenytoin, warfarian, and ethanol.

The Chilean population shows a gradient of ethnicity, Amerindian admixture, genetic markers, socioeconomic strata, and prevalence of NSCLP [Valenzuela, 1988; Palomino et al., 1997]. The families included in this study belong to low to middle-low socioeconomic strata which present the

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TABLE I. *IRF6* SNPs Information and Allele and Haplotype TDT Results

SNP	Alleles[a]	MAF[b]	T/NT[c]	*P*-value[d]	*P*-values for[e] 2 SNPs	3 SNPs	4 SNPs
rs764093	T/C	0.363	47/44	0.753	0.094	0.022	0.068
rs2236909	G/A	0.442	73/58	0.190	0.013	0.139	—
rs2235375	C/G	0.448	56/70	0.212	0.307	—	—
rs2235371	C/T	0.262	46/41	0.592	—	—	—

[a]Mayor allele listed first.
[b]MAF: minor allele frequency was estimated using 160 control individuals.
[c]T/NT: transmission/nontransmission count from heterozygous parents are given for the minor allele.
[d]*P*-value for TDT.
[e]Global *P*-value for haplotype analysis. In each case, the *P*-value is place on the line of the first SNP of the haplotype.

highest degree of Amerindian admixture. The study was approved by the Institutional Review Board of the School of Medicine of the University of Chile.

Genomic DNA was extracted from peripheral blood lymphocytes according to the method described by Lahiri and Nurnberger [1991]. Four *IRF6* SNPs (supporting information Table SI may be found in the online version of this article) were chosen based on the *HapMap* database (http://www.hapmap.org/cgi-perl/gbrowse/hapmap_b35/) [Carlson et al., 2004] with a selection criterion of $r^2 > 0.8$ and minor allele frequency (MAF) > 0.2. We used PCR-RFLP for genotyping *IRF6* SNPs. Primers were designed based on the SNP flanking sequence (http://www.ncbi. nlm.nih.gov) and using *Primer3* (http://frodo.wi.mit. edu/cgi-bin/primer3/primer3_-www.cgi). Adequate restriction endonucleases for each SNP were selected using the software *DNA for Windows version 2.2* (http://www.dna-software.co.uk/).

Simple proportions were used to estimate allele frequencies for the four SNPs (Table I). All the SNPs were in Hardy–Weinberg equilibrium (data not shown) according to an exact test [Schneider et al., 2000]. Lewontin's standardized disequilibrium coefficient (D′) [Devlin and Risch, 1995] was calculated using STATA 8 (http://www.gene.cimr.cam.ac.uk/clayton/software/stata). Strong linkage disequilibrium between pairs of SNPs was observed (Table II).

The Transmission Disequilibrium Test (TDT) [Spielman et al., 1993] was used to assess the differential pattern of excess allele over transmission. TDT analysis of the four individual SNPs showed that none of them presented a significant transmission distortion from heterozygous parents to affected progeny (Table I). Additionally, HBAT, the haplotype extension of FBAT program [Laird et al., 2000], was used to examine in extended haplotypes possible transmission distortions. The results of the Global *P*-value for TDT haplotype analysis showed significant results for rs764093-rs2235375 ($P = 0.022$) and rs2236909-rs2235375 ($P = 0.013$) (Table I). Table III presents haplotypes that showed significant transmission distortion in two-, three-, and four-marker haplotype analyses. The haplotypes which showed the highest significance shared the allele combination C-A-C for the first three SNPs rs764093-rs2236909-rs2235375 (C-A-C-X, $P = 0.009$ and C-A-C-C $P = 0.008$). Haplotypes X-A-C-X ($P = 0.039$), C-A-X-C ($P = 0.046$), C-X-C-C ($P = 0.039$) were also over transmitted and partially share the allele combination C-A-C. It is likely that the significant haplotypes are not the risk factors, but rather in linkage disequilibrium with the susceptibility changes in *IRF6*.

Our results differ from previous studies that reported altered allele and haplotype transmission in Asians and Caucasians, which tested SNPs rs2235371 (p.V274I) and rs2235375 [Zucchero et al., 2004; Blanton et al., 2005; Scapoli et al., 2005; Srichomthong et al., 2005; Morkūnienė et al., 2006;

TABLE II. Linkage Disequilibrium Between *IRF6* SNPs Estimated in 160 Control Individuals

	rs764093	rs2236909	rs2235375	rs2235371
rs764093		0.882	0.855	0.853
rs2236909	<0.001		0.943	0.952
rs2235375	<0.001	<0.001		0.953
rs2235371	<0.001	<0.001	<0.001	

D′ are located above the diagonal; *P*-values are below the diagonal.

TABLE III. Two, Three, and Four SNPs Haplotypes Demonstrating Significantly Altered Transmission

Haplotype	Observed[a]	Expected[b]	*P*-value
Two markers			
X-A-C-X	149	128	0.039
X-G-X-C	89	100	0.050
Three markers			
C-A-C-X	115	78	0.009
C-A-X-C	90	79	0.046
C-X-C-C	95	84	0.039
T-X-C-C	14	19	0.027
Four markers			
C-A-C-C	110	71	0.008
C-A-G-C	8	12	0.038

Order of SNPs: rs764093-rs2236909-rs2235375-rs2235371. An X indicates that the SNP was not included in creating the haplotype.
[a]Number of individuals that presented the haplotype.
[b]Expected number of individuals that should have presented the haplotype.

Park et al., 2007; Vieira et al., 2007]. The present findings can be partially explained given that the patients are from an admixed Caucasian-Amerindian population. For example, the p.V274 allele frequency of p.V274I in our sample was approximately 74%, greater than in Asians but lower than in Caucasians [Zucchero et al., 2004; Scapoli et al., 2005; Blanton et al., 2005; Srichomthong et al., 2005; Park et al., 2007]. Zucchero et al. [2004] proposed that p.274V allele shows a three-fold increased risk of recurrence of NSCLP in families with one affected child in Caucasian, Asian, and South American populations. Nevertheless, Hering and Grundmann [2005] do not consider p.274V as a risk allele given that its frequency in Asians, Europeans, Africans, and Pakistani is close to 100%.

Our findings are consistent with the association of *IRF6* gene variations in NSCLP. Functional further studies are needed to establish the role of *IRF6* in the etiology of NSCLP.

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