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The Association Between Interferon Regulatory Factor 6 (*IRF6*) and Nonsyndromic Cleft Lip With or Without Cleft Palate in a Honduran Population

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Objectives/Hypothesis: Interferon regulatory factor 6 (*IRF6*), the gene that causes van der Woude syndrome (VWS), is a candidate gene for nonsyndromic cleft lip with or without cleft palate (NSCLP) because a number of studies have supported an association between NSCLP and single nucleotide polymorphisms (SNPs) in *IRF6* in several populations. This project investigated the contribution of *IRF6* to NSCLP in the Honduran population, a previously unstudied group with a high prevalence of NSCLP.

Study Design: Family-based joint linkage and association study.

Methods: A set of five SNPs in and around *IRF6* previously reported to be associated with NSCLP were tested for association with NSCLP in 276 affected and unaffected Honduran individuals from 59 families with at least two members affected by clefting and at least one member with confirmed NSCLP.

Results: We observed support of linkage for three SNPs—rs1856161, rs2235371, and rs2235377—under a dominant model (log of odds [LODs] = 1.97, 1.56, 1.73, respectively). Subsequent single-point, haplotype, and joint linkage and association analyses continued to support the association with NSCLP ($P \leq .05$) at these three SNPs. When analysis was restricted to NSCLP cases, excluding cleft palate only cases, support for association strengthened.

Conclusions: This is the first study to demonstrate that three candidate SNPs within *IRF6* are significantly associated with NSCLP in the Honduran population, providing the first genetic clue to NSCLP observed in the Honduran population and confirming findings from populations in other parts of the world. Further studies are needed to identify the putative variant(s).

Key Words: Nonsyndromic, cleft lip, cleft palate, Van der Woude syndrome, candidate gene, *IRF6*, Honduras, Central America.

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INTRODUCTION

Cleft lip with or without cleft palate (CLP) and isolated cleft palate (CP) are common congenital malformations, occurring in roughly 1 per 1,000 Caucasian births, with higher prevalence rates of roughly 2.1 per 1,000 and 3.6 per 1,000 in Asian and North American Indian populations, respectively.[1] These abnormalities are associated with significant morbidity and mortality, particularly in countries with limited resources to care for these children.[2] CLP is a complex disease that has been linked to environmental and genetic risk factors; because orofacial development is a complex process, it is likely that many genes and signaling processes are involved.[3] Genetic linkage and association studies have identified several candidate genes and chromosomal regions associated with CLP, although findings have not been replicated consistently.

Interferon regulatory factor 6 (*IRF6*), located on chromosome 1 between 1q32.3 and q41 has been associated with both syndromic and nonsyndromic forms of CLP. Frameshift and nonsense mutations resulting in haploinsufficiency of *IRF6* are responsible for the majority

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TABLE I.
Summary of Study Families.

Total number of families	59
Genotyped family members	
Range	1–12*
Mean ± SD	4.7 ± 3.3
Number of affected individuals per family by report	
Range	2–5
Mean ± SD	2.5 ± 0.8
Distribution of affecteds	
2	38 families
3	14 families
4	6 families
≥5	1 family
Number of affected individuals with DNA sample per family	
Range	1–3
Mean ± SD	1.5 ± 0.6

*All samples from families with ≥2 affected members by report. SD = standard deviation.

of cases of van der Woude syndrome (VWS), an autosomal dominant disorder characterized by oral clefting, pitting of the lower lip, and occasional hypodontia.[4–6] It has been estimated that 15% of patients with VWS have isolated CLP without other syndromic markers.[4,5] This observation, in addition to in situ hybridization studies demonstrating the expression of *IRF6* in developing palatal tissue, suggests that *IRF6* may also be a candidate gene for nonsyndromic CLP (NSCLP).[6] Several recent studies have provided evidence for an association between polymorphic variations in *IRF6* and nonsyndromic clefting in populations from Asia, Europe, and South America.[5–9]

The present investigation examined a cohort of patients with NSCLP and their family members recruited in Honduras to evaluate the involvement of *IRF6* in cases of NSCLP in Central America. To date, the genes responsible for NSCLP have not been studied in the Honduran population. While no population-based epidemiologic studies of NSCLP have been conducted in Honduras to our knowledge, our experience suggests that the Honduran population has an increased prevalence of cleft lip and palate. This is presumably because of the Hondurans' Amerindian ancestry, which has its roots in Northeast Asia; both Native American Indian and Asian populations have an increased prevalence of orofacial clefting.[1] This, combined with the fact that the Honduran population is stable, with little influx of other populations, makes it well suited for investigation of a common genetic etiology for NSCLP.

MATERIALS AND METHODS

Subjects

In an effort to identify families with a genetic predisposition to NSCLP, our study was designed to include families with more than one member affected by clefting. Overall, 276 Honduran individuals from 59 families were studied. All study subjects were members of families with at least two members reported to have clefting and, of those affected, at least one case of NSCLP. Individuals with clefts and their family members were identified through a cleft clinic affiliated with the Honduran Medical Institute and the Department of Plastic Surgery of Hospital Escuela, a large public teaching hospital affiliated with the University of Honduras, located in Tegucigalpa, Honduras' capital city. All persons with clefts were screened for the presence of associated anomalies or syndromes, and only those determined to have nonsyndromic clefts were included in the study. This study was approved by the Institutional Review Board at Columbia University Medical Center, as well as by the Office of the Hospital Director at Hospital Escuela.

After written informed consent was obtained, 7.5 mL of whole blood was collected from study participants for genetic analysis and processed using DNA-isolation kits (Flexigene 250 mL kit, Qiagen, Valencia, CA). A systematic and thorough family history of affected and unaffected members and their relationships was also obtained to construct pedigree information. A summary of studied families and subjects is provided in Tables I and II.

Genotyping and Quality-Control Measure

Prior to single nucleotide polymorphism (SNP) genotyping, reported relationships were checked for nonpaternity. For this purpose, 39 unlinked microsatellite markers, including 38 autosomal markers and one X chromosome marker, were genotyped by Prevention Genetics (Marshfield, WI). A genetic map from the Marshfield Medical Research Foundation was used for the location of microsatellite markers.[10] In addition, five SNPs in and around *IRF6* were genotyped by Prevention Genetics. Genotyping was carried out using allele-specific polymerase chain reaction (PCR) with universal molecular beacons. DNA sequencing of positive control DNA samples was completed to assure correct assignment of alleles.

Subsequently, a combined set of all microsatellite and SNP markers were used to evaluate relationships among family members by computing allele sharing among relative pairs as implemented in the Pedigree Relationship Statistical Test (PREST, University of Chicago) software.[11] Based on the results, relationships in 21 families were corrected and 23 individuals who were not biologically related to any family were excluded (detailed information is available from the authors upon request). Reported relationships were resolved in all families. Subsequently, errors in Mendelian inheritance were analyzed using PedCheck (University of Pittsburgh).[12] Genotypes that failed to mendelize were treated as missing. Allele frequencies were estimated using all family members. The estimates from this approach are reported to be robust and closely approximate allele frequencies based on founders. Hardy-Weinberg equilibrium was assessed for all SNPs using unaffected individuals with Haploview, version 4.0,[13] which estimates

TABLE II.
Summary of Study Participants.

	Total n (%)	Female n (%)	Male n (%)
Participants	276	162 (58.7%)	114 (41.3%)
Affection status			
Unaffected	185 (67%)	124 (67%)	61 (33%)
Affected	91 (33%)	38 (41.8%)	53 (58.2%)
Cleft lip only	17 (18.7%)	9 (52.9%)	8 (47.1%)
Cleft lip with cleft palate	64 (70.3%)	24 (37.5%)	40 (62.5%)
Cleft palate only	4 (4.4%)	4 (100%)	0 (0%)
Cleft type unknown	6 (6.6%)	1 (16.7%)	5 (83.3%)

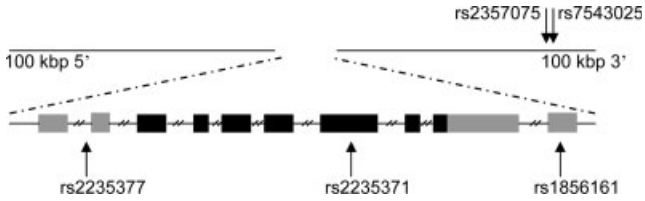

Fig. 1. Diagram of *IRF6* gene locus. Black denotes coding regions and gray untranslated regions. Single nucleotide polymorphisms (SNPs) are identified by their rs identifier, and the location of each SNP is indicated by an arrow.

probability of deviation explained by chance (*P* values).[14] Linkage disequilibrium plots and coefficients (D′) were also generated utilizing Haploview.[13]

SNP Selection and Genotyping

We selected candidate SNPs in the *IRF6* gene that have been previously associated with clefting from the literature. Specifically, rs7543025, rs2357075, rs1856161, rs2235371, and rs2235377 at the *IRF6* locus were chosen because they showed a strong association ($P < .01$) with NSCLP in the Filipino population.[5] Three SNPs (rs1856161, rs2235371, and rs2235377) were within *IRF6*, and two (rs7543025 and rs2357075) were within 100 kbp 3′ of the gene. *IRF6* and the position of candidate SNPs are shown in Figure 1. These SNPs were selected from a study of clefting in the Filipino population by Zucchero et al. in 2004[5] because the Honduran population, which is primarily Mestizo (an admixture of Amerindian and Spanish), shares a common ancestral background with the Asian population to some extent and because the Asian population has a high prevalence of CLP.

Statistical Analysis

Linkage Analysis and Allelic and Haplotype Association. Four SNPs were used in linkage and association analyses and one SNP (rs2357075) was excluded because it was found to be monomorphic in the study population. Linkage as well as joint linkage and linkage disequilibrium analyses were performed using Pseudomarker (Columbia University), version 1.0.5.[15] We assumed a disease gene frequency of 0.01 and used an affecteds only model with penetrance of 0.0, 0.1, and 0.1 for dominant models, and 0.0, 0.0, 0.1 for recessive models. To estimate recombination fraction, the physical distance in base pairs between markers was converted to genetic distance in cM, assuming 1 cM is comparable to ~1 mega base.

Because three contiguous SNPs—rs1856161, rs2235371, and rs2235377—had suggestive linkage and association, a haplotype analysis was performed using these three SNPs (Table V). Single-point and haplotype association analyses were performed under an additive model using version 2.0.2c of Family Based Association Test (FBAT, Harvard University) software.[16] Rare (≤5) haplotypes were dropped.

For all above models, the data first were analyzed using all family members, then analysis was repeated after removing individuals affected by cleft palate only (n = 4) from the data set to reduce genetic heterogenity. The results from both analyses are presented.

RESULTS

Tables I and II show the distribution of demographic and clinical characteristics of families and their members. Of the 59 families, on average, 4.7 members participated in the study, and 2.5 persons were reported to be affected by clefting. Through direct examination, an average of 1.5 persons per family were confirmed to have a cleft. Twenty-one families reported three or more members with clefting (at least one affected by NSCLP), and 38 reported two members with clefting (at least one affected by NSCLP). Of the 276 evaluated individuals, 33% were affected by a cleft. Of the affected individuals, 89% had CLP, 4.4% had CP, and 6.6% had uncertain cleft type.

None of the SNPs deviated from Hardy-Weinberg equilibrium among unaffected individuals (Table V). One of the five markers, rs2357075, was found to be monomorphic in the study population and was therefore excluded from linkage and single-point analyses. Three SNPs, rs1856161, rs2235371, and rs2235377, were found to be in strong linkage disequilibrium (D′ = 1) (Fig. 2).

Linkage analysis under a dominant model of inheritance supported suggestive linkage for two SNPs, rs1856161 and rs2235377, with log of odds (LOD) scores of 1.97 and 1.73, respectively (Table III). However, support for rs2235371 was modest, with an LOD score of 1.56. Evidence for linkage with rs7543025 was absent (LOD = 0.03). When analysis was restricted to CLP only (not shown) individuals, LOD scores decreased slightly to 1.65, 1.47, and 1.41 for rs1856161, rs2235371, and rs2235377, respectively, under the dominant model.

Linkage analysis was followed by a joint linkage and linkage disequilibrium analysis to determine whether significant allelic association exists given the suggestive linkage with the disease locus (Table IV). For all three contiguous SNPs, the model under a recessive model of inheritance provided stronger support for association compared with the model under a dominant model. Subsequently, when CLP only individuals were

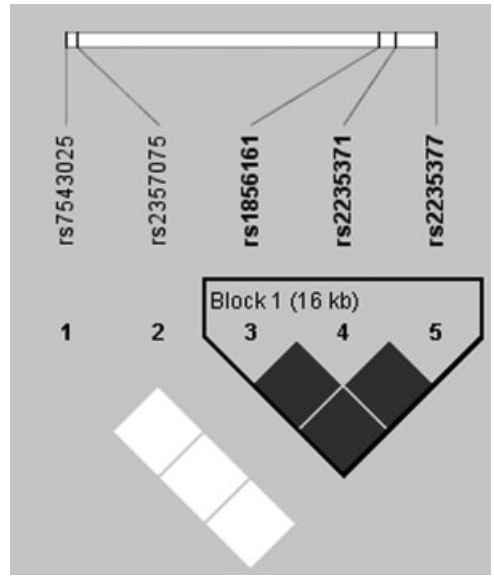

Fig. 2. Linkage disequilibrium (LD) plot of markers evaluated in *IRF6*. LD plot was generated using Haploview. Dark gray indicates strong evidence of linkage disequilibrium, and white denotes areas with strong evidence of recombination. D′ values of 1 within area of linkage disequilibrium are not pictured (boxes are empty). As illustrated, rs1856161, rs2235371, and rs2235377 are in tight linkage disequilibrium (D′ = 1) with one another.

TABLE III.
Single-Point Linkage Analysis Results.

SNP	Location	Intermarker Distance in bp	LOD Score Statistic*	
			Dominant Model	Recessive Model
rs7543025	207,936,042		0.030	0.021
rs2357075†	207,939,337	3,295		
rs1856161	208,025,791	86,454	1.969	1.317
rs2235371	208,030,703	4,912	1.555	1.024
rs2235377	208,042,015	11,312	1.728	1.214

*Calculated by Pseudomarker using model-based parameters: disease gene frequency 1%, recessive model penetrance (++ 0.0, D+ 0.0, DD 0.1), dominant model penetrance (++ 0.0, D+ 0.1, DD 0.1), theta based on intermarker distance between SNPs (bp converted to cM).

†Found to be monomorphic in study population; not tested for linkage analysis. SNP = single nucleotide polymorphism; LOD = log of odds.

examined, support for linkage for the three contiguous SNPs remained comparable, but support for allelic association conditional on linkage improved somewhat for both recessive and dominant models ($P \leq .05$).

A single-point family-based association test using FBAT was then performed and confirmed the positive association with rs1856161, rs2235371, and rs2235377 observed in Pseudomarker analysis, as expected (Table V). Even stronger evidence of association with these three SNPs was found when subanalysis was performed after exclusion of individuals affected by cleft palate only (P = .009, .012, and .010, respectively). A haplotype analysis using rs1856161, rs2235371, and rs2235377 was performed to improve information content from these three contiguous SNPs (Table VI). We observed that individuals carrying the haplotype TTC at rs1856161, rs2235371, and rs2235377 were at higher risk of having NSCLP (z = 2.251; P = .024). When analysis was restricted to CLP-affected individuals, the support for association strengthened ($z = 2.751$; $P = .006$).

DISCUSSION

IRF6 was significantly associated with NSCLP in this Honduran cohort with familial NSCLP, and the association was even stronger when individuals with cleft palate only were removed from analysis. Specifically, two SNPs within *IRF6*—rs1856161 and rs2235377—showed suggestive linkage with the disease locus, and rs2235371 showed modest support for linkage, as well as a strong allelic association. The present investigation, which is the first study of the genetic etiology for NSCLP in the Honduran population, confirms reports from the study of *IRF6* in other populations, including Italian, Asian, European-American, and Belgian CLP families.[7–9,17]

To date, the most comprehensive study of *IRF6* was performed by Zucchero et al.[5] The authors examined 36 SNPs in and around *IRF6* in 10 populations from Asia, Europe, and South America. Of these, nine SNPs were found to be significantly associated with clefting in individuals from the Philippines, which has a high incidence of clefting. We selected five of these markers for investigation and found three to be significantly associated with clefting in the Honduran population.

Of interest, one of these markers, rs2235371, is a common polymorphic variant where isoleucine replaces valine at position 274 in *IRF6*'s protein binding domain (V274I). Zucchero and colleagues hypothesized that because valine is highly conserved among species, the variant might affect gene function. In their overall population, as well as in the Filipino population, Zucchero et al. confirmed the variant's association with NSCLP. Additionally, in the overall population, Zucchero et al. demonstrated a recessive effect of the V allele and an increase in the frequency of the V/V genotype in probands compared with unaffected controls.[5] Additional studies of populations from Norway and Taiwan have provided further support for an association between clefting and rs2235371.[9,18]

TABLE IV.
Joint Linkage and Linkage Disequilibrium (LD) Analysis.

SNP	*P* Value Statistics*							
	All Affected				Cleft Palate Only Excluded			
	Recessive Model		Dominant Model		Recessive Model		Dominant Model	
	Linkage	LD/Linkage	Linkage	LD/Linkage	Linkage	LD/Linkage	Linkage	LD/Linkage
rs7543025	.379	.484	.355	.661	.329	.542	.261	.416
rs1856161	.007	.016	.001	.052	.009	.006	.003	.029
rs2235371	.015	.009	.004	.041	.012	.003	.005	.022
rs2235377	.009	.013	.002	.060	.012	.005	.005	.034

*Calculated by Pseudomarker using model-based parameters: disease gene frequency 1%, recessive model penetrance (++ 0.0, D+ 0.0, DD 0.1), dominant model penetrance (++ 0.0, D+ 0.1, DD 0.1), theta based on intermarker distance between SNPs (bp converted to cM).

TABLE V.
Single-Point Analysis.

SNP	Alleles*	Minor Allele Frequency	HW *P* Value	All Affected		Cleft Palate Only Excluded		Risk Allele
				Informative Families	*P* Value†	InformativeFamilies	*P* Value†	
rs7543025	G:A	0.262	.108	21	.954	20	.906	
rs2357075‡	A:A	0.00						
rs1856161	C:T	0.325	.127	24	.028	23	.009	T
rs2235371	C:T	0.326	.363	20	.040	19	.012	T
rs2235377	T:C	0.329	.089	24	.032	23	.010	C

*Minor allele listed first.
†Using additive model.
‡Found to be monomorphic in study population; excluded from single-point analysis. SNP = single nucleotide polymorphism; HW = Hardy-Weinberg.

Our results suggest that the V274I variant is associated with NSCLP; however, we cannot conclude whether this variant is the causal variant or whether it is in linkage disequilibrium with the *IRF6* mutation responsible for clefting in this Honduran cohort. In the study population, a group of markers flanking the V274I variant—rs1856161-rs2235371-rs2235377—demonstrated tight linkage disequilibrium ($D' = 1$), and these three SNPs were associated with CLP, suggesting that this is an important candidate region. However, because this strong association with flanking SNPs may merely represent high LD in the region, it will be necessary to perform further refined analysis of this region to identify putative variant(s).

Despite its limitations (relatively small sample size and limited number of SNPs), this is the first study to report the association between specific SNP markers in *IRF6* and NSCLP in a Central American population. The populations of Central America, given their Amerindian ancestry, have a high incidence of cleft lip and palate. This study suggests that *IRF6* may play a role in the etiology of clefting in the Honduran population and that it may be a gene of interest in studying other Central American populations. Detailed studies with a dense coverage of SNPs are needed in the Honduran population, as well as other Central American populations, to further evaluate the association between NSCLP and *IRF6* in Central America.

CONCLUSIONS

Although SNP coverage of *IRF6* and the number of study subjects was limited, this study suggests that *IRF6* is associated with nonsyndromic clefting in the Honduran population and that it is a gene of interest in the study of the genetic etiology of clefting in Central America. These results are consistent with previous reports of an association between *IRF6* and clefting in other non-Hispanic populations.

TABLE VI.
Haplotype Analysis*

rs1856161	rs2235371	rs2235377	All Affected		Cleft Palate Only Excluded	
			Z Score†	*P* Value†	Z Score†	P Value†
T	T		2.224	.026	2.722	.007
C	C		−2.224	.026	−2.722	.007
T	C					
C	T					
	T	C	2.251	.024	2.751	.006
	C	T	−2.251	.024	−2.751	.006
	C	C				
	T	T				
T	T	C	2.251	.024	2.751	.006
C	C	T	−2.251	.024	2.751	.006
T	C	C				
T	T	T				
C	T	T				

*Rare haplotypes (<5) excluded from analysis.
†Using additive model.

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