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Association of Common Variants, Not Rare Mutations, in *IRF6* With Nonsyndromic Clefts in a Honduran Population

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Objectives/Hypothesis: Cleft lip with or without cleft palate (CL/P) is a common birth defect throughout the world. Linkage studies have shown interferon regulatory factor 6 (*IRF6*) to be associated with CL/P in multiple populations, including one in Honduras. It is unknown, however, whether rare sporadic mutations or common variants are the cause of this association, and reports exist supporting both hypotheses. Thus, it is important to determine the cause for this association in a Honduran population.

Study Design: Case-control and family-based association studies.

Methods: Families with two or more members affected by CL/P were identified. We collected DNA from affected and unaffected family members (608 total), and from 100 gender-matched controls from Honduras. We sequenced the exons of *IRF6* for mutations in probands and controls. All patients were genotyped for single nucleotide polymorphisms (SNPs) rs642961 and rs2235371, which are proposed to have potential biological significance to *IRF6* expression and function.

Results: We found no mutations in *IRF6* in our CL/P probands. We found a risk association with the G allele of rs2235371 in both case-control ($P = .01$) and family-based association ($P = .01$) studies. We found no association with either allele of rs642961.

Conclusions: This study suggests that common variants, rather than rare mutations, are the cause for association between *IRF6* and nonsyndromic CL/P. rs2235371, but not rs642961, shows association with CL/P, suggesting a functional role for this polymorphism in our Honduran population. rs642961 has been previously reported to have an effect in other populations, suggesting that different populations may be affected by different polymorphisms.

Key Words: Nonsyndromic cleft palate, IRF6, cleft lip, cleft palate, Honduras.

Level of Evidence: 2b.

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INTRODUCTION

Cleft lip with or without cleft palate (CL/P) is a common disease throughout the world, affecting between 1:500 to 1:1000 newborns, with a higher prevalence in Native American, Asian, and Hispanic populations.[1] Clefting is associated with increased morbidity (speech problems, malnutrition, infection, psychiatric disease) and mortality throughout childhood and into adulthood.[2]

Cleft lip with or without cleft palate can exist in syndromic and nonsyndromic forms, with nonsyndromic forms comprising the majority of cases (~70%).[3] The etiology for nonsyndromic CL/P is thought to be due to a combination of genetic and environmental factors. Recently, numerous genes and chromosomal loci have been found to be associated with nonsyndromic CL/P in multiple populations, of which the most validated has been interferon regulatory factor 6 (*IRF6*).[4–7] A role in nonsyndromic CL/P is plausible for this gene given that mutations in *IRF6* have been shown to cause Van der Woude syndrome (VWS),[8] an autosomal dominant syndrome comprised of cleft lip and/or cleft palate along with lower lip pits.

The association between single nucleotide polymorphisms (SNPs) and disease from genome-wide association studies is thought to be due either to rare mutations creating a large association effect or common variants/polymorphisms carrying less effect. It is unclear which of these scenarios applies to the association between nonsyndromic CL/P and *IRF6*. Whereas some studies have found rare *IRF6* mutations in nonsyndromic CL/P cases,[9]

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others have not.[5] For potential causal polymorphisms, two candidate SNPs in the *IRF6* gene have been identified: 1) rs642961, which is in a transcription enhancer region for *IRF6* and alters a binding site for the *AP-2α* gene (a transcription factor, in which mutations cause a syndrome that includes CL/P[10]), and 2) rs2235371, which is in exon 7 of *IRF6* and codes for a V274I change in a conserved protein-binding domain.

Honduras is useful for CL/P studies due to its high prevalence of CL/P (given its Amerindian ancestry) and from little influx of other ethnic populations. We restricted our study to families with two or more members affected by CL/P to enhance the genetic contribution to disease. Previously, we described an association between nonsyndromic CL/P and SNPs in and around *IRF6*.[4] In addition, we identified unique exonic missense, nonsense, and frameshift mutations in *IRF6* in families with VWS.[11] The goal of this study was to characterize the association between *IRF6* and nonsyndromic forms of CL/P, and to understand better the pleiotropic effects of *IRF6* in leading to both VWS and nonsyndromic CL/P. Thus, we aimed to characterize the cause of the association in this population with *IRF6*: to determine whether rare, unique mutations or common variants were the cause of the association between *IRF6* and non-yndromic CL/P in a Honduran population.

MATERIALS AND METHODS

Human Subjects

Subjects were identified from a Honduran patient population at Hospital Escuela, in Tegucigalpa, Honduras. We identified families in which two or more members were affected by CL/P. Patients were physically screened and family histories were taken to ensure the absence of syndromic characteristics. We then constructed pedigrees of each family and took venous blood samples from all available consenting family members. We restricted our study to CL/P because isolated cleft palate is thought to have different genetic etiologies.

There were 100 sex-matched controls taken from pediatric patients in general and plastic surgery departments who were undergoing minor procedures at Hospital Escuela. All control patients were screened to ensure no family history of clefting, congenital, or genetic diseases.

This study was approved by the institutional review board of Columbia University Medical Center.

Direct Genomic Sequencing of IRF6

Genomic DNA was isolated from whole blood samples using Qiagen Flexigene kits (Qiagen, Valencia, CA). Primers were designed for each of the nine exons and flanking intronic regions of *IRF6* (see Supplemental Table online for primer sequences). Each exon was amplified separately by polymerase chain reaction from genomic DNA. Amplified products were then sent for sequencing (Macrogen, Rockville, MD). Sequences were analyzed using ABI Sequence Scanner (ABI, Foster City, CA) to identify any mutations.

SNP Genotyping

We had previously examined five SNPs (rs7543025, rs2357075, rs1856161, rs2235371, and rs2235377) and reported an association between nonsyndromic CL/P and *IRF6* in our Honduran cohort.[4] SNPs rs642961 and rs2235371 were selected for this study based on their potential biologic impact on *IRF6* expression and function. rs2235371 was previously genotyped in a smaller subset of this population, but new subjects were included in this study. Genomic DNA was isolated using Qiagen Flexigene kits (Qiagen). SNP genotyping was then performed (Prevention Genetics, Marshfield, WI). We ensured there was a >95% genotyping rate, with 99% confidence for all SNPs.

TABLE I.
Summary of Study Families.

Total No. of Families	106
Genotyped family members	
Total	608
Range per family	1–17
Mean ± SD	5.9 ±3.9
Number of affected per family by report	
Range per family	2–7
Mean ±SD	2.5 ± 0.8
Distribution of affected (families)	
2	72
3	24
4	9
≥5	1

SD = standard deviation.

Statistical Analysis

Hardy-Weinberg equilibrium for rs642961 and rs2235371 was tested using control patients as well as unaffected parents of probands. Haploview software (Broad Institute, MIT/Harvard, Cambridge, MA) was used with unaffected family members to test for linkage between rs642961 and rs2235371. Case-control analyses were performed using an additive allelic model and Armitage's test for trend.[12] Dominant and recessive allelic case-control models were also used for comparison. Family-based association test was performed using software from Harvard's Department of Biostatistics (http://www.biostat.harvard.edu/~fbat/default.html).

RESULTS

Patient Demographics

Overall, we studied 106 families and obtained DNA from 137 members affected by CL/P and from 471 unaffected family members. Family characteristics and patient demographics are listed in Table I and Table II.

IRF6 *Exon Sequencing*

By direct genomic sequencing, no exonic mutations were found in *IRF6* in any of the nonsyndromic CL/P patients. As a reference, 100 control patients were sequenced. No exonic mutations were identified in *IRF6* in any of the controls.

SNP Analysis

Hardy-Weinberg equilibrium was first calculated to ensure that the major and minor allele frequencies for SNPs rs642961 and rs2235371 had a normal distribution in members unaffected by disease in our cohort. We

TABLE II.
Study Participants.

	Total	Female	Male
Total participants	608	344	264
Affected status			
Affected	137	51	86
Unaffected	471	293	178

found that Hardy-Weinberg equilibrium was maintained for both rs642961 and rs2235371 in control patients and in unaffected parents of the probands (data not shown). Because Hardy-Weinberg equilibrium is maintained in these groups, they represent an unbiased population and can be used for comparison to the affected patients.

Minor allele frequencies (MAF) for both rs642961 and rs2235371 were examined and compared to known MAF in a Mexican cohort (from a HapMap database) to determine genetically how similar our Honduran cohort is to other Central American populations. For rs642961, the MAF was 0.187 and 0.173 in controls and unaffected parents, respectively, compared to 0.202 in the Mexican population. For rs2235371, MAF was 0.374 and 0.328 in controls and unaffected parents, respectively, compared to 0.167 in the Mexican population. Thus, there are differences between Honduran and Mexican heritages at these SNPs, particularly in the allele frequencies of rs2235371, with the rs2235371 minor allele being more common in our cohort.

The Haploview program was used to test whether rs642961 and rs2235371 are in close proximity within the *IRF6* gene and segregate together (i.e., are in linkage disequilibrium). Using unaffected family members, we found that rs642961 and rs2235371 are in linkage disequilibrium ($D' = 1.0$) in our Honduran cohort, suggesting that the portion of DNA containing these two SNPs is generally inherited as one block.

Affected probands (cases) versus control patients were compared to uncover any association between rs642961 and rs2235371 and clefting. In particular, we looked for overtransmission of particular alleles for rs642961 and rs2235371 in CL/P patients. Using an additive genetic model, where each copy of an allele is considered a risk for disease, a significant association between the G allele of rs2235371 and CL/P was identified (Table III). This suggests the G allele to be a risk allele for CL/P. No association was present between rs642961 alleles and CL/P.

For comparison, we used other genetic models to determine which model might best fit for the risk pattern for the G allele at rs2235371. Using a dominant genetic model, with CL/P modeled to act as a dominant trait, we found statistical significance for the G allele for rs2235371 ($P = .014$). Using a recessive genetic model, we found a trend toward significance for the G allele for rs2235371 ($P = .053$). Neither model identified an association between rs642961 alleles and CL/P (dominant $P = .223$, recessive $P = .130$).

TABLE III.
Additive Case-Control Model.

SNP	Armitage Statistic	*P* Value
rs642961	0.335	.563
rs22353371	6.54	.011

TABLE IV.
Genotypic Odds Ratio for rs2235371.

Genotype	Odds Ratio	95% CI
A/A	1.0	Reference
G/A	3.07	0.94 to 10.06
G/G	4.50	1.38 to 14.65

CI = confidence interval.

To further characterize the risk of the rs2235371 G allele, the genotypic odds ratio was computed using probands and control patients. Our results suggested a dosage effect for the G allele, with heterozygotes (odds ratio [OR] = 3.07, trending to significance) and homozygotes (OR = 4.50, significant) having a greater likelihood of CL/P compared to those with no G allele (Table IV).

Next, we examined how rs2235371 and rs642961 were inherited in families in relation to CL/P inheritance patterns. Family-based association statistic was performed to determine whether rs642961 and rs2235371 SNPs associated and segregated with CL/P within our Honduran family cohorts. Family-based association testing showed association between G allele of rs2235371 and nonsyndromic CL/P (Table V), supporting our findings from our case-control analysis. No association was established for rs642961, similar to our case-control analysis results.

DISCUSSION

By direct sequencing of exons in *IRF6*, we did not find any exonic mutations in patients with nonsyndromic CL/P, suggesting that such mutations are very rare and are unlikely to be the cause of association for nonsyndromic CL/P and *IRF6* in our Honduran population. In previous studies, Zucchero et al.[5] sequenced 80 Iowans and 80 Filipino CL/P cases and did not find any mutations in *IRF6* exons, whereas Jehee et al.[9] sequenced 108 CL/P cases from families with two or more affected members in Brazil and identified four unique *IRF6* mutations. Our results show an absence of mutations in *IRF6* in nonsyndromic CL/P, suggesting that rare missense, nonsense, and frameshift mutations

TABLE V.
Family-Based Association Test.

SNP	Allele	Allele Frequency	*Z*	*P*
rs642961	G	0.822	−1.245	.213078
rs642961	A	0.178	1.245	.213078
rs2235371	G	0.698	2.478	.013202
rs2235371	A	0.302	−2.478	.013202

in *IRF6* lead to a VWS phenotype and inheritance pattern, and not a nonsyndromic CL/P phenotype and pattern. It is likely, then, that common variants in *IRF6* contribute to the association with nonsyndromic CL/P in Honduras.

Recent studies have highlighted that the rs642961 SNP (in an enhancer binding site for transcriptional regulation of *IRF6*) may play a key role for the association of CL/P and *IRF6* in European, Filipino, and Chinese populations.[13–15] Pan et al.[14] reported that the risk allele for rs642961 resulted in decreased *IRF6* mRNA levels in tissue samples. These studies favor the hypothesis that rs642961 is the causal SNP in *IRF6*.

However, different SNPs and genetic factors may play different roles in other populations. The allele frequency of rs2235371 varies among different ethnic groups, ranging from MAF of 0.032 (in cohorts of European ancestry) to 0.411 (in Chinese in Beijing). Thus, studies on certain populations (European, in particular) may not properly account for the risk of rs2235371 given such largely monomorphic populations.

In addition, some reports suggest independent significant effects for both rs642961 and rs2235371 in certain populations[15] (Central European), and a strong effect for rs2235371 but not rs642961 in others[16] (Hispanics from Mexico). This suggests each SNP may play a role in risk for CL/P, with different degrees of influence in different ethnicities.

Here, we demonstrated in our Honduran population that there is no association between the rs642961 SNP and nonsyndromic CL/P, either in case-control analysis or in family-based association testing. We found an association between the G allele of rs2235371 and CL/P in our population, both in cases versus controls and in family-based association tests. These results are consistent with findings of overtransmission of the G allele in CL/P patients in other populations.[5,13–16] Interestingly, the G risk allele is the major allele and is conserved throughout multiple species. It is possible that the minor allele (A) developed later as a protective allele against CL/P.

The strength of our study is the inclusion only of families with multiple CL/P cases to increase the genetic contribution to CL/P. In addition, we collected DNA samples from extensive family pedigrees and were able to perform more rigorous family-based association testing. It will be important to continue to expand this population to increase our power in future studies and to confirm current associations.

CONCLUSION

These results suggest that in our Honduran population, rare mutations are not the cause for association between *IRF6* and nonsyndromic CL/P. Moreover, we find an association between the rs2235371 SNP (but not rs642961), suggesting that rs2235371 may play a role in the association between *IRF6* and nonsyndromic CL/P in the Honduran population and may have implications in *IRF6* protein function. Follow-up studies are needed to characterize the rs2235371 SNP (which causes a V274I coding change), and effects it may have on protein function. It will also be important to search for other genes, genetic variants, and environmental modifiers that contribute to *IRF6* function to fully characterize this gene. By doing so, we will better understand *IRF6*'s role in clefting, as well as other genes and risk factors involved.

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