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Report

Strong Evidence of Linkage Disequilibrium between Polymorphisms at the *IRF6* Locus and Nonsyndromic Cleft Lip With or Without Cleft Palate, in an Italian Population

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Cleft lip with or without cleft palate (CL/P) is one of the most common birth defects, but its etiology is largely unknown. It is very likely that both genetic and environmental factors contribute to this malformation. Mutations in the gene for interferon regulatory factor 6 (*IRF6*) have been shown to be the cause of Van der Woude syndrome, a dominant disorder that has CL/P as a common feature. Recently, it has been reported that genetic polymorphisms at the *IRF6* locus are associated with nonsyndromic CL/P, with stronger association in Asian and South American populations. We investigated four markers spanning the *IRF6* locus, using the transmission/disequilibrium test. A sample of 219 Italian triads of patients and their parents were enrolled in the study. Strong evidence of linkage disequilibrium was found between markers and disease in both single-allele ($P = .002$ at marker *rs2235375*) and haplotype ($P = .0005$) analyses. These findings confirm the contribution of *IRF6* in the etiology of nonsyndromic CL/P and strongly support its involvement in populations of European ancestry.

Orofacial development is a complex process that involves many genes and signaling pathways (Murray and Schutte 2004). Alterations in one or more of these genes could cause one of the most common malformations in humans: cleft lip with or without cleft palate (CL/P). Although CL/P and isolated cleft palate are features of >300 syndromes, >70% of cases present with cleft as the sole anomaly. Nonsyndromic CL/P (MIM 119530) is a complex multifactorial disease that affects >1/1,000 live births in Europe. Genetic linkage and association analyses have provided evidence to support the involvement of several genes and chromosomal regions, although difficulties have been encountered in replicating previous findings (Carinci et al. 2003).

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Recent advances in gene mapping have resulted in the identification of genes responsible for Mendelian-inherited clefting syndromes (Celli et al. 1999; Suzuki et al. 2000; Kondo et al. 2002). It is likely that mutations having a mild effect on the function of genes causing the syndromes could cause phenotypes not distinct from nonsyndromic CL/P, which, in turn, means that those genes could be involved in the etiology of nonsyndromic CL/P. An increasing amount of evidence seems to support this hypothesis.

Van der Woude syndrome (VWS [MIM 119300]) is an autosomal dominant disorder in which lower-lip pits and occasional hypodontia are the only features distinguishing the disorder from nonsyndromic CL/P. Lip pits are variable findings in VWS and may not be present at all in some affected individuals belonging to kindred with VWS. It has been shown that deletions involving the gene coding for interferon regulatory factor 6 (*IRF6*) or mutations causing haploinsufficiency are responsible for VWS (Kondo et al. 2002). Recently, some original evidence of a significant association between nonsyndromic CL/P and SNPs at the *IRF6* locus was reported (Zucchero et al. 2004). In that study, strong evidence of

Table 1

Marker Information and TDT Results

dbSNP	Alleles[a]	Physical Location[b]	Genetic Location	Allele Frequency[c]	T/NT[d]	*P*[e]	*P* for[f] 2 SNPs	3 SNPs	4 SNPs
rs1319435	G/A	207127007	70 kb 5′ of *IRF6*	.85	43/61	.077	.0004	.0014	.0005
rs2013162	G/T	207048073	In *IRF6*	.67	118/78	.004	.0098	.0087	…
rs2235375	C/G	207044976	In *IRF6*	.67	111/80	.002	.0376	…	…
rs2235543	A/G	206940057	100 kb 3′ of *IRF6*	.87	53/47	.55	…	…	…

[a] Base change in coding strand, with major allele listed first.
[b] Physical locations are from the UCSC Human Genome Browser, July 2003 assembly.
[c] Major-allele frequency was calculated using all parental chromosomes.
[d] Transmission/nontransmission (T/NT) counts from heterozygous parents are given for the common allele.
[e] *P* value for TDT.
[f] Global *P* value for haplotype analyses. In each case, the *P* value is placed on the line of the first SNP of the haplotype.

overtransmission of the ancestral allele of the V274I polymorphism to patients with nonsyndromic CL/P was observed in Asians but not in groups of European descent. However, analysis of additional SNPs showed that linkage disequilibrium with CL/P extended from 40 kbp in the 5′ portion of *IRF6* to 135 kbp in the 3′ portion of *IRF6* and was also detectable in the Iowan and Danish populations.

The complexity of CL/P genetics has been evident in the last decade. Several genes and loci have been claimed to be etiologically involved, although difficulties have been encountered in replicating positive findings, and different results have been obtained for the different populations investigated. In the present investigation, we examined a sample of patients with nonsyndromic CL/P recruited in Italy, to verify the involvement of *IRF6* in cases of CL/P in southern Europe.

Table 2

Two-Marker and Three-Marker Haplotypes Showing Significant Transmission Distortion

Haplotype	T/NT	Frequency in Haplotypes T	NT	*P*
Two markers, with allele:				
G-G-X-X	95/66	.53	.37	.0420
G-T-X-X	38/86	.21	.48	.0001
X-G-C-X	83/49	.63	.37	.0098
X-T-G-X	49/83	.37	.63	.0098
X-X-G-A	40/66	.27	.44	.0227
X-X-C-A	82/49	.55	.33	.0120
Three markers, with allele:				
G-T-G-X	37/79	.22	.46	.0004
X-G-C-A	81/43	.57	.30	.0029
X-T-G-A	35/66	.25	.46	.0050
Four markers, with allele:				
G-G-C-A	80/52	.49	.32	.0261
G-T-G-A	25/61	.15	.38	.0003
A-G-C-A	33/17	.20	.10	.0277

Note.—T = transmitted; NT = nontransmitted.

Our study group consisted of 219 unrelated patients with nonsyndromic CL/P and their parents. All of them were white and of Italian ancestry. Among them, 132 were sporadic, whereas 87, who had relatives affected with CL/P, were classified as familial cases. CL/P was the sole disorder affecting these patients. To assess the nonsyndromic status and to exclude known teratogenic influences, the patients and their relatives were asked specific questions about the presence in the family of any other somatic and neurological disorders and the use of clefting drugs—such as phenytoin, warfarin, and ethanol—during pregnancy. After informed consent was obtained, peripheral blood samples were drawn from each individual. Clinical analyses, blood collection, and DNA extraction were performed using a consolidated protocol described elsewhere (Pezzetti et al. 2004). To facilitate comparisons between studies, we selected polymorphisms that had been used before to investigate other populations (Zucchero et al. 2004). Four SNPs at the *IRF6* locus, with high heterozygosity in whites, were chosen because they showed a very strong association with the disorder (Zucchero et al. 2004). Marker information is included in table 1. Genotypes were obtained using an ABI PRISM 7700 Sequence Detection System and TaqMan chemistry. Reagents and SNP genotyping assays were supplied by Applied Biosystems.

Hardy-Weinberg equilibrium was observed for all polymorphisms in both patients and parents. Single-marker results were analyzed by the transmission/disequilibrium test (TDT), which examines the transmission of alleles from heterozygous parents (Spielman et al. 1993). Significant linkage disequilibrium with the disease locus was apparent for markers *rs2013162* and *rs2235375* ($P = .004$ and .002, respectively); in both cases, the frequent allele was significantly overtransmitted to the affected individuals. Haplotype analyses were

Table 3

Linkage Disequilibrium between Markers Genotyped in the Study

Marker	*rs1319435*	*rs2013162*	*rs2235375*	*rs2235543*
rs1319435	...	.280	.316	.001
rs2013162	.007	...	.995	.866
rs2235375	.008	.968	...	.868
rs2235543	.000	.237	.245	...

NOTE.—D' is above the diagonal; r^2 is below the diagonal.

performed, because they enhance the power of linkage disequilibrium studies by increasing the number of heterozygous parents that are processed by TDT statistics. Haplotype analysis was performed using the Web-based interface GLUE and the program TDTPHASE (Dudbridge 2003), part of the UNPHASED package, available at the Human Genome Mapping Project Web site. The analysis was restricted to phase-certain haplotypes, in a conditional logistic regression model. Rare haplotypes with a frequency <0.03 were excluded from statistical analysis. Highly significant transmission distortion was observed in two-, three-, and four-marker haplotype analyses. Global *P* values, most of which are <.01, are reported in table 1. Haplotypes carrying the frequent alleles at markers *rs2013162* and *rs2235375* were always found to be overtransmitted to patients, whereas those carrying the rare alleles were negatively associated (table 2).

Linkage disequilibrium between markers was calculated by D' and r^2 statistics from parental haplotypes, by use of the program ldmax from the GOLD software package (Abecasis and Cookson 2000). The results are shown in table 3, with D' values above and r^2 values below the diagonal. A comparison of these data with those regarding Filipinos, available in the report by Zucchero et al. (2004), shows that linkage disequilibrium between markers was stronger among Italians.

In the report by Zucchero et al. (2004), a significant association was reported between nonsyndromic CL/P and the V274I polymorphism in *IRF6*. In particular, the V allele was overtransmitted to patients from Asian and South American populations. Moreover, it was reported that the I allele was very rare or absent among Africans and Europeans. Specifically, it was not detected in 76 chromosomes from Italy. On the strength of this result, our study did not investigate the V274I polymorphism. On the other hand, it is very unlikely that V274I, or the SNPs investigated in the present study, may be directly implicated in CL/P etiology. The genetic alterations increasing the susceptibility to the disease are as yet unknown, and further investigations are needed to address this important issue.

Above all, in the present investigation, we found strong evidence supporting the involvement of *IRF6* in nonsyndromic CL/P. This is the first study to confirm the original finding reported by Zucchero et al. (2004) and to report an *IRF6* contribution to the etiology of CL/P in southern Europe.

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Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

Human Genome Mapping Project, http://www.rfcgr.mrc.ac.uk/Registered/Webapp/glue/ (for GLUE Web-based interface with linkage computer programs)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for nonsyndromic CL/P and Van der Woude syndrome)

dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/ (for markers *rs1319435, rs2013162, rs2235375,* and *rs2235543*)

UCSC Human Genome Browser, http://genome.ucsc.edu/cgi-bin/hgGateway (for July 2003 assembly)

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