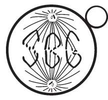

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Short Communication

Assessment of the association between *SMAD1* and *HHIP* gene variation and non-syndromic cleft-lip palate in Chilean case-parent trios

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Abstract

Nonsyndromic cleft lip/palate (NSCLP) is a congenital malformation with features of a complex genetic trait. Several studies have reported positive association and linkage between NSCLP and microsatellite markers in the 4q28-4q33 region particularly with the D4S192 (4q31) marker. We hypothesized that the candidate genes SMAD1 and HHIP (4q31) could be involved in the etiology of NSCLP based on previous positive linkage results and their important role in maxillofacial development. We evaluated the possible association between microsatellite markers located at less than 1 cM from these genes and NSCLP using a sample of 58 Chilean case-parent trios. Microsatellite markers were analyzed using the polymerase chain reaction (PCR) with fluorescent labeled primers. Electrophoresis of the PCR products was performed on a laser-fluorescent automatic DNA sequencer. The extended transmission disequilibrium test (ETDT) was used to analyze allelic transmissions from the parents to their affected progeny. No significant association due to linkage disequilibrium was detected between both markers and NSCLP.

Key words: candidate genes, case-parents trio design, linkage disequilibrium, nonsyndromic cleft lip/palate.

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Nonsyndromic cleft lip/palate (NSCLP), is a common birth defect with features of a genetically complex trait. Attempts to localize NSCLP loci in the human genome have generated considerable but sometimes discordant information (Murray, 1995). Thus, the nature of the genetic contribution to the etiology of NSCLP is still being studied and remains unresolved. Several candidate genes and loci mapping in various chromosome regions have been claimed to be involved in cleft determination by parametric and non-parametric linkage and association analysis (Carinci *et al.* 2000; Murray, 2002). In this context, some studies have reported positive and negative findings between NSCLP and the 4q28-4q33 region. Beiraghi *et al.* (1994) reported the first study in a single five-generation family where two markers (D4S175 and D4S192) were informative for linkage. Mitchell *et al.* (1995) and Paredes *et al.* (1999) found significant association between D4S192 and NSCLP in case-control studies. Additionally, Marazita *et al.* (2002) and Wyszynski *et al.* (2003) in genome wide scan studies also proposed the involvement of a cleft susceptibility locus in 4q region. Notwithstanding, Blanton *et al.* (1996), Prescott *et al.* (2000), and Wong *et al.* (2000) report exclusion of linkage between NSCLP and this chromosomal region. Recently, our group screened the 4q24-q33 region using five short tandem repeat (STR) microsatellite markers but found no evidence for linkage and association between them and NSCLP in the Chilean population using a case-parents trio design (Blanco *et al.*, 2005).

In a previous study in the Chilean population (Paredes *et al.*, 1999), we reported a positive association between D4S192 and NSCLP. Based on this finding, we searched for possible candidate genes within the 4q31 region using as a reference point the aforementioned STR. A search in genomic databases, showed that there were two possible candidate genes both centromeric and telomeric to D4S192. Moreover, experimental evidence has shown that these two genes are involved in craniofacial development. The *SMAD1* gene is a homolog of the drosophila gene denominated mothers against decapentaplegic 1 (*MAD1*) and encodes a protein which participates in a signaling cascade triggered by transforming growth factor betas (TGFβs) and bone morphogenetic proteins (BMPs) (Cohen, 2003). Nonaka *et al.* (1999) reported that SMAD1 serves as a point of convergence for the integration of two different growth fac-

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tor signaling pathways, BMP4 and epidermal growth factor (EGF), during chondrogenesis. Hatakeyama *et al.* (2003) showed that in chondroprogenitor cells, BMP stimulates differentiation through mechanisms mediated by SMAD1. On the other hand, the hedgehog-interacting protein (*HHIP*) gene encodes a protein which is a member of the sonic hedgehog (SHH) signaling pathway (Jiang *et al.*, 2006). Also Cobourne and Sharpe (2002) have postulated that a role for HHIP may be to allow differential responses to the SHH signal within different regions of odontogenic-specific cells in regions of the mandibular process. Rice *et al.* (2006) studying the expression patterns of hedgehog signaling pathway members during mouse palatal development reported that at 13 dpc, *HHIP* was expressed at a low level throughout the palatal mesenchyme and that the highest levels of transcripts localize adjacent to the thickened palatal oral epithelium in the anterior sections. At 14.5 dpc *HHIP* was expressed in the developing palatine bones.

The purpose of the study described in this paper was to use a case-parents trio design to assess a Chilean population for a possible association due to linkage disequilibrium between STR D4S1586 located at 0.3 cM telomeric from *SMAD1* and STR D4S2998 located at 0.1 cM centromeric from *HHIP* and NSCLP. These markers had not been included in previous association and linkage studies of Chilean populations (Paredes *et al.*,1999; Blanco *et al.* 2005).

Our sample consisted of 58 case-parents trios selected because they had an offspring affected with NSCLP. The unrelated NSCLP probands were identified and interviewed during the course of clinical examinations at the Cleft Lip/Palate Clinic of the School of Dentistry of the University of Chile, at the Dr. Alfredo Gantz Foundation located in the city of Santiago, Chile. One of the authors (RB) identified the affected individuals in the course of examinations conducted between 2004 and 2005. In-depth interviews of at least three family members were conducted to provide detailed information for pedigree construction. All the families enrolled were of Chilean ancestry including subjects presenting NSCLP as the unique familiar disease. Families using clefting drugs, such as phenytoin, warfarin and ethanol were excluded from the study. The pedigree history corresponds to individuals belonging to low to middle low socioeconomic strata given the genetic composition of the Chilean population which presents a relationship between ethnicity, Amerindian admixture, genetic markers, socioeconomic strata and prevalence of NSCLP (Valenzuela, 1988; Palomino *et al.*, 1997). The Institutional Review Board of the School of Medicine of the University of Chile approved the study and all participants gave their informed consent. Genomic DNA was extracted from peripheral blood cells (Poncz *et al.*, 1982). The microsatellite markers were D4S1586 located at 0.3 cM telomeric from the SMAD1 gene and D4S2998 located at 0.1 cM centromeric from *HHIP*. The polymerase chain reaction (PCR) was carried out according to a standard amplification protocol (Suazo *et al.*, 2004) using the primers described by Dib (1996). Annealing temperatures were 62 °C for D4S1586 and 52 °C for D4S2998. The amplification reaction was performed using a fluorescent-dye-labeled forward primer. The products were analyzed in an ABI PRISM 310 genetic analyzer (Applied Biosystems). The electrophoretic results were processed by GENESCAN 3.1.2 software, and allele assignation was carried out using Genotyper software, version 2.5. The Extended Transmission Disequilibrium Test (ETDT) for multiple alleles was carried out to assess the differential pattern of excess transmission of alleles from heterozygous parents to diseased children (Sham and Curtis, 1995). By sampling case-parent trios through an affected child, the association between alleles of genetic markers and the disease would cause transmission to appear different from the expected probability of 0.5. When cases are unrelated probands, ETDT represents a valid test of association even if population stratification is present. Given the relatively reduced sample size of our study and the low a priori statistical power to detect weak associations we also computed p-values based on simulations (10,000 per marker) made with the Monte Carlo ETDT program (MCETDT) (Zhao *et al.*, 1999), which avoids the problems associated with p-values based on chi-square distributions applied to sparse transmission tables. Additionally, individual transmission of each allele with respect to the rest of the alleles was evaluated (this procedure would imply applying a correction for multiple comparisons). Exact p-values were evaluated to assess significance of individual alleles (Cleves *et al.*, 1997) using a method implemented in the statistical package STATA 8.2 (Stata Statistical Software, 2004).

Multiple-allele ETDT analyses for marker-disease association based on case-parents trios yielded a p-value of 0.13 for the D4S1586 marker and 0.10 for the D4S2998 marker. Individual allele variants also showed no preferential transmissions. The Monte-Carlo ETDT yielded

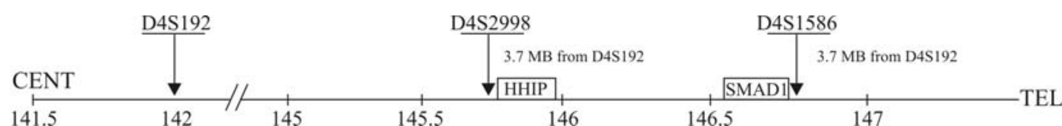

Figure 1 - Schematic representation of the position of the microsatellite markers analyzed in this study in relation to candidate genes *HHIP* and *SMAD.* In a scale of megabases, the boxes indicate the position of the genes within the 4q31 region and the arrows indicate the D4S2998 and D4S1586 microsatellite position. In addition the position of marker D4S192 is indicated. For each gene their distance relative to D4S192 is indicated. See text for the distance of microsatellite markers relative to the candidate genes.

p-values of 0.17 for D4S1586 and 0.20 for the D4S2998 (Tables 1 and 2).

The genes *SMAD1* and *HHIP* are located in 4q31.21 at a distance of 3.7 and 2.6 cM respectively from D4S192 (Figure 1). Several authors have reported positive findings in this chromosomal region for NSCLP. The results of experimental evidence cited in the preceding paragraphs support the role of these genes in craniofacial development but, nevertheless, the results of our study did not show positive evidence of the involvement of these genes in NSCLP in the Chilean population studied. It must also be taken into consideration the low a priori statistical power to detect weak associations and therefore we cannot exclude the possibility that these genes may have a role in NSCLP either as low susceptibility or modifier genes.

Table 1 - Transmission Disequilibrium Test (TDT) analysis for the association between the D4S1586 marker and nonsyndromic cleft lip/palate (NSCLP) in 58 case-parents triads.

D4S2998 alleles (bp)	Transmitted	Non transmitted	p-value*
97	6	2	0.29
99	24	20	0.65
101	29	19	0.19
103	3	10	0.09
105	2	5	0.45
107	12	15	0.70
109	10	12	0.83
111	0	2	0.5
113	0	1	1.0
Multi-allele TDT **			0.13

*Statistical significance for individual alleles was assessed using exact p-values. **Multi-allele TDT p-value based on simulations was computed as 0.17.

Table 2 - Transmission Disequilibrium Test (TDT) analysis for the association between the D4S2998 marker and nonsyndromic cleft lip/palate (NSCLP) in 58 case-parents triads.

D4S2998 alleles (bp)	Transmitted	Non transmitted	p-value*
143	19	21	0.87
145	10	6	0.46
149	3	3	1.0
151	14	16	0.86
153	2	4	0.69
155	3	2	1.0
157	1	3	0.63
159	0	2	0.5
161	4	0	0.13
163	12	19	0.28
165	19	9	0.09
167	5	5	1.0
169	0	2	0.5
Multi-allele TDT**			0.10

*Statistical significance for individual alleles was assessed using exact p-values. **Multi-allele TDT p-value based on simulations was computed as 0.20.

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