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J. Suazo[1], J.L. Santos[2], H. Carreño[1], L. Jara[1], and R. Blanco[1]*

[1]Human Genetics Program, Institute of Biomedical Sciences, School of Medicine, University of Chile, Av. Independencia 1027, PO Box 70061, Santiago, Chile; and [2]Laboratory of Genetic Epidemiology, Institute of Nutrition and Food Technology (INTA), University of Chile; *corresponding author, rblanco@med.uchile.cl

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Linkage Disequilibrium between MSX1 and Non-syndromic Cleft Lip/Palate in the Chilean Population

ABSTRACT

Non-syndromic cleft lip/palate (NSCLP) is a complex genetic trait. Linkage and association studies have suggested that a clefting locus could be located on chromosome 4p. Sixty Chilean families were recruited for this study; from these, we used unrelated trios to evaluate the possible linkage disequilibrium between MSX1 and NSCLP. An intragenic marker, MSX1-CA, and an extragenic marker, D4S432 at a distance of 0.8 cM from MSX1, were analyzed by means of polymerase chain-reaction with fluorescent-labeled forward primers, followed by electrophoresis on a laser-fluorescent sequencer. We carried out a transmission/disequilibrium test (TDT) for multiple alleles to evaluate the presence of linkage disequilibrium. Results showed a preferential transmission of the 169-bp allele of MSX1 ($p = 0.03$). Although there was no preferential transmission for the D4S432 marker, the overall extended TDT (ETDT) showed a significant result ($p = 0.01$). The authors' findings support the hypothesis of the contribution of MSX1 in the etiology of NSCLP in the Chilean population.

KEY WORDS: STR, non-syndromic cleft lip/palate, association, linkage disequilibrium.

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INTRODUCTION

Oral-facial clefts, particularly non-syndromic cleft lip with or without cleft palate (NSCLP), are common congenital anomalies readily observable at birth, with characteristics of genetically complex traits. Epidemiological studies and complex segregation analysis have well-established the importance of genetic factors in clefting, even though environmental influences have also been described (Murray, 1995). Attempts to localize NSCLP loci in the human genome have generated considerable, but sometimes discordant, information (Murray, 1995). Therefore, 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 association and linkage approaches (see reviews by Carinci *et al.*, 2000; Murray, 2002). The great majority of the studies mentioned in the previous reviews have been performed in Caucasian populations. More recently, some reports have been published for other ethnic groups: Wyszynski *et al.* (1997) in Mexico; Kanno *et al.* (2002) in Japan; Marazita *et al.* (2002a,b) in China; Schultz *et al.* (2004) in the Philippines; and Moreno *et al.* (2004) in Colombia. To date, no one locus has clearly emerged as 'necessary' for the development of NSCLP. On the contrary, the genetic etiology of oral-facial clefts appears more complex, with several loci showing significant results in at least some studies. Loci on chromosomes 1q, 2p, 4p, 4q, 6p, 14q, 17q, 19q, and 22q have all had positive findings, primarily in populations of Caucasian descent. None of these candidate regions and loci has shown a major involvement in non-Caucasian population, with the possible exception of the 19q31 region in the Chinese population (Marazita *et al.*, 2002a).

In recent years, a role for MSX1 in craniofacial development is emerging as an especially strong candidate gene supported by studies in both mice and humans. The predominant phenotype in mice homozygous for an Msx1-null allele is cleft palate. In addition, abnormalities of nasal, frontal, and parietal bones were observed, and teeth failed to develop (Satokata and Maas, 1994; Kaartinen *et al.*, 1995). These findings suggest that MSX1 plays an important role in epithelial-mesenchymal interactions during craniofacial development. Association and linkage studies further support a role for MSX1 in different populations. Case-control studies have reported both positive (Blanco *et al.*, 2001) as well as negative results. Parametric linkage studies also have communicated positive (Stein *et al.*, 1995) and negative (Scapoli *et al.*, 2002) results. Some studies using non-parametric methods (Transmission Disequilibrium Test [TDT] and Affected Family-Based Association Control [AFBAC]) have reported positive linkage disequilibrium (Lidral *et al.*, 1998; Beaty *et al.*, 2002; Fallin *et al.*, 2003; Vieira *et al.*, 2003), while others have not (Jugessur *et al.*, 2003). Recently, Jezewski *et al.* (2003) reported the complete sequencing of the

Table 1. Transmission Disequilibrium Test (TDT) Analysis for the Association between MSX1-CA Marker and Non-syndromic Cleft Lip/Palate in 60 Case-Parent Trios

MSX1-CA Alleles	Transmitted	Non-transmitted	p-value*
169 base pairs	28	13	0.03
171 base pairs	4	11	0.12
173 base pairs	10	17	0.25
175 base pairs	5	6	1
Multi-allele TDT**			0.09

* Statistical significance for individual alleles was assessed through exact p-values (not corrected for multiple comparisons).
** Multi-allele TDT p-value based on simulations was computed as 0.10.

MSX1 gene, demonstrating that some mutations in this gene are potentially etiological in NSCLP.

Our purpose in this study was to test the hypothesis that MSX1, located in 4p16.2, is involved in the etiology of NSCLP, using the case-parent trio design to determine if this gene is in linkage disequilibrium with NSCLP in the Chilean population.

SUBJECTS & METHODS

Families

The sample of 60 unrelated case-parent trios was obtained from our data bank from the corresponding extended pedigrees. The 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, a private clinic for the rehabilitation of cleft patients, and at the Cleft Children Center. Affected patients attend these three institutions for rehabilitation procedures. The first two institutions are located in Santiago, Chile (the capital of the country, a city of over five million inhabitants). The third center is located in the city of Talca, 250 km south of Santiago, with approximately 300,000 inhabitants located in a predominantly agricultural area. One of the authors (RB) identified the affected individuals in the course of examinations conducted between 1991 and 2001 in the three institutions mentioned above. We conducted in-depth interviews of the propositi, their parents, and at least three family members to provide detailed information for pedigree construction. All the families enrolled were of Chilean ancestry, without immigrants, and included subjects presenting NSCLP as the unique familial disease. Moreover, those 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 socio-economic strata, given the genetic composition of the Chilean population that presents a relationship among ethnicity, Amerindian admixture, genetic markers, socio-economic strata, and incidence of NSCLP (Valenzuela, 1988).

DNA Analysis

For every individual participating in the present study, an informed written consent was requested. The informed consent had been previously approved by the Institutional Review Board of the School of Medicine of the University of Chile. In the case of children under ten years of age, authorization was requested from their parents or from the individual legally in charge of the child. After the informed consent was given, genomic DNA was extracted from peripheral blood cells (Poncz *et al.*, 1982). The microsatellite markers were two dinucleotide repeats: the intragenic marker, MSX1-CA (4p16.2) (Padanilam *et al.*, 1992); and the extragenic marker, D4S432 (4p16.3) (Gyapay *et al.*, 1994), located at 0.8 cM from the MSX1 gene. Polymerase chain-reaction (PCR) was carried out in a total volume of 25 μL containing 50 ng of genomic DNA, 1.5 mM of $MgCl_2$, 50 mM of KCl, 10 mM of Tris-HCl (pH = 9.0), 0.2 mM of DNTPs, 1.5 units of Taq polymerase, and 10 pmol of each primer. For this study, the amplification reaction was performed with the use of a fluorescent-dye-labeled forward primer. Thirty-three cycles of amplification were performed, one included 1 min at 94°C for denaturation, 1 min at 55°C for alignment of primers, and 30 sec at 72°C for extension by the Taq polymerase for the two microsatellites. The products were analyzed by means of capillary electrophoresis in an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA). This method allows for the co-loading of different dye-labeled PCR products, with a size standard, in the same capillary. The electrophoretic results were processed by GENESCAN 3.1.2 software, and allele assignation was carried out with the use of Genotyper software, version 2.5.

Statistical Methods

We used the ETDT for multiple alleles to evaluate association due to linkage disequilibrium between MSX1-CA and D4S432 markers with NSCLP (Sham and Curtis, 1995). When unrelated affected children are sampled, associations between the genetic markers and the disease cause the probability of transmission to differ from the expected value (probability of transmission = 0.5), avoiding the distorting effect of population stratification by ethnicity. Given the relatively reduced sample size of our study and the low *a priori* statistical power to detect weak associations, we have also computed p-values based on simulations (10,000 *per* marker) through the Monte Carlo ETDT program (MCETDT) (Zhao *et al.*, 1999). MCETDT avoids the problems of p-values based on chi-square distributions applied to sparse transmission tables. After the evaluation of different statistical approaches, including goodness-of-fit tests, we have focused on allele rather than genotype analysis. 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). We evaluated exact p-values to assess the significance of individual alleles (Cleves *et al.*, 1997), using a method implemented in the statistical package STATA 8.2 (Stata Statistical Software, 2004).

RESULTS

Table 1 shows multiple-allele TDT analysis and uncorrected allele-specific p-values for markers-disease associations based on case-parent trios for the MSX1-CA marker. The 169-base-pair allele was transmitted in 28 occasions and non-transmitted in 13 occasions, giving an uncorrected exact p-value of 0.03. Multiple-allele TDT for the MSX1-CA marker yielded p-values of 0.09 (ETDT) and 0.1 (MCETDT). In contrast, the intragenic marker D4S432 showed significant overall association with NSCLP with a multiple-TDT, p-values of 0.01 (ETDT) and 0.02 (MCETDT). Given that the D4S432 marker is highly polymorphic (14 alleles), none of the individual allelic variants

Table 2. Transmission Disequilibrium Test (TDT) Analysis for the Association between D4S432 and Non-syndromic Cleft Lip/Palate in 55 Case-Parent Trios

D4S432 Alleles	Transmitted	Non-transmitted	p-value*
220 bp	1	1	1.0
222 bp	6	1	0.13
228 bp	26	20	0.46
230 bp	2	0	0.50
232 bp	1	0	1.0
238 bp	0	1	1.0
240 bp	25	25	1.0
244 bp	1	3	0.63
246 bp	0	1	1.0
250 bp	20	22	0.88
252 bp	0	1	1.0
254 bp	0	5	0.06
258 bp	0	1	1.0
260 bp	0	1	1.0
Multi-allele TDT**			0.01

* Statistical significance for individual alleles was assessed through exact p-values (not corrected for multiple comparisons).
** Multi-allele TDT p-value based on simulations was computed as 0.02.

showed a preferential transmission, and therefore no statistical significance was found when exact p-values were computed (Table 2).

DISCUSSION

Some authors have argued that population-based, case-control designs in which candidate genes are used are more suitable than a case-parent design to assess the effects of risk factors, a crucial step in disease prevention and health promotion (Caporaso *et al.*, 1999; Khoury, 1999). However, family-based studies still may be useful if population stratification is present. The case-parent trio design avoids concerns about spurious results due to population stratification within the sample, primarily because the observed case is always compared with ethnically matched 'pseudocontrols' (parents) (Beaty *et al.*, 2002). Therefore, given the known population stratification that has been detected in the Chilean population (Valenzuela, 1988), the case-parent trio design seems to be the most appropiate method to test for linkage dissequilibrium (LD).

Our study was based on case-parent trios with unrelated cases as probands. Such trios were used to assess the association between the intragenic marker MSX1-CA and the extragenic marker D4S432 located at 0.8 centiMorgans from MSX1. The results for the multi-allele TDT (all alleles simultaneously) showed significant association between NSCLP and D4S432 ($p = 0.01$). Nevertheless, the results for the intragenic marker MSX1-CA did not reach significance ($p= 0.09$). However, only the 169-bp allele of the intragenic marker showed a preferential transmission ($p = 0.03$). The observation that the 169-base-pair allele, of the MSX1-CA marker, is preferentially transmitted in our case-parent study is concordant with the results reported by Vieira *et al.* (2003). It must be noted that Vieira *et al.* used a sample of 217 mother-child pairs ascertained through the Latin American Collaborative Study of Congenital Malformations (ECLAMC), whereas our sample of case-parent trios was smaller. Nonetheless, our results and those of Vieira *et al.* suggest a possible common genetic origin of NSCLP in different South America regions. The significant association with D4S432 provides additional support for the involvement of MSX1 in the etiology of NSCLP.

It is worth noting that the interpretation of the results of case-parent studies relies on the assumption that the proportions of offspring genotypes follow Mendelian probabilities. However, a word of caution is necessary, since it has been recently suggested that transmission distortions occur in some human genome loci (Zollner *et al.*, 2004).

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