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MSX1 gene polymorphisms in Mexican patients with non-syndromic cleft lip/palate

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ABSTRACT

Objective: Non-syndromic cleft lip/palate malformation (CL/P) is one of the most common birth defects in humans and has a complex etiology involving genetic and environmental factors. Mutations in the *MSX1* gene are critical during craniofacial development. The purpose of this study was to investigate the contribution of *MSX1* gene polymorphisms to the risk of developing CL/P in a sample of Mexican patients.
Methods: The sample consisted of 282 subjects (69 cases and 213 relatives). Four single-nucleotide polymorphisms (SNP1, P147Q, SNP5 and P278S) were tested for association with CL/P in triad and case-pseudo-control analyses. Polymorphism typing was performed by restriction fragment length polymorphism and dot-blot techniques. Allele and genotype frequencies were calculated between patients and pseudo-controls and compared using the Chi square test with Yates correction. Odds ratios and 95% confidence intervals were obtained using SPSS software (v19). Triad analysis was also performed using the program HAPLIN (v5.3).
Results: In the cases and pseudo-controls, an association was found between CL/P and the SNP1-G allele ($P = 0.031$) and the SNP1-G/G genotype ($P = 0.032$), a polymorphism located near *MSX1*. Triad analysis showed a tendency toward CL/P susceptibility for the genotype SNP1-G/G ($P = 0.075$) and an association between CL/P and the haplotype GCTC ($P = 0.037$). No associated haplotype was found in the cases and pseudo-controls. Two partial haplotypes, GT (SNP1-SNP5) ($P = 0.032$) and GC (SNP1-P278S) ($P = 0.033$), were associated with susceptibility in the heterozygous and homozygous types, respectively. In contrast, haplotype AT (SNP1-SNP5) was associated with protection ($P = 0.012$) in the homozygous type.
Conclusions: The results of this study suggest an association between CL/P susceptibility and SNP1, located near the *MSX1* gene, in the Mexican population.

1. Introduction

Non-syndromic cleft lip/palate (CL/P) is the most common craniofacial birth defect in humans, mainly appearing as an isolated condition without other major birth defects (70% of cases) [1]. The overall prevalence is approximately 1 in 700 live births, with differences among ethnic groups and geographic regions. Patients with CL/P have speech and feeding difficulties, malnutrition, hearing injury, and infections as well as psychiatric diseases [2]. Affected individuals require multidisciplinary care from birth until adulthood.

Normal development of the lip and palate involves a series of closely coordinated events during the development of facial primordia, including cell migration, growth, differentiation and apoptosis, and perturbations in these processes may affect proper morphogenesis of facial structures, resulting in disease manifestation [3,4]. Essentially, CL/P results from failure of fusion of the maxillary or palatal shelves, both of which occur between the 4th and 12th weeks of embryogenesis [3].

Although CL/P has a complex etiology, including genetic and environmental causes, no specific genetic cause has been

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confirmed to date for isolated, nonsyndromic oral clefts [5]. Nonetheless, some of the risk factors described for CL/P are environmental teratogens during the first trimester of pregnancy, such as maternal alcohol intake and exposure to tobacco, retinoic acid, and folate antagonists [2].

Identification of genetic factors involved in CL/P has been the subject of intensive research in recent years. The candidate genes described thus far include *IRF6, TGFA, MSX1, TGFB3,* and *FOXE1* [6].

The gene muscle segment homeobox 1 (*MSX1*) has been extensively studied and has been demonstrated to be critical during craniofacial development. *MSX1*, which is located at 4p16, encodes a DNA-binding protein expressed in spatially restricted regions of the head during early development. Mutations in this gene are known to be associated with Witkop and Wolf-Hirschhorn syndromes, CL/P, and autosomal dominant hypodontia [7–10].

Complete sequencing of *MSX1* in humans has revealed several mutations, and it is estimated that approximately 2% of CL/P patients carry mutations in this gene [7,9,11]. Jezewski et al. described the complete sequence of *MSX1* (two exons, one intron, and two untranslated regions), reporting mutations in individuals of European, Asian, and native South American ancestry. Additionally, these authors identified specific polymorphic variants in a panel of 18 populations and established 48 variant sites, of which 43 are single-nucleotide polymorphisms (SNPs); the nucleotide position of each is indicated in GenBank entry AF426432 [9]. Following this approach, Suzuki et al. defined SNPs 1–5 and reported their relative position in GenBank [7] (Fig. 1). Tongkobpetch et al. described primers and typing methodology for SNP1 (−8796 A > G) and SNP5 (452–402 G > T) and for polymorphisms P147Q (440 C > A) and P278S (832 C > T) [11].

Based on these data, the purpose of this study was to investigate the contribution of 4 *MSX1* gene polymorphisms to the risk of nonsyndromic cleft lip/palate in Mexican patients.

2. Material and methods

2.1. Subjects

Sixty-nine CL/P cases and 213 relatives studied between 2009 and 2011 were included. The cases were diagnosed as nonsyndromic cleft palate by expert geneticists. The protocol was approved be the Ethics in Research and Research Committees of the General Hospital “Dr. Manuel Gea González”. Written informed consent was obtained from each family.

2.2. Extraction of genomic DNA and genotyping

Genomic DNA was obtained from 20 mL of ethylenediamine tetraacetic acid-treated peripheral blood using proteinase K and phenol/chloroform extraction methods [12]. The analyzed SNPs of the *MSX1* gene were SNP1, SNP5, P147Q and P278S; the primers and probes are described in Table 1 [7,11,13].

The polymerase chain reaction (PCR) mixture contained 10 mM Tris, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.5 mM each primer, 2 U *Taq* polymerase (Epicentre Biotechnologies; Madison, WI, USA) and approximately 200 ng DNA in a final reaction volume of 50 μL. Standard thermocycling was as follows: 96°C/60 s, 72°C/60 s, and 72°C/60 s for 30 cycles. The amplified products were detected by electrophoresis on a 2% agarose gel with 0.5 μg/mL ethidium bromide and visualized under ultraviolet light. The P147Q polymorphism was detected using the dot blot-chemiluminescence technique [14]. The other polymorphisms were examined by PCR-RFLP (restriction fragment length polymorphism) (Table 1).

2.3. Statistical analysis

Allele frequencies (AFs) and genotype frequencies (GFs) were calculated by direct counting and were compared between the patients and pseudo-controls using a Chi-squared test with Yates correction or a two-tailed Fisher's exact test when the expected frequency in at least one cell was less than 5. Relative risks for alleles, genotypes and haplotypes were calculated as the odds ratio (OR), and 95% confidence intervals (95%CI) were obtained using SPSS software (v19). Pseudo-controls are defined as “individuals who have two parental genes not transmitted to their diseased offspring, thus could be used as the control sample.” This would ensure that both samples of the patient's genes and the matched control would originate from the same genetic population [15,16].

Triad analysis considering the child, mother and father was also performed using a log linear method to test for asymmetric distribution of a particular variant allele among the affected offspring and their biologic parents, assuming Mendelian inheritance with or without Hardy-Weinberg equilibrium (HWE) [17–19]. The program HAPLIN was used for this analysis (v5.3) [20]. The most frequent haplotype was use as a reference for estimating the effect in a multiplicative model.

To reconstruct haplotypes and estimate relative risk associated with a single or double dose of each haplotype among mother-father-child triads, pairwise linkage disequilibrium (LD) was determined using Haploview (v4.2). Association analyses were performed under additive or genotype-wise models using family-based conditional logistic regression analysis [20–22].

To establish the most informative model of Mendelian association (dominant, co-dominant, recessive, overdominant or log additive), the online program SNPStats was used for case-pseudo-controls and triad analyses [23].

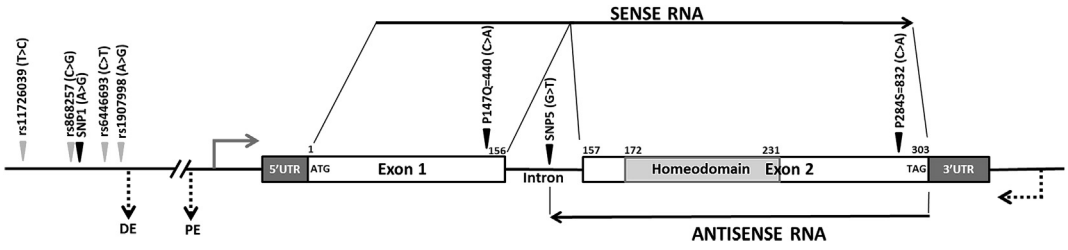

Fig. 1. Organization of the *MSX1* gene. The colorless boxes represent exon 1 and exon 2; the line between the two exons represents the intron; the light grey box is the homeodomain-encoding region; ATG, start codon; TAG, stop codon. Numbers represent amino acid residues. The *MSX1* S RNA (top) and AS RNA (bottom) are indicated by black horizontal arrows; a grey arrow (left) and dashed arrow (right) represent the S and AS promoters, respectively [50]. Dashed straight arrows at the left indicate proximal (PE, −2630 to −2553 kb) and distal (DE, −4670 to −4420 kb) enhancer positions [39]. SNPs rs11726039 (−11.9 kb), rs868257 (−9.2 kb) rs6446693 (−6.3 kb), and rs1907998 (−4.8 kb) analyzed by Gurramkonda et al. [45] are in light grey. The polymorphisms studied in this work, SNP1 (−8796 kb), P147Q (440 kb), SNP5 (2845 kb), P278S (832 kb), are in black. The 5′ UTR and 3′ UTR are in dark grey.