Archives of Oral Biology 91 (2018) 91–95

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/archoralbio

Gene-gene interaction for nonsyndromic cleft lip with or without cleft palate in Chilean case-parent trios

José Suazo[a,*], José Luis Santos[b], Alicia Colombo[c,d], Rosa Pardo[e,f,g]

[a] Instituto de Investigación en Ciencias Odontológicas, Facultad de Odontología, Universidad de Chile, Sergio Livingstone #943, Santiago, Chile
[b] Departamento de Nutrición, Diabetes y Metabolismo, Escuela de Medicina, Pontificia Universidad Católica de Chile, Lira #44, Santiago, Chile
[c] Programa de Anatomía y Biología del Desarrollo, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Independencia #1027, Santiago, Chile
[d] Servicio de Anatomía Patológica, Hospital Clínico de la Universidad de Chile, Santos Dumont #999, Santiago, Chile
[e] Sección de Genética, Hospital Clínico Universidad de Chile, Santos Dumont #999, Santiago, Chile
[f] Unidad de Neonatología, Hospital Clínico Universidad de Chile, Santos Dumont #999, Santiago, Chile
[g] Unidad de Genética, Hospital Dr. Sótero del Río, Concha y Toro #3459, Santiago, Chile

ARTICLE INFO

Keywords:
Nonsyndromic cleft lip with or without cleft palate
TGFB3
MSX1
Case-parents trios
GxG interaction

ABSTRACT

Objective: Nonsyndromic cleft lip with or without cleft palate (NSCL/P) is a birth defect for which several genes susceptibility genes been proposed. Consequently, it has been suggested that many of these genes belong to common inter-related pathways during craniofacial development gene-gene interaction. We evaluated the presence of gene-gene interaction for single nucleotide polymorphisms within interferon regulatory factor 6 (*IRF6*), muscle segment homeobox 1 (*MSX1*), bone morphogenetic protein 4 (*BMP4*) and transforming growth factor 3 (*TGFB3*) genes in NSCL/P risk in Chilean case-parent trios.

Design: From previous studies, we retrieved genotypes for 13 polymorphic variants within these four genes in 152 case-parent trios. Using the *trio* package (R) we evaluate the gene-gen interaction in genetic markers pairs applying a 1°-of-freedom test (1df) and a confirmatory 4°-of-freedom (4df) test for epistasis followed by both a permutation test and a Benjamini-Hochberg test for multiple comparisons adjustment.

Results: We found evidence of gene-gene interaction for rs6446693 (*MSX1*) and rs2268625 (*TGFB3*) (4df $p = 0.024$; permutation $p = 0.015$, Benjamini-Hochberg $p = 0.001$).

Conclusions: A significant gene-gene interaction was detected for rs6446693 (*MSX1*) and rs2268625 (*TGFB3*). This finding is concordant with research in animal models showing that *MSX1* and *TGFB3* are expressed in common molecular pathways acting in an epistatic manner during maxillofacial development.

1. Introduction

Expression and susceptibility to complex traits are characterized by the interaction of genes and environmental factors and by gene-gene (GxG) interaction (also known as epistasis). Thus, any study aiming to elucidate the genetic architecture of a complex trait, should consider GxG interaction analysis (Carlborg & Haley, 2004). Nonsyndromic cleft lip with or without cleft palate (NSCL/P) is a complex disorder representing the most common birth defect affecting human craniofacial development (Jugessur & Murray, 2005; Watkins, Meyer, Strauss, & Aylsworth, 2014). Consequently to its complex etiology, NSCL/P potential causal mutations have been detected within regulatory and coding regions if at least 20 candidate genes encoding products involved in different molecular pathways (Dixon, Marazita, Beaty, & Murray, 2011; Leslie & Marazita, 2013; Vieira, 2008).

In the best of our knowledge, there is no genome-wide association studies for this birth defect analyzing exclusively Chilean subjects. However, our research group has been assessed the association between *loci* interferon regulatory factor 6 (*IRF6,* 1q32.2), muscle segment homeobox 1 (*MSX1,* 4p16.2), bone morphogenetic protein 4 (*BMP4,* 14q22.2) and transforming growth factor 3 (*TGFB3*, 14q24.3) and NSCL/P based on previous reports in other populations (Paradowska-Stolarz, 2015; Tang, Wang, Han, Guo, & Wang, 2013; Watkins et al., 2014; Wattanawong, Rattanasari, McEvoy, Attia, & Thakkinstian, 2016). Thus, in single-gene studies we found cleft risk haplotypes composed by polymorphic variants (single nucleotide polymorphisms or SNPs) within these *loci* using a sample of NSCL/P case-parent trios from a Chilean population (Suazo, Santos, Jara, & Blanco, 2008; Suazo,

Abbreviations: BMP4, bone morphogenetic protein 4; GxG interaction, gene-gene interaction; IRF6, interferon regulatory factor 6; MSX1, muscle segment homeobox 1; NSCL/P, nonsyndromic cleft lip with or without cleft palate; SNP, single nucleotide polymorphism; TGFB3, transforming growth factor beta 3

* Corresponding author.
E-mail address: jsuazo@odontologia.uchile.cl (J. Suazo).

https://doi.org/10.1016/j.archoralbio.2018.04.009
Received 2 January 2018; Received in revised form 12 April 2018; Accepted 13 April 2018

Santos, Jara, & Blanco, 2010; Suazo, Santos, Jara, & Blanco, 2010; Suazo, Santos, Scapoli, Jara, & Blanco, 2010).

There is evidence of interaction among the products of *IRF6*, *MSX1*, *BMP4* and *TGFB3* during maxillofacial development in animal models. Thus, the expression of *Msx1* and *Bmp4* seems to be reciprocally regulated in the mesenchymal tissues of murine palatal shelves where the expression of the Bmp4 protein reverts *Msx1* deficiency and rescues the cleft palate phenotype (Smith, Lozanoff, Yyyanar, & Nazarali, 2013; Zhang et al., 2002). On the other hand, a mice knock-out line for *Tgfb3* exhibits an expression reduction of *Msx1* and a proliferation deficiency of the palatal shelves which possibly explain the cleft palate observed in these animals (Del Río et al., 2011). Based on these evidences and in the fact that, as we previously mentioned, we found NSCL/P-risk haplotype in our triads population, instead single-marker association, we decided to evaluate GxG interaction between haplotypes composed by SNPs from these four genes. Unfortunately, in the best of our knowledge, there are no developed methods for haplotype interaction for case-parent trio samples. Alternatively, in a Chilean case-control sample we recently assessed haplotype-based interaction of these same *loci* for NSCL/P expression and found GxG interaction between haplotypes composed by SNPs of *BMP4* and *IRF6* (Blanco, Colombo, Pardo, & Suazo, 2017). However, population-based design (such as case-control) may generate spurious associations due to population stratification by ethnicity, a phenomenon detected in the contemporary urban Chilean population (Palomino, Palomino, Cauvi, Barton, & Chacraborty, 1997; Santos, Perez, Carrasco, & Albala, 2002; Valenzuela, 1988).

The case-parent trio design is insensible to population stratification due to the construction of genetic matched pseudo-controls based on the non-transmitted alleles from parents to affected progeny (Santos et al., 2002). Using case-parent trio design, for the current study we propose to assess the single-marker GxG interaction between 13 SNPs within *IRF6*, *MSX1*, *BMP4* and *TGFB3* genes in the risk of NSCL/P in a sample of 152 Chilean case-parent trios. In order to achieve our aim, we propose to apply a step-wise method based in two statistical tests. First, we have used a Wald test of 1°-of-freedom (1df) in order to identify pairs of interacting SNPs. These interactions must be confirmed due to 1 df test may detect significant results not only due to epitasis but also due to of independence between *loci* (Cordell, 2002; Xiao et al., 2016). Thus, a 4°-of-freedom (4df) test was used as a confirmatory test of these SNP pairs (Cordell, 2002). To detect GxG interaction based on several genes and/or markers is always complex due to it is necessary to assess multiple hypotheses with the need of multiple comparisons affecting the statistical power of the tests, even for large sample sizes studies (Xiao et al., 2016). This is the reason why we decide to confirm our 4df test using two alternative methods for multiple comparisons correction.

2. Materials and methods

2.1. Subjects

The sample was composed of 152 unrelated NSCL/P Chilean case-parent trios. Cases were 38% females and 69% of them had no family history of orofacial clefts. According to the clinical classification, 92% were cataloged as cleft lip and palate whereas 8% exhibited only a cleft lip. These cases and their parents were obtained between the years 2008 and 2011 after in depth interviews of at least three family members in order to reconstruct a family history. Interviews considered maternal exposure to teratogenic substances (phenytoin, warfarin and ethanol) during pregnancy. These nuclear families were recruited at the following centers: Craniofacial Malformations Unit, School of Dentistry, Universidad de Chile; Cleft Lip/Palate Center, Hospital Exequiel Gonzalez Cortes; Dental Service, Hospital Roberto del Rio; Maxillofacial Service, Hospital San Borja-Arriaran; and Maxillofacial Service, Hospital Sótero del Rio (all of them located in the city of Santiago, Chile). Our study was approved by School of Medicine (Universidad de Chile) Institutional Review Board (Protocols #2005-11 and #018-2009) and all participants or its legal representative gave their informed consent.

Table 1
IRF6 *MSX1*, *BMP4* and *TGFB3* SNPs considered for the current study.

Gene	SNP	Physical Location[a]	MAF[b]	HW *P* value[c]
IRF6	rs2235371 (C > T)	Chr1: 209790735	0.197	0.870
	rs2235375 (C > G)	Chr1: 209792242	0.515	0.148
	rs2236909 (G > A)	Chr1: 209798310	0.498	0.272
	rs764093 (T > C)	Chr1: 209809986	0.289	0.014
MSX1	rs6446693 (T > C)	Chr4: 4853353	0.473	0.528
	rs3775261 (A > C)	Chr4: 4862018	0.403	0.853
	rs12532 (G > A)	Chr4: 4863419	0.490	0.090
BMP4	rs2855532 (T > C)	Chr14: 53953247	0.318	0.018
	rs762642 (T > G)	Chr14: 53956335	0.455	0.079
	rs1957860 (T > C)	Chr14: 53962637	0.413	0.667
TGFB3	rs3917201 (A > G)	Chr14: 75963212	0.286	0.853
	rs2268625 (A > G)	Chr14: 75973291	0.239	0.957
	rs2268626 (A > G)	Chr14: 75978424	0.415	0.383

[a] Chromosome location according to GRCh38 genomic assembly at dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/).
[b] MAF: minor allele frequency estimated in an all parental genotypes.
[c] HW P value: significance for deviations from Hardy-Weinberg equilibrium for genotype frequency distribution in the parental sample.

2.2. Genotype data extraction

For GxG interaction estimation, genotype data for 13 SNPs (Table 1) were extracted from previous studies including the same Chilean case-parent trios population here described (Suazo et al., 2008; Suazo et al., 2010a, 2010b; Suazo, Santos and Scapoli et al., 2010). All SNP genotypes were obtained by a polymerase chain reaction followed by a restriction fragment length polymorphism analysis as was described in our previous reports (Suazo et al., 2008; Suazo et al., 2010a, 2010b; Suazo, Santos and Scapoli et al., 2010). As quality control, amplicons for a subset of samples were directly sequenced confirmed the genotypes obtained by polymerase chain reaction followed by a restriction fragment length polymorphism analysis and then used as internal controls (data not shown). Polymorphisms considered in the current report, their alleles frequencies and genomic positions are listed in Table 1.

2.3. Statistical analyses

We calculated allele frequencies based on simple proportions using parental genotypes. Departures from the Hardy-Weinberg equilibrium in genotype distributions in parents were evaluated through a chi-square goodness-of-fit test implemented in STATA 12 statistical package. In order to select candidate SNPs pairs for GxG interaction, we performed a conditional logistic regression (1df Wald test). This conditional model is based in the assumption of an additive mode of inheritance for a pair of SNPs randomly selecting one among three pseudo-controls and, therefore, containing one interaction parameter (Li et al., 2010), providing a rapid analysis of pairwise GxG interactions (Xiao et al., 2016). As we previously mentioned, 1df Wald test may be considered as evidence of epistasis but also may reflect a violation of interaction model (Cordell, 2002; Xiao et al., 2016). Thus, these results were confirmed applying the 4df likelihood ratio test which considers the genotypes for the case and three possible genotypes (pseudo-controls) based on both parental genotypes (Cordell, 2002; Schaid, 1999; Xiao et al., 2016). Due to the multiple hypotheses evaluated in this study, it is necessary to adjust the significant results by a multiple comparison correction. The most popular method, named Bonferroni correction, can increase the false-positive rate when SNPs pairs are not independent (i.e. they are in linkage disequilibrium) reducing the statistical power (Nyholt, 2004). Therefore, we have opted for a