Research Article

Preliminary Analysis of the Nonsynonymous Polymorphism rs17563 in *BMP4* Gene in Brazilian Population Suggests Protection for Nonsyndromic Cleft Lip and Palate

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Cleft lip with or without palate (CL±P) is common congenital anomalies in humans. Experimental evidence has demonstrated that bone morphogenetic protein 4 gene (*Bmp4*) is involved in the etiology of CL±P in animal models. The nonsynonymous polymorphism rs17563 T>C (p.V152A) in the *BMP4* gene has been associated to the risk of nonsyndromic CL±P in Chinese population and microforms from different ethnic backgrounds. The aim of this study was to investigate the role of *BMP4* gene in CL±P in Brazilian sample using genetic association approach. Our sample was composed by 123 patients with nonsyndromic CL±P and 246 controls, in which absence of CL±P was confirmed in 3 generations. The rs17563 polymorphism was genotyped by PCR-RFLP technique. Logistic regression was performed to evaluate allele and genotype association. Our data showed statistical power to detect association (86.83%) in this sample. Logistic regression results showed significant association between C allele and CL±P ($P = 0.00018$, OR = 0.40, and 95% CI = 0.25–0.65), as well as CC genotype and CL±P ($P = 0.00018$, OR = 0.35, and 95% CI = 0.19–0.66). So, there is a strong association between nonsyndromic CL±P and *BMP4* rs17563 polymorphism in our sample and the C allele had a protective effect against the occurrence of nonsyndromic CL±P.

1. Introduction

Nonsyndromic cleft lip with or without palate (CL±P) is a congenital defect with multifatorial transmission. It is a birth defect that occurs with a prevalence of 1 in 500 to 1 in 2500 live births in different populations, varying with geographic location, ethnic group, and socioeconomic conditions [1–3]. Populations of either American native descent or Asian descent have the highest birth prevalence [4]. In Latin America, according to the ECLAMC (Latin American Collaborative Study of Congenital Malformations), the incidence of this malformation is 1 in 850 births [5]. CL±P has a complex etiology that includes strong genetic and environmental factors.

Clinically, CL±P are classified as nonsyndromic (isolated) or syndromic based on the presence of other congenital anomalies or mental disabilities [1, 6]. Around 400 recognizable syndromes that have CL±P as part of the

phenotype are described [7, 8], however, 70% of CL±P cases are nonsyndromic. Approximately 12–25% of the genetic variations associated with nonsyndromic CL±P have been identified [1, 6]. Although genetic studies have identified a number of candidate genes and chromosomal regions associated with CL±P, findings from different studies have been inconsistent [9, 10].

Candidate genes for CL±P have been proposed as result of studies using knockout mice models. One interesting candidate gene is the Bone morphogenetic protein 4 (*BMP4*; MIM#112262). Experimental evidences have postulated that *Bmp4* is involved in the etiology of CL±P in mice. Bilateral cleft lip was presented in all knockout mice for *Bmp4* at 12 days after conception, however after 14.2 days only 22% of the embryos showed cleft lip. Moreover, Bmp signaling is also required for cell proliferation at earlier stages, in the maxillary process mesenchyme [11].

BMP4 gene, located in 14q22-q23, is a member of the BMP family and a member of the transforming growth factor-b (TGF-b) of secretory signaling molecules that play essential roles in embryonic development [12]. This gene is a well-characterized mammalian growth factor. Expression of the *Bmp4* gene occurs in a wide spectrum of normal tissue in mouse embryos, as cardiac [11], limb [13], and face tissue [11, 14].

In addition, possible association between *BMP4* gene and nonsyndromic CL±P in humans was firstly suggested in a case-control study performed with Chinese children in 2008 [15]. Authors analyzed the single-nucleotide polymorphism (SNP) rs17563 (p.Val152Ala) and found an increased risk of nonsyndromic CL±P among carriers of the C allele.

A missense and nonsense mutations were found in *BMP4* gene in a sample of patients from different genetic background (Mongolia, Philippines, United States of America, Colombia, Guatemala, and Europe) diagnosed with subepithelial, microform, and overt cleft lip. These mutations were not found in a control population [16].

Furthermore, Suazo et al. [17] reported a mutation screening analysis of *BMP4* in a sample of 150 Chilean nonsyndromic CL±P case-parent trios. Significant deviations from expected transmissions were observed for haplotypes conformed by rs1957860 and rs762642. These polymorphisms delimitate a genomic region where a promoter and an enhancer of *BMP4* are located [18]. Then, Suazo et al. [19] searched for nonsyndromic CL±P risk variants within the two *BMP4* promoters by direct sequencing in a 167 Chilean nonsyndromic CL±P cases and 336 controls. They found three novel variants considered as cleft risk factors, as they are not present in controls.

Lately, Chen et al. [20] provided further evidence of association between *BMP4* gene and nonsyndromic CL±P. They tested for possible association between markers in and around the *BMP4* gene and nonsyndromic CL±P in Asian and Maryland trios. Nominal significant evidence of linkage and association was observed for three SNPs (rs10130587, rs2738265, and rs2761887) in 221 Asian trios and for one SNP (rs762642) in 76 Maryland trios.

Brazilian population presents a heterogeneous ethnic origin which includes Native Americans, Europeans, Africans, and some degree of Asians. Relative contribution of each one of these ethnic groups is variable according to the geographic region of the country. This characteristic gives a unique and richly blended genetic and cultural background to the country [21–25].

Therefore, since Brazilian population presents a diverse genetic background, the purpose of this study was to investigate the role of the rs17563 polymorphism of *BMP4* gene in CL±P in a sample of Brazilian population.

2. Materials and Methods

2.1. Study Population. The sample consisted of 369 individuals enrolled in Craniofacial Brazil Project. All cases were evaluated by clinical geneticist using the same clinical protocol, including family history. Subjects were recruited between 2009 and 2011 from five centers of research involved in Craniofacial Brazil Project located in six cities from three different regions of Brazil (Southeast region: Campinas and São José do Rio Preto, South region: Porto Alegre and Curitiba, Northeast region: Maceió). Case group was compound of 123 individuals (62 males and 61 females) with nonsyndromic CL±P (98 cleft lip and palate and 25 cleft lip only). Control group were recruited from Southeast region by universitary e-mail included 246 phenotypically normal unrelated individuals (102 males and 144 females) without family history of oral cleft in three generations and no Japanese or Chinese ancestry. Their family history was collected by a participant researcher.

The study was approved by the local Institutional Review Boards and the National Research Ethics Committee (CEP 059/2008). All participants provided informed consent. The informed consent for patients under 18 years old was obtained from their parents.

2.2. Molecular Analysis. Genomic DNA was extracted from peripheral blood lymphocytes according to the method described by De Araujo et al. [26].

SNP genotyping was performed using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. The PCR primers were designed based on the SNP flanking sequence described in the Ensembl Genome Browser (http://www.ensembl.org/index.html).

Primer sequences for PCR reactions were 5′AGTTTGGCTGCTTCTCCC3′ (forward) and 5′AGTTTGGCTGCTTCTCCC3′ (reverse). The PCR reaction were performed in a total volume of 15 μL containing 1.5 μL KCl$_2$ (10X), 0.6 μL MgCl$_2$ (25 μM), 1.5 μL dNTP (2 μM), 0.35 μL of each primer *primer sense* (5pmoles), 0.5 μL DMSO$_4$, 0.2 μL Taq DNA Polimerase (5U) (New England BioLabs, Ipswich, MA, USA), and 1 μL DNA (200 ng/μL) e 9 μL H$_2$O. The PCR cycle conditions consisted of an initial denaturation step at 94°C for 5 min followed by 35 cycles of 1 min at 94°C, 1 min at 56°C, 1 min at 72°C, and a final elongation at 72°C for 7 min.

Adequate restriction endonuclease for SNP was selected using the Gene Runner software (Hastings Software Inc., Hastings, NY, USA; http://www.generunner.net/). Primers, restriction enzyme, and the length of digested fragments for the SNP are listed in Table 1.

TABLE 1: Primers, restriction enzyme, and fragment lengths for rs17563 of *BMP4* gene.

SNP	Primer	Annealing temperature	Restriction enzyme	Fragment lengths
rs17563	Forward 5′CACCATTCATTGCCCAAC3′	56°C	HphI	T Allele: 195pb + 229pb
	Reverse 5′AGTTTGGCTGCTTCTCCC3′			C Allele: 424pb

TABLE 2: The genotype and allele distribution of rs17563 in nonsyndromic CL±P patients and controls and result of logistic regression.

SNP rs17563		Controls n (%)	Cases n (%)	χ^2	P	OR	95% CI
Genotype	TT[1]	52 (21,1)	49 (39,8)	—	—	1.00	—
	TC	130 (52,8)	53 (43,1)	10.645	0.00110	0.43	0.26–0.71
	CC	64 (26,1)	21 (17,1)	11.217	0.00081	0.35	0.19–0.66
Allele	T[1]	234 (47,6)	151 (61,4)	—	—	1.00	—
	C	258 (52,4)	95 (38,6)	13.992	0.00018	0.40	0.25–0.65

[1] Reference factor.

The digestion reaction were performed in a total volume of 20 μL containing 15 μL PCR, 0.5 μL enzyme, and 2.0 μL buffer e 2.5 μL H$_2$O. The PCR products were digested overnight at 37°C with *Hph*I enzyme (New England BioLabs, Ipswich, MA, USA). PCR products were electrophoresed through 1% agarose gel in the presence of ethidium bromide and visualized by fluorescence in UV light; the digested PCR products were resolved on 12% polyacrylamide gels and visualized by a silver staining protocol.

2.3. SNP Association Analysis. Gender distribution was evaluated using Fisher's exact test in *R* statistical environment [27]. Minor allele frequency (MAF) and Hardy-Weinberg equilibrium were estimated using Haploview software [28] (http://www.broadinstitute.org/haploview). Allele and genotype association analysis were performed by logistic regression using *R* statistical environment [27]. We evaluated posthoc statistical power of our sample by GPOWER software [29], using the following parameters: logistic regression test; two-tail; OR = 1.5; statistical significance level α = 0.05; n = 369.

2.4. In Silico Test. *In silico* tests were performed to analyze whether the substitution c.538T>C in the *BMP4* gene (rs17563) modifies the structure or function of the encoded protein. The sequence of amino acids used in the tests was obtained from NCBI database (http://www.ncbi.nlm.nih.gov/).

Three *in silico* tests were used: Grantham scale [30], PolyPhen [31] (http://genetics.bwh.harvard.edu/pph/), and SNAP [32] (http://rostlab.org/services/snap/) for the analysis of rs17563.

3. Results

The gender distribution was similar between groups (Fisher's exact test P = 0.1198). MAF was 0.478 and SNP rs17563 was in Hardy-Weinberg equilibrium (P = 0.9664) in the sample. There were significant differences in the genotypes and allele frequencies of the rs17563 between nonsyndromic CL±P and control groups.

Logistic regression revealed association between C allele and CL±P (P = 0.00018, OR = 0.40, and 95% CI = 0.25–0.65). Genotype logistic regression analysis showed rs17563 CC genotype was associated with decreased of CL±P susceptibility (P = 0.00081, OR = 0.35, and 95% CI = 0.19–0.66) (Table 2). The sample analyzed showed statistical power to detect genetic association (86.83%).

The results obtained of *in silico* tests were similar for the three. Grantham's scale showed that SNP was considered "moderately conservative" (value 64). The PolyPhen algorithm aligned sequences "wild" and "altered" to 75 homologous sequences and estimated the change as "benign" (score 0416). In addition, SNAP demonstrated that p.Val152Ala is neutral (reliability index = 3, expected accuracy = 78%).

4. Discussion

Mutant mice for *Bmp4* gene with CL±P phenotype suggest a possible role for this gene in lip and palate development [11]. Moreover, have been reported few studies of association between *BMP4* gene and CL±P in population.

Lin et al. [15] found significant differences in genotype distribution and allele polymorphism rs17563 of *BMP4* gene between 184 nonsyndromic CL±P cases and 205 controls. In a study with 1614 individuals from different countries (Mongolia, Philippines, United States of America, Colombia, Guatemala, and Europe), missense and nonsense mutations in *BMP4* gene were detected in 5 of 968 individuals with overt CL±P, 1 of 30 cases with microforms and 2 of 87 cases with orbicular oris muscle. They also found a borderline difference in the frequency of SNP rs17563 in cases compared with controls [16]. Thus, the results of this study support the hypothesis that this polymorphism might be clinically important in the genesis of CL±P.

Recently, Chen et al. [20] tested for possible association between markers in *BMP4* gene and nonsyndromic CL±P in 297 Asian and Maryland trios. Their results does not support the evidence of linkage and association for the SNP rs17563.

However, in the present study the polymorphism rs17563 in *BMP4* gene was associated with decreased susceptibility for nonsyndromic CL±P ($P = 0.00081$, OR = 0.35, and 95% CI = 0.19–0.66). This result was the opposite found by Lin et al. [15] and Suzuki et al. [16]. The difference could be attributed to the clinical selection of individuals, the well-defined control group, and the statistics power herein used. As well, this study obtained a strong association between nonsyndromic CL±P and *BMP4* rs17563 polymorphism and suggested that C allele had a protective role in the occurrence of nonsyndromic CL±P in the Brazilian sample.

The rs17563 polymorphism replaces the amino acid valine by alanine at position 152 of the protein. It would seem that the structure and function of the protein are not significantly affected by the substitution due to physicochemical similarities between the involved amino acids. In our study, results of the *in silico* test using Grantham's scale, PolyPhen, and SNAP were similar and showed the moderately conservative, benign, and neutral character of the rs17563 polymorphism. These results corroborate the hypothesis of a protective effect of this polymorphism.

Although cleft lip with cleft palate and cleft lip only are usually grouped into the same category, Dixon et al. [33] suggested that they could be etiologically distinct. Thus, further genetic association studies designed to examine these two conditions separately would be helpful in elucidating the role *BMP4* rs17563 in orofacial clefts.

5. Conclusions

This study found a strong association between nonsyndromic CL±P and *BMP4* rs17563 polymorphism and a possible protective effect of the C allele against nonsyndromic CL±P in a Brazilian sample. In this regard it could be speculated that rs17563 polymorphism plays different roles in oral cleft pathogenesis in specific populations. It would be interesting to replicate this study in individuals with similar and different ethnicity. As well, this association reinforces the importance of this gene as candidate for oral cleft.

Acknowledgments

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