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Association of *WNT9B* gene polymorphisms with nonsyndromic cleft lip with or without cleft palate in a Brazilian Population

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Abstract

Objective—Nonsyndromic cleft lip with or without cleft palate (NSCL/P) is a common craniofacial anomaly in humans of complex etiology. WNT pathway genes have important roles during craniofacial development and the association of WNT genes with NSCL/P has been demonstrated in different populations. The aim of this study was to evaluate the association between polymorphisms in *WNT3* and *WNT9B* genes and CL/P in a Brazilian population.

Patients—Seventy nuclear families composed by an affected individual and their unaffected parents were examined clinically and saliva samples were collected for molecular analyses.

Design—Five single nucleotide polymorphisms (SNPs) including three in the *WNT3* gene and two polymorphisms in *WNT9B* were investigated in real-time PCR using TaqMan chemistry. The Family-Based Association Test (FBAT) and the transmission disequilibrium test (TDT) were used to verify the association between each marker allele and NSCL/P. The level of significance was established at $P \leq 0.05$.

Results—A positive association was detected between NSCL/P and SNP rs1530364 in *WNT9B* gene. Haplotype analysis showed association of *WNT3* and *WNT9B* haplotypes. No association was detected between NSCL/P and individual SNPs in *WNT3*.

Conclusion—Our study further supports the involvement of *WNT9B* as a cleft susceptibility gene in Brazilian NSCL/P families. Although additional studies are still necessary to unveil the exact mechanism by which WNT genes would contribute to NSCL/P, allelic polymorphisms in these genes and their interactions may partly explain the variance of individual susceptibility to NSCL/P.

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Keywords

cleft lip/palate; genetic polymorphisms; association

Introduction

Human development is a complex event and requires the integration of various mechanisms to allow proper formation of all the structures. Cleft lip with or without cleft palate is one of the most common craniofacial anomalies in humans, and may occur as part of a syndrome or isolated, when the affected individuals do not present any associated structural anomalies. Over 300 syndromes, including some that are either chromosomal or Mendelian, might present a cleft of the lip and/or the palate as a feature, and comprise about 30% of all cleft cases. The remaining 70% are attributed to isolated, nonsyndromic oral clefts, without any associated structural anomaly (Gorlin *et al.*, 2001). Nonsyndromic cleft lip with or without cleft palate (NSCL/P) has a prevalence of 1–2 per 1000 live births depending on the population, and the etiology includes genetic and environmental factors acting individually or interactively (Murray, 2002).

The wingless-type MMTV integration site family (Wnt) signaling pathway plays an important role in craniofacial development. Wnt signaling genes are conserved among species and are essential to the development of several processes, including face morphogenesis (Fossat et al., 2011). Studies in animal models have revealed that these genes are expressed in the midface of mice and chicken (Lan et al., 2006; Brugmann et al., 2007; Song et al., 2009; Reid et al., 2011) and that Wnt signaling plays an important role in various aspects of craniofacial development in many species. Loss of function of WNT genes is associated with defects in the facial region, incomplete penetrance of cleft lip and defects in kidney morphogenesis in homozygous in mice mutants (Carroll et al, 2005). Variations in *Wnt5a* that cause the loss of expression have been associated with complete clefting of the secondary palate, and altered expression of several genes, including sonic hedgehog (*Shh*), bone morphogenetic protein 4 (*Bmp4*), msh homeobox 1 (*Msx1*), patched 1 (*Ptch1*), in the palate of *Wnt5* knockout mice (He et al., 2008). Further, *Wnt3* and *Wnt9b* genes are located on chromosome 11 within the *clf1* locus associated with the spontaneous development of CL/P in A/WySn mouse strains (Juriloff et al., 2005; 2006). Interestingly, this region is syntenic to the human chromosome 17q21, which is known to be associated with NSCL/P in humans (Carinci et al., 2007).

Studies in humans also point towards a likely role of Wnt pathway genes in the etiology of craniofacial defects in humans. A study of a consanguineous family with tetra-amelia, a disorder characterized by complete limb agenesis and other anomalies, including cleft lip with or without cleft palate has been associated to a nonsense mutation (Q83X) in the *WNT3* gene (Niemann et al., 2004). In addition, polymorphic variants in *WNT3, WNT3A, WNT5A, WNT9B,* and *WNT11* genes have been associated with NSCL/P in different populations (Chiquet et al., 2008; Nikopensius et al., 2011; Yao et al., 2011; Mostowska et al., 2012), including a case-control study in a Brazilian population (Menezes et al., 2010). The aim of

this study was to evaluate the association between polymorphisms in *WNT3* and *WNT9B* genes and NSCL/P in Brazilian families with NSCL/P.

Material and methods

Subjects

Seventy nuclear families, each consisting of a child with cleft lip/palate (probands) and unaffected parents (210 individuals), were recruited for this study at Hospital Nossa Senhora do Loreto (Rio de Janeiro, Brazil). Only individuals with NSCL/P were recruited for the study, as determined by patient records. The average age of individuals with NSCL/P was 3.1 ± 2.8 years (minimum age 2 months, maximum 13 years). The average maternal age was 23.4 ± 13.2 years (minimum age 13 years, maximum 40 years) and the average paternal age was 25 ± 17 (minimum age 18 years, maximum 56 years). Written informed consent to participate in the study was obtained from all probands and their parents/legal guardians. This study was approved by the Human Ethics Committee of the Health Department of the City of Rio de Janeiro, Rio de Janeiro, Brazil (Process #113/09).

Genotyping

Saliva samples were collected as source of genomic DNA using Oragene saliva collection kits (DNA Genotek, Ontario, CA, USA). DNA extraction followed using the manufacturer's recommendations.

We selected five single nucleotide polymorphisms (SNPs) in *WNT3* and *WNT9B* genes (Table 1) to be genotyped, based on previously published findings for association with NSCL/P in humans (Chiquet et al., 2008; Menezes et al., 2010). Genotyping was performed using 5ng/uL DNA in a 10uL final reaction volume using TaqMan chemistry (Ranade et al., 2001), and detected on a ViiA7^{TM} Sequence Detection System (Applied Bio-systems, Foster City, CA, USA). Genotyping was performed blinded to sample status.

Statistical analyses

Power calculations were performed using the Genetic Power Calculator and indicated that the sample size would provide approximately 98% statistical power to detect an association with an alpha of 0.05, if the markers selected were in linkage disequilibrium with the causal factor ($D' = 0.8$) and their frequencies were around 20% (Purcell et al., 2003) (data not shown).

The transmission disequilibrium test (TDT) was used to examine the transmission of alleles from heterozygous parents to affected offspring, as implemented in FBAT (Family Based Association Test) software (http://biosun1.harvard.edu/»fbat/fbat.htm) (Horvath et al., 2001). FBAT uses a score test statistic to compare expected genotypes among offspring under the assumption of no association between observed genotype counts and the phenotype (Laird et al. 2000; Rabinowitz and Laird, 2000). The level of significance was established at $P \leq 0.05$. Haplotype analyses was performed to test for linkage between the WNT3 and WNT9B loci and NSCL/P using the 'hbat' function in FBAT.

Results

We found strong evidence of genotypic and allelic association for a SNP (rs1530364) in *WNT9B* with NSCL/P (P=0.001). This polymorphism is located in an intronic region at position 42306776. No association was found for SNPs in *WNT3* and NSCL/P in our population (Table 2). Haplotype analysis of polymorphisms in the *WNT3* and *WNT9B* genes revealed SNP combinations associated with a risk *for* NSCL/P. Namely, a haplotype comprising *WNT3* rs199525-T, rs111769-C, rs3851781-C and *WNT9B* rs1260243-C and rs1530364-G, was also associated with NSCL/P (global P-value=0.012).

Discussion

Wnt signaling molecules constitute a family of conserved secreted glycoproteins that play fundamental roles in developmental and biological processes (Wodarz et al., 1998). The WNT genes are involved in regulating midface development and upper lip fusion and are therefore candidates for an etiologic role in NSCL/P. Evidence supporting WNT genes as possible clefting loci come from studies with the inbred A/WySn mouse strain, in which *Wnt3* and *Wnt9B* genes are located in the *clf1* region and contribute to a clefting phenotype (Juriloff et al., 2004; Juriloff et al., 2005). In humans, variations in WNT genes have been described in cases with syndromic (Niemann et al., 2004) and nonsyndromic CL/P (Chiquet et al., 2008; Menezes et al., 2010; Nikopensius et al. 2011; Yao et al., 2011; Mostowska et al., 2012).

In this study, we investigated SNPs in *WNT3* and *WNT9B* as possible candidates in the etiology of NSCL/P in Brazilian families. We observed a strong association of an intronic SNP (rs1530364) in *WNT9B* with NSCL/P, whereas no association was found for SNPs in *WNT3*. The families in the present study consisted of 70 parent-case trios from the Southeastern region of Brazil which constitute mostly European descents. Power calculations indicated that the sample size would provide approximately 98% statistical power to detect an association.

The association of *WNT9B* with NSCL/P has also been shown in previous family-based studies with NSCL/P families and cases and controls of European ancestry (Chiquet et al., 2008; Menezes et al., 2010; Nikopensius et al., 2011). Further, and similarly to these studies where haplotypes including markers in *WNT9B* showed strong association with NSCL/P, we also observed significant association of haplotypes including markers in *WNT9B* and *WNT3* with NSCL/P in our families. Evidence for an epistatic interaction was also identified between SNPs in *WNT9B* and *MSX1*, another established cleft susceptibility gene (Nikopensius et al., 2011).

WNT9B lies approximately 32 kb telomeric from the start codon of *WNT3*, which is required at the earliest stages of human limb formation and for craniofacial morphogenesis. In our study, the associated intronic SNP (rs1530364) in *WNT9B* associated does not appear to alter transcription factor-binding sites or to have potential detrimental effects, due to the fact that introns are transcribed into the pre-mRNA but removed during formation of the mature mRNA and therefore not translated into the peptide. Nevertheless, although the associated

SNP may not have a direct effect on the etiology of NSCL/P, it may be in linkage disequilibrium with a true causal variant, within *WNT9B* or in other potential candidate genes.

NSCL/P is a complex disorder and may exhibit different clinical subphenotypes associated (Weinberg et al., 2006). The use of a broadened phenotype description, including information on the cleft type and side, presence of lip whorls, dental anomalies and discontinuity of the oral orbicularis muscle in NSCL/P cases, has been suggested to increase the likelihood of finding a causative gene in association with the condition (Weinberg et al., 2006; Letra et al., 2007; Marazita et al., 2007; Neiswanger et al., 2009). Moreover, these cleft subphenotypes could be used to verify if a particular gene is preferentially associated with a certain subphenotype (Letra et al., 2007). In this context, the preferential association of a SNP in *WNT5A* (rs566926) with cases of unilateral left-sided NSCL/P has been described, although no association was detected for the overall NSCL/P group (Menezes et al., 2010). This suggests that specific genes may contribute differently to each NSCL/P phenotype. In this study, we only performed analysis for a combined NSCL/P group, as stratifying our analysis by different CL/P subgroups (unilateral, bilateral, left or right) would decrease statistic power due to the reduced sample size. This may explain any discrepancies between our results and previous reports (Menezes et al., 2010). Nevertheless, a combined NSCL/P group as used in this study is still the most widely used approach in genetic studies of NSCL/P (Grant et al., 2009; Birnbaum et al., 2010; Letra et al., 2012; Beaty et al., 2013).

In summary, a number of observations suggest that WNT genes, particularly *WNT9B* and *WNT3*, may be involved in the etiology of NSCL/P (Chiquet et al., 2008; Menezes et al., 2010; Yao et al., 2011; Mostowska et al., 2012). Our study further supports the involvement of *WNT9B* as a cleft susceptibility gene in Brazilian NSCL/P families. Although additional studies are still necessary to unveil the exact mechanism by which WNT genes would contribute to NSCL/P, allelic polymorphisms in these genes and their interactions may partly explain the variance of individual susceptibility to NSCL/P.

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Table 1

Details of the studied polymorphisms in *WNT3* and *WNT9B* genes.

Gene	Chromosome	SNP	Bp Position	Alleles	Function
WNT3	17	rs199525	42203002	GT	intron
WNT3	17	rs111769	42227151	CT	intron
WNT3	17	rs3851781	42246300	CT	intron
WNT9B	17	rs12602434	42283052	CG	Near-5′UTR
WNT9B	17	rs1530364	42306776	AG	intron

SNP, single nucleotide polymorphism; UTR, untranslated region; Bp, base pair.

Table 2

Results of association tests with *WNT3 and WNT9B* genes in NSCL/P families.

Gene	SNP	Allele	MAF	Base	P value	Reference*
WNT3	rs199525	1	0.199	G	0.149	G: 10.99% (241 / 2192)
WNT3	rs199525	2	0.801	T	0.149	T: 89.00% (1951 / 2192)
WNT3	rs111769	1	0.648	C	0.309	C: 67.52% 1765 / 2614)
WNT3	rs111769	2	0.352	T	0.309	T: 32.47% (849 / 2614)
WNT3	rs3851781	1	0.574	C	0.507	C: 50.66% (2181 / 4305)
WNT3	rs3851781	2	0.426	T	0.507	T: 49.33% (2124 / 4305)
WNT9B	rs1260243	1	0.156	C	0.723	C: 28.00% (1285 / 4588)
WNT9B	rs1260243	2	0.844	G	0.723	G: 71.99% (3303 / 4588)
WNT9B	rs1530364	1	0.315	A	0.001	A: 29.35% (1411 / 4806)
WNT9B	rs1530364	2	0.685	G	0.001	G: 70.64% (3395 / 4806)

*Reference: Allele frequency in general population based on UCSC Human Genome Browser, February 2009 Human Reference Sequence (GRCh37).

SNP, single nucleotide polymorphism; MAF, minor allele frequency.

Table 3

Results of haplotype analysis for markers in *WNT3* and *WNT9B* genes in the studied families.

WNT3			***WNT9B***		**P-value**
rs199525	rs111769	rs3851781	rs1260243	rs1530364	
T	C	C	G	G	**0.0125**
T	C	T	G	G	0.1039
T	C	C	G	A	0.1068
T	C	T	G	A	0.1525
T	T	C	G	G	0.3017