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Studies with WNT Genes and Nonsyndromic Cleft Lip and Palate

Renato Menezes[1,3,#], **Ariadne Letra**[1,3,#], **Ana H. Kim**[5], **Erika C. Kuchler**[6], **Alicia Day**[7], **Patricia N. Tannure**[8], **Luise Gomes da Motta**[9], **Katiucia Batista Paiva**[10], **Jose M. Granjeiro**[6], and **Alexandre R. Vieira**[1,2,3,4,*]

[1]Department of Oral Biology, School of Dental Medicine, University of Pittsburgh

[2]Department of Pediatric Dentistry, School of Dental Medicine, University of Pittsburgh

[3]Center for Craniofacial and Dental Genetics, School of Dental Medicine, University of Pittsburgh

[4]Department of Human Genetics, Graduate School of Public Health, University of Pittsburgh

[5]Department of Biological Sciences, Carnegie Mellon University

[6]Department of Cell and Molecular Biology, Fluminense Federal University, Rio de Janeiro, Brazil

[7]Honors Biology Program, University of Michigan-Flint

[8]Department of Pediatric Dentistry and Orthodontics, Federal University of Rio de Janeiro, Brazil

[9]Department of Restorative Dentistry and Dental Materials, Fluminense Federal University, Rio de Janeiro, Brazil

[10]Laboratory of Molecular Pathology, Department of Oral Pathology, Dental School, University of São Paulo, Sao Paulo, SP, Brazil

Abstract

Background—Clefts of the lip and/or palate (cleft lip/palate) are notable for their complex etiology. The WNT pathway regulates multiple developmental processes including craniofacial development and may play a role in cleft lip/palate and other defects of craniofacial development such as tooth agenesis. Variations in WNT genes have been recently associated with cleft lip/palate in humans. In addition, two WNT genes, *Wnt3* and *Wnt9B*, are located in the *clf1* cleft locus in mice.

Methods—We investigated 13 SNPs located in *WNT3A*, *WNT5A*, *WNT8A*, *WNT11*, *WNT3* and *WNT9B* genes, for association with cleft lip/palate subphenotypes in 500 cleft cases and 500 unrelated controls. Genotyping of selected polymorphisms was carried out using Taqman assays. PLINK 1.06 software was used to test for differences in allele frequencies of each polymorphism between affected and unaffected individuals. Haplotype analysis was also performed.

Results—Individuals carrying variant alleles in *WNT3* presented an increased risk for cleft lip/palate (P=0.0003; OR=1.61 95% C.I: 1.29 -2.02) in the population studied.

Conclusion—Our results continue to support a role for WNT genes in the pathogenesis of cleft lip/palate. Although much remains to be learned about the function of individual WNT genes during craniofacial development, additional studies should focus in the identification of potentially functional variants in these genes as contributors to human clefting.

[*]Corresponding author: Alexandre R. Vieira, DDS, MS, PhD, Assistant Professor, 614 Salk Hall, Department of Oral Biology, Center for Craniofacial and Dental Genetics, School of Dental Medicine, University of Pittsburgh, 3501 Terrace Street, Pittsburgh, PA 15261, Phone (412) 383-8972, FAX (412) 624-3080, arv11@pitt.edu.
[#]These authors contributed equally to this work.

Keywords

WNT pathway; polymorphisms; cleft lip/palate; tooth agenesis; subphenotype

INTRODUCTION

Nonsyndromic oral facial clefts are notable for their complex etiology with interaction of genetic and environmental components (Murray, 2002). Genes involved in craniofacial development are plausible candidates for oral clefts. Wnt signaling is critical for proper development of the head and face in the mouse embryo, playing important roles in various aspects of craniofacial development ranging from axis formation to survival of cranial neural crest cells to patterning of the brain (Mani et al., 2009).

Several studies support a role for the Wnt gene family in the etiology of cleft lip/palate. *Wnt3* and *Wnt9b* genes are expressed in the developing facial ectoderm in mice (Lan et al., 2006). A *Wnt9b*$^{-/-}$ mutant mouse was described presenting a phenotype that included incomplete penetrance of cleft lip/palate (Carroll et al., 2005). Both these genes are located within the *clf1* cleft locus in chromosome 11 of A/WySn cleft susceptible mouse strains (Juriloff et al., 2005; 2006). This region is syntenic to human chromosome 17q21, a region that has been associated with nonsyndromic oral facial clefts in humans (Carinci et al., 2007). A nonsense mutation (Q83X) in the *WNT3* gene has also been described in a case of tetra-amelia and cleft lip/palate in a large consanguineous family (Niemann et al., 2004). Recently, variations in WNT genes have been recently associated with human nonsyndromic oral clefts. In this particular family-based study (Chiquet et al., 2008), a variety of WNT genes (*WNT3A*, *WNT5A*, *WNT8A*, *WNT11*) showed association with cleft lip/palate in either European American or Hispanic families. Gene-gene interaction between *WNT3A* and both *WNT3* and *WNT5A* was also suggested.

To investigate a role for WNT genes in nonsyndromic oral facial clefts, we interrogated thirteen SNPs in six WNT genes that had been previously associated with cleft/lip palate in humans and in animal models for association with cleft subphenotypes.

MATERIAL AND METHODS

Subjects

The subjects of this study have been described in part in previous studies (Letra et al., 2007; 2009). A total of 766 individuals of Caucasian ancestry, 463 cases with nonsyndromic clefts and 303 unrelated control individuals without clefts or family history of clefting, were ascertained at the Dental Clinics of the Hospital of Rehabilitation and Craniofacial Anomalies and Bauru Dental School, both of the University of São Paulo, Bauru, SP, Brazil and at the Center for Treatment of Craniofacial Anomalies (CTAC), Rio de Janeiro, Brazil. Individuals were considered as Caucasians when there was no history of African, Native Amerindians or Japanese descent. Subjects with clefts were examined clinically and through their medical records to determine their individual cleft status. Cleft status was based on cleft completeness (comprised of primary and secondary palates entirely) or incompleteness, and on laterality (left, right, bilateral). An “unknown” cleft status indicated that either cleft type or side could not be determined, even after medical records were reviewed. The presence of tooth agenesis was assessed clinically and through radiographs by dental professionals.

The study was conducted with the consent of the participants and approved by the Research and Ethics Committee of the aforementioned institutions. In the case of children under 15

years of age, authorization was also requested from their parents or from the individual legally in charge of the child.

Clefts subphenotypes

Individuals with oral clefts were divided by subphenotype as: **All Clefts** (Cleft lip + Cleft Lip and Palate + Cleft Palate), **Cleft lip with or without cleft palate** (CLP), and **Cleft Palate only** (CP). The CLP group was further divided into Bilateral CLP, Unilateral CLP, Right Unilateral CLP, and Left Unilateral CLP subgroups.

We also considered here a new dental subphenotype (unsuccessful bilateral). This trait was suggested for individuals presenting unilateral clefting and agenesis of the lateral incisor on the noncleft side (Letra et al., 2007). The absence of the incisor would represent a microform of the cleft (Figure 1). Notwithstanding, instances of tooth agenesis adjacent to the cleft area (affecting maxillary central incisors, lateral incisors, or canines) were not considered, because the absence of such teeth is likely the consequence of developmental anomalies at the cleft area.

Single Nucleotide Polymorphism (SNP) selection and genotyping

We selected thirteen SNPs spanning six WNT genes that had been previously associated with cleft/lip palate in humans (Chiquet et al., 2008) or in animal models (Juriloff et al., 2005; Juriloff et al., 2006; Lan et al., 2006) to test for association with CL/P subphenotypes in our population (Table 1).

Genomic DNA was obtained from saliva samples as previously described (Menezes et al., 2008). Genotyping of selected polymorphisms was carried out using Taqman assays and reagents in an ABI 7900 automatic instrument (Applied Biosystems, Foster City, CA). We used PLINK 1.06 software (Purcell et al., 2007) to test for differences in allele frequencies of each polymorphism between affected and unaffected individuals. Haplotype analysis using “all clefts” phenotype was also performed using PLINK. We considered the P-values adjusted with Bonferroni correction as implemented in PLINK.

We used the online software program FASTSNP (Function Analysis and Selection Tool for Single Nucleotide Polymorphisms) to predict the functionality of the SNPs. FASTSNP is a web server that allows users to efficiently identify the SNPs most likely to have functional effects. It prioritizes SNPs according to putative functional effects, such as changes to the transcriptional level, pre-mRNA splicing, protein structure, etc. the prediction of functional effects is based on information extracted from external web servers. We analyzed the investigated SNPs and found that although some are located in putative regulatory regions, no information with regards to functionality is known so far.

RESULTS

A summary of the most significant results are presented in Tables 2 and 3. Genotype and allele distributions were within Hardy-Weinberg equilibrium (data not shown).

We found association between the *WNT3* gene and the phenotypes “All Clefts” (CLP + CP) and “Cleft lip with or without cleft palate” in the studied population. SNP rs142167, located in the 5′ UTR of *WNT3*, showed the most significant association with the phenotypes “All clefts” [(P=0.0003; OR=1.61 (1.29-2.02)], “CLP” [P=0.001; OR=1.6 (1.26-2.02)], and “unilateral CLP” [P=0.002; OR=1.65 (1.27-2.13)]. Under a nominal value of 0.05, SNP rs9890413 in *WNT3* also showed an association with the phenotypes “All clefts” [*P*=0.03; OR=1.4 (1.12 -1.74)] and with “CLP” and “unilateral CLP” [P=0.02; OR=1.46 (1.16-1.84) and P=0.04; OR=1.46 (1.13-1.89), respectively] (Table 2).

When analyzing the "unsuccessful bilateral" cleft subphenotype, we also observed an association for *WNT3* SNP rs142167 [P=0.03; OR= 1.57 (95% CI: 1.17-2.11] (Table 2).

The results of the haplotype analysis further support the associations found for the individual SNPs (Table 3). We observed overtransmission of *WNT3* haplotypes rs142167-rs199498 (P=0.008), rs111769-rs9890413 (P=4.407e-005), rs142167-rs199498-rs111769 (P=0.001), rs199498-rs111769-rs9890413 (P=0.001), and also *WNT3*-*WNT9B* haplotypes rs9890413-rs2165846 (P=0.009) and rs111769-rs9890413-rs2165846 (P=0.001) (Table 3).

DISCUSSION

The Wnt genes are involved in regulating midface development and upper lip fusion and are therefore candidates for an etiological role in nonsyndromic cleft lip with or without cleft palate. Evidences supporting Wnt genes as possible clefting loci come from the inbred A/WySn mouse strain (Juriloff et al., 2004). *Wnt3* and *Wnt9B* are located in the *clf1* region and could contribute to the clefting phenotype (Juriloff et al., 2005). In humans, variations in WNT genes have been described in cases with syndromic (Niemann et al., 2004) and nonsyndromic oral clefts (Chiquet et al., 2008).

In this study, we investigated SNPs *in WNT3*, *WNT3A*, *WNT5A*, *WNT8A*, *WNT9B*, and *WNT11*, for association with cleft lip/palate in a case-control population. Studies with cases and controls in populations characterized by significant immigration such as Brazilians deserve special caution in the interpretation of results because of possible effects of admixture. The option of using only Caucasian individuals in the genetic analyses should overcome any major stratification effects since these individuals have been shown to have major contribution of European ancestry (Lins et al., 2009). In contrast to Chiquet et al. (2008), where the authors report the association of *WNT3*, *WNT3A*, *WNT7A*, *WNT8A*, *WNT9B* and *WNT11* with cleft lip/palate in their European American sample, we only found association with two genes. SNPs in *WNT3* and *WNT5A* had nominal P-values of 0.05 or less, although only one SNP in *WNT3* (rs142167) remained significant after Bonferroni correction. Our results corroborate with the results of Chiquet et al. (2008) for European-American families and further support the suggestion that *WNT3* may be a cleft susceptibility gene. Although the SNP showing the strongest association in our study (rs142167) was not associated in the study of Chiquet et al. (2008), the fact that both studies reveal association of *WNT3* with clef lip/palate warrants further investigations and reinforces a role for *WNT3* in the etiology of nonsyndromic clefts. *WNT3* showed association with all clefts and all cleft subphenotypes except for cleft palate alone, although our sample size with cleft palate only may be too small to draw definite conclusions. Haplotypes in *WNT3* and *WNT9B* genes were also associated with the cleft phenotype.

The association with *WNT5A* was found in cases with unilateral left CL/P. We subdivided unilateral CLP into right or left because the authors believe that stratifying cleft cases into specific cleft subsets may increase homogeneity. Moreover, there may be some genetic differences in the etiology of left and right clefts. The acknowledged larger prevalence of unilateral left clefts (one third of all cleft cases) suggests that some genes may be preferentially involved in cleft side. This gene was also associated with nonsyndromic clefts in the Hispanic population described in Chiquet et al. (2008). During craniofacial development, *Wnt5A* signals were detected in the mesenchymal cells and around Meckel's cartilage and in only in the mesenchyme of the elevating palatal shelves (Paiva et al., 2009).

In a similar context, we considered that individuals with unilateral clefts who also present tooth agenesis of the lateral incisor on the noncleft side may in fact have a bilateral cleft, and the absence of the lateral incisor represents a microform of the cleft, namely an unsuccessful

bilateral cleft (Letra et al., 2007). Comparison of this cleft subphenotype with controls also revealed association with *WNT3*. WNT intercellular signaling molecules have been implicated in the regulation of murine tooth development, where *Wnt3* shows specific expression in the enamel knot at the cap stage. The importance of *Wnt3* during odontogenesis is further highlighted by the progressive loss of ameloblasts from postnatal incisor teeth in transgenic mice (Millar et al., 2003).

One may argue that most investigated SNPs are in intronic or intergenic regions and do not seem to alter transcription factor binding sites or have any other potentially damaging effect. Overall, *in silico* analysis predicted that none of the SNPs tested would abolish the protein domain, although some SNP alleles could arguably have a deleterious effect. For example, *WNT5A* SNP rs566926, associated with unilateral left CLP, is located at a transcription factor binding site with intronic enhancer function for which the A allele creates two additional binding sites for *SOX5*. Both these transcription factors are involved in the regulation of embryonic development and in the determination of the cell fate. The encoded protein may also play a role in chondrogenesis (Woods et al., 2007), which is a critical step in palatogenesis. We should also consider that these SNPs, although putatively neutral, may also be in linkage disequilibrium with an etiologic variant which could explain the results observed here.

In summary, our results continue to support the involvement of WNT genes in human clefting. Although much remains to be learned about the function of individual WNT genes during craniofacial development, additional studies should focus in the identification of potentially functional variants in these genes as contributors to human clefting.

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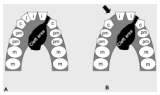


Figure 1.
Schematic representation of unilateral cleft (A) and unilateral cleft with agenesis of the lateral incisor (arrow) on the opposite side of the cleft (B), also referred to as “unsuccessful or occult cleft” according to Letra et al. (2007).
(i=Central Incisor; l= Lateral Incisor; c= Canine; pm= Pre-molar; m= Molar)

Table 1

Summary of candidate genes and SNPs studied

SNP	Chr.	Gene	Base pair position	SNP function	Base change [§]	MAF
rs708111	1	WNT3A	226,257,988	Promoter/regulatory	**C**/T	0.47 (C)
rs3094912	1	WNT3A	226,276,438	Intronic	T/**A**	0.47 (A)
rs752107	1	WNT3A	226,313,974	3′ UTR	C/T[*]	0.32 (T)
rs1745420	1	WNT3A	226,318,355	3′ UTR	C/**G**	0.13 (G)
rs566926	3	WNT5A	55,495,818	Intronic	A/**C**	0.21 (A)
rs2040862	5	WNT8A	137,447,888	Intronic	**C**/T	0.16 (T)
rs1533767	11	WNT11	75,583,448	Silent Mutation	A/**G**	0.39 (A)
rs142167	17	WNT3	42,150,418	Dowstream	A/**G**	0.19(G)
rs199498	17	WNT3	42,220,763	Intronic	**C**/T	0.23 (C)
rs111769	17	WNT3	42,227,151	Intronic	**C**/T	0.35 (T)
rs9890413	17	WNT3	42,256,448	Upstream	**A**/G	0.32 (G)
rs2165846	17	WNT9B	42,296,365	Intron	**A**/G	0.44 (G)
rs197915	17	WNT9B	42,345,521	Upstream	**A**/G	0.33 (G)

MAF, minor allele frequency.

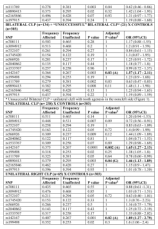
[§] Ancestral allele listed in bold.

[*] not available.

Table 2

Results of association tests with WNT genes in cleft lip/palate and control individuals, according to cleft subphenotype

ALL CLEFTS* (n=463) X CONTROLS (n=303)					
SNP	Frequency Affected	Frequency Unaffected	P value	Adjusted P value §	OR (95%CI)
rs708111	0.497	0.465	0.21	1	1.14 (0.93 - 1.4)
rs3094912	0.476	0.531	0.03	0.47	0.8 (0.65 - 0.99)
rs752107	0.273	0.294	0.36	1	0.9 (0.72 - 1.13)
rs1745420	0.149	0.122	0.14	1	1.25 (0.92 - 1.7)
rs566926	0.288	0.2374	0.03	0.4	1.3 (1.02 - 1.65)
rs2040862	0.119	0.1174	0.9	1	1.02 (0.74 - 1.4)
rs1533767	0.283	0.2583	0.27	1	1.14 (0.9 - 1.44)
rs142167	0.371	0.2673	2.80E-05	**0.0003 (A)**	**1.61 (1.29 - 2.02)**
rs199498	0.311	0.2533	0.01	0.18	1.34 (1.06 - 1.68)
rs111769	0.309	0.3812	0.003	0.05	0.73 (0.59 - 0.90)
rs9890413	0.369	0.2953	0.002	**0.03 (G)**	**1.4 (1.12 - 1.74)**
rs2165846	0.486	0.4281	0.02	0.37	1.26 (1.02 - 1.56)
rs197915	0.406	0.3943	0.63	1	1.05 (0.85 - 1.3)
* All cleft cases including unknown type clefts (n=22).					
CLP* (n=372) X CONTROLS (n=303)					
SNP	Frequency Affected	Frequency Unaffected	P value	Adjusted P value§	OR (95%CI)
rs708111	0.5101	0.465	0.1	1	1.2 (0.96 - 1.49)
rs3094912	0.4596	0.5319	0.009	0.11	0.75 (0.60 - 0.93)
rs752107	0.2617	0.2947	0.18	1	0.85 (0.67 - 1.07)
rs1745420	0.1634	0.1225	0.03	0.47	1.4 (1.02 - 1.92)
rs566926	0.2994	0.2374	0.01	0.16	1.37 (1.07 - 1.76)
rs2040862	0.1202	0.1174	0.88	1	1.03 (0.73 - 1.44)
rs1533767	0.2853	0.2583	0.28	1	1.15 (0.9 - 1.47)
rs142167	0.3678	0.2673	8.91E-05	**0.001 (A)**	**1.6 (1.26 - 2.02)**
rs199498	0.3076	0.2533	0.03	0.38	1.31 (1.03 - 1.67)
rs111769	0.3062	0.3812	0.004	0.05	0.72 (0.57 - 0.9)
rs9890413	0.3795	0.2953	0.001	**0.02 (G)**	**1.46 (1.16 - 1.84)**
rs2165846	0.4697	0.4281	0.14	1	1.18 (0.95 - 1.48)
rs197915	0.4125	0.3943	0.51	1	1.08 (0.86 - 1.35)
* CLP= cleft lip with or without cleft palate					
BILATERAL CLP (n=134) X CONTROLS (n=303)					
SNP	Frequency Affected	Frequency Unaffected	P value	Adjusted P value§	OR (95%CI)
rs708111	0.508	0.465	0.25	1	1.19 (0.88 -1.6)
rs3094912	0.519	0.468	0.17	1	1.23 (0.92 - 1.65)
rs752107	0.263	0.294	0.35	1	0.85 (0.62 - 1.18)
rs1745420	0.161	0.122	0.13	1	1.38 (0.91 - 2.09)
rs566926	0.295	0.237	0.08	1	1.34 (0.96 - 1.88)
rs2040862	0.119	0.117	0.92	1	1.02 (0.64 - 1.62)
rs1533767	0.242	0.258	0.62	1	0.92 (0.65 - 1.29)
rs142167	0.363	0.267	0.004	**0.05 (A)**	**1.57 (1.15 - 2.13)**
rs199498	0.299	0.253	0.16	1	1.26 (0.91 - 1.74)



rs111769	0.338	0.381	0.36	1	0.83 (0.56 - 1.24)
rs9890413	0.385	0.285	0.04	0.6	1.49 (1.01 - 2.21)
rs2165846	0.405	0.428	0.63	1	0.91 (0.61 - 1.34)
rs197915	0.439	0.394	0.38	1	1.2 (0.8 - 1.8)
UNILATERAL LEFT CLP (n=173) X CONTROLS (n=303)					
SNP	**Frequency Affected**	**Frequency Unaffected**	**P value**	**Adjusted P value[§]**	**OR (95%CI)**
rs708111	0.539	0.465	0.03	0.38	1.35 (1.03 -1.77)
rs3094912	0.421	0.531	0.001	0.01	0.64 (0.49 - 0.83)
rs752107	0.276	0.295	0.55	1	0.91 (0.68 - 1.23)
rs1745420	0.167	0.122	0.06	0.74	1.44 (0.99 - 2.11)
rs566926	0.326	0.237	0.003	**0.04**	**1.56 (1.15 - 2.09)**
rs2040862	0.114	0.117	0.87	1	0.97 (0.63 - 1.48)
rs1533767	0.306	0.258	0.12	1	1.27 (0.94 - 1.71)
rs142167	0.363	0.267	0.002	**0.02 (A)**	**1.56 (1.18 - 2.08)**
rs199498	0.305	0.253	0.09	1	1.3 (0.96 - 1.74)
rs111769	0.312	0.381	0.05	0.65	0.76 (0.57 - 1.01)
rs9890413	0.378	0.295	0.009	0.12	1.45 (1.09 - 1.92)
rs2165846	0.485	0.428	0.1	1	1.26 (0.96 - 1.65)
rs197915	0.382	0.394	0.71	1	0.95 (0.72 - 1.25)
CLEFT PALATE ONLY (n=69) X CONTROLS (n=303)					
SNP	**Frequency Affected**	**Frequency Unaffected**	**P value**	**Adjusted P value**	**OR (95%CI)**
rs708111	0.431	0.465	0.49	1	0.87 (0.59 -1.28)
rs3094912	0.441	0.468	0.57	1	0.89 (0.62 - 1.3)
rs752107	0.294	0.294	0.99	1	0.99 (0.66 - 1.5)
rs1745420	0.092	0.122	0.33	1	0.73 (0.38 - 1.38)
rs566926	0.246	0.2374	0.83	1	1.05 (0.68 - 1.62)
rs2040862	0.128	0.117	0.72	1	1.11 (0.63 - 1.96)
rs1533767	0.313	0.258	0.19	1	1.31 (0.87 - 1.97)
rs142167	0.345	0.267	0.07	0.87	1.45 (0.97 - 2.15)
rs199498	0.289	0.253	0.38	1	1.20 (0.80 - 1.81)
rs111769	0.347	0.381	0.46	1	0.86 (0.59 - 1.27)
rs9890413	0.351	0.295	0.21	1	1.29 (0.87 - 1.92)
rs2165846	0.543	0.428	0.01	0.18	1.59 (1.09 - 2.31)
rs197915	0.369	0.394	0.59	1	0.90 (0.61 - 1.33)

[§]Letter in parentheses indicates associated allele.

Table 3

Most significant sliding window haplotype association results observed for markers in WNT3 and WNT9B genes located in chromosome 17 and cleft lip/palate in a Brazilian population

WNT3				WNT9B	
rs142167	**rs199498**	**rs111769**	**rs9890413**	**rs2165846**	**rs197915**
0.008 (AT)					
	0.07 (CT)				
		4.407e-005 (TG)			
			0.009 (GG)		
				0.09 (GG)	
0.001 (ATT)					
	0.001 (TTG)				
		0.001 (TGG)			
			0.03 (GGA)		
0.05 (ATTGGA)					