RESEARCH REPORTS
Clinical

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Association of *AXIN2* with Non-syndromic Oral Clefts in Multiple Populations

ABSTRACT

We have previously shown the association of *AXIN2* with oral clefts in a US population. Here, we expanded our study to explore the association of 11 *AXIN2* markers in 682 cleft families from multiple populations. Alleles for each *AXIN2* marker were tested for transmission distortion with clefts by means of the Family-based Association Test. We observed an association with SNP rs7224837 and all clefts in the combined populations ($p = 0.001$), and with SNP rs3923086 and cleft lip and palate in Asian populations ($p = 0.004$). We confirmed our association findings in an additional 528 cleft families from the United States ($p < 0.009$). We tested for gene-gene interaction between *AXIN2* and additional cleft susceptibility loci. We assessed and detected *Axin2* mRNA and protein expression during murine palatogenesis. In addition, we also observed co-localization of Axin2 with Irf6 proteins, particularly in the epithelium. Our results continue to support a role for *AXIN2* in the etiology of human clefting. Additional studies should be performed to improve our understanding of the biological mechanisms linking *AXIN2* to oral clefts.

KEY WORDS: cleft lip and palate, genetics, gene expression, gene-gene interactions, wnt pathway, craniofacial anomalies.

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INTRODUCTION

Oral-facial clefts are common birth defects of complex etiology involving the interplay of genetic predisposition and environmental exposures. Most oral-facial clefts are non-syndromic, isolated defects, which can be separated into two different phenotypes: cleft lip with or without cleft palate (CL/P) and cleft palate only (CPO). Genetics and embryology suggest that CL/P and CPO are different entities (Murray, 2002). CL/P is more common, with a prevalence of 1 to 2/1000 births, with Asians and Native Americans having the highest rate and Africans the lowest, whereas CPO affects approximately 1/1500 to 2000 births and does not vary in different racial backgrounds (Mossey and Little, 2002). Numerous lines of evidence now suggest that the phenotypic spectrum of non-syndromic CL/P is more complex than previously realized and should include a variety of subclinical phenotypic features observed in either an individual with a cleft and/or his/her 'unaffected' relatives (Weinberg *et al*., 2006; Letra *et al.,* 2007).

Several genes—including, but not limited to, *MSX1*, *IRF6, CRISPLD2, FOXE1,* and members of the WNT and FGF gene families—have been associated with oral clefts (Jezewski *et al*., 2003; Zucchero *et al*., 2004; Chiquet *et al*., 2007; Riley *et al*., 2007; Menezes *et al*., 2009, 2010; Moreno *et al.,* 2009). Recently, genome-wide association studies have implicated additional loci on 1p22, 8q24, 10q25.3, 17q22, and 20q12 in the etiology of oral clefts (Birnbaum *et al*., 2009; Beaty *et al.,* 2010; Mangold *et al*., 2010). Current efforts now include unveiling the etiologic variants and their biological functions that may contribute to the cleft phenotype (Dixon *et al*., 2011).

Members of the WNT gene family have been associated with clefts in humans and mice (Lan *et al*., 2006; Chiquet *et al*., 2008; Menezes *et al*., 2009). The *AXIN2* (axis inhibition protein 2) gene is a negative regulator of the WNT pathway due to its role in the degradation of β-catenin. Mutations in *AXIN2* have been associated with increased susceptibility to cancer (Lammi *et al*., 2004; Kanzaki *et al*., 2006; Yook *et al*., 2006), and in some cases have been detected segregating together with familial tooth agenesis (Lammi *et al*., 2004). We have previously reported the association of a missense mutation in *AXIN2* (rs2240308, P50S) with CL/P in US cleft families (Menezes *et al*., 2009).

Table. Details of the Studied Populations and Number of Pedigrees in Each Cleft Subgroup

						Lip Involvement			No Lip Involvement	
Group	Population	Mean Pedigree Size	Min-Max	Unaffected Types	Affected Types	CLO	CLP	CL/P	CPO	All
Latin America	Argentina	5.24 ± 2.9	1-16	192	81	11	60	71	7	78
	Guatemala	5.82 ± 3.6	1-20	377	115	21	99	120	3	123
Europe	USA	5.07 ± 2.6	1-14	303	141	14	89	103	14	117
	Madrid	3.82 ± 0.9	3-7	88	45	5	28	33	3	36
	Hungary	4.96 ± 1.1	3-8	154	79	20	53	73	16	89
	Turkey	9.68 ± 2.7	3-17	72	44	7	20	27	11	38
Asian	Shanghai	10.61 ± 4.7	3-28	480	171	12	54	66	0	66
	Beijing	3	3-3	168	84	14	46	60	24	84
India	India	14.00 ± 6.7	3-38	222	100	22	29	51	0	51
All populations		6.51 ± 4.5	1-38	2,056	860	126	478	604	78	682

We formed cleft groups by looking at the type(s) of cleft(s) present in each pedigree.
Cleft subgroups include:
CLO (pedigrees with Cleft Lip only)
CLP (pedigrees with Cleft Lip with Cleft Palate)
CL/P (pedigrees with Cleft Lip with or without Cleft Palate)
CPO (pedigrees with Cleft Palate only)
All (combination of CL/P and CPO groups)

Here, we performed a family-based association study of *AXIN2* polymorphisms and oral clefts in multiple populations. Additionally, an independent family-based dataset was used for confirmation of the association findings. We investigated *Axin2* expression at critical periods during mouse palatogenesis and looked for statistical evidence of gene-gene interaction with additional cleft susceptibility genes.

MATERIALS & METHODS

Sample Population

Data and samples from 682 non-syndromic cleft pedigrees (2,916 individuals) were ascertained from study sites in the United States, Argentina, Guatemala, Spain, Hungary, Turkey, Beijing, Shanghai, and India. This study was approved by the institutional review boards at each site and by the University of Pittsburgh. Pedigrees were ascertained through probands presenting overt clefts. Individuals provided written informed consent and saliva samples as source of genomic DNA. Details of the study families are summarized in the Table.

Association Analysis

Details of the investigated genes and polymorphisms are provided in the Appendix. Alleles at each *AXIN2* SNP were tested for association with all clefts, cleft lip and palate (CLP), and cleft lip with or without cleft palate (CL/P) phenotypes according to the Family-based Association Test (FBAT) (Horvath *et al*., 2001). We performed analyses for each population individually, combined, and in the following population groups: Asians (Beijing and Shanghai), Europeans (US, Spain, Hungary and Turkey), Latin Americans (Argentina and Guatemala), and non-Asians (*i.e.,* European plus Latin American). India was included only in the combined population. We performed haplotype analysis to test for linkage disequilibrium between haplotypes in each cleft group. We used Bonferroni correction considering the number of variables and tests performed (19), and a $p \leq 0.003$ was considered statistically significant.

Confirmation Dataset

We tested 6 SNPs of interest in *AXIN2* for confirmation of association findings in an independent dataset of 528 CL/P nuclear families ascertained in Texas (344 non-Hispanic white and 184 Hispanic). These included SNPs rs2240308 and rs7224837 associated in the original dataset and other suggestive SNPs. The Pedigree Disequilibrium test (PDT), Geno-PDT, and Association in the Presence of Linkage (APL) were used to test for association as previously described (Chiquet *et al*., 2007). APL was used to test for overtransmission of pairwise haplotypes. A $p \leq 0.006$ was considered statistically significant under Bonferroni correction.

Statistical Analysis of Gene-Gene Interactions

We tested for *AXIN2* SNP (rs3923086 and rs7224837) interactions with loci previously associated with oral clefts: ***IRF6*** (rs2235371 and rs642961), ***ABCA4*** (rs560426) and ***MAFB*** (rs13041247), ***FOXE1*** (rs3758249 and rs4460498), and chromosome **8q24** (rs987525 and rs16903544). Allele and haplotype Transmission Disequilibrium Tests (TDT) were used to detect Mendelian transmission of alleles from heterozygous parents to affected offspring. Analyses were performed in FBAT, utilizing the extended family option (*i.e.*: -e) to adjust for multiple affected individuals in a pedigree. Interaction calculations were performed with the *per*-pedigree TDT Z-score for each SNP, and the correlation between pairs of individual SNPs in specific genes was assessed.

Expression Analysis

Details regarding sample collection, experimental procedures, and analysis are available in the Appendix. In brief, we analyzed *Axin2* mRNA and protein expression during mouse palatogenesis using *in situ* hybridization, real-time PCR, and immunohistochemistry. We also analyzed mRNA and protein expression of *Irf6,* an acknowledged cleft susceptibility gene, to see if it correlated with *Axin2* expression pattern.

RESULTS

Association Analysis

Association results for the original dataset are presented in Fig. 1. The most significant P value and the greatest degree of over-transmission were for a variant located in intron 10 [SNP rs7224837] and all clefts in non-Asian populations (p = 0.0009) and in the combined populations (p = 0.002). Although not formally significant, a suggestive association with this SNP and all clefts was also observed in Europeans (p = 0.005) (Fig. 1). This same SNP was associated with CL/P (p = 0.001) in the combined populations and in non-Asians (p = 0.004) (Fig. 1), and with CLP in the combined populations (p = 0.006) (Fig. 1). In Asians, the best association results were observed for SNP rs3923086 and CLP (p = 0.004) (Fig. 1).

When the individual populations were considered, SNP rs2240308 was associated with CL/P in the US (p = 0.003); meanwhile, additional SNPs showed marginal association values ($0.01 \leq p \leq 0.05$) with cleft phenotypes (Appendix Table 2).

Haplotype analysis showed that the allelic combination of SNPs was significant only in those groups where the individual association with SNP rs7224837 was significant, namely, for SNP alleles rs7224837 (A) - rs4128941 (G) with all clefts (p = 0.002), and with CL/P (p = 0.006) in non-Asians. Haplotypes including our previously associated variant, SNP rs2240308, showed nominal association only in Asian families with CLP ($p \leq 0.05$) (Appendix Tables 3-5).

Confirmation of Association

Individual SNP analysis showed association of SNP rs11655966 (located at ~1.5Kb from rs7224837) in both Caucasian (p = 0.008) and Hispanic samples (p = 0.009), whereas rs4791171 was also associated in Caucasians (p = 0.008), although these p-values do not meet conservative criteria for multiple testing. In addition, analysis of two-window haplotypes revealed the significant association of SNPs rs7224837 (A) and rs11655966 (T) in the Caucasian group (p = 0.006). Additional haplotypes in both non-Hispanic white and Hispanic populations showed nominal significance values ($0.02 \leq p \leq 0.05$) (Appendix Tables 6 and 7).

Interaction Analysis

We found evidence of statistical interaction between *AXIN2* rs3923086 (T) and *IRF6* rs2235371 (C) alleles with CLP (p = 0.007) in Asian families, whereas results in non-Asians suggest interaction of *AXIN2* rs7224837 (A) and *IRF6* rs2235371 (C) alleles with CL/P (p = 0.02). A trend was also observed for *AXIN2* rs7224837 (A) and *IRF6* rs642961 (A) (p = 0.06) and with *IRF6* rs2235371 (C) (p = 0.07) with CLP in non-Asians.

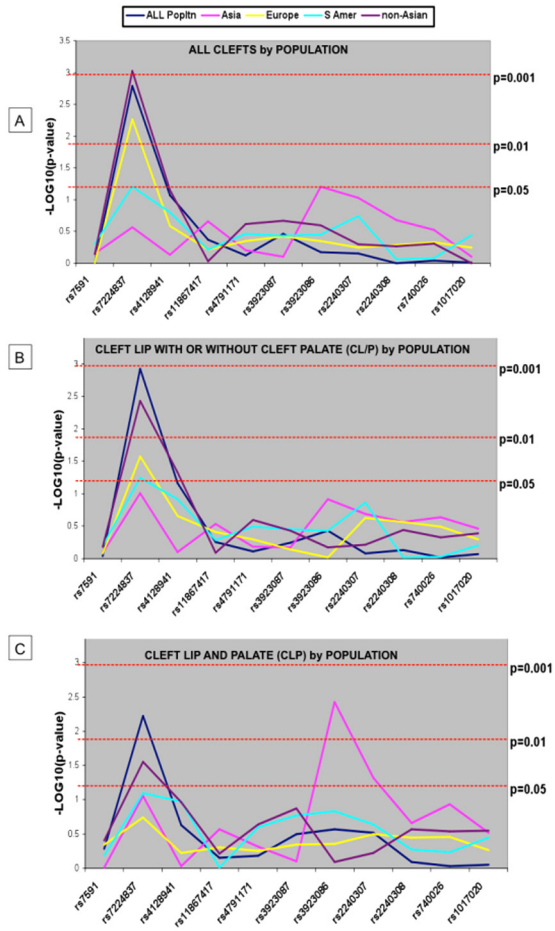

Figure 1. Results of TDT analyses of *AXIN2* SNPs and oral clefts by population groups. **(A)** For all clefts. [Note strongest association for SNP rs7224837 in non-Asians (p = 0.0009) and Europeans (p = 0.005) and in the combined populations (p = 0.002). The lack of association in the other population groups indicates that the association in the combined population is primarily due to contributions from non-Asians.] **(B)** For CL/P (cleft lip with or without cleft palate). [Note strongest association with rs7224837 in non-Asians (p = 0.004) and in the combined populations (p = 0.001).] **(C)** For CLP (cleft lip with cleft palate). [Note strongest association for SNP rs3923086 in Asians and rs7224837 in non-Asians (p = 0.004) and in the combined populations (p = 0.006).]

Expression Results

Analysis of *Axin2* mRNA expression by *in situ* hybridization in E12.5 mice showed expression in the mesenchyme of the palatal shelves, tongue, in neural crest cells that surround the myogenic core, as well as the optic nerve, and telencephalon. Near the end of palatogenesis, at E15.5, *Axin2* mRNA was markedly expressed in the palatal mesenchyme, oral epithelia, and in the molar tooth buds (Fig. 2). Similarly, real-time PCR showed *Axin2* mRNA

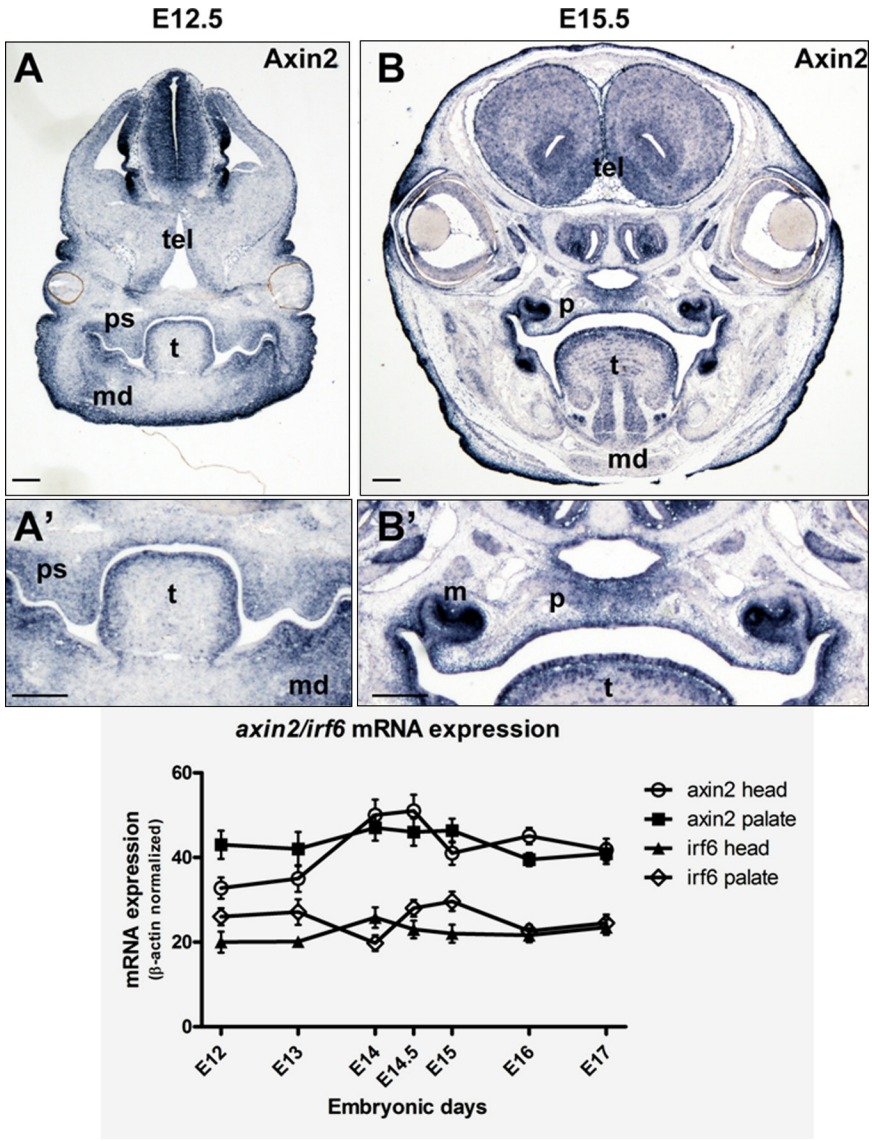

Figure 2. Axin2 is expressed in the mouse palate. Top, mouse embryos were sectioned coronally. (**A**) At E12.5, Axin2 expression (blue) is found in the palatal shelf (ps) and tissues of the developing head, including the mandible (md), tongue (t), and telencephalon (tel). (**A'**) Magnified view of the palatal shelves. (**B**) After palatal fusion at E15.5, Axin2 expression is maintained in the palate (p). (**B'**) Magnified view of the palate. Axin2 expression is also found in the molar tooth buds (m). Scale bars = 125 microns (bottom). Graphical representation of Axin2 and Irf6 mRNA expression levels by real-time PCR. Both gene transcripts are expressed in the murine head and palate at embryonic days (E) 12-17.

expression in the mouse head and palate throughout the experimental periods, although inversely correlated. At the initial stages, while *Axin2* expression increased from E12 to E13 in the mouse head, it decreased in the palate. At the later stages, *Axin2* expression in both the head and palate appeared to reach similar levels gradually. This was also observed for *Irf6*. Although not significant, an increase in *Axin2* mRNA expression was observed at later stages of palatogenesis (E14-15), decreasing thereafter. *Axin2* mRNA expression was significantly higher when compared with that of *Irf6* ($p = 0.0001$) (Fig. 2).

Analysis of Axin2 protein expression showed a pattern consistent with its levels of mRNA expression, including epithelial expression in the palate shelves, teeth, tongue, and also palate and facial mesenchyme, and tongue musculature (Fig. 3). Expression in the brain, eye, olfactory epithelium, nasal cartilage, ocular muscles, and optic nerve was also evident (Appendix Fig. 2). Irf6 protein expression was restricted to epithelial cell layers of the oral cavity, teeth, and palate shelves. Although Axin2 appears to be expressed in a more dispersed pattern than Irf6, both proteins appear to co-localize in relevant orofacial epithelial cell layers critical to normal palate development (Fig. 3).

DISCUSSION

In this study, we confirmed our previously reported association of *AXIN2* with oral clefts (Menezes *et al.,* 2009) with additional variants in multiple populations. While the associated variant in our previous study in a US population was comprised of a missense mutation (rs2240308) in exon 2 of the *AXIN2* gene, in the present study, a variant located in intron 10 (SNP rs7224837) yielded the most significant association values in families presenting CL/P in non-Asian and European populations, and in the overall combined populations as well. Meanwhile, SNP rs3923086, located at the 3′ UTR, was most significant in Asian families with CLP. When compared with the individual populations, our previously associated SNP (rs2240308) continued to show association with CL/P in an expanded sample set from the same US population. An independent dataset of CL/P families and additional SNPs were used for confirmation of the association findings with *AXIN2*. At ~1.5-kb bp from rs7224837, another intronic SNP rs1165596 showed association in both Caucasian and Hispanic populations, whereas SNP rs4791171 was also associated in Caucasians. Intriguingly, in both original and confirmation datasets, the haplotype analysis was most interesting in views of 2- and 3-SNP sliding windows across the *AXIN2* gene in the area of SNP rs7224837 [for non-Asians with all clefts and CL/P in the original dataset, and for Caucasians in the confirmation dataset], and rs3923086 [for Asian families with CLP]. Of note, the associated haplotypes are always those composed of alleles showing positive association in the individual

SNP analysis, regardless of whether the TDT result was significant. Haplotypes including our previously associated variant rs2240308 also showed nominal association with Asian families with CLP.

We also performed statistical interaction analysis between *AXIN2* and other cleft susceptibility loci. In complex diseases, gene-gene interactions should be considered as additional thresholds for genetic predisposition. The results suggested possible allelic interactions between *AXIN2* and *IRF6*. *IRF6* is one of the largest contributors to the underlying genetics of human non-syndromic CL/P, contributing to as much as 12% of cleft cases in the background of other genes (Zucchero *et al.,* 2004). *AXIN2* SNP rs7224837 appears to interact with 2 *IRF6* SNPs, rs2235371, and rs642961. The associated A and C alleles in *AXIN2* rs7224837 and *IRF6* rs2235371, respectively, are both the reference alleles for these loci. For rs2235371, which facilitates a Valine to Isoleucine substitution at position 274 (V274I) of *IRF6*, the common C allele is also overtransmitted in cleft families (Zucchero *et al*., 2004). In contrast, the trend for interaction with *IRF6* rs642961 is with the rare A allele, which was significantly overtransmitted in cleft families, particularly those with cleft lip (Rahimov *et al.,* 2008).

AXIN2 belongs to the WNT pathway, which plays a critical and evolutionary conserved role in directing cell fates during craniofacial morphogenesis (Parr *et al*., 1993). *AXIN2* mutations have been linked to cancer and developmental defects as well (Lammi *et al*., 2004; Yu *et al*., 2005; Yook *et al*., 2006). Intriguingly, *Axin2*-null mice present skeletal defects resembling craniosynostosis but do not present a cleft (Yu *et al*., 2005; Liu *et al*., 2007)—hence our question if Axin2 was expressed in the palatal tissues during murine palatogenesis. We observed *Axin2* mRNA expression throughout palatogenesis stages, and particularly around E14.5, when the palatal shelves fuse in the midline. These results corroborate previous findings where *Axin2* expression was observed most intensely in the mesenchyme underlying the oral and dental epithelium of mouse embryos at E11-13 (Lammi *et al*., 2004), at which time-points the lip and primary palate have formed. At more advanced stages of tooth morphogenesis (*i.e.,* E17), *Axin2* expression was also detected in the odontoblasts and in the enamel knot (Lammi *et al*., 2004). Unlike *Axin* mRNA, which is expressed ubiquitously throughout embryogenesis, Axin2 protein expression has been described in a more restricted pattern during mouse embryogenesis, such as in the dorsal neural tube and branchial arches from embryonic days 8.5 to 11.5, and at later stages in the epithelial components of several developing organs (Jho *et al.,* 2002; Yu *et al.,* 2007). In our study, Axin2 protein expression was markedly evident at E14.5 in the epithelial cell layers of the palate shelves, teeth, tongue, facial mesenchyme, and tongue musculature.

We also investigated the expression of Axin2 protein and its possible co-localization with Irf6 in the developing palatal tissue, to verify if the genetic interaction data in humans would correlate with a biological scenario. We found Axin2 and Irf6

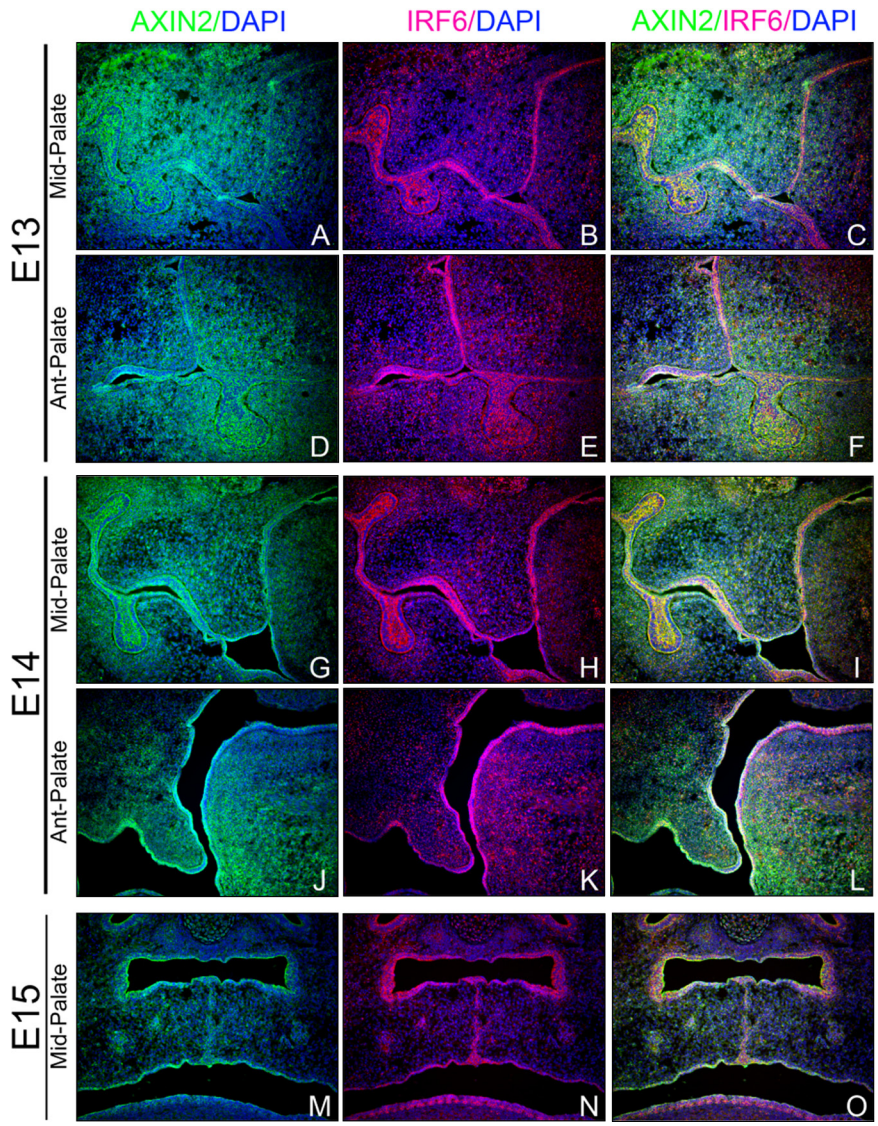

Figure 3. Axin2 co-localizes with Irf6 in mouse palate epithelium. Coronal sections through mid- and anterior-palate regions of wild-type embryos pre- (E13, A-F; E14, G-L) and post-palate fusion (E15, M-O) were subject to immunofluorescent labeling with Axin2- and Irf6-specific polyclonal antibodies. Axin2 expression (green) is evident in palate and tooth epithelium and mesenchyme, in addition to tongue and oral epithelium and tongue musculature throughout palatogenesis **(A, D, G, J,** and **M)**. Irf6 (pink) is expressed primarily in palate, tooth, oral, and tongue epithelium **(B, E, H, K,** and **N)**. Merged images (yellow; **C, F, I, L,** and **O)** show co-localized expression of Axin2 and Irf6 in these epithelial cell layers critical to normal palate and tooth development.

proteins co-localizing particularly in the developing secondary palate, nasal, oral, and eye epithelia. Of note, these observations of simultaneous expression of Axin2 and Irf6 in these relevant tissues do not necessarily implicate these molecules as interacting during their programmed functions. Previous evidence has shown that interaction between *Axin2* and *Axin1* genes, through targeted deletion of *Axin2* in an *Axin1* heterozygous background in mice (*Axin2*$^{-/-}$; *Axin1*$^{+/-}$), results in severe craniofacial deformities with truncation of the neural-crest-derived skeletal structures (Yu *et al.,* 2007). Hence, *Axin2* may play a role in craniofacial development, individually or interactively with other genes. Nevertheless, the role of Axin2 as well as Axin2/Irf6 interactions in lip fusion still needs to be defined in animal models.

Overall, our results support a likely role for *AXIN2* in the etiology of human clefting. The different variants associated with distinct population groups, and the observed differences in minor allele frequencies of the investigated polymorphisms, justify the individual associations in the particular populations, and also highlight the importance of allelic heterogeneity when results of genetic studies are interpreted. It is possible that more than one variant might contribute to a particular condition, and that these variants may be different across populations. Additionally, a specific combination of variants on a single chromosome may be required for a person to exhibit a particular phenotype. While the reported genetic interactions between *AXIN2* and *IRF6* reflect statistical calculations, additional research should be performed to verify if correlate biological interactions exist in animal models. Nonetheless, the contribution of variants in single genes to oral clefts is an important consideration in genetic counseling.

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