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Association of *IFT88* gene variants with nonsyndromic cleft lip with or without cleft palate

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Abstract

Background: Nonsyndromic cleft lip with or without cleft palate (NSCLP) is a common birth defect with multifactorial etiology. Genetic studies have identified numerous gene variants in association with NSCLP. *IFT88* (intraflagellar transport 88) has been suggested to play a major role in craniofacial development, as *Ift88* mutant mice exhibit cleft palate and mutations in *IFT88* were identified in individuals with NSCLP.

Objective: to investigate the association of *IFT88* single nucleotide gene variants (SNVs) with NSCLP in a large family dataset consisting of non-Hispanic white (NHW) and Hispanic families.

Methods: Nine SNVs in/nearby *IFT88* were genotyped in 482 NHW families and 301 Hispanic NSCLP families. Genotyping was performed using TaqMan® chemistry. Single- and pairwise-SNV association analyses were performed for all families stratified by ethnicity and family history of NSCLP using the Family Based Association Test (FBAT), and Association in the Presence of Linkage (APL). Bonferroni correction was used to adjust for multiple testing and p values ≤ 0.0055 were considered statistically significant.

Results: Significant association was found between *IFT88* rs9509311 and rs2497490 and NSCLP in NHW all families (P = 0.004 and 0.005, respectively), while nominal associations were

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found for rs7998361 and rs9509307 ($P < 0.05$). Pairwise association analyses also showed nominal associations between NSCLP in both NHW and Hispanic datasets ($P < 0.05$). No association was found between individual variants in *IFT88* and NSCLP in the Hispanic dataset.

Conclusions: Our results suggest that variation in *IFT88* may contribute to NSCLP risk, particularly in multiplex families from a non-Hispanic white population.

Keywords

nonsyndromic; cleft lip/palate; association; gene; IFT88

Introduction

Development of the craniofacial complex is a highly coordinated process that requires interactions between tissues from various embryonic origins including the neuroectoderm, neural crest and surface ectoderm. Defects in any step of this process can affect proper formation of the face and lead to severe craniofacial abnormalities(Mossey, Little, Munger, Dixon, & Shaw, 2009).

Nonsyndromic cleft lip with or without cleft palate (NSCLP) is a common craniofacial abnormality in humans, occurring in 1–2 per 1000 live births(Tolarova & Cervenka, 1998). NSCLP arises early in embryonic development and has multifactorial etiology, with both genetic and environmental factors contributing to the abnormal midfacial development phenotype(Dixon, Marazita, Beaty, & Murray, 2011; Mossey et al., 2009). Variations in numerous genes have been implicated in NSCLP etiology, most of which reflect genes with critical roles in facial development(Dixon et al., 2011). However, the genetic contributions to NSCLP are still largely unknown.

Previous evidence suggested a link between ciliary function and NSCLP(Schock et al., 2017; Tian et al., 2017). Primary cilia are cell surface, microtubule-based organelles that dynamically extend from cells to receive and process molecular and mechanical signaling cues(Singla & Reiter, 2006). Signaling in the primary cilia coordinates key processes during development and in tissue homeostasis, including cell migration, differentiation and/or re-entry into the cell cycle, cell division, and apoptosis(Satir, Pedersen, & Christensen, 2010). Moreover, primary cilia have been shown to regulate essential developmental signaling pathways (*i.e.*, Wnt and Shh)(Tian et al., 2017). Loss of functional primary cilia has been shown to have a strong effect on the developing craniofacial complex, resulting in a variety of abnormalities including craniosynostosis, midfacial dysplasia, dental abnormalities and NSCLP(Schock et al., 2017). Mutations in *TMEM107* (transmembrane protein 107), an important regulator of ciliary protein composition, were previously associated with a range of ciliary syndromes featuring distinct craniofacial abnormalities, including oral-facial-digital syndrome, Meckel-Gruber syndrome, and Joubert syndrome (Lambacher et al., 2016; Shaheen et al., 2015; Shylo, Christopher, Iglesias, Daluiski, & Weatherbee, 2016). Consistent with these findings in humans, *Tmem107*$^{-/-}$ mice also present with numerous skeletal and craniofacial defects including exencephaly, microphtalmia or anophthalmia, shorter and wider snout, misshapen and smaller tongue, and cleft lip and palate (Cela et al., 2018).

IFT88 (intraflagellar transport protein) is an essential protein for ciliogenesis(Pazour et al., 2000), and was recently suggested as a candidate gene for NSCLP(Tian et al., 2017). A rare missense mutation in *IFT88* (c.915G>C; p.E305D) was found segregating in autosomal dominant form in one NSCLP family(Tian et al., 2017). In mice, complete loss of *Ift88* resulted in defects in neural tube patterning, craniofacial abnormalities, polydactyly and left–right axis determination defects(Ohazama et al., 2009; Pazour et al., 2000); whereas mice with *Ift88* haploinsufficiency exhibit craniofacial abnormalities including cleft palate and supernumerary teeth(Ohazama et al., 2009). Disruption of primary cilia in neural crest cells due to loss of *Ift88* in the facial mesenchyme also resulted in severe craniofacial midline defects and cleft palate(Tian et al., 2017). These observations suggest that IFT88 may have an important role in proper ciliary function that when disrupted lead to NSCLP.

In this study, we investigated the association of *IFT88* gene variants with NSCLP in our large and well-characterized family-based dataset.

MATERIALS AND METHODS

Study Population

The study population consisted of 783 simplex and multiplex families of non-Hispanic white (NHW) (n = 482) and Hispanic (n = 301) ethnicities totaling 2,428 individuals (Table 1). Families were ascertained through probands, and additional relatives were recruited. All individuals were evaluated for the presence or absence of NSCLP by clinical geneticists. Individuals presenting with additional structural abnormalities suggesting syndromic forms of clefting were excluded. Individuals with cleft palate only or unknown cleft types were also excluded. After obtaining written informed consent, saliva and/or peripheral blood samples were collected as source of genomic DNA. DNA extraction followed established protocols. This study was approved by the University of Texas Health Science Center Committee for Protection of Human Subjects.

Genotyping

Nine single nucleotide variants (SNVs) in/nearby the *IFT88* gene (Table 2) were selected for genotyping based on: 1) tagSNVs considering the linkage disequilibrium block surrounding each gene(Carlson et al., 2004) (Suppl. Fig. 1), 2) location within the gene, and 3) availability of minor allele frequencies in European Caucasian populations in the 1000 Genomes Project Database (http://www.internationalgenome.org/1000-genomes-browsers). Genotypes were generated using TaqMan chemistry(Ranade et al., 2001) on an automatic sequence-detection instrument (Viia7, Applied Biosystems, Foster City, CA). A genotype call rate >95% was deemed acceptable. Genotypes were entered into Progeny2000 database and checked for Mendelian errors using Pedcheck(O'Connell & Weeks, 1998).

Data Analysis

Analyses were performed for all families stratified by ethnicity and by family history of NSCLP in each population. Single SNV association tests were performed using Family-Based Association Test (FBAT), with the "-e" option that accounts for complex pedigree structures(Horvath, Xu, & Laird, 2001), and using the Association in the Presence of

Linkage (APL) test(Chung, Hauser, & Martin, 2006). Pairwise SNV haplotype analysis was performed using APL. Bonferroni correction was used to adjust for multiple testing considering the number of SNVs genotyped; P-values ≤ 0.0055 (0.05/9) were considered statistically significant.

RESULTS

Significant association was found between *IFT88* variants and NSCLP in our NHW families. In the individual SNV association analysis, two intronic variants (rs9509311 and rs2497490) were significantly associated in the NHW dataset (P=0.003 and P=0.004, respectively); these associations appear to be driven by the multiplex families (P=0.009 and P=0.002). Nominal associations for additional *IFT88* missense and intronic variants were also noted in NHW families (P<0.05) (Table 3). No significant associations were found between NSCLP and individual *IFT88* variants in the Hispanic families.

Pairwise association analyses of *IFT88* variants revealed multiple nominal associations between NSCLP in the NHW dataset, particularly in the multiplex families, whereas three SNV combinations also showed nominal association in Hispanic multiplex families (P<0.05) (Table 4).

DISCUSSION

In this study, we investigated the association of *IFT88*, recently suggested as a NSCLP candidate gene, in a large and well-characterized family dataset. Our most significant results showed positive association between two noncoding intronic variants in *IFT88* and NSCLP in a NHW population, thus suggesting potential ethnicity-specific effects. Additional population-specific associations with various genes and gene variants have been reported for NSCLP(Beaty, Marazita, & Leslie, 2016; Dixon et al., 2011; Letra et al., 2011; Vieira, 2008; Zucchero et al., 2004). For example, *IRF6* has been consistently identified as a NSCLP susceptibility gene although with different variations reported in association in a population/ethnicity specific manner(Wattanawong, Rattanasiri, McEvoy, Attia, & Thakkinstian, 2016; Xia, Hu, Chen, Zheng, & Song, 2017; Zucchero et al., 2004).

Variants in noncoding regions have been consistently associated with NSCLP as well as with other complex diseases, and it is generally expected that the true pathogenic variant is one that is in linkage disequilibrium with the associated noncoding variant(s)(Liu et al., 2017). The *IFT88* variants associated in this study (rs9509311 and rs2497490) are considered eQTLs and significantly associated with IFT88 expression in several tissues. eQTLs are polymorphisms that affect the expression level of a gene and provide useful information towards interpretation of how genetic variants could translate to differences in biological function(Gamazon et al., 2018).

IFT88 was recently suggested as a candidate gene for NSCLP after a missense mutation (c.915G>C; p.E305D), resulting in a glutamate to aspartate substitution in the IFT88 protein, was found segregating in autosomal dominant form in a multiplex NSCLP family(Tian et al., 2017). This same study also showed that conditional deletion of *Ift88* (*Wnt1-Cre;Ift88*$^{fl/fl}$) resulted in loss of primary cilia in the cranial neural crest-derived palatal mesenchyme and

early lethality in mice due to severe craniofacial defects including bilateral cleft lip and palate and tongue agenesis(Tian et al., 2017). Ift88 expression was not detected in the palatal mesenchyme of *Wnt1-Cre;Ift88*$^{fl/fl}$ mice and both the number of ciliated cells and cilia lengths were decreased upon loss of *Ift88*(Tian et al., 2017). Additional craniofacial abnormalities such as supernumerary teeth have also been described in Ift88 transgenic (*Ift88*^{orpk}) mice(Zhang et al., 2003).

The IFT88 protein is essential for proper formation and function of primary cilia. Primary cilia are microtubule-based organelles located on the cell surface of most mammalian cell types and are considered key regulators of signaling pathways during tissue development and homeostasis(Satir et al., 2010; Schock et al., 2017). Defects in primary cilia (i.e., ciliopathies) have been regarded as a major cause of human disorders and diseases(Satir et al., 2010; Suzuki, Sangani, Ansari, & Iwata, 2016). Mutations in additional IFT genes such as *IFT43* and *IFT44* were identified in patients with Jeune and Sensenbrenner syndromes, rare disorders caused by increased Shh signaling, featuring craniofacial defects including hypertelorism, short limbs and polydactyly and small ribs. Mouse mutants for *Ift44* also present with multiple skeletal and craniofacial abnormalities, resembling human phenotypes(Ashe et al., 2012).

Primary cilia are generated through a process called intraflagellar transport (IFT); since protein synthesis does not occur in the cilia the proteins that compose the ciliary structure must be transported into the cilium via microtubules(Haycraft & Serra, 2008). Two intraflagellar transport complexes, IFT-A and IFT-B, consisting of 6 and 14 subunits, mediate transport of cargoes from the tip to the base of the cilium and from the base to the tip of the cilium, respectively(Haycraft & Serra, 2008). IFT88 belongs to complex IFT-B, and was shown as most robustly expressed in the embryonic head, kidney, lung and pancreas(Cano, Murcia, Pazour, & Hebrok, 2004; Taulman, Haycraft, Balkovetz, & Yoder, 2001), whereas its expression was nearly undetectable in the heart, spleen, and liver(Taulman et al., 2001).

Studies have shown that Wnt, Shh, and Fgf signaling pathways play a crucial role in craniofacial and palate development by regulating the underlying cellular interactions that occur during these processes(Haycraft & Serra, 2008; Serra, 2008; Snedeker et al., 2017; Suzuki et al., 2016). In the absence of *Ift88*, a non-functional cilium led to altered Wnt, Shh, and Fgf signaling(Haycraft & Serra, 2008; Serra, 2008; Snedeker et al., 2017; Suzuki et al., 2016). More specifically, the expression of *Ptch1* and *Gli3*, receptors and targets of Shh signaling, was significantly downregulated in the palatal mesenchyme of *Ift88* mutant (*Wnt1-Cre;Ift88*$^{fl/fl}$) mice while expression in the palatal epithelium was unchanged. In contrast, expression of *Axin2*, a negative regulator of the Wnt pathway, was increased in the palatal mesenchyme of *Ift88* mutant mice(Tian et al., 2017). Furthermore, it has been reported that *IFT88* interacts with *GLI2* and *GLI3*; interestingly mutations in these genes have also been associated with craniofacial disorders featuring an oral cleft(Ge et al., 2015).

The link between NSCLP, *IFT88*, and ciliary function may be related to disturbances in cranial neural crest cell (NCC) function. NCCs are a pluripotent, migratory population of cells that are intimately involved in the development of the craniofacial complex. Many

studies have shown that NCCs are highly orchestrated to receive and follow specific signals from adjacent tissues, particularly those of ectodermal origin(Bronner & LeDouarin, 2012).

Formation of many of the facial bones including the palatal bone derive from NCCs and consist of continuous reciprocal epithelial-mesenchymal interactions involving cell migration, proliferation, and apoptosis in the NCC-derived ecto-mesenchyme and palatal epithelium. Migrating NCCs harbor primary cilia and defects of migration of these cells resulted in disruption of non-canonical Wnt signaling and craniofacial defects in zebrafish embryos(Tobin et al., 2008). Loss of primary cilia also affected NCC polarity, and NCCs without cilia presented a more randomized cell polarity compared to wild type NCCs(Schock & Brugmann, 2017). Polarity defects in NCCs have been previously linked to abnormal facial growth in response to ectopic FGF signaling(Li et al., 2013). Furthermore, studies have demonstrated an important relationship between primary cilia and NCC cell cycle (Ching-Fang Chang). *Ift88* has been linked to centrosome behavior during the cell cycle(Robert et al., 2007), and in fact is required for correct spindle position during mitosis(Delaval, Bright, Lawson, & Doxsey, 2011). Normal primary cilia on NCCs are required for regulating cell proliferation, specifically in the developing face; overexpression of *Ift88* leads to abnormal cell cycle and hinders the G_1–S phases inducing apoptosis, meanwhile knockdown of *Ift88* promotes cell cycle progression(Robert et al., 2007). As presented, while different mechanisms have been proposed to explain how NCCs are negatively affected in the absence of a normal primary cilia, the exact mechanism for how this occurs remains unclear.

Our results support a likely role for *IFT88* variants in NSCLP susceptibility. NSCLP is complex with many genes and environmental factors playing a role. The nature and function of associated genes vary widely, and highlight the distinct craniofacial developmental pathways that when perturbed may contribute to the occurrence of NSCLP. Further studies are necessary to confirm the role of *IFT88* and additional ciliary genes in NSCLP in other populations and to explore their roles in craniofacial development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1.

Study families

Dataset	Families				Individuals			
	Simplex	Multiplex	Mean Pedigree Size (min-max)	Total	Unaffected	Affected	Unknown	Total
NHW	332	150	3.22 (3–32)	482	965	572	12	1549
Hispanic	206	95	2.92 (3–11)	301	548	325	6	879
Total	538	245	3.07 (3–32)	783	1513	897	18	2428

Table 2.

IFT88 SNPs investigated

SNP ID.	Base position[†]	Location/function	Alleles[‡]	MAF CEU[§]
rs4465462	20529556	Upstream (~44kb)	G/**A**	0.22 (A)
rs3809357	20566642	5' near gene (~1.3kb)	**T**/A	0.20 (A)
rs7998361	20614716	Intron 15	**T**/A/C	0.28 (T)
rs2442455	20615802	Missense (exon 16)	**G**/A	0.14 (A)
rs3002172	20616095	Intron 16	**A**/T	0.14 (T)
rs9509307	20631053	Missense (exon 18)	**G**/A	0.28 (G)
rs2497490	20633875	Intron 18	**C**/T	0.18 (T)
rs9509311	20635498	Intron 18	**A**/G	0.47 (A)
rs2149769	20692088	Downstream (~117kb)	**T**/C	0.21 (C)

[†]According to NCBI dbSNP Assembly GRCh38.p7, build 151

[‡]Ancestral allele in bold (forward strand)

[§]MAF CEU, minor allele frequency in European Caucasian population (1000 Genomes Project Phase 3 allele frequencies)

Table 3.

Summary of association results

SNP	Base position	Location	FBAT†			APL‡		
			NHW All	NHW Multiplex	NHW Simplex	NHW All	NHW Multiplex	NHW Simplex
rs4465462	20529556	Upstream (~44kb)	---	---	---	---	---	---
rs3809357	20566642	5' near gene (~1.3kb)	---	---	---	---	---	---
rs7998361	20614716	Intron 15	0.0493	0.0280	---	0.027	0.049	---
rs2442455	20615802	Missense (exon 16)	---	0.0384	---	---	---	---
rs3002172	20616095	Intron 16	---	0.0276	---	---	0.032	---
rs9509307	20631053	Missense (exon 18)	---	0.0245	---	0.033	0.038	---
rs2497490	20633875	Intron 18	0.0115	**0.0019**	---	**0.004**	0.023	---
rs9509311	20635498	Intron 18	0.0219	0.0096	---	**0.003**	0.013	---
rs2149769	20692088	Downstream (~117kb)	---	---	---	---	---	---

†FBAT, Family-based association test

‡APL, Association in the presence of linkage test

§Only P≤0.05 are reported; P≤0.0055 indicates statistical significance under Bonferroni correction (in bold)

Table 4.

Results of pairwise analyses

SNP1	SNP2	P-value*					
		NHW All	NHW Multiplex	NHW Simplex	HISP All	HISP Multiplex	HISP Simplex
rs3809357	rs7998361	0.0109	0.0166	---	---	---	---
rs3809357	rs9509307	0.0141	0.0402	---	---	---	---
rs9509307	rs2149769	0.0233	---	---	---	---	---
rs9509311	rs2149769	0.0330	---	---	---	---	---
rs7998361	rs2497490	0.0389	---	---	---	---	---
rs2497490	rs9509311	0.0397	0.0293	---	---	0.0334	---
rs9509307	rs2497490	0.0401	---	---	---	---	---
rs7998361	rs9509311	0.0404	0.0392	---	---	---	---
rs3002172	rs9509311	0.0466	0.0387	---	---	---	---
rs3809357	rs2497490	0.0471	---	0.0412	---	---	---
rs2442455	rs9509311	0.0485	---	---	---	---	---
rs2497490	rs2149769	0.0499	---	---	---	0.0407	---
rs3809357	rs2149769	---	0.0225	---	---	---	---
rs9509307	rs9509311	---	0.0365	---	---	---	---
rs7998361	rs2149769	---	0.0491	---	---	---	---
rs2442455	rs2497490	---	---	---	---	0.0108	---

NHW, non Hispanic whites; HISP, Hispanics

*APL, Association in the presence of linkage test, only P≤0.05 are reported