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Further Evidence Suggesting a Role for Variation in *ARHGAP29* in Nonsyndromic Cleft Lip/Palate

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Abstract

Nonsyndromic cleft lip with or without cleft palate (NSCL/P) is a common birth defect of complex etiology. Several genes have been implicated in the etiology of NSCL/P, although only a few have been replicated across datasets. *ARHGAP29* was suggested as a candidate gene for NSCL/P as it is located in close proximity to *ABCA4* (1p22), a gene previously identified in a GWAS of NSCL/P. Rare, potentially damaging, coding variants in *ARHGAP29* were found in NSCL/P cases, and its expression was detected during murine craniofacial development. In this study, we investigated whether variations in *ARHGAP29* were associated with NSCL/P in our family based dataset. Five SNPs flanking and within *ARHGAP29* were genotyped in our NSCL/P datasets consisting of simplex and multiplex families of non Hispanic white (NHW, primarily European) and Hispanic ethnicities. Results showed strong association of three *ARHGAP29* SNPs with NSCL/P in the NHW families. Two intronic SNPs (rs1541098 and rs3789688) showed strong association with NSCL/P in all NHW families (P=0.0005 and P=0.0002, respectively), and simplex NHW families (P=0.003 for both SNPs). A SNP in the 3' UTR (rs1576593) also showed strong association with NSCL/P in all NHW families (P=0.002), and the multiplex subset (P=0.002). *ARHGAP29* SNP haplotypes were also associated with NSCL/P. Evidence of gene-gene interaction was found between *ARHGAP29* and additional cleft susceptibility genes. This study further supports *ARHGAP29* as a candidate gene for human NSCL/P in families of Caucasian descent.

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Keywords

cleft lip/palate; *ARHGAP29*; association; haplotype; gene-gene interaction

INTRODUCTION

Nonsyndromic cleft lip with or without cleft palate (NSCL/P) is a common birth defect, notable for its significant lifelong morbidity and complex etiology. It affects 135,000 newborns worldwide with wide variability related to geographic origin and socioeconomic status. In general, Native American and Asian populations present the highest frequencies, sometimes at 1/500 or higher, followed by Caucasian, and African-derived populations showing the lowest frequencies at approximately 1/2500 births (Mossey et al., 2009).

The complex etiology of NSCL/P reflects multiple genetic and environmental factors acting individually or in concert. A variety of research approaches including candidate gene, genome-wide linkage, and genome-wide association studies (GWAS), have been used to identify the etiologic genes contributing to NSCL/P. While numerous genes and specific polymorphic variants have been suggested to confer an increased risk of NSCL/P, only a few have been replicated across datasets (Dixon et al., 2011). Of note, the association of *IRF6* gene variants with NSCL/P has been confirmed in multiple populations (Dixon et al., 2011) and have been suggested to explain about ~12% of the genetic contribution to NSCL/P (Zucchero et al., 2004). Additionally, a gene desert region on chromosome 8q24 was consistently associated with NSCL/P in different GWA studies (Beaty et al., 2010; Birnbaum et al., 2009; Grant et al., 2009; Mangold et al., 2010), and independently replicated in various populations (Bagordakis et al., 2013; Beaty et al., 2013; Blanton et al., 2010; Brito et al., 2012; Lennon et al., 2012; Mostowska et al., 2010; Nikopensius et al., 2009; Rojas-Martinez et al., 2010; Velazquez-Aragon et al., 2012; Wang et al., 2012).

ARHGAP29 was suggested as a candidate gene for NSCL/P because it is located in close proximity to *ABCA4* (1p22), a gene identified in a previous GWAS of NSCL/P (Leslie et al., 2012). Despite identifying a number of missense mutations in *ABCA4* in individuals with NSCL/P, *ABCA4* is unlikely to be the etiologic gene for clefting because expression is restricted to the retina (Beaty et al., 2010). Hence, it has been hypothesized that a neighboring gene, *ARHGAP29*, may be the true etiologic gene or locus. Rare, potentially damaging, coding variants in *ARHGAP29* were found in NSCL/P cases, and its expression was detected during craniofacial development in mice (Leslie et al., 2012). *ARHGAP29* encodes a Rho GTPase activating protein (GAP) 29 and is involved in essential cellular functions that are critical for craniofacial development (Mossey et al., 2009), and may be a downstream effector of Tgfb and Wnt (Kardassis et al., 2009; Schlessinger et al., 2009) signaling pathways, which are also involved in craniofacial development. This suggests that perturbation of *ARHGAP29* may play a role in NSCL/P. In this study, we investigated whether variations in *ARHGAP29* were associated with NSCL/P in families of Hispanic and non Hispanic white (NHW) ethnicities.

MATERIALS AND METHODS

Study Population

Our study population consisted of simplex and multiplex families of NHW (primarily European) (n=507) and Hispanic (primarily Mexican) (n=314) ethnicities. Details of the study families are presented in Table I. Families were ascertained through probands, and additional relatives were recruited. Individuals presenting with syndromic clefts, cleft palate only, or unknown cleft types were excluded. This study was approved by the University of Texas Health Science Center Committee for Protection of Human Subjects.

Genotyping

Five flanking and intragenic *ARHGAP29* SNPs were genotyped in our NSCL/P datasets (Table II). SNPs were selected as tag-SNPs, considering heterozygosity values, gene structure, and the linkage disequilibrium block surrounding each gene, as previously described (Carlson et al., 2004) (Figure 1). Genotypes were generated using Taqman chemistry (Ranade et al., 2001) on an automatic sequence-detection instrument (ABI Prism 7900HT, Applied Biosystems, Foster City, CA).

Statistical Analysis

Family-based single SNP association tests were performed using CAPL (Chung et al., 2011). Analysis were performed for all families stratified by ethnicity and then stratified by ethnicity and family history of NSCL/P. Pairwise haplotype analysis was performed using APL (Chung et al., 2006). Bonferroni correction was used to adjust for multiple testing and P-values ≤0.01 were considered statistically significant for the single SNP analyses.

Gene-Gene Interaction Analysis

We examined *ARHGAP29* SNP interactions with genes recently suggested to be involved with NSCL/P in humans and/or mice: *IRF6* (Zucchero et al., 2004), *P63* (Leoyklang et al., 2006), *PBX1* and *PBX2* (Ferretti et al., 2011), *WNT3* and *WNT9B* (Juriloff and Harris, 2008). Transmission of all possible intergenic 2-SNP pairs was examined with APL (Chung et al., 2010; Chung et al., 2011). Genotype data for twenty-three SNPs in these genes were used for the gene-gene interaction analyses (Supplementary Material).

In Silico Prediction of SNP Function

AliBaba2.1 and PATCH (www.gene-regulation.com), SNP function prediction methods, were used to determine whether the genotyped *ARHGAP29* SNPs harbored transcription factor binding sites.

RESULTS

Overall, we found strong association with individual *ARHGAP29* SNPs and haplotypes in our NHW dataset. One SNP in the 3'UTR (rs1576593) and two intronic SNPs (rs1541098 and rs3789688) showed evidence for association (p=0.002, p=0.0005, and p=0.0002, respectively) with NSCL/P in the NHW dataset. When stratified by family history, only the association with rs1576593 remained significant in the multiplex families (p=0.002),

whereas rs1541098 and rs3789688 remained significant in the simplex families (p=0.003 for both) (Table III). No associations were found for *ARHGAP29* SNPs and NSCL/P in Hispanics (Table III).

Haplotype analyses detected modest evidence for association of *ARHGAP29* SNP alleles with NSCL/P in the NHW dataset. In the pooled dataset, associations were detected for several haplotypes: rs1576593 and rs3789688 (p=0.003), rs1541098 and rs3814019 (p=0.006), and rs1541098 and rs3789688 (p=0.008) (Table IV). There was evidence for altered transmission of the rs1541098 and rs3789688 haplotypes in the multiplex families (p=0.003), whereas in simplex families there was evidence for altered transmission of several haplotypes: rs1576593 and rs1048854 (p=0.004), rs1048854 and rs3789688 (p=0.009), and rs1048854 and rs1541098 (p=0.009).

Gene-gene interaction analyses for *ARHGAP29* and additional cleft susceptibility genes showed evidence for statistical interaction in the pooled NHW families. Significant interactions were observed between *ARHGAP29* rs1576593 and *PBX1* rs6426870 (p=0.0002) in simplex families, as well as between *ARHGAP29* rs3789688 and *P63* rs4575879 (p=0.00001), *PBX2* rs204993 (p=0.0004), *WNT3* rs7216231 (p=0.0001), and *WNT9B* rs12602434 and rs1530364 (p=0.0001 and p=0.0004, respectively) in all NHW families (Table V).

The results of the *in silico* analyses showed differential binding partners for each allele in rs3814019 and in rs1576593 located in the 5’ and 3’ UTR of *ARHGAP29*, respectively (Table VI). While the ancestral allele of rs3814019 was predicted to bind to the transcription factors GATA-3, CPC1, and p40X, the alternate allele did not harbor biding sites for these transcription factors. Rather, it was predicted to bind to ELF-1, ISGF-3, NF-Kb, and RelA. For rs1576593, the ancestral allele T, associated with NSCL/P, was predicted to bind to a micro-RNA (mIR-1194), whereas analyses for the alternate allele using both prediction programs failed to identify a transcription factor-binding site.

DISCUSSION

Recent evidence suggests a role for *ARHGAP29* in NSCL/P based on craniofacial expression during murine development and identification of several variants in *ARHGAP29*, that collectively were overrepresented in cases with NSCL/P from Filipino and US populations, compared with unaffected controls (Leslie et al., 2012). In this study, we replicate the association of *ARHGAP29* gene variants with NSCL/P in our large family-based NHW dataset, further supporting the suggestion that *ARHGAP29* may be a cleft susceptibility gene. A SNP in the 3’UTR (rs1576593) and two intronic SNPs (rs1541098 and rs3789688) showed strong association with NSCL/P in all NHW families. Notably, the association with rs1576593 was stronger in multiplex families, whereas the association with rs1541098 and rs3789688 was stronger in simplex families. Although these latter two SNPs are located in different LD blocks (Figure 1), they are in strong LD with each other and may be transmitting the same information. No individual associations were observed for SNPs rs3814019 and rs1048854, nonetheless altered transmission of haplotypes including these SNPs were also associated with NSCL/P in the NHW dataset. While rs3814019 is located in

the 5' UTR of the *ARHGAP29* gene with potential regulatory effects on gene transcription, rs1048854 is a synonymous variant (Gln891Gln) in exon 21 with no known impact on the final protein.

Although intronic variants are unlikely to have effects on gene transcription and/or on the final protein structure, variants located in regulatory regions in many cases are known to have deleterious effects. Our *in silico* analysis of potential regulatory SNPs in the 5' and 3' UTR of *ARHGAP29* (rs3814019 and rs1576593, respectively) showed distinct allele-specific binding partners. The ancestral allele A was predicted to bind to GATA-3 (GATA binding protein 3), a member of the GATA family of transcription factors, which acts as an enhancer-binding protein and regulator of T-cell development with an important role in endothelial cell biology (Song et al., 2009). In contrast, the alternate allele G was predicted to bind to transcription factors (ISGF3, NFKB, and RELA) that act both as enhancers and as repressors to regulate transcription of various genes. ISGF3 (interferon stimulated gene factor 3) comprises a gene complex, which in response to stimuli such as cytokines and growth factors, are phosphorylated and translocate to the cell nucleus to act as transcriptional activators (Bluyssen et al., 1996). Similarly, NFKB (nuclear factor kappa-B) and RELA (v-rel avian reticuloendotheliosis viral oncogene homolog A) belong to the NFKB family, a protein complex that controls DNA transcription and represent the endpoint of a series of signal transduction events that are initiated by a vast array of stimuli related to many biological processes such as inflammation, immunity, differentiation, cell growth, tumorigenesis and apoptosis. NFKB binds to the Rel-like domain-containing proteins (i.e., RELA/p65, RELB, REL) and form dimers that bind at kappa-B sites in the DNA of their target genes; and the different dimer combinations act as transcriptional activators or repressors, respectively (Rayet and Gelinas, 1999). For the 3' UTR SNP rs1576593, the ancestral allele T, associated with NSCL/P, was predicted to present a microRNA (mIR-1194) binding site, whereas analyses for the alternate allele using both prediction programs failed to demonstrate a transcription factor-binding site. This suggests that rs1576593 may have a regulatory role in post-transcriptional regulation of *ARHGAP29* gene expression. In mouse, mIR-1194 presents 169 conserved sites including a site for fibroblast growth factor 5 (*FGF5)* (Calabrese et al., 2007). *FGF5* is a member of the fibroblast growth factor signaling pathway, which is involved in several aspects of craniofacial development, including formation of the lip and the palate (Ferguson, 1988). Many FGF ligands and receptors are expressed at specific stages and at precise locations during normal palatogenesis and an absolute requirement of some has been demonstrated by their (conditional) inactivation resulting in a cleft palate phenotype (Lee et al., 2001; Rice et al., 2004). In humans, FGF gene variants have also been associated with NSCL/P (Menezes et al., 2008; Nikopensius et al., 2011; Riley et al., 2007; Riley and Murray, 2007; Wang et al., 2013).

In addition to the allele and haplotype associations found for *ARHGAP29* with NSCL/P, we detected evidence of statistical interaction between *ARHGAP29* and other cleft susceptibility genes *(IRF6, P63, PBX1, PBX2, WNT3, and WNT9B*) identified in previous human and mouse studies (Ferretti et al., 2011; Juriloff and Harris, 2008; Leoyklang et al., 2006; Zucchero et al., 2004). In complex disorders such as NSCL/P, gene-gene interactions should

be considered as additional thresholds for genetic predisposition (Cordell, 2009). While most genetic studies have used a single-locus analysis strategy, whereby each variant is tested individually for association with some complex phenotypes, an oft-cited reason for the lack of success in genetic studies of these disorders is the challenge of identifying interactions between loci. If a genetic factor operates primarily through a complex mechanism involving multiple other genes, the concern is that the effect will be missed if one examines it in isolation, without allowing for its potential interactions with other genetic factors (Cordell, 2009). Therefore testing for gene-gene interactions might increase the power to detect effects, and also statistical interactions between loci that are informative about the biological and biochemical pathways underpinning NSCL/P. Our results suggested possible allelic interactions between *ARHGAP29* and *WNT3, WNT9B, PBX1, PBX2*, and *P63* genes in the NHW dataset. *WNT3* and *WNT9B* belong to the wingless-type MMTV integration site (Wnt) signaling pathway, which 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 (Jiang et al., 2006). *Wnt3* and *Wnt9b* are located on chromosome 11 within the *clf1* locus, which is associated with the spontaneous development of cleft lip and/or cleft palate in A/WySn mouse strains (Juriloff et al., 2005, 2006). In addition, polymorphic variants in numerous WNT genes including *WNT3* and *WNT9B* have been associated with NSCL/P in different populations (Chiquet et al., 2008; Fontoura et al., 2014; Menezes et al., 2010; Mostowska et al., 2012; Yao et al., 2011). Recent evidence has suggested that *PBX1* and *PBX2* genes are clefting genes in mice, possibly through interactions with *Wnt9b* and *p63* (Ferretti et al., 2011). Inactivation of Pbx genes in the epithelia and mesenchyme of the craniofacial region, as observed in compound *Pbx1*$^{-/-}$*; Pbx2*$^{+/-}$ (*Pbx1/2)* mutants, resulted in animals with broader face, mandibular hypoplasia, cleft lip, and cleft palate. *Wnt3* and *Wnt9b* were markedly downregulated contributing to perturbed p63 expression in the midface of Pbx compound mutants. Further, a Pbx-dependent Wnt-p63-Irf6 regulatory module was suggested, which when disrupted, led to localized suppression of apoptotic programs and CL/P (Ferretti et al., 2011). Taken together, these observations highlight the complex etiology of NSCL/P and point towards the need for considering genetic interactions in studies of NSCL/P. Of note, no evidence of interaction was found between *ARHGAP29* and *IRF6. IRF6* is suggested to be one of the largest contributors to the underlying genetics of human NSCL/P, contributing to as much as 12% of the genetic risk for clefting (Zucchero et al., 2004). This is an intriguing finding as *Arhgap29* was reported to act downstream of *Irf6*, showing decreased expression in the palatal epithelium and skin of *Irf6* deficient mice (Leslie et al., 2012).

In summary, we provide further evidence for the role of *ARHGAP29* in NHW families with NSCL/P but not Hispanic families. Studies in additional families from other populations are needed to determine the role of this gene across populations. Importantly, we found evidence of genetic interactions involving *ARHGAP29, PBX1, PBX2, P63, WNT3*, and *WNT9B* indicating that variants in these genes and their interactions should also be evaluated across populations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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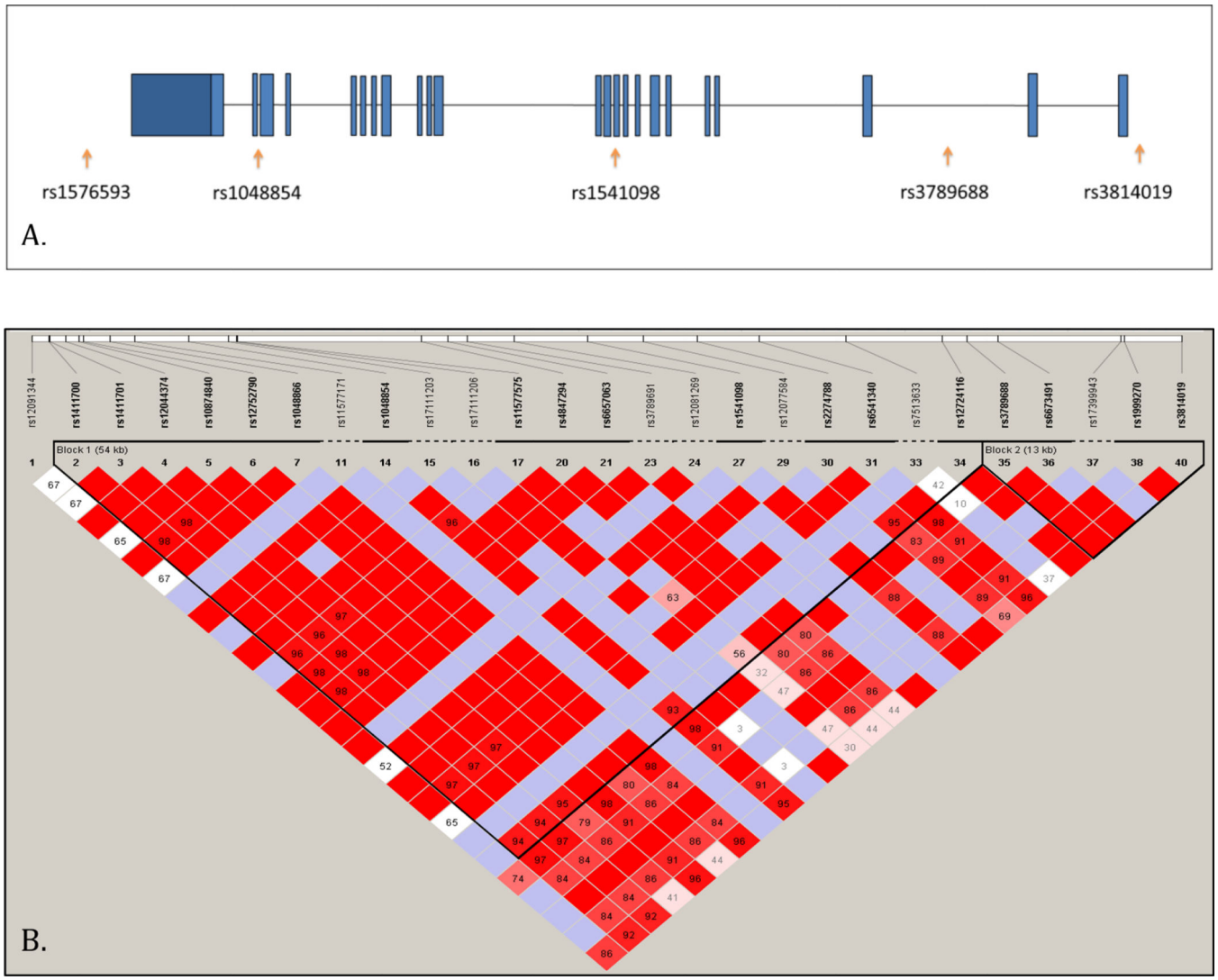

Figure 1.

Schematic representation of the *ARHGAP29* gene structure and linkage disequilibrium plot. **A**. Gene structure of *ARHGAP29* with colored bars representing exons, horizontal lines representing introns. Arrows denote location of genotyped SNPs. **B**. Linkage disequilibrium plot with boxes representing the marker pair relationship plotted between two markers. The color of the boxes (intensity of the color) is based on the raw score for that marker pair. Squares are colored darker if the D' value is high (i.e., LD is strong), lighter shades indicate less LD. Empty dark squares mean D'= 1 (i.e., complete LD between two SNPs).

Table I

Description of NSCL/P families

Ethnic group	Mean pedigree size (min-max)	Pedigrees	Unaffecteds typed	Affecteds typed	Total individuals
Hispanic	2.8 (2–11)	314	327	564	891
NHW	3.1 (2–32)	507	575	998	1573

NHW = non Hispanic white.

Table II

ARHGAP29 SNPs genotyped

SNP Id.	Base position*	Function	Alleles**
rs3814019	94704396	5' near gene	A/G
rs3789688	94691240	Intron	A/G
rs1541098	94667970	Intron	T/C
rs1048854	94643531	Coding synonymous	A/G
rs1576593	94631751	3' UTR	T/C

*According to NCBI dbSNP Build 137.

**Ancestral allele listed first.

Table III

Association results by ethnicity

Families	SNP	P-value*	
		NHW	Hispanic
All	rs1576593	0.002 (T)	0.13
	rs1541098	0.0005 (T)	0.96
	rs3789688	0.0002 (A)	0.79
Multiplex	rs1576593	0.002 (T)	0.03 (C)
Simplex	rs1541098	0.003 (T)	0.58
	rs3789688	0.003 (A)	0.86

* APL test, $p<0.01$ indicates statistical significance; Letters in parenthesis indicate overtransmitted allele.

NHW = non Hispanic white

Table IV

NHW haplotype results

Families	*ARHGAP29*		Haplotypes with Greatest Altered Transmission	Global p-value*
	SNP1	SNP2		
ALL	rs1541098	rs3814019	C -G↓	0.008
	rs1541098	rs3789688	T - G↑ C - A↓	0.006
	rs1576593	rs3789688	T - A↓	0.003
MULTIPLEX	rs1541098	rs3789688	T - G↑	0.003
SIMPLEX	rs1048854	rs3789688	A - A↓ G - G↑	0.009
	rs1048854	rs1541098	G - T↑ A - C↓	0.009
	rs1576593	rs1048854	T - A↓ C - G↑	0.004

*APL test, all p<0.01 included.

Table V

NHW gene-gene interaction results

Gene/SNP		*ARHGAP29 SNP*					
		rs3789688		**rs1541098**		**rs1576593**	
		ALL	**Simplex**	**ALL**	**Simplex**	**ALL**	**Simplex**
PBX1	rs6426870	--- -	0.003	----	0.003	----	**0.0002**
P63	rs4575879	**0.00001**	0.001	0.001	0.003	0.003	----
PBX2	rs204993	**0.0004**	0.0045	----	0.007	0.003	----
WNT3	rs7216231	**0.0001**	0.002	0.004	0.008	0.007	----
WNT9B	rs12602434	**0.0001**	----	0.006	----	----	----
WNT9B	rs1530364	**0.0004**	0.004	0.001	----	0.007	----

* Only interactions with at least one P-value ≤ 0.005 are reported.

NHW = non Hispanic white