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Variation in WNT genes is associated with non-syndromic cleft lip with or without cleft palate

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Non-syndromic cleft lip with or without cleft palate (NSCLP) is a common birth defect. Genetic and environmental factors have been causally implicated and studies have begun to delineate genetic contributions. The Wnt genes are involved in regulating mid-face development and upper lip fusion and are therefore strong candidates for an etiological role in NSCLP. Furthermore, the clf1 region in A/WyN clefting susceptible mice contains the Wnt3 and Wnt9B genes. To assess the role of the Wnt family of genes in NSCLP, we interrogated seven Wnt genes (Wnt3, Wnt3A, Wnt5A, Wnt7A, Wnt8A, Wnt9B and Wnt11) in our well-defined NSCLP dataset. Thirty-eight single nucleotide polymorphisms were genotyped in 132 multiplex NSCLP families and 354 simplex parent–child trios. In the entire dataset, single-nucleotide polymorphisms (SNPs) in three genes, Wnt3A ($P = 0.006$), Wnt 5A ($P = 0.002$) and Wnt11 ($P = 0.0001$) were significantly associated with NSCLP after correction for multiple testing. When stratified by ethnicity, the strongest associations were found for SNPs in Wnt3A ($P = 0.0007$), Wnt11 ($P = 0.0012$) and Wnt8A ($P = 0.0013$). Multiple haplotypes in Wnt genes were associated with NSCLP, and gene–gene interactions were observed between Wnt3A and both Wnt3 and Wnt5A ($P = 0.004$ and $P = 0.039$, respectively). This data suggests that alteration in Wnt gene function may perturb formation and/or fusion of the facial processes and predispose to NSCLP.

INTRODUCTION

Non-syndromic cleft lip with or without cleft palate (NSCLP) is a common birth defect with a prevalence of 1/700 live births (1–3). NSCLP is a complex malformation caused by the interaction of multiple genes and environmental factors (4). Genetic causality has been slow to be identified, but variations in a small number of genes have been shown to contribute to a non-syndromic orofacial clefting phenotype, including interferon regulatory 6 (IRF6), Msh homeobox homolog 1 (MSX1), transforming growth factor alpha (TGFα), and cysteine-rich secretory protein Limulus factor C-related region (LCCL) domain containing 2 (CRISPLD2) (4–12). Approximately 12–25% of the genetic variation causing NSCLP is estimated to have been identified (4,10,13,14).

In an effort to identify additional NSCLP genetic loci, we interrogated the Wnt gene family members. These genes were chosen because Wnt3A, Wnt5A, Wnt8A and Wnt11 play important roles in murine craniofacial embryogenesis; Wnt expression is observed in the upper lip and primary and secondary palates and Wnts are involved in regional specification of the vertebrate face (15–22). Further evidence supporting Wnt genes as possible clefting loci comes from the inbred A/WySn mouse strain where ~5–30% are born with a cleft lip and palate (CL/P) (23). Two cleft loci, clf1 and clf2, were mapped in these mice (23). Wnt3 and Wnt9B genes are in the clf1 region; however, no causative variation within these two genes has been identified. An inserted transposable element, unique to the A/WySn strain, was found 6.6 kb downstream of the Wnt9B gene. This insertion is thought to inhibit normal Wnt9B expression and contribute to the A/WySn clefting phenotype (24). Additionally, mice heterozygous for the clf1 locus and Wnt9B-null alleles exhibit penetrance of up to 67% for cleft lip (25). Wnt expression and A/WySn murine studies indicate that the Wnt

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family of genes plays a critical role in the development of the lip and palate. This study was undertaken to interrogate seven Wnt genes (Wnt3, Wnt3A, Wnt5A, Wnt7A, Wnt8A, Wnt9B and Wnt11) to determine if variation in members of this gene family is associated with NSCLP.

RESULTS

All single-nucleotide polymorphisms (SNPs) were in Hardy–Weinberg equilibrium. Comparisons of SNP allele frequencies, after Bonferroni correction, showed significant differences between European Americans and Hispanics ($P < 0.001$) in three of the seven genes (Table 1). Therefore, in addition to an overall analysis of the complete dataset, the data were stratified by ethnicity and examined separately. There was no evidence for linkage under any of the tested parametric models or with non-parametric methods for any of the regions (data not shown).

Pedigree disequilibrium test (PDT) and Geno-PDT results for the entire dataset are presented in Table 2. Out of the seven genes interrogated, five have SNPs that meet a nominal threshold of 0.05. If we correct the significance level for the number of genes tested, then SNPs in three genes (Wnt3A, Wnt5A and Wnt11) still yield significant results ($P < 0.007$). Table 3 and Supplementary Material, Tables S1 and S2 contain the results of PDT, Geno-PDT a]nd association in the presence of linkage (APL) analyses when the families were grouped by ethnicity and also subdivided according to the presence or absence of family history (FH). SNPs meeting a nominal threshold (P-value < 0.05) were found in each gene, generally by multiple methods. In the overall European American sample, and when stratified by FH, associations were identified with multiple SNPs in Wnt3, Wnt5A and Wnt11 (Table 3). In addition, SNPs in Wnt7A were significant in the total European American sample and in the positive FH subgroup, while an SNP in Wnt9B was significant only in the positive FH subgroup. Multiple SNPs in Wnt3A were significant in the total European American sample and in the negative FH subgroup, and an SNP in Wnt8A was significant in the negative FH subgroup.

When examining the entire Hispanic sample or when stratifying by FH, associations were identified for SNPs in Wnt3A and Wnt9B. Two SNPs were significant in the total sample; one of these was significant in the negative FH subgroup. One SNP in Wnt11 was significant in the positive FH subgroup and when the sample was considered as a whole. Lastly, an SNP in Wnt3 was significant in the positive FH subgroup. Overall, across ethnicity, the same SNPs were significant in four genes: Wnt3A (two SNPs), Wnt7A (four SNPs), Wnt11 (one SNP) and Wnt9B (one SNP) and both ethnicities had significant SNPs in Wnt3, although there was no overlap.

The results of haplotype analysis are shown in Table 4. Because of the small sample size, data were not stratified by FH. In both European American and Hispanic samples, four haplotypes in Wnt3A and one in Wnt8A were significantly associated (Table 4). Wnt11 haplotypes were only significant in the European American sample (Table 4). While some of the SNPs within genes were in significant linkage disequilibrium (LD), most of the SNPs in the significantly overtransmitted haplotypes had $r^2 < 0.3$ (Supplementary Material, Table S3).

Generalized estimating equations (GEE) analysis identified significant gene–gene interactions for SNPs in Wnt3A and Wnt3 ($P = 0.0037$) in the European American sample, and SNPs in Wnt3A and Wnt5A in the Hispanic sample ($P = 0.0388$) (Table 5). Models for interaction considering dominant and recessive main effects were tested in the European American sample. The best model for increased susceptibility was when at least one copy of the rarer allele was present at each locus ($P = 0.03$, data not shown). Interestingly, when there was homozygosity for the common allele in Wnt3, the rare allele in Wnt3A was protective ($P = 0.007$, data not shown). The Hispanic sample was too small to evaluate the main effects.

DISCUSSION

In this study, we interrogated Wnt3, Wnt3A, Wnt5A, Wnt7A, Wnt8A, Wnt9B and Wnt11 to determine if any of them are implicated in the etiology of NSCLP. In the overall dataset (Table 2), SNPs in five of the genes (Wnt3A, Wnt7A, Wnt5A, Wnt8A and Wnt11) had nominal P-values of 0.05 or less. Three of these, Wnt3A, Wnt5A and Wnt11 remained significant after correction for multiple testing. Among the subsets of data, the strongest evidence for an association was found in the European American sample for SNPs primarily in Wnt3, Wnt3A, Wnt7A, Wnt8A, Wnt9B and Wnt11 (Table 3), although only SNPs in Wnt3, Wnt3A, Wnt5A, Wnt9 and Wnt11, meet the more restrictive P-value of 0.007. In the Hispanic sample, the strongest associations were found with SNPs in Wnt3A, although some evidence for association was also observed in Wnt3, Wnt7A, Wnt9B and Wnt11 (Table 3). SNPs in five genes (Wnt3, Wnt3A, Wnt7A, Wnt9B and Wnt11) had at least suggestive evidence for association in both ethnicities; in four of these genes (excluding Wnt3), eight of the associated SNPs were identical. Moreover, with the exception of Wnt7A, multiple haplotypes in these same genes were also associated with NSCLP (Table 4). This pattern is quite striking, although the analyses performed are not independent and the haplotype results were not corrected for multiple testing.

The issue of correcting for multiple tests is complicated when multiple methodologies are performed, and neither the tests nor all the SNPs are independent. Moreover, which correction factor to use is further complicated when the SNPs are in genes considered viable candidates from other scientific evidences. Therefore, we elected to report results meeting a nominal P-value of 0.05. In addition, for the association studies of the individual SNPs, we have employed a correction factor of 7 ($0.05/7 = 0.007$) to take into consideration the number of genes evaluated. This represents a generally acceptable correction strategy; one not as stringent as a strict Bonferroni, yet still taking the number of totally independent analyses into account. This correction is not employed in the haplotype or gene–gene interaction analyses, as these are truly exploratory in nature and are restricted by sample size. Clearly, replication of these studies is critical, as well as

Table 1. SNP alleles and frequencies by ethnicity

Gene	SNP	Chr	Base pair	Order in gene	Alleles[a]	European American MAF[b]	Hispanic corresponding frequency[c]	*P*-value
Wnt3	rs142167	17	42150418	1	A/G	0.258	0.523	<0.0001
Wnt3	rs7216231	17	42170907	2	A/G	0.055	0.409	<0.0001
Wnt3	rs199525	17	42203002	3	G/T	0.22	0.159	0.07
Wnt3	rs70602	17	42214876	4	C/T	0.219	0.149	0.041
Wnt3	rs199501	17	42217772	5	A/G	0.256	0.538	<0.0001
Wnt3	rs199498	17	42220763	6	C/T	0.241	0.489	<0.0001
Wnt3	rs111769	17	42227151	7	C/T	0.416	0.258	<0.0001
Wnt3	rs3851781	17	42246300	8	C/T	0.474	0.657	<0.0001
Wnt3	hCV1139279	17	42248220	9	C/G	0.198	0.11	0.005
Wnt3	rs9890413	17	42256448	10	A/G	0.363	0.215	0.0002
Wnt3A	rs708111	1	226257988	1	C/T	0.483	0.524	0.290
Wnt3A	rs708114	1	226264119	2	C/T	0.469	0.557	0.034
Wnt3A	rs3094912	1	226276438	3	A/T	0.454	0.5	0.228
Wnt3A	rs3121310	1	226291447	4	A/G	0.308	0.382	0.055
Wnt3A	rs752107	1	226313974	5	C/T	0.308	0.356	0.175
Wnt3A	rs1745420	1	226318355	6	C/G	0.144	0.178	0.193
Wnt5A	rs566926	3	55487719	1	A/C	0.261	0.333	0.55
Wnt7A	rs1124480	3	13832970	1	C/T	0.459	0.418	0.274
Wnt7A	rs9840696	3	13840076	2	A/G	0.375	0.392	0.651
Wnt7A	rs6778046	3	13846680	3	C/T	0.369	0.415	0.212
Wnt7A	rs9863149	3	13858361	4	C/T	0.315	0.216	0.004
Wnt7A	rs934450	3	13862730	5	A/G	0.329	0.257	0.037
Wnt7A	rs1433354	3	13872246	6	A/G	0.305	0.453	<0.0001
Wnt7A	rs6442414	3	13881839	7	A/G	0.312	0.221	0.008
Wnt7A	rs11128663	3	13887851	8	C/G	0.4	0.297	0.005
Wnt7A	rs4685048	3	13897736	9	A/C	0.487	0.412	0.046
Wnt8A	rs4835761	5	137445768	1	A/G	0.469	0.489	0.619
Wnt8A	rs2040862	5	137447888	2	C/T	0.172	0.108	0.030
Wnt8A	rs2306110	5	137455986	3	A/C	0.477	0.489	0.765
Wnt9B	rs2165846	17	42296365	1	A/G	0.421	0.720	<0.0001
Wnt9B	rs1530364	17	42306776	2	A/G	0.261	0.317	0.128
Wnt9B	rs197915	17	42345521	3	A/G	0.434	0.432	0.972
Wnt11	rs663746	11	75571777	1	C/T	0.498	0.474	0.524
Wnt11	rs1533763	11	75578175	2	A/T	0.209	0.172	0.272
Wnt11	rs1533767	11	75583448	3	A/G	0.267	0.279	0.718
Wnt11	rs689095	11	75591735	4	A/G	0.307	0.324	0.667
Wnt11	rs596339	11	75594155	5	A/G	0.338	0.412	0.06
Wnt11	rs1568507	11	75596967	6	A/T	0.315	0.324	0.82

[a]Most common allele are listed first.
[b]Minor allele frequency.
[c]Frequency in Hispanic of European American minor allele.

examining gene sequences to identify etiologic susceptibility mutations.

Interestingly, three of these genes, Wnt3A, Wnt11 and Wnt8A, are involved in neural crest cell (NCC) differentiation and migration. NCCs are derived in the neural tube and migrate into the pharyngeal/branchial arches and differentiate into connective tissue and bone of the head and neck (26,27). Several mouse studies have demonstrated that genes inactivated in NCCs (i.e. Tgfbr2, Tcof1 and Ap-2alpha) cause orofacial clefting, as well as other malformations (28–30).

Wnt3A, in mice, has been shown to control the fate of both mesenchymal and NCCs in the craniofacial processes and to regulate palatal fusion (22). Embryonic expression of Wnt3A is upregulated by fibroblast growth factor 8, which is highly expressed in the facial primordia and is also an important regulator of craniofacial development (31,32). Additionally, Wnt3A signaling is also decreased when fibroblast growth factor receptor 1, which regulates the epithelial-mesenchymal transformation necessary for normal palatal development, is inactivated (31). We found that variation in Wnt3A and Wnt3A haplotypes are associated with NSCLP (Tables 2–4). Additionally, we showed that variation in Wnt3A interacts with other Wnt genes expressed in NCCs in NSCLP (Table 5). Of the six SNPs genotyped in Wnt3A, five were found to be associated with NSCLP. rs752107 is in the 3′-UTR region of the gene and the other SNPs were either intronic or flanked the gene. Therefore, while none of these SNPs code for the Wnt3A protein, the results suggest that Wnt3A is involved in the etiology of NSCLP.

Embryogenic studies have shown that Wnt11 is necessary for directing migrating NCCs, which will later form ectomesenchyme of the developing face (17,26). Also, bone morphogenic protein 4 (BMP4), which functions in the same pathway as Msx homeobox genes, is necessary for upper labial fusion in mice (32,33), and downregulates Wnt11 during normal urogenital development (34). Inactivation of BMP4 causes cleft lip in mice; therefore, the relationship between BMP4 and Wnt11 suggests that variation in Wnt11 could play an

Table 2. Results of association testing in complete dataset[a]

Gene	SNP	PDT	Geno-PDT
Wnt3	rs142167	0.147	0.278
Wnt3	rs7216231	0.359	0.584
Wnt3	rs199525	0.353	0.636
Wnt3	rs70602	0.132	0.302
Wnt3	rs199501	0.190	0.249
Wnt3	rs199498	0.269	0.220
Wnt3	rs111769	0.498	0.331
Wnt3	rs3851781	0.914	0.219
Wnt3	hCV1139279	0.299	0.519
Wnt3	rs9890413	0.853	0.741
Wnt3A	rs708111	0.127	0.310
Wnt3A	rs708114	0.378	0.570
Wnt3A	rs3094912	0.334	0.352
Wnt3A	rs3121310	0.034	0.088
Wnt3A	rs752107	0.021	0.022
Wnt3A	rs1745420	**0.006**	0.009
Wnt7A	rs1124480	0.290	0.314
Wnt5A	D3S3719	0.320	0.340
Wnt5A	rs566926	**0.002**	0.008
Wnt5A	D3S2408	0.757	0.358
Wnt7A	rs9840696	0.272	0.483
Wnt7A	rs6778046	0.172	0.049
Wnt7A	rs9863149	0.183	0.415
Wnt7A	rs934450	0.209	0.454
Wnt7A	rs1433354	0.228	0.437
Wnt7A	rs6442414	0.665	0.091
Wnt7A	rs11128663	0.229	0.332
Wnt7A	rs4685048	0.010	0.049
Wnt8A	rs4835761	0.490	0.067
Wnt8A	rs2040862	0.306	0.045
Wnt8A	rs2306110	0.535	0.164
Wnt9B	rs2165846	0.962	0.971
Wnt9B	rs1530364	0.869	0.939
Wnt9B	rs197915	0.469	0.149
Wnt11	rs663746	0.083	**<0.001**
Wnt11	rs1533763	0.199	0.378
Wnt11	rs1533767	0.099	0.009
Wnt11	rs689095	0.386	0.151
Wnt11	rs596339	0.747	0.747
Wnt11	rs1568507	0.124	0.178

[a]All SNPs with $P < 0.01$ are shown and $P \leq 0.007$ are bolded.

etiological role in NSCLP (33). Of the four SNPs in Wnt11 that were associated with our European American sample, irrespective of FH, the most significant association was with rs1533767, a synonymous SNP in exon 3 ($P = 0.001$; Table 3). The other three SNPs were in non-coding regions. While synonymous SNPs do not alter protein sequence, these SNPs may be in linkage disequilibrium with a functional mutation or they might alter amino acid translation time. Either scenario could be detrimental to gene function (35). Haplotypes of Wnt11 SNPs were also associated with clefting in the European American sample (Table 4), giving further support that Wnt11 may play an etiological role in the development of NSCLP in that population.

We previously identified a region on chromosome 3p21–14 between two short-tandem repeat (STR) markers that may harbor a clefting gene (36). Wnt5A is a candidate gene in this region because *in situ* hybridization studies have shown that Wnt5A is expressed in the frontonasal prominence and maxillary process, which fuse to form the primary palate (21). Wnt5A intronic SNP rs566926 ($P = 0.001$) is associated with NSCLP in the overall European American sample. While this SNP does not alter protein sequence, recent studies have shown associations with intronic SNP variants in other complex disorders (5,7,10,11,37–39). Gene–gene interaction between Wnt5A and Wnt3A was observed in the Hispanic sample ($P = 0.039$; Table 5), suggesting that variation in genes that regulate neural crest cells (NCCs) from cell fate determination (Wnt3A) to differentiation (Wnt5A) may predispose to an orofacial cleft.

While there is clearly overlap in the associated SNPs between ethnicities and across FH, there are some differences. These differences may be the result of a number of factors. The sample sizes in some stratifications are not extensive, so that genes with a smaller effect may not be detected. It is also likely that there are different genes acting in different populations. Our negative FH subgroups are likely to be composed of two different susceptible populations. One group has a higher genetic risk, and by chance, there is only a single affected individual and another group that has a lower genetic risk. The genes may be different between the two groups. Additionally, this model suggests that variation in individual Wnt genes and/or multiple genes acting to direct craniofacial morphogenesis, but on different genetic backgrounds, may ultimately be used to define those at high and low risk for NSCLP. This would explain the multiplex families in which NSCLP is present in several relatives but not in a Mendelian pattern. It would also explain those families that appear to have a lower liability.

Given the extensive role that the Wnt signaling pathway genes plays in craniofacial development, and the number of orofacial clefting syndromes caused by mutations in Wnt genes, it is not surprising that we found association with SNPs in the Wnt genes in our NSCLP sample (15,16,18,19,40–42). Of importance is the finding that variation within Wnt genes specifically involved in NCC development and migration (Wnt3A, Wnt5A and Wnt11) were the most strongly associated with NSCLP. Wnt genes clearly play a critical role during cephalogenesis and our results suggest that variation in the Wnt family of genes plays an etiological role in NSCLP.

MATERIALS AND METHODS

Study population and sample preparation

The study sample was composed of 132 multiplex NSCLP families and 235 simplex parent–child trios and 199 duos which have been described previously (36,43,44). Each family was ascertained through a proband affected with NSCLP from one of three craniofacial centers: Children's Hospital, Boston, Texas Children's Hospital, Houston, and the University of Texas Craniofacial Clinic, Houston. Probands and relatives were examined to exclude syndromes known to be associated with any type of orofacial clefting. Ethnicity (Hispanic versus European American) was determined by self-report. After obtaining informed consent, blood or saliva samples were collected and DNA was extracted using either the Roche DNA Isolation Kit for Mammalian Blood (Roche, Switzerland) or the Oragene Purifier for

Table 3. Results of association testing by ethnicity and FH[a]

Gene	SNP	All			Positive FH			Negative FH		
		PDT	Geno-PDT	APL	PDT	Geno-PDT	APL	PDT	Geno-PDT	APL
European Americans										
Wnt3	rs70602	0.154	0.308	0.041	0.309	0.585	0.009	0.299	0.426	0.365
Wnt3	rs199498	0.162	0.239	0.161	0.087	0.139	0.006	1.000	0.941	0.947
Wnt3	rs9890413	0.948	0.698	0.593	0.932	0.216	0.504	0.840	0.005	0.835
Wnt3A	rs752107	0.023	0.027	**0.003**	0.31	0.441	0.125	0.0178	0.027	0.009
Wnt5A	rs566926	**0.005**	0.016	**0.004**	0.043	0.107	0.049	0.0462	0.144	0.02
Wnt8A	rs2040862	0.352	0.055	0.061	0.581	0.372	0.085	0.009	0.016	0.001
Wnt9B	rs197915	0.160	0.051	0.573	0.072	0.180	0.003	0.926	0.060	0.310
Wnt11	rs663746	0.067	0.001	0.449	0.104	0.047	0.278	0.361	0.014	0.849
Wnt11	rs1533767	0.114	0.012	0.001	0.744	0.010	0.100	0.006	0.016	0.003
Hispanics										
Wnt3A	rs708111	NS	NS	NS	**0.034**	0.135	**0.007**	0.297	0.426	**0.005**
Wnt3A	rs3094912	0.343	0.572	**0.031**	NS	NS	NS	0.162	0.240	**<0.001**
Wnt3A	rs1745420	0.083	0.157	**<0.001**	0.096	0.264	**0.009**	0.346	0.376	**0.008**

[a]All SNPs with $P < 0.01$ are shown and $P \leq 0.007$ are bolded.

Table 4. Overtransmitted haplotypes by ethnicity

Gene	SNP1	SNP order[a]	SNP2	SNP order	Overtransmitted haplotype	*P*-value[b]
European Americans						
Wnt3	rs142167	1	rs199525	3	2.1	0.049
Wnt3	rs7216231	2	rs199525	3	2.1	0.033
Wnt3	rs199525	3	rs70602	4	1.2	0.05
Wnt3A	rs708111	1	rs752107	5	2.1	0.010
Wnt3A	rs708114	2	rs752107	5	1.1	0.014
Wnt3A	rs3094912	3	rs752107	5	1.1	0.037
Wnt3A	rs3121310	4	rs752107	5	2.1	0.017
Wnt3A	rs752107	5	rs1745420	6	1.2	**0.001**
Wnt8A	rs4835761	1	rs2306110	3	1.1	0.05
Wnt11	rs663746	1	rs1533767	3	2.2	0.047
Wnt11	rs1533763	2	rs596339	5	2.2	0.012
Wnt11	rs1533767	3	rs1568507	6	2.2	0.013
Hispanics						
Wnt3	rs199498	6	rs9890413	10	2.2	0.047
Wnt3A	rs708111	1	rs3121310	4	1.1	0.022
Wnt3A	rs708111	1	rs752107	5	1.1	0.008
Wnt3A	rs708111	1	rs1745420	6	1.2	**0.007**
Wnt3A	rs708114	2	rs3094912	3	2.1	**0.004**
Wnt3A	rs708114	2	rs1745420	6	2.2	**<0.001**
Wnt3A	rs3094912	3	rs3121310	4	1.1	**<0.001**
Wnt3A	rs3094912	3	rs752107	5	1.1	**<0.001**
Wnt3A	rs3094912	3	rs1745420	6	1.2	**0.003**
Wnt3A	rs3121310	4	rs752107	5	1.1	0.027
Wnt3A	rs3121310	4	rs1745420	6	1.2	**<0.001**
Wnt3A	rs752107	5	rs1745420	6	1.2	**<0.001**
Wnt8A	rs4835761	1	rs2306110	3	2.2	0.049

[a]SNP order: order of SNP within the gene.
[b]All SNPs with $P < 0.05$ are shown and $P \leq 0.007$ are bolded.

saliva (DNA Genotek, Inc., Ontario, Canada) following the manufacturer's protocol.

Genotyping

Thirty-eight SNPs in seven candidate genes [Wnt3 (three flanking and seven intragenic), Wnt3A (two flanking and four intragenic), Wnt5A (one intragenic), Wnt7A (two flanking and seven intragenic), Wnt8A (two flanking and one intragenic), Wnt9B (one flanking and two intragenic) and Wnt11 (2 flanking and 4 intragenic)] and two STRs flanking Wnt5A were evaluated in this study (Table 1). SNPs were selected based on heterozygosity, tagging ability and position in or around the gene as previously described (5). SNPs were genotyped using ABI TaqMan Genotyping Assays following manufacturer's protocol and were detected on an ABI 7900HT Sequence Detection System (Foster City, CA, USA). Two microsatellite markers flanking Wnt5A were genotyped, as

Table 5. Results of gene–gene interaction analysis[a]

Ethnicity	Gene1/SNP	Gene2/SNP	GEE *P*-value
European American	Wnt3A/rs752107	Wnt3/rs70602	**0.004**
Hispanic	Wnt3A/rs1745420	Wnt5A/rs566926	0.039

[a]SNPs with $P < 0.05$ are shown and $P < 0.007$ is bolded.

previously described (UCSC database – genome.ucsc.edu) (36).

Statistical methods

In the initial association analysis, the entire data set was considered. In addition, probands were stratified by ethnicity alone or by ethnicity and FH. Allele frequency differences and Hardy–Weinberg equilibrium were calculated using SAS (v9.1). Pairwise linkage disequilibrium values (D' and r^2) were calculated using GOLD (graphical overview of linkage disequilibrium) (45). To extract the maximum amount of information from the dataset, multiple approaches for assessing linkage and/or association were used. Parametric and non-parametric linkage analyses were conducted using multipoint engine for rapid likelihood inferences (MERLIN) (46) and linkage parameters were defined, as previously reported (43). The PDT, Geno-PDT and APL test were used to evaluate evidence for association (47–49). This panel of analytic tools was chosen because each has different strengths and assumptions. PDT is an extension of the transmission disequilibrium test and allows for the incorporation of extended pedigrees in the analysis of allelic association. The Geno-PDT examines association between marker genotypes and disease while APL allows for missing parental genotypes. Each program may be more powerful for certain genetic models and for different pedigree structures. In addition, APL can be used to examine multi-marker haplotypes; it was employed to look for overtransmission of two-marker haplotypes within genes. However, since APL makes genotype assignments based on allele frequencies, it is not appropriate for analyzing multi-ethnic datasets where the allele frequencies can vary greatly between the specific groups. Lastly, gene–gene interactions of SNPs found to be significant with association studies were examined by GEE as implemented in SAS (50). Only two-way interactions were tested with GEE because of the limited sample size.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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