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Search for Genetic Modifiers of *IRF6* and Genotype-Phenotype Correlations in Van der Woude and Popliteal Pterygium Syndromes

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Abstract

Van der Woude syndrome is the most common form of syndromic orofacial clefting, accounting for 1-2% of all patients with cleft lip and/or cleft palate. Van der Woude and popliteal pterygium syndromes are caused by mutations in *IRF6*, but phenotypic variability within and among families with either syndrome suggests that other genetic factors contribute to the phenotypes. The aim of this study was to identify common variants acting as genetic modifiers of *IRF6* as well as genotype-phenotype correlations based on mutation type and location. We identified an association between mutations in the DNA-binding domain of *IRF6* and limb defects (including pterygia). Although we did not detect formally significant associations with the genes tested, borderline associations suggest several genes that could modify the VWS phenotype, including *FOXE1*, *TGFB3*, and *TFAP2A*. Some of these genes are hypothesized to be part of the *IRF6* gene regulatory network and may suggest additional genes for future study when larger sample sizes are also available. We also show that families with the Van der Woude phenotype but in whom no mutations have been identified have a lower frequency of cleft lip, suggesting there may be locus and/or mutation class differences in Van der Woude syndrome.

Keywords

Modifier gene; Van der Woude; popliteal pterygium; cleft; lip pit

INTRODUCTION

Van der Woude syndrome (VWS; OMIM #119300) is the most common form of syndromic clefting, accounting for approximately 2% of all patients with cleft lip and/or palate, with a

prevalence of 1/34,000 live births [Burdick 1986]. VWS is highly penetrant, with penetrance estimated to range from 89-99%, but with remarkably variable expressivity [Burdick *et al.* 1985]. Individuals with VWS can present with a range of clefting phenotypes, including cleft lip (CL), cleft lip and palate (CLP), and cleft palate only (CP). The primary feature distinguishing VWS from nonsyndromic clefting is the presence of paramedian lower lip pits or mounds [Van Der Woude 1954]. Lip pits are observed in 83% of individuals diagnosed with VWS and are the only phenotypic feature seen in 44% of affected individuals [Burdick *et al.* 1985]. Lip pits and orofacial clefts are also observed in popliteal pterygium syndrome (PPS; OMIM #119500), but unlike VWS, PPS is further characterized by skin folds, genital anomalies, syndactyly, oral adhesions, and ankyloblepharon [Gorlin *et al.* 1968]. Even within families, there can be a broad range of severity in each of these two disorders, suggesting that other genetic or environmental factors play roles in determining the phenotypic features of VWS and PPS.

VWS and PPS are allelic, autosomal dominant disorders caused by mutations in *Interferon Regulatory Factor 6 (IRF6)* [Kondo *et al.* 2002]. IRF6 belongs to a family of nine transcription factors with a highly conserved DNA-binding domain and a less conserved protein-binding domain [Tamura *et al.* 2008]. Mutations in patients with VWS are divided between missense and truncation mutations (including whole gene deletions), suggesting that haploinsufficiency is a likely mechanism underlying VWS [de Lima *et al.* 2009]. On the other hand, there is a strong correlation between PPS and missense mutations at residues of the DNA-binding domain predicted to contact the DNA, suggesting that PPS-causing mutations have a different effect on IRF6 function than do VWS-causing mutations. Despite these general genotype-phenotype differences between PPS and VWS, notably, mutations causing VWS are enriched in the DNA-binding domain and can also occur at residues contacting DNA [de Lima *et al.* 2009; Little *et al.* 2009]. Therefore, the presence and severity of the additional PPS-associated phenotypic features may in part be due to additional genetic or environmental factors.

We hypothesized that genetic variation in or around genes contributing to other forms of clefting contribute to the variable phenotype of individuals affected with VWS and PPS. Although hundreds of genes have been implicated in clefting through genetic and expression studies in humans and mice [Dixon *et al.* 2011; Marazita 2012], here we perform a candidate gene association study of VWS and PPS to identify common genetic variation associated with these phenotypes using genes identified in genome wide association or human mutation searches as high priority candidates. We also examined phenotypic correlations with the type and location of *IRF6* mutations in these families.

MATERIALS AND METHODS

Candidate Genes

Twenty-eight SNPs from 12 candidate genes or loci (*IRF6*, 8q24, *MAFB*, *ABCA4*, *VAX1*, *FOXE1*, *TGFA*, *TFAP2A*, *FGFR2*, *BMP4*, *TGFB3*, and *RIPK4*) were chosen for analysis. SNPs for *IRF6*, 8q24, *MAFB*, *ABCA4*, and *VAX1* were selected from SNPs with the lowest p-values from previous genome wide association studies (GWAS) [Beatty *et al.* 2010; Birnbaum *et al.* 2009; Ludwig *et al.* 2012; Mangold *et al.* 2010]. Several SNPs near *TFAP2A* and *RIPK4* were selected because mutations in these genes cause clefting syndromes with phenotypes that overlap VWS or PPS [Kalay *et al.* 2012; Milunsky *et al.* 2008; Mitchell *et al.* 2012]. *FOXE1*, *TGFA*, *FGFR2*, *BMP4*, and *TGFB3* were selected because of compelling statistical or supporting biological evidence [Dixon *et al.* 2011; Marazita 2012]. Supplemental Table I summarizes the genes selected and types of supporting data available in the literature. The SNPs for *TFAP2A*, *RIPK4*, *FOXE1*, *TGFA*,

FGFR2, *BMP4*, and *TGFB3* were chosen to interrogate the haplotype block structure of the relevant gene while also considering practicality and cost.

Samples

A total of 1,057 DNA samples from 225 families were used, with a total of 567 affected individuals. The families are from the U.S., Philippines, Brazil, Colombia, and other European countries (Supplemental Table II). Approval for all protocols was obtained from the Institutional Review Boards at the University of Iowa. All subjects were examined by a clinical geneticist or genetic counselor, and diagnoses were made as described [Kondo *et al.* 2002]. Affected subjects were given a phenotypic classification of cleft lip (CL), cleft lip with cleft palate (CLP), cleft palate (CP), lip pits only (PO), CL with lip pits (PCL), CLP with lip pits (PCLP), or CP with lip pits (PCP). Additional phenotypic classifications included the presence of dental anomalies (hypodontia, dental aplasia, or malocclusion), limb anomalies (cutaneous syndactyly, polydactyly, or contractures), or popliteal pterygia (Table I). Finally, family level phenotypes were assigned for each of these classifications based on the presence or absence of each phenotype in the affected family members (including family members without a DNA sample, there were 816 affected individuals).

Genotyping, sequencing, mutation classification

Mutation analysis for *IRF6* was performed as described [de Lima *et al.* 2009]. Mutations for affected individuals in this study are available in Supplemental Table III. Genotype analysis was performed as described [Rahimov *et al.* 2008]. *IRF6* mutations were classified as truncating (large deletions, frameshift, nonsense, splicing, and those predicted to alter the translation start site) or non-truncating (missense mutations and small in-frame deletions or insertions). Mutations were also classified by domain [de Lima *et al.* 2009]. Within the DNA-binding domain, mutations were divided into those that contact DNA (P12, W13, L14, W40, A43, R45, H46, W60, K66, E69, A77, K80, Q82, R84, C85, N88, K89, K101, and E102) and those that do not contact DNA.

Analysis

All SNPs were tested for departures from Hardy-Weinberg equilibrium (HWE) using a threshold of $p = 0.001$ but recognizing that the mixed ancestry of our populations make HWE testing a poor metric for data quality. (Table II).. The Family Based Association Test (FBAT) [Horvath *et al.* 2001] was used to test for association of both single markers and haplotypes with VWS phenotypes. We utilized the $-e$ option in FBAT, which allows for the adjustment for multiple affecteds or multiple nuclear families within a pedigree by adjusting the variance used in the Z-score calculation. Table I summarizes the phenotypes of affected individuals. Larger groups for analysis were made by combining all families; all individuals with a cleft lip (CL/P: CL or CLP); all individuals with a cleft palate (CP/L: CLP or CP); all individuals with lip pits (LP: PO, PCL, PCLP, and PCP); and those with clefts but no lip pits. The threshold p-value for modifier analysis was determined using a Bonferroni correction ($p=0.05/196$ ($12 \text{ loci} \times 14 \text{ phenotypes}$)= 0.0003).

We performed genotype-phenotype correlation analyses between *IRF6* mutations and family level phenotypes with Fisher's exact test using STATA. Exclusion criteria for this analysis included all individuals diagnosed with VWS but without a known *IRF6* mutation, individuals without specific descriptions of the craniofacial features (i.e., cleft type or presence of lip pits), and individuals with a cleft phenotype but where the familial *IRF6* mutation was not identified by direct sequencing (i.e., potential phenocopies). We then assumed that all affected individuals within the same family shared the same mutation. A broader genotype-phenotype correlation analysis compared family-level phenotypes between *IRF6* mutation-positive families and *IRF6* mutation-negative families. The

threshold p-value for these genotype-phenotype correlation analyses was also calculated using a Bonferroni correction ($p=0.05/11$ phenotypes= 0.005).

RESULTS

Genetic modifiers of the VWS phenotype

To test for genetic modifiers of the VWS phenotype, we classified the VWS phenotype into 11 phenotypic groups and genotyped 225 families for 28 SNPs (Table II). The results of the TDT analyses are depicted in Figure 1, and the most significant results are detailed in Table III (all results are available in Supplemental Table IV). None of the results reached formal levels of significance; however, several results were interesting in that they had p values (uncorrected) of less than 0.05. For example, rs3758249 (*FOXE1*) was associated with CL/P ($p=0.03$) as was the G-C haplotype of *FOXE1* ($p=0.007$). Similarly, rs2235371 (*IRF6*) and rs7278103 (*RIPK4*) were also associated with CL/P ($p=0.02$ for both SNPs).

Lip pits are a distinguishing feature of VWS but are not always present, so we looked for associations with the presence or absence of lip pits (Figure 1C). The SNPs rs2235371 (*IRF6*), rs2902345 (*TGFA*), and rs2268625 (*TGFB3*) were associated with lip pits, without regard for cleft phenotype ($p=0.03$) while rs2268625 (*TGFB3*) and rs7278103 (*RIPK4*) were associated with PO ($p=0.03$ for both SNPs). Although neither *TFAP2A* SNP was significant independently, the T-T haplotype was associated with PO ($p=0.01$). We did not observe any associations with clefts without lip pits.

Another indicator of genetic modifiers of the VWS phenotype could be an association between ancestry and these phenotypes. Genetic markers can be used to distinguish continental population groups on the basis of differing minor allele frequencies between populations [Rosenberg *et al.* 2002]. Therefore, variants with differing allele frequencies between populations could act as modifiers of the VWS phenotype in a population-specific manner. To determine if VWS phenotypic heterogeneity was associated with ancestry/population, we analyzed the phenotypes of VWS families with *IRF6* mutations by population, when known. While there was variation in the frequency of these phenotypes between populations, these differences were not significantly different.

Mutations in the DNA-binding domain are associated with limb defects

We next performed association analyses in 124 families to determine if either the type or location of *IRF6* mutations was correlated with any VWS or PPS phenotypes (Table IV). Neither missense mutations nor truncation mutations were associated with any of the clefting phenotype categories. In five of the 124 families, the only phenotype present was lip pits; there was no association between the mutation type or domain and the risk of having a cleft.

Limb defects were associated with missense mutations ($p=0.03$). Families with missense mutations were 3.1 times more likely to have limb defects than those with truncation mutations (95% confidence interval (CI) 1.05-9.56). We found that mutations in the DNA binding domain, and more specifically in residues contacting DNA, were significantly associated with limb defects ($p=0.002$ and $p=0.001$, respectively). Families with mutations at these residues were 13 times more likely to have limb defects than those with mutations elsewhere in the DNA-binding domain (CI: 2.6-65.2). Interestingly, these associations were not driven by the *IRF6* p.Arg84Cys or p.Arg84His mutations, which commonly cause PPS, as families with missense mutations and limb defects were evenly split between p.Arg84Cys or p.Arg84His and other mutations. Although PPS is highly associated with mutations in the DNA-binding domain, we did not detect strong associations with the pterygia phenotype.

This result was likely due to insufficient power as there were only eight families with pterygia.

We also detected a nominally significant association between mutations in the protein-binding domain and PO ($p=0.01$). Families with mutations in this domain were 2.9 times more likely to have family members with PO than families with mutations in the rest of *IRF6* ($p=0.008$, $OR=2.9$; $CI: 1.3-6.8$). Within the families that had PO and mutations in this domain, the mutation type was evenly divided between truncation and missense; no single mutation predominated.

Mutation negative families are less likely to have cleft lip

Of the 225 families included in this study, 56 (25%) did not have detectable mutations in *IRF6*. We compared the phenotypes of these mutation-negative families with the 124 families with *IRF6* mutations (Table V, Figure 2). Mutations in *IRF6* were significantly associated with all cleft lip phenotypes ($p=2.1 \times 10^{-4}$ for CL/P, $OR=3.5$; $CI: 1.8-6.9$). In addition, while 95.2% of families with *IRF6* mutations had lip pits, only 89.3% of mutation-negative families had lip pits ($p=0.14$). While this difference was not statistically significant, the differences in the lip pit phenotype led to the question of whether these mutation-negative families would exhibit linkage between their phenotypes and the *IRF6* locus. The average number of individuals per family was small (5.2), with small numbers of affected individuals (average=2.5), so there was insufficient power to perform linkage analyses. We used TDT to look for association between syndrome occurrence in these 56 families and the 12 candidate genes/loci. This analysis showed a non-significant association with rs10483873 (*TGFB3*, $p=0.02$), but the number of informative families was too low to draw conclusions (N informative families < 18).

DISCUSSION

VWS and PPS have considerable variability in phenotypes within and between families, prompting us to evaluate genetic variation in genes other than *IRF6* (that are known to be involved in craniofacial development) as modifiers of the VWS phenotype. Orofacial clefts are a heterogeneous group of disorders, and epidemiological [Grosen *et al.* 2010] and biological [Rahimov *et al.* 2008] data suggest that CL and CLP may have separate genetic etiologies. However, except for several interesting but not formally significant associations with *IRF6*, we did not detect associations between the candidate genes and the major clefting phenotypes (CL, CLP, CP). The rs2235371 SNP (*IRF6*) was associated using uncorrected values for significance with several phenotypes, but the minor allele frequency of this SNP was low (5%). Furthermore, the observed association was with the minor allele (A), which is contrary to the previous associations with nonsyndromic clefting [Rahimov *et al.* 2008; Zuccherro *et al.* 2004]. This result may reflect linkage of VWS to this SNP, rather than suggesting a role for this SNP as a modifier.

Clefts involving the lip are also heterogeneous, and may be either unilateral or bilateral; to date, no SNPs have been specifically associated with unilateral or bilateral CL. We found non-significant associations of *FOXEL* and *RIPK4* with CL/P and bilateral CL. *FOXEL* was identified as a cleft candidate gene by a genome-wide linkage scan and subsequent fine-mapping [Marazita *et al.* 2004; Moreno *et al.* 2009]. Expression of *Foxe1* has been described in the epithelium during fusion of the medial nasal and maxillary processes, consistent with a role in CL.

The feature distinguishing VWS from nonsyndromic clefting is the presence of paramedian lower lip pits. The association of *TGFB3* with lip pits regardless of the presence of a cleft suggests a role for *TGFB3* in the development of lip pits, although the role of *TGFB3* in lip

development has not been specifically examined. The *TGFB3* gene has been associated with clefting through animal models, in which *Tgfb3*^{-/-} mice have a cleft palate [Kaartinen *et al.* 1995; Proetzel *et al.* 1995]. We found evidence for *TGFA* as a modifier of the lip pit phenotype in VWS; recently *IRF6* and *TGFA* were shown to interact to increase risk for nonsyndromic CL/P [Letra *et al.* 2012]. A role for *TFAP2A* in lip pits is not surprising as mutations in *TFAP2A* cause branchiooculofacial syndrome, which has overlapping features with VWS, including cleft lip, cleft palate, and upper lip pits [Milunsky *et al.* 2008]. In addition, *TFAP2A* binds to an enhancer element upstream of *IRF6* [Fakhouri *et al.* 2012; Rahimov *et al.* 2008]. In contrast, a role for *RIPK4* in lip pits is unclear because a feature distinguishing Bartsocas-Papas syndrome (BPS) from PPS is the absence of lower lip pits in BPS. However, *Ripk4* is a transcriptional target of p63, which also regulates *Ir6*, so it is possible that variability in this pathway modifies the VWS or PPS phenotype, including the presence of lip pits.

We did not identify associations with SNPs near 8q24, *MAFB*, *ABCA4*, or *VAX1*, all of which have been associated with nonsyndromic clefting by GWAS [Beaty *et al.* 2010; Birnbaum *et al.* 2009; Mangold *et al.* 2010], or with candidate genes *FGFR2* or *BMP4*. While these results could indicate that common genetic variation at these loci does not contribute to VWS phenotypic variation, they may also reflect a lack of power. One limitation of this study was the sample size. Although we included a large number of families with VWS, the number of informative families for the various analyses (ranging from 0 to 76) depended on both the frequency of each phenotype in these families and on the minor allele frequencies of the SNPs. Another limitation was the ethnic heterogeneity of the samples, which included families from Brazil, Colombia, the Philippines, and the US, but 33% of the families were from unspecified populations (Supplemental Table II).

We also examined the types and locations of *IRF6* mutations to determine if these contributed to specific VWS phenotypes. We would have predicted that truncation mutations, which are most obviously damaging to the protein, would be associated with more severe phenotypes such as CLP. We did not find such an association, suggesting that both missense and truncation mutations result in haploinsufficiency of *IRF6* and result in similar phenotypes. The exception to this is the strong association between missense mutations in the DNA-binding domain (and more specifically, residues that contact DNA) with limb defects. This result was expected and was consistent with previous data showing a strong association between mutations at residues contacting DNA and PPS [de Lima *et al.* 2009]. In addition, we found a non-significant association between mutations in the protein-binding domain and lip pits only. There was no association when we grouped all individuals with lip pits together, suggesting that mutations in the protein-binding domain may not be related to the development of lip pits but rather may protect against the development of clefts.

Although statistically we only detected associations that met non-stringent ($p < 0.05$) significance, it is notable that they tended to be with genes related to the *IRF6* gene regulatory network, including *TFAP2A* [Rahimov *et al.* 2008], *FOXE1* [Venza *et al.* 2011], and *TGFB3* [Knight *et al.* 2006]. Recently, the *IRF6* gene-regulatory network has been expanded to include Pbx-1, 2, and 3 regulating p63 through Wnt9b and Wnt3 [Ferretti *et al.* 2011]. We propose that these genes and *TP63*, which regulates *IRF6* through the enhancer MCS9.7 [Fakhouri *et al.* 2012; Moretti *et al.* 2010; Rahimov *et al.* 2008; Thomason *et al.* 2010], be investigated in future modifier studies.

The MCS9.7 variant was not associated in this study (through rs642961); however, its expression pattern does not completely recapitulate the expression of *IRF6* [Fakhouri *et al.* 2012], suggesting that *IRF6* expression is controlled by additional regulatory elements. It is

possible that variation in these yet to be discovered regulators and regulatory elements contribute to the variable expressivity of VWS and PPS. We also showed that the mutation-negative families are less likely to have individuals with cleft lip. The causal mutations in the *IRF6*-mutation-negative families may lie in these undiscovered regulatory elements. A second locus for VWS has been reported on 1p34 by linkage [Koillinen *et al.* 2001], and a mutation has been described in *WDR65*, although not in the linkage family [Rorick *et al.* 2011]. Locus heterogeneity could explain the phenotypic differences in the mutation-negative families. Although the association with *TGFB3* was not formally significant, this gene may contribute directly or indirectly (as a modifier) to the development of VWS in the families in which a mutation has not been identified.

Although VWS is highly penetrant, there are individuals who are obligate carriers with no phenotypic expression, and VWS family members with only a cleft or only lip pits may have additional subtle phenotypes. There is increasing evidence that subclinical phenotypes are part of the expression of nonsyndromic clefting risk genes in family members with no overt cleft. For example, sub-epithelial discontinuities in the *orbicularis oris* muscle (OOM) [Neiswanger 2007; Rogers *et al.* 2008] and lip print whorls [Neiswanger *et al.* 2009] are significantly increased in relatives of individuals with clefts versus controls with no family history of craniofacial anomalies. Notably, we found that the unaffected father of an individual with VWS had OOM discontinuities and carried the same mutation as his child (unpublished results, Schutte, Marazita). Also, lip print whorls are seen in the same paramedian region where lip pits are found, even when no pitting or mounding is evident [Neiswanger *et al.* 2009]. Therefore, additional assessment of such subclinical phenotypes could also be instrumental in the genetic dissection of VWS phenotypic expression.

Here, we hypothesized that common variation contributes to VWS and PPS phenotypic expression, but it is equally likely that rare variants play a role. As sequencing costs have decreased, exome and whole genome sequencing have been used to uncover the causes of dozens of Mendelian disorders [Bamshad *et al.* 2011]. We still do not have a good understanding of the variable expressivity seen in many of these disorders, including VWS. Eventually, we will need to revisit the families in which a mutation has already been identified using deep phenotyping and exome sequencing, targeted sequencing, or whole genome sequencing, to fully understand the genetic contributions to these Mendelian phenotypes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Results of TDT for phenotypic subgroups

(A) TDT for main clefting phenotypes: VWS - Van der Woude syndrome, CL - cleft lip, CLP - cleft lip and palate, CP - cleft palate. (B) TDT for cleft lip phenotypes: CL±P - Cleft lip with or without cleft palate, BL CL - bilateral cleft lip, UL CL - unilateral cleft lip. (C) TDT for lip pit phenotypes: no lip pits, lip pits only, and lip pits with or without a cleft.

Figure 2. Comparison of phenotypes between mutation-positive and mutation-negative VWS families

56 mutation-negative families (white) and 124 families with *IRF6* mutations (black) were compared for 11 phenotypes. CL - cleft lip, CLP - cleft lip and palate, CL/P - cleft lip with or without cleft palate, CPO - cleft palate only, CP/L - cleft palate with or without cleft lip.

** $p < 0.01$ *** $p < 0.0001$

Table I

Phenotypic classification of affected individuals.

<u>Main Phenotypes</u>	<u># Individuals</u>
Cleft lip and lip pits (PCL)	55
Bilateral CL	20
Unilateral CL	21
Cleft lip and palate and lip pits (PCLP)	182
Bilateral CL	77
Unilateral CL	32
Cleft palate and lip pits (PCP)	128
Lip pits only (PO)	184
Cleft palate (CP)	87
Cleft lip and palate (CLP)	54
Bilateral CL	19
Unilateral CL	7
Cleft lip (CL)	24
Bilateral CL	4
Unilateral CL	1
TOTAL MAIN PHENOTYPES	714
<u>Other Phenotypes</u>	
Dental anomalies	64
Limb anomalies	57
Pterygia	15
TOTAL with other phenotypes	136

Note: There were individuals with unknown laterality (14 PCL, 73 PCLP, 28 CLP, 19 CL).

Table II

Alleles and frequencies of SNPs studied

Chr	SNP	bp (Hg19)	Gene	Minor Allele	Major Allele	MAF ^a	N ^b	HWE
1	rs560426	94553438	<i>ABCA4</i>	G	A	0.433	339	0.023
1	rs481931	94570016	<i>ABCA4</i>	A	C	0.389	291	0.122
1	rs4147811	94575056	<i>ABCA4</i>	A	G	0.408	289	0.112
1	rs2235371	209964080	<i>IRF6</i>	A	G	0.05	339	0.117
1	rs642961	209989270	<i>IRF6</i>	A	G	0.187	329	0.162
2	rs2902345	70716599	<i>TGFA</i>	T	C	0.45	319	0.031
2	rs454305	70736219	<i>TGFA</i>	G	A	0.316	307	0.038
6	rs303050	10403183	<i>TFAP2A</i>	C	T	0.176	322	0.383
6	rs1675414	10412188	<i>TFAP2A</i>	C	T	0.408	298	0.082
8	rs987525	129946154	8q24	A	C	0.203	308	0.006
9	rs3758249	100614140	<i>FOXE1</i>	A	G	0.312	316	0.034
9	rs4460498	100620412	<i>FOXE1</i>	T	C	0.285	279	0.002
10	rs7078160	118827560	<i>VAX1</i>	A	G	0.186	269	0.003
10	rs3750817	123332577	<i>FGFR2</i>	T	C	0.398	324	0.027
10	rs2981582	123352317	<i>FGFR2</i>	T	C	0.392	297	0.580
14	rs17563	54417522	<i>BMP4</i>	T	C	0.486	322	0.044
14	rs2268625	76439634	<i>TGFB3</i>	C	T	0.20	330	0.101
14	rs2284792	76443579	<i>TGFB3</i>	C	T	0.299	308	0.049
14	rs10483873	76508770	<i>TGFB3</i>	A	G	0.343	312	0.163
20	rs17820943	39268516	<i>MAFB</i>	T	C	0.385	312	0.683
20	rs13041247	39269074	<i>MAFB</i>	C	T	0.385	321	0.592
20	rs11696257	39270816	<i>MAFB</i>	T	C	0.399	301	0.585
21	rs3171532	43160015	<i>RIPK4</i>	A	G	0.409	285	0.272
21	rs7278103	43163288	<i>RIPK4</i>	A	C	0.133	290	0.359
21	rs6586235	43174436	<i>RIPK4</i>	C	A	0.493	283	0.594
21	rs3850705	43180208	<i>RIPK4</i>	C	T	0.260	275	1.000
21	rs2309107	43184597	<i>RIPK4</i>	A	G	0.241	276	0.452
21	rs4919944	43185765	<i>RIPK4</i>	A	C	0.310	295	0.294

^a minor allele frequency^b number of founders genotyped

Table III

Most significant results from single marker and haplotype analyses

Gene	SNP	Associated Allele	VWS	CL/P	CP/L	Bilateral CL	PO	Lip Pits
Single Marker Analysis								
IRF6	rs2235371	A	0.05	0.02	0.04	0.01	0.39	0.02
TGFA	rs2902345	C	0.095	0.63	0.33	0.51	0.14	0.03
FOXE1	rs3758249	G	0.25	0.03	0.21	0.01	0.48	0.41
FGFR2	rs2981582	T	0.68	0.08	0.72	0.04	0.56	0.32
TGFB3	rs2284792	T	0.02	0.40	0.34	0.43	0.03	0.02
RIPK4	rs7278103	T	0.322	0.02	0.26	0.04	0.03	0.66
Haplotype Analysis *								
IRF6	rs2235371-rs642961	A-G	--	0.01	--	--	--	--
TGFA	rs2902345-rs454305	C-A	--	--	--	--	--	0.05
TFAP2A	rs303050-rs1675414	T-T	--	--	--	--	0.01	--
FOXE1	rs3758249-rs4460498	G-C	--	0.007	--	--	--	--
RIPK4	rs3171532-rs7278103	A-A	--	--	--	--	0.05	--

--: global p-value > 0.05

CL/P: cleft lip with or without cleft palate; CP/L: cleft palate with or without cleft lip; CL: cleft lip; PO: lip pits only

* global p-values < 0.05

Table IV

Association analysis of VWS phenotypes with types and locations of IRF6 mutations in 124 families

Phenotype	Mutation Type		Mutations contacting DNA	IRF6 Domains	
	p-value	Associated Mutation Type	p-value	p-value	Associated Domain ^b
Clefting ^a					
Cleft lip	0.05	truncation	1.00	0.27	
Cleft lip and palate	0.70		0.16	0.71	
Cleft palate	0.05	missense	1.00	0.71	
Lip Pits					
Cleft only	0.36		1.00	0.62	
Lip pits only	0.86		0.26	0.01	PBD
Lip pits	0.69		0.10	0.30	
Other Features					
Limb defects	0.03	missense	0.002	0.002	DBD
Pterygia	0.30		0.02	0.04	DBD
Dental anomalies	0.46		1.00	0.54	

^aincludes individuals with or without lip pits^bDBD: DNA-binding domain; PBD: protein-binding domain

Table V

Comparison of mutation-positive and mutation-negative VWS families

Phenotype	% Mutation-positive families (N=124)	% Mutation-negative families (N=56)	p-value	OR [95% CI]
Clefting^a				
Cleft lip	30.7	8.9	0.001	4.51 [1.7-12.2]
Cleft lip and palate	66.9	44.6	0.006	2.51 [1.3-4.8]
Cleft palate	54.0	48.2	0.47	
Lip Pits				
Cleft only	39.5	42.9	0.74	
Lip pits only	44.4	41.1	0.68	
Lip Pits	95.2	89.3	0.20	
Other Features				
Limb defects	20.2	14.3	0.41	
Pterygia	6.5	3.6	0.73	
Dental anomalies	16.1	10.7	0.49	

^a includes individuals with or without lip pits